Utility of models of the gastrointestinal tract for assessment of the digestion and absorption of engineered nanomaterials released from food matrices

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Abstract

Engineered metal/mineral, lipid and biochemical macromolecule nanomaterials (NMs) have potential applications in food. Methodologies for the assessment of NM digestion and bioavailability in the gastrointestinal tract are nascent and require refinement. A working group was tasked by the International Life Sciences Institute NanoRelease Food Additive project to review existing models of the gastrointestinal tract in health and disease, and the utility of these models for the assessment of the uptake of NMs intended for food. Gastrointestinal digestion and absorption could be addressed in a tiered approach using in silico computational models, in vitro non-cellular fluid systems and in vitro cell culture models, after which the necessity of ex vivo organ culture and in vivo animal studies can be considered. Examples of NM quantification in gastrointestinal tract fluids and tissues are emerging; however, few standardized analytical techniques are available. Coupling of these techniques to gastrointestinal models, along with further standardization, will further strengthen methodologies for risk assessment.

Introduction

Nanotechnology approaches are allowing the development of deliberately engineered nanomaterials (NMs), presenting promising new applications for conventional foods, such as the enhancement of texture, color, flavor, nutrient stability and food packaging safety (Sozer & Kokini, 2009). Some NMs possess unique properties, and novel uses in food or food contact materials could require a separate risk assessment from the equivalent bulk and ionic counterparts. Thus, there is heightened interest in the identification, validation and standardization of scientific methodologies to assess the uptake, and therefore internal absorbed dose, of NMs that are relevant to human dietary sources (Bouwmeester et al., 2009; Card et al., 2011). Identification and quantification of changes to these materials during digestion in the gastrointestinal (GI) tract lumen, and of absorption through the GI tract epithelium, can be important in their risk assessment (Cockburn et al., 2012; Dekkers et al., 2011; Hagens et al., 2007). The oral absorption of drugs in the gastrointestinal tract fluids and tissues are emerging; however, few standardized analytical techniques are available. Coupling of these techniques to gastrointestinal models, along with further standardization, will further strengthen methodologies for risk assessment.

Keywords

Alimentary canal, bioaccessibility, bioavailability, nanotechnology

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For food travelling through the GI tract, the bioaccessible fraction is the proportion of a component that is released from the food matrix into the digestive juice (Rein et al., 2013). Then, the bioaccessible fraction has the potential to either be digested or remain intact, and either be absorbed through the GI tract wall or be excreted. Bioavailability, or fraction absorbed, refers to the portion of the bioaccessible fraction that crosses through GI epithelial cells into lymph and blood. For a NM, bioavailability may refer either to the fraction of particles still in the nanometer size range, or to the atoms that make up the NMs. Another key definition for the topics covered in this review is the distinction of particle aggregation and agglomeration. In an aggregate, the primary particles are tightly bound by chemical forces, such that disintegration is very difficult even when applying physical forces. Agglomeration is the loose association of primary particles by weak forces such as van der Waals forces. Agglomerates can be disintegrated by physical forces such as agitation and sonication. The scope of this article does not include models of systemic distribution, metabolism, elimination, toxicity or nutrition. Rather, the objectives were to (a) identify models of the human GI tract that are used to quantify the digestion and absorption of conventional chemicals in states of health and disease, (b) consider the relevance of the models to NM digestion and absorption pathways, (c) provide examples where food-relevant NMs have been tested and (d) identify research, validation and standardization needs specific to the assessment of food-relevant NMs.

**Models of the healthy gastrointestinal tract**

Various models closely simulate human physiology and have been used for decades to assess the solubility, digestion and epithelial permeability of conventional food and drug components in the buccal cavity, stomach, small intestine and colon. The potential utility of these experiments in the assessment of food-relevant NMs is detailed below.

**In vivo animal models**

The processes of digestion and absorption of nutrients are similar in humans and other mammalian species. Therefore, toxico-kinetic studies in animals have historically been used as one way of assessing these parameters for conventional chemicals (Table 1). However, ethical consideration and approval is required for the use of animals. The concepts of replacement, reduction and refinement have formed the basic tenants of animal welfare in experimentation since their introduction by Russell and Burch in 1959 (Liebsch et al., 2011). Replacement involves the use of *in vitro* or *in silico* models instead of animals, when such methods are available and sufficiently predictive of outcomes in humans. Reduction maximizes data from the lowest number of animals possible, without compromising the required statistical strength of the study. Reduction strategies include completing *in vitro* mechanistic studies before deciding if further animal testing is required, the maintenance of public databases to share non-proprietary historical *in vivo* information (e.g. the Nanomaterial Registry at https://www.nanomaterialregistry.org/), and forming consortia in order to avoid duplicative testing. Refined protocols incorporate advances in animal husbandry and scientific procedures to reduce the pain and suffering experienced by animals. Examples include species-specific enrichment and housing conditions, the use of non-invasive techniques, appropriate pain relief and humane endpoints (OECD, 2000).

The scientific quality and potential extended usefulness of *in vivo* animal studies is improved with standardized protocols. The Organization for Economic Co-operation and Development (OECD) maintains guidelines for toxico-kinetic feeding studies in animals, including rodents. With these protocols, test compounds are orally administered either in the food or drinking water, or via single or repeated gastric gavage. Whereas dosing in food or water is more realistic, it might be less accurate. The gavage procedure can increase accuracy. However, limitations include the bolus effect, lack of exposure of the buccal cavity, lack of the physical chewing forces that are encountered in humans, potential tissue damage, potential accidental aspiration into the lungs, stress, non-specific binding of the test substance to the gavage tubing and abnormal food transit times (Powell et al., 2010). Following administration, detection methods scan for test material residues in feces, blood, lymph and GI tissues. It is not yet known if it will be necessary to modify the current OECD guidelines for toxico-kinetics studies to accommodate NMs (OECD, 2010) and it is currently not clear whether administration via gavage versus introduction into food or water is more appropriate. Despite this, some oral feeding studies in the literature demonstrate that several toxico-kinetic parameters can be modeled for NMs.

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### Table 1. *In vivo* techniques that have been commonly used to assess the digestion and absorption of conventional chemicals in the gastrointestinal tract, and could be relevant to the assessment of NMs.

| Model                          | Species                        | Characteristics and applications for conventional chemicals                                                                 | Limitations                                                                 | References on conventional uses |
|--------------------------------|--------------------------------|---------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------|---------------------------------|
| Toxicokinetic feeding or gavage studies | Primarily rat, mouse, hamster or guinea pig, unless another species such as pigs or primates is required for regulatory purposes | Assesses systemic absorption, excretion, and persistence of the chemical                                                   | Gavage does not represent a realistic exposure scenario; recovery and quantification of administered compounds in animal tissues is a challenge; some species do not accurately predict chemical bioavailability in humans | OECD (2010)                     |
| *In situ* intestinal perfusion   | Rats or other species          | Cannulation of both ends of a segment of the gastrointestinal tract in a live, anesthetized subject allows infusion of a solution and collection of the perfusate at the distal end; determines permeability through the gastrointestinal tract wall | Requires surgery and anesthesia; can result in abnormal transit times or paralysis of the bowel, digestion is not modeled; not a high-throughput model | Lennernas et al. (1997), Kim et al. (2006) |
Silver nanoparticles (NPs) are a unique example with regard to their chemistry, dissolution and interaction. The absorption of 14 nm silver NPs has been indirectly characterized following a 28-day repeated oral exposure study in rats, and was compared with silver ion exposure (Loeschner et al., 2011). The silver atoms that made up both silver sources were detected in the intestinal lamina propria and submucosa of exposed rats. Interestingly, electron microscopy energy dispersive X-ray emission spectroscopy detected nanometer-sized granules, containing silver, selenium and sulfur, in the intestinal basal lamina propria of rats exposed to either the NPs or the ions. The level of silver atoms in the stomach and intestinal wall, normalized to silver exposure dose, was significantly higher in the ion-exposed rats than in the NP-exposed rats (van der Zande et al., 2012). Levels of silver atoms in the feces represented 99% of the administered dose. A single oral exposure of rats to 8 nm citrate-coated silver NPs, using OECD toxicokinetic guideline 417, confirmed that most of the silver content was excreted via feces within 24 h of the treatment (Park et al., 2011). Bioavailability, quantified as blood concentration of silver, was 1.2% following treatment with 1 mg/kg body weight NPs and 4.2% following treatment with 10 mg/kg. Pioneering work in this field investigated other insoluble particles, such as 500 nm rutile titanium dioxide, and demonstrated uptake by gut-associated lymphoid tissue (Jani et al., 1994). Other NM categories such as vitamin B1-coated NPs have also been tested in vivo (Salman et al., 2007). Similarly, Yuan et al. (2007) characterized fluorescently labeled engineered lipid-based particles following intragastric administration to rats by gavage. Fluorescence in blood and lymph was detected by spectrophotometry, as an indirect measure of particle absorption through the GI tract. The 202 nm solid–lipid NPs modified with stearic acid showed 30% absorption efficiency into the bloodstream during the 48 h following administration. The fluorescent label alone was not absorbed, ruling out false-positive absorption of label leaked from the particles.

Together, these studies provide a useful stage in addressing the need to better quantify uptake of NM from the gastrointestinal tract into blood over time, and their subsequent biodistribution. More work is required to extend this information to a wider array of NMs with different physicochemical properties, including those for which solubility may be an issue.

**In situ** perfusion models in live, anesthetized subjects can specifically quantify the permeability of a compound through a segment of the gastrointestinal tract over the course of several hours. Rat intestinal permeability coefficients for chemicals have been shown to correlate well with those in humans; particularly for substances that are passively transported (Lennernas, 2007). It is not known if this will hold true for NMs. However, relevant experiments are emerging. Lipid-based NMs were studied in situ with ligated intestinal jejunum loops; an approach similar to perfusion (Yuan et al., 2013). Following necropsy, microscopy with gut sections qualitatively demonstrated that 200 nm solid–lipid particles modified with polyethylene glycol were able to penetrate through the mucus coating the epithelial cells and gain contact with those cells, positioning them for absorption. Unmodified particles were trapped in the mucus above the epithelium. Fluorescence from polysaccharide macromolecule-based 290 nm chitosan particles administered in situ in rats were observed in intestinal enterocyte cells, in Peyers’ patches, and in the underlying lamina propria (Behrens et al., 2002). More work is required to identify why some NM become trapped in the mucus layer while others appear to penetrate effectively.

A major advantage of in vivo studies is the presence of intact anatomical features and physiological processes. The absorption of conventional chemicals is often similar to humans; however, discrepancies can result from the differences in anatomy, food transit times, digestive enzymes, transporters and metabolism (Kim et al., 2006). The differences between humans and rodents in terms of uptake and how such parameters modify uptake are not yet understood for NM. When alternative in vitro and in silico models were not available for particular purposes, in vivo animal studies were used as the status quo method of predicting human absorption. Classical in vivo and in situ toxico-kinetic study designs may be valid to study the uptake of NMs in GI tissues. However, most relevant analytical tools will need to be validated and adopted internationally in order to be able to quantify the uptake. This presents an opportunity for simultaneous validation of the analytical tools with human-relevant in vitro or in silico methods that show potential for the replacement of current animal testing methods.

**Ex vivo tissue models**

Ex vivo models use tissue samples that are excised from an anesthetized organism and maintained under conditions supporting certain aspects of normal function. Ex vivo methods are sometimes used to assess chemical permeability (Table 2). Ex vivo gastrointestinal models suffer the disadvantage of lacking digestive juices. However, they are higher throughput than in vivo or in situ studies. The following paragraphs summarize how ex vivo models have been applied to study NMs.

The everted gut sac was used to quantify the permeability of radiolabeled 500 nm tomato lectin-coated polystyrene particles (Carreno-Gomez et al., 1999). At an administered concentration of 20 μg/mL, the particles transferred through the gut wall to the serosal fluid at a rate of 22.7 ng/mg tissue protein per hour. However, overall this represented only a small percentage of the administered material. The kinetics of particle and agglomerate sedimentation was not determined. The effects of NM agglomeration and sedimentation on ex vivo models such as the gut sac need to be investigated. In another study, segments of rat small intestine were incubated with fluorescent chitosan. Mucus adhesion, penetration and particle disintegration were studied by fluorescence image visualization of tissue slices and by fluorescence quantification in mucus scraped from the tissue (Dunnhaupt et al., 2011). There was significantly greater mucoadhesion and stability of thiol-conjugated than non-thiolated chitosan. The non-thiolated particles more readily penetrated through the mucus, contacted the underlying epithelial cells and disintegrated. Going one step further, mucoadhesion and tissue permeation of larger 800 nm fluorescent chitosans with different surface chemistries was quantified on isolated rat intestinal mucosa using a Franz diffusion cell (Sandri et al., 2007). Adhesion to the tissue was indirectly quantified from the residual fluorescence in the donor and receiver buffers following exposure. Particles with higher mucoadhesion were identified by their higher residence time on the intestinal mucosa, but they had lower penetration through the tissue, as confirmed by microscopy. Again, the physicochemical characteristics of NM that influence their interaction with mucus and hence epithelial penetration requires further investigation. Such models provide a useful tool as they are more easily controlled than in vivo studies, but they still include the full complement of cell types within the barrier structure.

The analysis of specific segments of the GI tract provides a mechanistic focus, but is limited by the capacity to collect and maintain viable tissues from the area of interest (Lennernas, 2007). The lack of digestive enzymes and short viability time of ex vivo tissues limit their utility for the prediction of absorption in vivo. However, they are higher throughput than in vivo studies and they have allowed qualitative and quantitative determination of mucoadhesion and permeability of NMs through the intestinal wall.
Table 2. *Ex vivo* tissue models that have been commonly used to assess the absorption of conventional chemicals through the gastrointestinal tract, and that could be relevant to the assessment of NMs.

| Model                                      | Species and tissue of origin | Characteristics and applications for conventional chemicals | Limitations                                                                 | References on conventional uses |
|--------------------------------------------|-----------------------------|----------------------------------------------------------|----------------------------------------------------------------------------|----------------------------------|
| Everted gut sac                            | Excised intestinal segments from rat or other species | The intestine is everted, ligated at both ends and cultured in an oxygenated nutrient solution; adhesion of an administered compound to the outer mucus layer and permeability through the tissue to the inside of the sac is measured; relatively simple and inexpensive methodology | Careful tissue handling is critical to avoid mechanical damage; appropriate culture conditions must be used to maintain tissue viability; the static fluid in the serosal compartment does not accurately model *in vivo* circulation | Alam et al. (2012) |
| Transmucosal perfusion through isolated mucosa | Mucosal layer isolated from biopsies from the buccal cavity or intestine of rat, pig or other species | Perfusion device with a donor and receiver chamber, such as an Ussing chamber or Franz diffusion cell, determines permeability through the mucus layer; results compare favorably to human data, particularly for passively absorbed substances | Tissue viability declines rapidly | Roblegg et al. (2012), Rozenhal et al. (2012) |

A disadvantage of Caco-2 cells is that, when grown in monolayer, they lack a mucus layer, allowing easy access to the apical cell membrane. However, despite this, the rate of paracellular transport is typically more limited than in the human intestine, and total transport values for many conventional molecules through Caco-2 cells are similar to values obtained through human colon and rectum biopsies (Chen et al., 2010; Rubas et al., 1996). The limited paracellular transport will likely not be an issue for NMs since uptake via this pathway is negligible for materials larger than 1 nm in the healthy GI tract (Frohlich, 2012). Interlaboratory reproducibility with this model rests in careful attention to culture conditions, such as cell passage number, seeding density and length of time in culture (Natoli et al., 2012). In addition, Caco-2 cells have been demonstrated to produce mucus when grown in more human-relevant three-dimensional (3D) environments (Kim & Ingber, 2013). The uptake of food-relevant NMs into cells and transport to the basolateral chamber has been tested with several epithelial cell types. Caco-2 cells confirmed intestinal absorption of well-characterized hemin-coupled iron (III)-hydroxide NPs and of chitosan coated, iron loaded solid lipid NPs, as possible alternatives to oral ferrous salt supplements in iron mineral deficiency (Jahn et al., 2011; Zariwala et al., 2013). The permeability of 40 nm titanium dioxide NMs was also determined with Caco-2 cells using a dual chamber approach (Koeneman et al., 2010). The average diameter of the NMs in the donor medium was greater than 500 nm, indicating agglomeration. In general, the mass of larger NMs can increase the rate of settling and so the exposure dose to the cells becomes not only the concentration of particles administered, but also is a factor of how much of the total mass in that volume sediments (Cohen et al., 2013; Teeguarden et al., 2007). This might not hold true for agglomerates, of which the effective density can be smaller than their dispersed density (DeLoid et al., 2014). In any model, it is challenging to distinguish binding to the cell surface, which would slow transit time along the GI tract, from uptake into the cell. Koeneman et al. (2010) used scanning confocal microscopy to visualize the titanium dioxide inside the cells, demonstrating absorption by transcellular transcytosis.
Table 3. *In vitro* cell culture models that simulate the gastrointestinal tract epithelium, are commonly used to assess the absorption of conventional chemicals, and could be relevant for the assessment of NMs.

| Model                  | Species and tissue of origin                                                                 | Characteristics and applications for conventional chemicals                                                                 | Limitations                                                                                      | References on conventional uses                                      |
|------------------------|------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|------------------------------------------------------------------------|
| TR146 cells            | Human buccal carcinoma                                                                        | Epithelial origin; forms a multi-layered squamous tissue in culture; a model of chemical permeability through the buccal epithelium | Transport mechanisms require further characterization                                           | Teubl et al. (2013)                                                   |
| Caco-2 cells           | Human colorectal carcinoma                                                                      | Widely used model of small intestine enterocytes; develop tight junctions, polarization, brush border enzymes and membrane transporters that are responsible for the active uptake of chemicals | Cells vary in phenotype and function according to culture conditions; tight junction permeability is lower than in humans; expression level of some active transporters differs from the human intestine; lacks mucus | Sambuy et al. (2005), Prieto et al. (2010), Kim et al. (2012)          |
| T84 cells              | Human colon carcinoma cells that had metastasized to a pulmonary tumor                          | Less prone to differentiation than Caco-2 cells; an ideal model of the colon since the cells are capable of regulated chloride secretion and can secrete mucus | Few microvilli on the apical membrane; relatively low monolayer permeability                    | Donato et al. (2011)                                                  |
| 2/4/A1 cells, IEC-6 cells, or IEC-18 cells | Rat intestinal epithelial cells                                                                  | Good predictors of paracellular permeability and passive transport in the human duodenum | Lack several active transporters and efflux systems; a poor model of active transport          | Thomas & Oates (2002), Versantvoort et al. (2002), Tavelin et al. (2003) |
| Caco-2 with HT29 cells | Human colorectal carcinoma cell lines                                                           | Co-culture incorporates a mucus layer on the enterocytes and enhances paracellular permeability                             | The availability of different cell sub-clones results in interlaboratory variability           | Walter et al. (1996), Chen et al. (2010)                                |
| Caco-2 with Raji B cells, or Caco-2 with Peyer’s patch lymphocytes | Human colorectal carcinoma cells in combination with human Burkitt’s lymphoma cells or murine Peyer’s patch lymphocytes | Co-culture induces a fraction of the Caco-2 cells to develop the morphology of Peyer’s patch epithelial microfold cells, including few microvilli and a soft mucus layer | Variations in primary lymphocyte preparations may influence co-culture characteristics; quantitative correlation to human uptake not fully understood | Tyer et al. (2002), des Rieux et al. (2007)                             |
| Gut-on-a-chip          | Human colorectal carcinoma                                                                      | Two fluidic channels separated by a Caco-2 monolayer, with microcircuitry to detect absorption of substances | Cells vary in phenotype and function according to culture conditions; tight junction permeability is lower than in humans; expression level of some active transporters differs from the human intestine; lacks mucus | Kim et al. (2012), Vergeres et al. (2012)                              |

Inductively coupled-plasma mass spectrometry detected microgram quantities of titanium atoms in the basolateral compartment, representing 14% of the administered material at the 100 μg/mL dose, and 2% of the administered material at the 1000 μg/mL dose. Approaches should be developed to factor total surface area of the cell monolayer into quantitative determinations such as this. The lower percentage permeability at the high dose may have indicated saturation of cellular absorption mechanisms, but was more likely related to limited permeation through the culture apparatus; as the percentage of titanium that gained access to the basolateral compartment was low even in the absence of cells. While the data demonstrated total absorption of titanium, it did not distinguish between intact NPs and ions in the basolateral fluid. Again, total surface area of the cell monolayer must be indicated to allow validation by comparison with the *in vivo* surface area of the GI epithelium and total absorption. The demonstration of interlaboratory reproducibility can also aid in standardization. Fisichella et al. (2012a) also used Caco-2 cells in combination with simulated gastric digestion to study the permeability of titanium dioxide NMs consisting of a 10 nm wide and 50 nm long rutile core, surrounded by a thin aluminium hydroxide layer, and surface treated with polydimethylsiloxane polymer. Simulated gastric digestion of the NMs in acidic medium removed the polydimethylsiloxane coating, and the product agglomerated to between 720–1350 nm upon subsequent incubation in cell culture medium. Following 72-h exposure of the Caco-2 cells, microscopy visualized the agglomerates as tiny spots on the outer surface of the plasma membrane. However, no particles could be seen inside the cells. This difference from the previous study may have been related to the differences in the physico-chemical properties of the NMs (Fisichella et al. 2012b). The discrepancy highlights the need for the characterization of the NMs, both pristine to know what is being tested and in the model systems to understand the fate and stability in the exposure medium and cellular permeability of each type of NM.
Next to monocultures, \textit{in vitro} cell co-culture systems integrate several cell types, thus more adequately resembling the complex anatomy of the GI tract epithelium (Table 3). They model different pathways and mechanisms of chemical and NM permeability. The co-culture models most relevant to NM absorption pathways are discussed below.

HT29 cells secrete mucus and provide a useful model of the adhesive and barrier properties of the GI mucus layer. However, monoculture does not fully reflect the human intestinal epithelium, where goblet cells represent 10–25% of the cells. This discrepancy can be overcome by co-culturing HT29 cells with Caco-2 cells. Chemical permeability coefficients that correlate with the \textit{in vivo} situation have been obtained using optimized culture media, cell ratios, culture times and seeding densities (Walter et al., 1996). Several NPs have been tested in this model. For example, iron oxide NPs coated with oleic acid were not transported at a detectable level into or through a Caco-2/HT29 monolayer, despite the 10\,nm diameter of the primary particles (Kenzouei et al., 2012). However, solid–lipid NPs of approximately 200\,nm permeated the mucus and were absorbed by endocytosis (Yuan et al., 2013). Agglomeration or differences in surface charge and chemistry of the NMs in the media could be a factor in the differential permeability.

In the intestine, microfold (M) cells are localized in the epithelium of Peyer’s patch and lymphoid nodules, and are responsible for sampling antigens from the lumen. They are a primary route of particulate matter uptake. Thus, M cell models are important in the mechanistic investigation of particulate absorption (Shahbazi & Santos, 2013). Since the percentage of M cells in the epithelial barrier is low, co-culture models facilitate controlled studies. Caco-2 cells can be co-cultured with either Raji B lymphoma cells or freshly isolated murine Peyer’s patch lymphocytes in order to induce the differentiation of a fraction of the Caco-2 cells to an M cell phenotype. The Caco-2 and Raji B lymphoma co-culture dual chamber model characterized the permeability of silver NPs of 20, 34, 61 and 113\,nm diameters (Bouwmeester et al., 2011b). Before administration, the suspensions of silver NPs had very low levels of silver ions. However, 24-h incubation in the cell culture medium alone released approximately 5–20\% of the silver content to an ionic form. Thus, conventional silver ions were used as a control treatment. Following NP exposure of the cells, approximately 0.5\% of the silver content from the NPs had translocated from the apical to the basolateral chamber. There was a similar translocation rate when pure silver ions were applied, suggesting that the ions released from the NPs likely contributed to the majority of the translocated material. This observation is similar to that described above following \textit{in vivo} exposure of rats. This study also highlights the importance of dissolution testing and ionic controls for metals, when possible. The Caco-2 with Peyer’s patch lymphocyte co-culture model was similarly used to demonstrate M cell uptake of fluorescent chitosan – deoxyribonucleic acid (DNA) NMs of 50–100, 200 and 400–500\,nm (Kadiyala et al., 2010). The NMs did not agglomerate during incubation in the cell culture medium, and following 30\,min of cell exposure, fluorescence from the NMs was quantified in the basolateral chamber by flow cytometry, demonstrating that the 50–100\,nm particles had the highest permeability. The percentage of particles that permeated was 5-fold greater through this co-culture M cell model than through a Caco-2 enterocyte monolulture. Microscopy visualized the fluorescence in the cells, confirming the uptake and transepithelial transcytosis of the NMs.

Recently, \textit{in vitro} 3D cell culture models are emerging that more accurately mimic the structure of the GI mucosa \textit{in vivo}. For example, buccal TR146 or intestinal Caco-2 cells can be grown on a 3D collagen scaffold and overlaid with GI mucus and fluids (Moharamzadeh et al., 2008; Pusch et al., 2011; Yadev et al., 2011; Yu et al., 2012). Whereas the collagen scaffold in those examples can be a limiting barrier for rapidly transported substances, more permeable synthetic membranes can be incorporated in 3D human tissue models (Giovino et al., 2013). Further evaluation and comparison to \textit{ex vivo} tissue and animal studies, followed by standardization of these novel models will clarify their applicability and accuracy for the prediction of particle uptake rate in humans.

Interest in \textit{in vitro} cell lines is increasing for the toxicokinetic analysis of NMs due to their potential for high-throughput, cost-effective and good predictability of permeability potential. An additional advantage is the ability to use cell lines that are derived from humans. There are several limitations and possible solutions. Simple cell culture systems using established cell lines do not incorporate factors such as age and sex, and they lack systemic circulation pathways such as the enterohepatic secretion of absorbed materials back into the GI lumen. Emerging approaches to account for different genotypes could possibly be developed, such as the use of induced pluripotent stem cells (Gracz et al., 2013). The InLiveTox project (http://www.inlivetox.eu/) has developed a multi-tissue fluidic model that allows flow above and below a Caco2 differentiated confluent layer, with the basolateral flow linking to endothelial (HUVEC) and hepatocyte (C3A) culture. Further validation of this model is required to assess its usefulness for NM compared to simple \textit{in vitro} models and animals. The same approach could be used to link more tissues to better reflect pathways such as enterohepatic circulation.

Another limitation of simpler \textit{in vitro} systems is that standard culture media do not contain GI lumen factors. More physiologically relevant culture fluids can be used to reflect the fasted and fed states (Patel et al., 2006). Like with any model system, the suspension of NMs in aqueous administration fluids can be problematic (Bihari et al., 2008). If dispersion agents are used in the vehicle to solve this problem, effects on the integrity of the cells and the function of transporters must be ruled out by including the agents in control wells (Buckley et al., 2012). Nonetheless, \textit{in vitro} cellular models have many unique advantages in the mechanistic analysis of epithelial permeability. The limitations can often be overcome by combining the data from more than one \textit{in vitro} assay. With the rapidly generated data, mathematical approaches can be used to approximately equate the values to human \textit{in situ} transepithelial permeability (Avdeef & Tam, 2010; Pelkonen et al., 2001). Further research is required to confirm the accuracy of these equation approaches to NM permeability. If \textit{in vitro} systems are to be used to reflect NM bioavailability in humans, it is important to be able to characterize not only permeability, but also digestion in GI fluids.

\textbf{In vitro non-cellular fluid models}

Digestion is a complex process involving physical, chemical, enzymatic and microbial degradation of food as it travels through the buccal cavity, stomach, small intestine and large intestine. Various factors in the lumen influence digestion including ionic strength, pH, digestive enzymes, bile, microbiota, transit time, hydrodynamics and the composition of the food matrix (Brandon et al., 2006). Fluid models that simulate the human GI tract can provide useful ways of evaluating certain aspects of NM digestion including bioaccessibility, stability, dissolution and aggregation (Cockburn et al., 2012). Available models originate from pharmaceutical and food safety research (Table 4). Equipment such as the US Pharmacopeia (USP) I/II dissolution apparatus and basket stirred flask is routinely used to assess the dissolution characteristics of drug substance and dosage forms...
Table 4. *In vitro* non-cellular fluid models that simulate the gastrointestinal tract, are commonly used to assess the digestion and absorption of conventional chemicals, and could be relevant to the assessment of NMs.

| Model                          | Characteristics and applications for conventional chemicals                                      | Limitations                                                                                     | References on conventional uses                      |
|-------------------------------|------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-----------------------------------------------------|
| Static digestion models       | Synthetic fluids simulate the enzymatic and chemical digestive conditions in the various compartments of the gastrointestinal tract | Lack the mechanical forces that contribute to digestion *in vivo*; metabolites accumulate and can interfere with digestion | Versantvoort et al. (2005), Guhmann et al. (2013) |
| Dynamic digestion models      | Synthetic digestive fluids are mixed and propelled by mechanical forces similar to those encountered *in vivo*; bioaccessibility and digestion are modeled for the various compartments of the gastrointestinal tract | Specialized equipment required to generate the physical forces | Minekus et al. (1999), McAllister (2010)              |
| Natural and synthetic mucus   | Isolated gastrointestinal mucus incubated *in vitro*; models mucoadhesion and permeation through the mucus | Variations in mucus source and preparation can influence characteristics | Crater & Carrier (2010)                             |
| Artificial membrane permeability assays | Non-cellular synthesized membrane on a filter support models the cell membrane; lipid composition can be specified; passive transcellular permeability is modeled; both hydrophilic and hydrophobic compounds can be applied | Membrane lacks pores and transporter proteins; does not model active transport or paracellular permeability | Reis et al. (2010)                                   |

(McAllister, 2010). Most dissolution models are mixed with an impellor, a magnetic stirrer or a shaker. They mimic the chemical and enzymatic conditions in the human GI tract. However, many do not accurately mimic the complex physical forces that are an important factor in the disintegration of food particles *in vivo* (Minekus et al., 1999). More sophisticated models recreate the physical environments encountered in the GI tract.

*In vitro fluid systems that mimic the lumen of the buccal cavity*  
*In vivo*, the composition of salivary fluid changes in response to a number of stimuli (Yan et al., 2010). Despite this, most simulated salivary fluid experiments use set concentrations of salts, amylase and pH. Since changes in ionic strength and pH may influence engineered NM stability and agglomeration, it may be more ideal to use several fluids with different osmotic, amylase and pH values (Guhmann et al., 2013). Moreover, the chewing and swallowing forces encountered *in vivo* can be modeled using devices with mobile parts, that faithfully reproduce shear, compression and tongue forces (Salles et al., 2007). Further research is required to standardize these models for the application of NMs.

*In vitro fluid systems that mimic the lumen of the stomach*  
Conditions in the gastric environment, such as pH, enzyme concentration, water content, temperature, pressure, microbial load and emptying rate are continually changing as food is ingested (Minekus et al., 1999). Static *in vitro* digestion assays using simulated gastric fluids have been well validated to approximate these conditions, for example for the assessment of novel protein stability (Thomas et al., 2004). This type of assay has also been used to determine how gastric conditions impact the behavior of NMs. Silver NPs in simulated gastric fluid agglomerated significantly and released ionic silver from their surface that re-precipitated and coated the agglomerates as insoluble silver chloride (Mwilu et al., 2013). Interestingly, the digestion of pure silver ions also resulted in the *de novo* formation of silver chloride agglomerates ranging in size from 1 micron to greater than 10 microns, with 50 nm particles visible on the surface by electron microscopy energy dispersive X-ray spectroscopy. Thus, the precipitation of silver chloride agglomerates was not a phenomenon specific to NMs. However, it was not a characteristic of bulk materials, as conventional silver particles of 1.6–2.6 microns did not significantly dissolve (Rogers et al., 2012). Zinc oxide NPs readily dissolved under gastric conditions (McCracken et al., 2013). Clearly, static *in vitro* digestion assays using simulated gastric fluids could be used to distinguish a variety of the possible outcomes for NMs.

Several dynamic gastric simulators reproducibly recreate the complex physical mixing and emptying conditions at work in the human stomach (Kong & Singh, 2010; Wickham et al., 2009). Various food matrices can be applied to determine their influence on the retention, release and breakdown of food particles (Kong & Singh, 2008; Kong et al., 2011). For example, a dynamic model provided a more accurate simulation of the digestion of a self-emulsifying drug delivery system in terms of droplet size than the conventional USP II dissolution apparatus (Mercuri et al., 2011). However, more studies are required to determine the utility of these systems for the evaluation of the behavior of NMs in food.

*In vitro fluid systems that mimic the lumen of the stomach and small intestine*  
Static digestion can sequentially simulate the buccal cavity, stomach and small intestine using ionic compositions, digestive enzyme concentrations and pH profiles close to human physiology (Versantvoort et al., 2005). The digestion of macronutrients has been validated with these models (Kopf-Bolanz et al., 2012), and the models have been applied to study the digestion of NMs by coupling the output material with robust analytical methods appropriate for complex matrices. For example, the fate of silver NMs was evaluated (Bohmert et al., 2013; Walczak et al., 2013). It appears that the presence of proteins and other food components has a crucial impact on the particle size kinetics during the different phases of digestion. In the absence of proteins, 60 nm silver NMs completely dissolved under stomach conditions, whereas in the presence of proteins they agglomerated to clusters composed of silver NMs and chlorine, as reviewed in another example above, and then disintegrated back into single silver NMs at the intestinal stage of digestion (Walczak et al., 2013). A comparable agglomeration and disintegration phenomenon was observed for silicon dioxide NPs (Peters et al., 2012), whereas 115 nm aggregates of 21 nm titanium dioxide NPs maintained a constant size in all of the fluids (McCracken et al., 2013). Simulated intestinal digestion of pure silver ions, again resulted in *de novo* formation of 20–30 nm NPs composed of silver, sulfur
and chlorine (Walczak et al., 2013). These data have allowed consideration that existing health effects data on silver ion exposure may be useful for extrapolation in the risk assessment of silver NPs (Bachler et al., 2013). Hydrophobic solid lipid particles with a 200 nm diameter agglomerated to approximately 500 nm during incubation in simulated intestinal fluid, whereas particles coated with poly(ethylene glycol)-stearic acid had limited aggregation (Yuan et al., 2013). Simulated GI fluids provide not only a means of assessing the degree of particle degradation and aggregation, but also of leakage of any fluorescent labels that had been incorporated into the particles (Llabot et al., 2011). Data on NM stability and label release are very useful in the design of subsequent in vitro cell culture studies. The pH and composition of simulated intestinal fluids are fairly similar to standard cell culture media, and the products of sequential digestion can either be directly applied to a permeability model or replicated in cell culture media (McCracken et al., 2013).

Again, physiological forces are important to consider in in vitro fluid digestion models, for which applicable models are available. TIM-1 is a dynamic in vitro model of the stomach and small intestine that simulates human digestive and physiological processes including appropriate pH, gastric lipase, pepsin and hydrochloric acid, small intestinal pancreatic juice, bile and sodium bicarbonate, peristaltic mixing and transit, temperature and removal of the products of digestion (Minekus et al., 1999). The jejenum and ileum compartments include a semi-permeable dialysis membrane that removes water and the dissolved small molecule products of digestion, allowing quantification of the bioaccessible fraction released from the food matrix (Van de Wiele et al., 2007). The peristaltic mixing and fluid transfer in TIM-1 allowed more accurate prediction of drug dissolution in healthy human volunteers than the USP II and USP IV models (Souliman et al., 2007). For conventional drug and nutritional compounds, TIM-1 has been extensively used to characterize their bioaccessibility from food and beverage product matrices, expressed as a percentage intake, under various physiological conditions and transit times along the GI tract (Blanquet et al., 2004). The profiles are highly consistent with preclinical jejunal data in both fasted and fed states (Souliman et al., 2006). However, the use of a dialysis membrane to provide an absorptive surface does not model active cellular transport. To incorporate this function, it is possible to further analyze the digested fractions, after ultracentrifugation, filtration and dilution, and with a Caco-2 cell permeability assay (Deat et al., 2009). This model could be very relevant for the assessment of both the bioaccessibility and digestion of food-relevant NMs.

In vitro fluid systems that mimic the lumen of the large intestine

A notable characteristic of the large intestine lumen is the high concentration of microbes. The simplest and most frequently used in vitro models of the human large intestine are static batch fermentations (Ghoddusi et al., 2007). These cultures of intestinal or fecal microbial strains are far from physiological. They suffer from confounding factors such as the accumulation of metabolites, which change pH and redox potential, inhibit microbial activity and change community structure. However, the high-throughput nature could make batch incubations valuable as an initial screening assay for NMs.

Continuous dynamic models of the large intestine allow more in-depth study of the effect of the gut microbiota on selected food molecules. Several models have been used to date (Cinquin et al., 2004; Macfarlane et al., 1998; Makivuokko et al., 2005; Minekus et al., 1999; Molly et al., 1993). The medium contains factors such as nutrients, resistant starch, vitamins, minerals, bile and pancreatic enzymes, reflecting in vivo conditions. TIM-2, for example, is a dynamic model of the colon that is based on the same technology as TIM-1, described above (Minekus et al., 1999). It is inoculated with a high-density fecal microflora and incorporates peristaltic movement and mixing forces. A dialysis membrane allows removal of microbial metabolites, preventing the inhibition of the fermentation assay. Additional studies with systems such as these can determine the fate of NMs during fermentation in the colon.

Various in vitro non-cellular digestion systems allow high-throughput mechanistic screening of the bioaccessibility, dissolution, aggregation and digestion of NMs in solid, semi-solid and liquid food matrices that reflect fed and fasted states. Clearly, this analysis is possible only when sound analytical methods are available. Several other non-cellular in vitro models similarly require enhanced analytical techniques to ensure their usefulness with NM.

In vitro fluid models mimicking mucus

The mucus layer represents the first barrier a chemical has to cross in the uptake process. Isolated mucus allows for rapid and convenient analysis of particle adhesion to, or diffusion through, this layer (Table 4). For example, fluorescent cationic chitosan NPs diffused more rapidly through isolated porcine intestinal mucus than mucoadhesive thiol-conjugated particles (Dunnhaupt et al., 2011). While adhesion in vivo can slow the transit toward fecal excretion and thereby increase residence time, it can prevent diffusion through the mucus and prevent absorption by the underlying epithelium. Isolated mucus models in general, and in use with NM, require further investigation, validation and then standardization.

In vitro fluid artificial membrane models

Models such as the parallel artificial membrane permeability assay analyze the passive permeability of a substance from a donor compartment, through a lipid-infused artificial membrane on a filter support, into an acceptor compartment (Table 4). pH can affect the surface charge of administered NMs, and can be specified to reflect the properties the NMs would display in the various compartments of the GI tract (Reis et al., 2010). Artificial membrane models could offer high-throughput screening of passive transcellular permeability. Active transport mechanisms would have to be addressed with other systems.

Overall, several categories of in vitro models are used for the assessment of conventional food and drug chemicals and, when standardized, have shown good in vitro to in vivo correlation. These high-throughput systems show promise for NM assessment (Nel et al., 2013). There is a need to further adapt and standardize the available models and the corresponding analytical methods to allow quantification of the digestive fate of engineered NMs (Misra et al., 2012). Furthermore, over time there is a need to streamline the choice of models required to reliably predict NM uptake across the gastrointestinal barrier, as it will be impossible to test all NM in all models (Stone et al., 2014).

In silico computational models

In silico programs driven by complex computational algorithms are designed to predict how novel substances will behave in humans. Table 5 summarizes relevant in silico models of the GI tract.

When existing solubility, permeability and clearance data for a chemical is available from in vivo animal, ex vivo or in vitro models, in silico physiologically based toxico-kinetics programs can be constructed to extrapolate bioavailability in humans (Paixao et al., 2012). Some data for a very limited number of NM
Table 5. *In silico* computational models that simulate the gastrointestinal tract, are increasingly used to assess the dissolution and absorption of conventional chemicals, and could be relevant to the assessment of NMs.

| Model                                      | Capabilities for conventional chemicals                                                                                                                                                                                                 | Limitations                                                                                                                                                                                                 | References on conventional uses                                                                 |
|--------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| Compartmental absorption and transit       | The dissolution and bioavailability of chemicals in different compartments of the human gastrointestinal tract is modeled based on inputted solubility and permeability data on that chemical from *in vivo*, *ex vivo* or *in vitro* studies; can incorporate food effects and physiological inter-individual variability | Uses generalized mathematical models; prediction of intrinsic solubility is a challenge                                                                                                                                                                     | Yu & Amidon (1999), Bolger et al. (2009), Jamei et al. (2009), Sugano (2009), Sinha et al. (2012) |
| Statistical machine learning and Quantitative Structure Property Relationship | Prediction of human intestinal chemical permeability or bioavailability, based on the observed or calculated physico-chemical properties of the molecule; trained with existing permeability data from similar molecules in the human intestine or in Caco-2 cells | Molecular descriptor selection is algorithm- and descriptor software package- dependent, leading to variability in prediction results among methods; training data sets limit the applicability domain; relies on correlations with property values only, not on structural features | Linnankoski et al. (2008), Ahmed & Ramakrishnan (2012) |
| Quantitative Structure-Activity Relationship | Toxicokinetic database that predicts human intestinal absorption based on chemical descriptors or fingerprints in 1-, 2-, and 3-dimensions; computes from patterns of chemical fragments or substructural features; uses multiple algorithmic techniques and decision-tree analysis | Training data sets limit the applicability domain; quality of training sets vary; different validation methods lead to different predictive performance characteristics | Suenderhauf et al. (2011), Moda & Andricopulo (2012), Winkler et al. (2012) |

is available, but more is required for a wider range of NM differing in their physicochemical properties. Some compartmental programs also model the relationship between an external dietary dose and internal organ, blood and excretion concentrations over time in animals or humans. Such a model has been developed for silver NMs, for example Bachler et al. (2013). The models are dependent upon the nature and quality of the data used to train and validate the software, and specialized expertise is required to generate and interpret the data. The predictive power of compartmental programs is reduced when estimation of the solubility, permeability and clearance values is required.

Sophisticated quantitative structure–activity relationship (QSAR) software can predict chemical dissolution and bioavailability purely by extrapolation from structural similarity with substances for which these activities are known. QSAR programs have similarly been used in toxicology risk assessment to predict the physiological targets and health effects resulting from human exposure to drugs (Choi et al., 2013; Valerio et al., 2013). In *silico* models are of growing interest for utility in product development and in safety assessments. They can be just as predictive of human drug bioavailability as data generated with *in vivo* rat, Caco-2 cell, 2/4/A1 cell and artificial membrane models (Linnankoski et al., 2008).

*In silico* models could be an ideal approach to aid in the primary screening of the dissolution and absorption of engineered NMs (Winkler et al., 2012). However, in order to train the software for the analysis of novel NMs, a larger database of experimental results is required to document the physico-chemical properties and structures of existing NMs, and their corresponding digestion and absorption values. Descriptors that can be characterized to construct QSAR models include two-dimensional attributes of particle size, surface area, ionization potential, zeta potentials and common physico-chemical properties such as lipophilicity and hydrogen bond donor or acceptor strength. In addition, three-dimensional descriptors of shape can be incorporated in several software programs.

*In silico* models are high-throughput, efficient for use in prioritization, cost-effective and easily shared if built upon public data. This new research area holds promise for the assessment of NM dissolution and absorption, but requires further critical assessment for specific uses. Further testing is needed to learn which descriptors and computational methods are more accurate for the elucidation of the non-linear relationships that NMs likely possess.

Whereas the aforementioned *in vivo*, *ex vivo*, *in vitro* and *in silico* models are intended to simulate physiological parameters reflective of the majority of the human population, models are also available that reflect the GI tract in states of varied or altered physiology.

**Models reflecting inter-individual variability and disease states of the gastrointestinal tract**

The human population has normal inter-individual variability in the function of the GI tract, resulting from genetic and environmental factors (Rappaport, 2012). For example, the transit time and the composition of the digestive fluids, including pH, enzyme levels and microbes, vary considerably in response to factors such as age, diet and health status (Russell et al., 1993). Significant sub-populations of humans also have inflammatory or pre-cancerous lesions, affecting large areas of the GI tract, and modifying the anatomy and permeability (Camilleri et al., 2012). As described below, early studies show that NM digestion, adhesion and absorption may be altered by these factors. Thus, these varied states can be important to consider and model in the assessment of NM uptake in humans.

As there is no need for inter-species extrapolation, tissues excised from human patients provide an ideal system in which to study the effects of NMs in humans. Human tissues can be used to study inter-individual variability and to focus on individuals of a specific gender, ethnicity or disease state. This allows for the accurate study of diseased human tissue as opposed to animal
tissue in which a disease condition has been artificially induced. For example, healthy and diseased sections of tissue from patients with Crohn’s disease, ulcerative colitis and colonic carcinoma have been examined for the presence of nanoparticles (Powell et al., 1996; Schmidt et al., 2013). In addition, intestinal tissues from humans have been used to examine transmucosal penetration of particles to the vascular endothelium (Nwokolo et al., 1992). Pending ethical approval, further studies of human tissue will be useful in better understanding the fate and effect of NMs in the body and for the validation of other model systems.

Models of inter-individual variability

With in vivo studies, inter-individual variability can be accounted for not only using an appropriate number of replicate animals per treatment group, but also integrating available data from different animal strains, ages, sexes, stages of pregnancy, diets and/or disease states (Paek et al., 2013; Tryndyak et al., 2012). In vitro this can be accomplished with replicate treatment samples, multiple cell lines or digestive conditions and various disease parameters. Often animal and in vitro models of human diseases are generated by artificially inducing similar states in the animals or cells, and thus when used, the relevance of the disease models to the human conditions needs to be referenced. The necessity of NM testing with such variables should depend on the prevalence of the variables in the general human population, literature that points to hypothesized risks and/or benefits to those sub-populations, and the availability of alternative approaches for accounting for inter-individual variability to protect vulnerable populations, such as uncertainty factors (Canady, 2010).

Models of inflammation

Inflammation associated with a variety of disease states can affect the integrity of the GI mucosal epithelium, from the oral cavity through to the colon, and can lead to maligestion and malabsorption (Camilleri et al., 2012; Lefebvre et al., 2006; Meresse et al., 2012). Inflammatory bowel disease includes ulcerative colitis and Crohn’s disease; it is one of the most common chronic conditions involving inflammation of the intestine. Numerous animal models of these diseases have been developed, with no single model fully reflecting the pathogenesis observed in humans (Mizoguchi, 2012; Neurath, 2012), suggesting a need for research to improve model relevance. Several models of inflamed intestine have been used to assess NM adhesion and absorption. A passive accumulation of fluorescent polystyrene latex beads specifically in inflamed intestinal areas was first observed in the trinitrobenzene sulfonic acid (TNBS)-induced rat colitis model, following oral exposure (Lamprecht et al., 2001). This was observed qualitatively by using microscopy, and was quantitatively determined from fluorescence in tissue homogenates. The adhesion was size dependent, as 100 nm particles showed higher levels compared to particles 1000 and 10,000 nm in diameter. A significant extent of the adhesion was to mucus, as scraping the mucus from the excised inflamed tissue before analysis significantly reduced the total fluorescence readout. Polystyrene NPs similarly adhered to gastric ulcers induced by acetic acid in mice (Hassani et al., 2009).

Ex vivo exposure systems can also model the binding and uptake of NMs to the inflamed intestine. Colonic mucosa excised from a rat model of dinitrobenzene sulfonic acid (DNBS)-induced colitis demonstrated adhesion of liposomes to the inflamed regions (Jubeh et al., 2004). A similar adhesion and permeability experiment, using ex vivo tissue biopsies from human volunteers with ulcerative colitis, helped to solve a discrepancy between observations in animals and humans. Although in vivo data from various animal studies had shown selective accumulation of fluorescein labeled poly(ε-lactide-co-glycolide) NPs on inflamed regions of the GI tract, Schmidt et al. (2013) could not observe this phenomenon using confocal laser endoscopy on ulcerated rectal areas in human patients with Crohn’s disease and colitis. Thus, it was hypothesized that the NPs had been absorbed across the weakened epithelial barrier in the patients, rather than remaining adhered. An ex vivo Ussing chamber assay with resected human biopsies showed a high translocation rate of the NPs through the inflamed tissue, confirming the hypothesis. Additional analytical methods have been used to identify the chemical composition of NMs in human biopsies (Powell et al., 1996).

The favorable comparisons between the rat model of DNBS-induced colitis, the excised tissue from such animals and the excised inflamed human tissue suggests that rodents appear to be useful for assessing the impact of gastrointestinal inflammation of uptake of NM. Either more work is required to use animals with an extended array of NMs, or more work is required to compare the human, in vivo or ex vivo models with in vitro models to generate more useful and reliable alternatives to animal testing. Some such examples are provided below.

In vitro cell culture can model an inflammatory state of the GI tract epithelium. For example, Caco-2 or T84 cells can be treated with tumor necrosis factor-alpha and interferon-gamma, or infected with enteropathogenic Escherichia coli, to induce barrier dysfunction (Zolotarevsky et al., 2002). Mannose-grafted polymeric NPs, for example, showed high accumulation on a Caco-2 pro-inflammatory cytokine model (Coco et al., 2013). 3D in vitro cellular models of oral and intestinal inflammation may also prove useful (Frontela-Saseta et al. 2013; Lambros et al., 2011). One such model uses Caco-2 cells co-cultured with primary macrophages and dendritic cells derived from human blood, and then treated with interleukin-1-beta to induce inflammation (Leonard et al., 2010). A fraction of fluorescently labeled 190 nm liposomal NPs that deposited on the apical surface of these cells translocated to the basolateral side. The penetration was deeper and of a greater quantity in the inflamed model, and the signal was more diffuse, providing a qualitative measure of the breakdown of the liposomes in the tissue (Leonard et al., 2012). Models of inflammation are very relevant for drug development, but the necessity for the kinetic testing of food-relevant NMs requires further consideration.

Models of epithelial malignancies

In humans, several compartments of the GI tract can be prone to cancer growth. Carcinogenesis is a multistep progression through defined pre-cancerous intermediaries to a cancerous state. Advanced tumors manifest as morphological entities defined to a particular area, and are usually present in small numbers. Premalignant lesions, on the other hand, are usually present as a population covering a large area. They are morphologically well-defined including features such as premalignant lesions in the oral cavity, polyps in the intestine and aberrant crypt foci in the colon (Gupta et al., 2007; Izumo, 2011; Will et al., 2008). Not only can these lesions modify barrier function, but high-dose chemotherapeutic regimens can also modify the digestive and absorptive function of the GI tract (Rubenstein et al., 2004). Several in vivo rodent models can be induced to develop premalignant lesions and tumors of the buccal cavity, esophagus, stomach, small intestine and colon that are similar to those observed in humans and are amenable to study interactions with dietary components (Chen & Lin, 2010; Garman et al., 2012; Lai et al., 2010; Raju, 2008; Raju & Bird, 2003; Tanaka, 2012; Zeinelddin & Neufeld, 2013; Zhang & Moss, 2012). Such models could be useful for assessing not only the influence of cancer on NM uptake from the...
and agglomerates during the exposure time. Mathematical models such as the In Vitro Sedimentation, Diffusion and Dosimetry (ISDD) model can calculate the apparent density of the NMs in the medium and the dose to the cells (Hinderliter et al., 2010; Teeguarden et al., 2007; Watson et al., 2014). Further research is required to demonstrate whether modifying dose according to these methods alters the conclusions made about the relative toxicity of different nanomaterials. In addition, research is required to compare these models to data from animal or human studies. While the models are an excellent idea in principle, they have not yet been widely adopted because of this lack of information.

Following exposure of the selected model, distinguishing non-labeled lipid and biochemical macromolecule NMs from other biochemicals already present in biological fluids and tissues is challenging. Non-labeled metal NMs can be more easily distinguished if their atomic constituents are not endogenous to the cells. Due to such challenges, labels are often used for detection.

Fluorescence is a common labeling method for the detection of NMs. In vivo, fluorescence detection of administered NMs can include not only post-mortem analysis of tissues, but also non-invasive imaging. For example, multimodal multiphoton optical microscopy imaging in mice can qualitatively localize fluorescent NPs such as chitosan in organs (Garrett et al., 2012). X-ray computed tomography and NIR fluorescent imaging can also detect NPs such as fluorescent silica-coated gold nanorods (Luo et al., 2011). Metallic nanoshells can be visualized inside a tissue in vivo and ex vivo with a photoacoustic molecular probe and optical projection tomography (Rouleau et al., 2013), and near-infrared similarly detects the biodistribution of gold and zinc oxide NPs deep in tissues (Lee et al., 2012). With cultured cells, the amount of fluorescently coated iron oxide nanomaterials taken up has been detected by flow cytometry (Wang et al., 2012). As described in previous examples, confocal microscope imaging can also detect fluorescent NMs taken up into the cells, although the resolution is limited to 200 nm (Gaiser et al., 2012). Alternatively, total internal reflection fluorescence (TIRF) imaging can resolve fluorescent proteins and vesicles smaller than 10 nm at the cell surface (Rickman et al., 2007). However, microscopic methods are often qualitative and so more quantitative approaches are required. As described earlier, quantitative analysis of the fluorescent signal from NPs in homogenized tissues or cells can be quantified against a standard curve (Llabot et al., 2011).

Isotope labeling of NMs is less practical than fluorescence, but allows for a greater sensitivity of detection. In addition, isotope labeling can be coupled with high precision detection techniques such as multiple collector inductively coupled-plasma mass spectrometry, bioimaging by coherent anti Stokes Raman scattering, and electron microscopy with energy dispersive X-ray spectroscopy. For example, these techniques can be used to detect zinc oxide against a natural zinc background (Larner et al., 2012). Positron emission tomography and neutron activation can detect a wide variety of radionabeled materials in vivo, ex vivo and in vitro (Gibson et al., 2011). Well-established specialized methodologies using radiolabeled materials have been more reluctantly used in recent years, but scientific questions should be answered using the best suited and available technologies.

As described earlier, the elements making up NMs can be detected as an indication of absorption. With tissue biopsies or cells, autometallography enhanced microscopy or elemental analysis of homogenized tissues can allow accurate quantification of metal NPs (Loeschner et al., 2011). However, if extensive homogenization and furnace preparation of the samples is performed, analyses that only detect the elements present will not indicate whether the NPs remained intact. Single particle inductively coupled-plasma mass spectrometry,
in combination with hydrodynamic chromatography or field flow fractionation separation allows quantitative determination of intact metal NPs in biological media. The lower particle size limit is approximately 10–20 nm, depending on the chemical composition (van der Zande et al., 2012; Walczak et al., 2013). With intact cellular sections, transmission electron microscopy – electron energy loss spectroscopy is also capable of identifying the chemical composition of individual NPs for many compounds. Refinement of these imaging and analytical detection techniques, and correspondingly of GI models, is an active area or research.

Before delivery of a NM to a model, sterilization may be required to reduce confounding effects from microbial and endotoxin contamination; particularly when using an in vitro cellular system (Neun & Dobrovolskaia, 2011). Irradiation or heat treatment can be used before administration, although the impact on physico-chemical properties would need to be assessed. Microbial contaminants in the NM product might modify gastrointestinal behavior and fate, and thus might be important to remain present in some systems.

Exposure conditions must be described in detail and should reflect those that would be encountered in humans as closely as possible (Bouwmeester et al., 2009; OECD, 2012). As mentioned above, the exposure vehicle may contain solutes, nutrients and/or serum to improve NM dispersibility, provide physiologically relevant conditions and/or maintain cell health. A vehicle control treatment should include all components except the NMs (Figure 1).

Some NMs have the capacity to bind non-specifically to the glass, plastic, collagen or other matrices that make up administration equipment and culture apparatuses, interfering with the dosing (Shahbazi & Santos, 2013). Mass balance calculation in the fluids following administration to the apparatus alone can detect this phenomenon. When the cells are present, quantification of the sum of the mass in the donor compartment, receiver compartment and the cells can also ensure that changes in concentration are not due to artifactual removal.

If a fraction of the NPs dissolves to ions, the chosen biological model should be tested by exposure to ions alone for comparison (Figure 1). If available, larger bulk particles of the same material are also useful for comparison.

When NMs are detected with an incorporated label, for example, with a fluorescent molecule or a radioisotope, a level of uncertainty is introduced since the label could dissociate and be absorbed alone (Yuan et al., 2007). As mentioned, this can be controlled for by testing the degree of dissociation of the label from the NM in the exposure media or in synthetic gastrointestinal fluids, and by the administration of the label alone to a control group (Figure 1).

For in vivo and in vitro toxico-kinetics studies, it is ideal if a range of NM concentrations is administered at known or anticipated human exposure levels. These realistic exposure doses can also allow coinciding, preliminary nutrition and toxicology analysis (Johnston et al., 2013; Teeguarden et al., 2007). Toxicity could be a confounding factor if it changes the permeability of the GI epithelium. Generalized changes in permeability can be quantified with compounds that are known to normally be excluded by the epithelium. For in vitro dual chamber systems, the trans-epithelial electrical resistance (TEER) value also provides a measure of cell monolayer integrity. Loss of TEER can indicate either cell death or the opening of tight junctions between the cells. For example, chitosan polymers transiently enhanced the paracellular absorption of co-administered small molecules (Sonaje et al., 2012).

All models use assumptions, and no method is perfectly accurate or precise. Thus, uncertainty factors are often applied to data when used in risk assessment. For example, an interspecies uncertainty factor is used to account for the level of uncertainty when extrapolating data obtained in animals to the human situation (Cao et al., 2006). An inter-individual uncertainty factor is also applied (Canady, 2010; Huizer et al., 2012). The standardization of methods to analytically quantify NM fate in GI fluids and tissues will reduce methodological uncertainties in the risk assessment of these chemicals.

Figure 1. Control treatments for in vivo, ex vivo and in vitro nanomaterial digestion and absorption assays. (A) Background levels of the nanomaterial in the model should be measured in a control well receiving the vehicle components. (B) The controls are compared with the nanomaterial treatment group. (C) If a fraction of the nanomaterial surface dissolved in simulated gastrointestinal fluids, that concentration of ions can be administered alone, to determine if the ions are responsible for any absorption. (D) If the label dissociated from the nanomaterials in simulated gastrointestinal fluids, that concentration of label can be administered alone, to control for false positives from its absorption.
**Conclusions and future research**

Research is accelerating on the application of human-relevant GI tract models to quantify the bioaccessibility, digestion, mucoadhesion, bioavailability and excretion of food-relevant engineered NMs. By integrating the examples provided in this review, we have summarized the current state of the science on models that could be relevant for these quantifications (Figure 2).

There likely will not be a single method that can address all necessary questions, albeit some models are better than others in addressing specific variables. Thus, a combination or battery of models may be required depending on the data gaps addressed. An example approach, for assessing NM uptake in the gastrointestinal tract was proposed earlier in the review. This included that for the testing of novel NMs, a tiered strategy with a thorough physical and chemical characterization in the matrix as dosed, taking into account NM changes over time, followed by alternative testing strategies (*in vitro* or *in silico*), would prioritize materials for further testing *in vivo*. This strategy is not necessarily novel or ground breaking, but instead follows the procedure used for other substances.

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**Figure 2.** Categories of gastrointestinal tract models that demonstrate potential utility for the assessment of the digestion and absorption of engineered nanomaterials released from food. Specific examples are provided of models that have been used, or could be further developed, to quantify digestion and absorption parameters relevant to nanomaterials. The general parameters that the models could measure are listed.
Before such an approach can be taken forward, considerable work is required to develop, validate and standardize such models for use with NMs. In discussing these issues, the previous sections have highlighted some of the gaps in knowledge from this tiered strategic approach, and how they might be addressed. In order to make a useful reference tool to help structure future research priorities in this area, we have generated a table to summarize the identified gaps and the approaches that could be used to address these gaps (Table 6). Additional factors to consider in the selection of models for further development include ease of use, the wide availability of required instrumentation, ethical issues and cost-effectiveness.

In addition to the need to address gaps in, the models available for assessing NM uptake there are also methodological considerations for nanomaterial detection and characterization that need to be addressed. Research is required to further validate and interpret the results from these models.

**Table 6. Gaps and proposed research strategies for models of the digestion and absorption of engineered nanomaterials in the gastrointestinal tract.**

| Identified gap                                                                 | Approaches to address gaps                                                                 |
|--------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|
| **Overarching strategy required to assess uptake of NMs via the gastrointestinal tract** | • A tiered strategy: (i) thorough physical and chemical characterization in the matrix as dosed, taking into account NM changes over time, (ii) high-throughput toxicokinetic approaches such as human-relevant *in vitro* or *in silico* systems, (iii) further testing *in vivo*. |
| **Require validated and standardized models and analytical tools for the detection of NMs in food, complex media, chyme and feces, as well as within cells and tissues of the GI tract** | • Assess reliability of analytical digestion and detection for routine use (e.g. Szakal et al., 2014b).  
• Radiolabeled materials have been used more reluctantly used in recent years, but these may need to be applied to answer scientific uptake questions. |
| **Require validation and standardization of GI models - general comments** | • Adapt test models used for conventional drugs. |
| **Require assessment of the appropriateness of *in vivo* models** | • Assess and modify if necessary the current OECD 2010 guidelines for toxicokinetics studies.  
• Assess relevance of administration via gavage versus introduction into food or water.  
• Either confirm relevance of existing techniques (e.g. neutron activation, radioactivity or fluorescent tags) or develop improved (or simpler) approaches to better quantify uptake of NM from the gastrointestinal tract into blood over time, and their subsequent biodistribution.  
• Extend existing and newly improved techniques to a wider array of NMs with different physicochemical properties.  
• Determine differences between humans and rodent models in terms of uptake and how parameters (e.g. food intake) modify uptake.  
• Utilize *in situ* perfusion models in live, anesthetized subjects to assess uptake. |
| **Need to understand factors affecting mucus penetration by NMs** | • Assess physicochemical characteristics of NM that influence their interaction with mucus. |
| **Require improved alternative models - cell based systems** | • Develop and utilize models to better represent the diversity of cell types in the gastrointestinal mucosa.  
• Incorporate permeable synthetic membranes into 3D human tissue models.  
• Determine the relationship between NM concentration in the media, internal dose and toxicity *in vitro* and relate this to the *in vivo* uptake.  
• Evaluate *in vitro* models compared to *ex vivo* tissue and animal models, followed by standardization of these novel models to clarify their applicability and accuracy for the prediction of particle uptake rate in humans.  
• Combine the data from more than one *in vitro* assay to address limitations of simple *in vitro* systems.  
• Simultaneously validate analytical tools with human-relevant *in vitro* or *in silico* methods.  
• Assess the effects of NM agglomeration and sedimentation on *ex vivo* models such as the gut sac.  
• NMs administered to test models can be either pristine particles, useful for mechanistic studies, or manipulated to more realistically reflecting the range of particles that might be found in food products. |
| **Simple cell culture systems using established cell lines do not incorporate factors such as age and sex.** | • Cell lines with different genotypes could be developed, e.g. via induced pluripotent stem cells. |
| **Simple cell culture systems using established cell lines lack systemic circulation pathways such as the enterohepatic secretion.** | • Develop and exploit multi-tissue fluidic model that allows flow. |
| **Standard culture media does not contain GI lumen factors** | • More physiologically relevant culture fluids can be used to reflect the fasted and fed states. |
| **Agglomeration and settling vary according to the physicochemical properties of NMs and this then influences the dose rate to cells.** | • Mathematical models such as the *In Vitro* Sedimentation, Diffusion and Dosimetry (ISDD) model can calculate the apparent density of the NMs in the medium and the dose to the cells (Hinderliter et al., 2010; Teeguarden et al., 2007; Watson et al., 2014).  
• Further research is required to demonstrate whether modifying dose according to the ISDD model alters the conclusions made about the relative toxicity of different NMs.  
• Research is required to compare the ISDD model to data from animal or human studies.  
• Research is required to assess whether mass is the best metric for dose, or whether other options (e.g. surface area or particle number) might be more useful. |
| **Require improved alternative models – non-cellular fluid models** | • Use fluid models that simulate the human GI tract to evaluate certain aspects of NM digestion including bioaccessibility, stability, dissolution and aggregation.  
• Static *in vitro* digestion assays using simulated gastric fluids could be used to distinguish a variety of the possible outcomes for NMs.  
• Dynamic gastric simulators which recreate physical mixing and emptying conditions at work in the human can be applied to determine their influence on the retention, release, and breakdown of food particles and NM, but requires validation for evaluation of the behavior of NMs in food. |

(continued)
| Identified gap                                                                 | Approaches to address gaps                                                                                   |
|-----------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|
| Many non-cellular fluid models do not accurately mimic the complex physical  | • NM administered to test models can be either pristine particles, useful for mechanistic studies, or          |
| forces that are an important factor in the disintegration of food particles   | manipulated to more realistically reflecting the range of particles that might be found in food products.       |
| in vivo                                                                     | • Chewing and swallowing forces encountered in vivo can be modeled using devices with mobile parts.          |
| In vitro fluid systems that mimic the lumen of the buccal cavity             | • Use several fluids with different osmotic, amylase and pH values.                                        |
|                                                                             | • Research is required to standardize in vitro fluid system models that mimic the lumen of the buccal cavity   |
|                                                                             | for the application of NMs.                                                                                 |
| Impacts of NM on microflora                                                 | • In vivo assess diversity of microorganisms in faeces over time after NM exposure.                         |
|                                                                             | • In vitro assess impact on commensal and pathogenic microorganisms.                                       |
|                                                                             | • Develop and validate in vitro models of epithelial and microorganism co-culture to investigate how such   |
|                                                                             | interactions influence NM uptake.                                                                            |
| Modelling of uptake                                                         | • Mathematical approaches can be used to approximately equate uptake values to human in situ transcellular    |
|                                                                             | permeability.                                                                                               |
| There are a lack of disease models relevant for studying inter-individual   | • Characterize not only permeability, but also digestion in GI fluids.                                       |
| variability in NM uptake from the gastrointestinal tract                    | • Development of models of specific disease states (e.g. inflammatory bowel disease, Crohn’s disease and    |
|                                                                             | cancer). Examples of such models include:                                                                     |
|                                                                             | ○ In vivo rodent models induced to develop premalignant lesions and tumors of the buccal cavity, esophagus,  |
|                                                                             | stomach, small intestine and colon.                                                                           |
|                                                                             | ○ The rat model of dinitrobenzene sulphonate acid-induced colitis.                                            |
|                                                                             | ○ Development of models that reflect differences in human digestion and permeability associated with factors  |
|                                                                             | such as age and pregnancy.                                                                                    |
|                                                                             | ○ Include alternative approaches to represent different physiological and diseases states Examples of such   |
|                                                                             | models include:                                                                                              |
|                                                                             | ○ The in vitro culture of neoplastic buccal cavity keratinocytes (Costea et al., 2005).                      |
|                                                                             | ○ Caco-2 or T84 cells treated with tumor necrosis factor-alpha and interferon-gamma, or infected with        |
|                                                                             | enteropathogenic Escherichia coli, to induce barrier dysfunction (Zolotarevsky et al., 2002).               |
|                                                                             | ○ Caco-2 cells co-cultured with primary macrophages and dendritic cells derived from human blood, and then    |
|                                                                             | treated with interleukin 1-beta to induce inflammation (Leonard et al., 2010).                              |
|                                                                             | ○ Use of ex vivo tissue biopsies from human volunteers with ulcerative colitis.                              |
|                                                                             | ○ Such models would not only identify how disease influences uptake, but could provide beneficial targeting of |
|                                                                             | ingested pharmaceutical or imaging NMs to gastrointestinal disease sites.                                      |

standardize models and analytical tools for the detection of NMs in food, complex media, chyme and feces, as well as within cells and tissues of the GI tract. These future research areas are being studied by the ILSI NanoRelease Food Additive project (Szakal et al., 2014a).

Such studies will require more work to ensure the reliability of a variety of techniques including analytical digestion and detection. Therefore, while a range of techniques exist to detect NMs in complex matrices and biological specimens, robust and cost effective protocols for routine use are still required. This element of detection, which is essential for estimating exposure, remains a major bottleneck in many risk assessment studies (Stone et al., 2014).

The standardization of GI models and NM detection methods can go hand-in-hand, and method suitability evaluation could include interlaboratory reproducibility experiments with standard food-relevant compounds. A suitability approach could be adapted from those used to test models with conventional drugs (Volpe, 2010). Analytical research needs that have been identified by the NanoRelease Food Additive project include methods to sample, separate, detect and measure inorganic and organic NMs at key time points in the food lifecycle, including during engineering, food preparation, packaging, consumption and GI tract transit (Szakal et al., 2014a).

The final decision regarding what is needed to assess NM uptake for risk assessment may also be influenced by local regulatory requirements. Different jurisdictions may or may not require data on these parameters as part of a safety assessment for the use of novel engineered NMs in food or food contact materials. The categories of models that can be used for such testing varies at the discretion of each jurisdiction.

The development of methodologies for the detection of engineered NMs during digestion and absorption will enhance the capacity to assess the fate of these products in the body, and will facilitate their safe and sustainable development in food applications. Finally, the results from any approach should be interpreted with caution in the risk assessment of NMs proposed for human consumption until there is a body of knowledge confirming the general relevance of the model to humans.

Overall, several categories of in vitro models are used for the assessment of conventional food and drug chemicals and, when standardized, have the potential to show good in vitro to in vivo correlation. There is a need to further adapt and standardize the available models and the corresponding analytical methods to allow quantification of the digestive fate of engineered NMs including uptake across the gastrointestinal barrier.

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