THE ROLE OF *in vitro* SIMULATED HUMAN DIGESTION MODELS IN ASSESSING POTENTIAL EXPOSURE TO NANOPARTICLES IN FOOD: AN ESSENTIAL TOOL FOR RISK ASSESSMENT OF NANOMATERIALS USED IN FOOD-RELATED APPLICATIONS

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Abstract–In the food sector, applications of nanotechnologies to (i) agricultural production, (ii) food processing, and (iii) food contact materials are rapidly developing. An appropriate nano-specific risk assessment has to be carried out for nanotechnology applications that result in the presence of nanoparticles in food. One essential issue in such an assessment is to ascertain whether there is potential for systemic exposure, *i.e.*, if nanoparticles may persist as such after gastrointestinal digestion and be absorbed in the gut. The importance of studying the dissolution of nanoparticles under conditions representative of the gastrointestinal tract is discussed, along with the *in vitro* models and the analytical methods that can be used for such an undertaking.

Keywords: *Nanotechnology, Nanomaterials, Risk Assessment, Human Dietary Exposure, Human Digestion Models.*

1. INTRODUCTION

The word 'nano' refers to a billionth of a metre (10^{-9} m) and nanotechnology can be understood as the application of scientific knowledge to manipulate and control matter in the size range <100 nm, *e.g.*, in the 'nanoscale'.

While nanoparticles, or nanomaterials consisting of such particles, are generally accepted as those with a size below 100 nm[1], this size limit is fairly arbitrary.

In particular, size-dependent properties and biological effects that are of potential concern for human health (e.g. the toxicokinetic behaviour, particle-cell interactions) are not rigidly related to specific size thresholds. They depend on exposure levels (i.e. dose) and normally occur even when the particles constituting the nanomaterial have a size above 100 nm. Furthermore, whereas physical, chemical and biological properties of materials may change with size, there is no scientific justification for a single size limit associated with these changes that can be applied to all nanomaterials [1].

Nanomaterials exhibit unique size- and structure-dependent properties distinct from those associated with larger sizes of the same material. This is mainly because of their small size and consequently their much larger surface to mass ratio as compared to larger sized materials. Nanotechnologies enable management of characteristics such as material size and morphology for the improvement or development of new process and product properties. In the food sector, three main categories of products/applications of nanotechnologies and nanomaterials can be identified, namely agricultural production (e.g. nano-formulated agrochemicals and animal feeds), food processing (nano-sized ingredients, nutritional supplements, additives), and food contact materials.

Although making materials smaller can generate novel and useful properties, concerns exist on potential risks related to the interactions of nano-sized materials with cellular components, which may ultimately harm human health. The unique physicochemical properties of engineered

1In the Recommendation on the definition of nanomaterial of the European Commission (2011/696/ EU) - currently under revision - a nanomaterial is a material with ≥50% of the particles in the number-based size distribution having one or more external dimensions in the size range 1 nm-100 nm. In specific cases derogations apply to the 50% threshold.

nanomaterials may influence the toxicological properties, first of all the toxicokinetic behaviour, and an appropriate nano-specific risk assessment has to be carried out for nanotechnology applications that result in the presence of nanoparticles in food. One essential issue in such an assessment is to ascertain whether there is potential for systemic exposure, *i.e.*, if nanoparticles may persist as such after gastrointestinal digestion and may be absorbed in the gut. Nanomaterials that quickly dissolve/degrade in the gastrointestinal tract do not give rise to nano-specific concerns since in the absence of exposure no risk is expected. The importance of studying the dissolution of nanoparticles under conditions representative of the gastrointestinal tract is discussed here under, along with the *in vitro models* and the analytical methods that can be used for such an undertaking.

2. *IN VITRO* MODELS FOR SIMULATED GASTROINTESTINAL DIGESTION

In vitro methods simulating human digestion processes are widely used to study the gastrointestinal behaviour of food (or pharmaceuticals) and specific substances therein.

Although human nutritional studies are still being considered the gold standard for addressing diet-related questions - in particular related to bioavailability - *in vitro* methods have the advantage of being more rapid, less expensive, less labour intensive, and do not have ethical restrictions. They also allow a relatively large number of samples to be measured in parallel for screening purposes.

Simulated digestion models typically include the oral, gastric and small intestinal phases, and only occasionally the large intestinal fermentation. These models try to mimic physiological conditions *in vivo*, taking into account the presence of digestive enzymes and their concentrations, pH, digestion time, and salt concentrations, among other factors. A typical scheme of a simulated *in vitro* digestion method is shown in Table 1. Solid foods are minced before the oral phase, whereas liquid foods can be directly exposed to the gastric phase if they do not contain starch (Table 1).

According to the physiology that is simulated (e.g. fasted vs. fed state) the conditions used may vary between models.

Static models of human digestion are relatively simple to be implemented and have been used to investigate the bioaccessibility of food components (e.g. nutrients or xenobiotics), i.e. to assess the amount of a compound that is released from the food matrix and becomes available for absorption through the gut wall [2,3]. Recently a static *in vitro* digestion method for food has been harmonised by an international network with the aim to aid the production of more comparable data in the future [4].

Table 1.A typical scheme of a simulated *in vitro* digestion method. SSF, SGF and SIF are Simulated Salivary Fluid, Simulated Gastric Fluid and Simulated Intestinal Fluid, respectively.

Digestion phase	**Short description**
Oral	Mix food 1:1 with SSF and salivary amylase. Duration 2 min, pH 7
Gastric	Mix oral bolus 1:1 with SGF and pepsin. Duration 2 h, pH 3
Intestinal	Mix gastric chyme 1:1 with SIF, enzymes and bile. Duration 2 h, ph 7. The enzymes are trypsin, chymotrypsin, pancreatic amylase, pancreatic lipase, and pancreatic colipase.

3. APPLICATION OF *IN VITRO* DIGESTION MODELS TO NANOMATERIALS

Most nanomaterials are not readily soluble/degradable and when dispersed in water or another liquid medium tend to maintain their particulate nature, i.e. form a suspension (and not a solution). However some of them (e.g. ZnO and Ag nanoparticles) have a certain solubility and might be expected to be at least partially dissolved or degraded after gastrointestinal digestion. If such dissolution is fast and complete, than the intestinal

epithelium of the host does not come in contact with nanoparticles meaning that absorption and internal exposure is zero.

In the case of a nanomaterial used in a food-related application, this implies that standard risk assessment - i.e. targeting the conventional (non-nano) chemicals resulting from nanoparticle dissolution - has to be carried out.

In general, fast and complete dissolution of a nanomaterial in conditions representative of the human gastrointestinal tract can be expected to be rare, which implies that a nano-specific risk assessment considering the peculiar nature of the (nano-sized) agent to be assessed has to be carried out. Such an assessment will focus on size-related properties associated with specific hazards. In this case, *in vitro* digestion methods are useful to give an insight about the possible transformations of the nanomaterial during human digestion, e.g. highlight if there is a tendency to agglomeration (i.e. primary particles interact to form larger particles) or on the contrary to formation of smaller particles. In general, smaller particles are more readily absorbed in the intestine compared to larger ones [5].

Even though *in vitro* digestion models for nanomaterials are of essential importance for the reasons stated above, there is a lack of validation and standardisation of these methods and comparisons of dissolution data from such models and *in vivo* data do not exist for nanomaterials. Lefebvre et al. concluded that *in vitro* digestion models are generally applicable for nanomaterials, as the basis of the models is mimicking the conditions of the gastrointestinal tract [6]. To date, these models have been applied to study the fate of SiO_2 and Ag particles in the human gastrointestinal tract [7,8]. For synthetic amorphous silica (SAS), it was shown that in the gastric digestion stage nano-sized particles disappear most likely due to the low gastric pH combined with high electrolyte concentrations, which lead to formation of large silica agglomerates [7]. However, in the subsequent intestinal stage the nano-sized SiO_2 particles reappeared again suggesting that, upon consumption of foods containing SAS, the gut epithelium is most likely exposed to nano-silica [7]. This study highlights that use of the gastric step alone is not sufficient to investigate the bioaccessibility of nanomaterials. A similar result was obtained in the case of Ag particles [8]. In our laboratory *in vitro* simulated human digestion models are presently being applied to assess the bioaccessible nanofraction of nutrient sources (ZnO) and food additives (SiO_2, TiO_2, iron oxides) currently used in a number of food and supplements.

In order to be able to assess the dissolution and particle size of nanomaterials after simulated digestion, state-of-the-art analytical techniques have to be used. Electron microscopy (EM) is the technique of choice as it enables imaging of particles, the study of their morphology, and elemental analysis when combined to Energy Dispersive Spectroscopy, even though quantitative data can be difficult to be obtained. For inorganic nanoparticles, single particle-ICP-MS and ICP-MS in combination with a fractionation technique like ultrafiltration, hydrodynamic chromatography (HDC) and field flow fractionation (FFF) can be used. FFF has the advantage of the possible combination with multiple detectors in addition to ICP-MS, such as UV, dynamic light scattering (DLS) and multiple angle light scattering (MALS). On the other hand, speed and sensitivity are the major advantages of single particle-ICP-MS; another advantage is that it determines number-based size distributions of the particles.

The situation is less favourable when organic nanoparticles composed of polymers, lipids, proteins and polysaccharides are dealt with. Imaging techniques are limited and EM is generally only useful if staining techniques are applied. Separation techniques as HDC and FFF can be used (care has to be taken to not disrupt the micelle-like structures), with UV detection for quantification and matrix assisted laser desorption ionization (MALDI) in combination with MS techniques for chemical characterization.

Independent of the chemical features of the particles (inorganic vs. organic), measurements are made complex by the nature of the matrix (the mouth, gastric and intestinal hydrolysates). Much remains to be done in order to develop fit-for-

purpose analytical methods, specific for each nanomaterial and with a sufficient detection power (i.e. acceptable limits of detection for both size and concentration). Multi-technique approaches, as it often happens with nanomaterials, appear to be the way forward for a successful characterization.

4. CONCLUSIONS

Nanotechnology applications in the food sector may bring benefits, e.g. lead to the production of improved nutrient sources (due to higher bioavailability or less severe side-effects upon ingestion). However potential risks have to be assessed and excluded. For applications that result in the presence of nanoparticles in food, the first issue to be considered is whether there is potential for systemic exposure to such particles. If nanoparticles persist as such after gastrointestinal digestion, they may be absorbed in the gut and a nano-specific risk assessment is required. Nanomaterials that quickly dissolve/degrade in the gastrointestinal tract do not give rise to nano-specific concerns since in the absence of exposure no risk is expected. The transformations and potential dissolution of nanoparticles under conditions representative of the gastrointestinal tract can be studied by means of *in vitro* methods simulating human digestion. Their use, along with application of fit-for-purpose, state-of-the-art analytical methods enabling the physicochemical characterization of the particles after the digestion process – and especially the assessment of their size distribution and agglomeration state – is a keystone of risk assessment of nanotechnology applications in the agri-food sector. Validation and standardisation of these methods for nanomaterials is an urgent research need.

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