16 Nanomaterials in Foods and Human Digestion: An Important Layer in the Assessment of Potential Toxic Effects

Carla Martins, Paula Alvito, and Ricardo Assunção

Abstract

The use of nanoparticles (e.g. titanium dioxide) in commercial food products to modify some properties, such as brightness and whiteness, increased in the last years and is nowadays widespread. Despite the inhalation of nanoparticles is already a topic of concern, the potential adverse health effects due to ingestion still present gaps of knowledge. In fact, gastrointestinal tract is the first interface between the body and the external environment and consequently could represent a target organ for compounds present in food, namely nanoparticles, that could exert toxic effects. The *in vitro* digestion models used to simulate the human digestion may contribute to fill these gaps. The applicability of the *in vitro* digestion methods is discussed concerning its potential use as a tool for addressing the toxicity of ingested nanomaterials or other food contaminants, mimicking the physiological processes.

Keywords

In vitro digestion · Digestion models · Ingested nanomaterials · Human digestion simulation · Gastrointestinal system

C. Martins (✉)
NOVA National School of Public Health, Public Health Research Center, Universidade NOVA de Lisboa, Lisbon, Portugal

Food and Nutrition Department, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal

CESAM, Centre for Environmental and Marine Studies, University of Aveiro, Aveiro, Portugal

Comprehensive Health Research Center (CHRC), Universidade NOVA de Lisboa, Lisbon, Portugal
e-mail: carla.martins@insa.min-saude.pt

P. Alvito
Food and Nutrition Department, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal

CESAM, Centre for Environmental and Marine Studies, University of Aveiro, Aveiro, Portugal

R. Assunção
Food and Nutrition Department, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal

CESAM, Centre for Environmental and Marine Studies, University of Aveiro, Aveiro, Portugal

Comprehensive Health Research Center (CHRC), Universidade NOVA de Lisboa, Lisbon, Portugal

16.1 General Introduction

Several challenges are posed nowadays through the development of novel foods and their by-products. The current food production system is making efforts to promote shifts to more sustainable products, guaranteeing simultaneously the food safety and nutritional quality. The inclusion of nanomaterials (NMs) in foods is one of these challenges. The increased shelf-life, flavor release and absorption of nutrients and other bio-

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active components have been referred as some of the beneficial effects of the use of NMs in foods [48]. As more NMs are being included in food production systems, a need to understand the potential risks and benefits of their use for consumers' health is essential. The knowledge of NMs' fate along the gastrointestinal system with the use of *in vitro* digestion models is a major contribution for this assessment.

16.2 Inclusion of Nanomaterials in Foods

According to the Novel Food Regulation (EU) 2015/2283 and referring to Regulation (EU) 1169/2011 on the Provision of Food Information to Consumers, the term ENM (Engineered Nanomaterials) means "any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale" [16, 18, 19].

Dietary intake of ENM can be an important pathway of human exposure to nanoparticles (NPs) and food additives, as other forms deliberately added in food industry, are considered the primary source of ingested exposure. ENM intake by food is not yet extensively characterized in developed countries; however, it is estimated to be considerable, as revealed by a study that reported an ingestion uptake estimate of ~10^{12} nanoparticles/person per day, consisting mainly of titanium dioxide (TiO_2), colloidal silica, and mixed silicates [7, 47]. The estimation of human daily intake of NPs has been a subject of interest in the last years. Rompelberg et al. [40] estimated the exposure of the Dutch population to TiO_2 NP through oral intake of several products (food, supplements and toothpaste). The median estimates of exposure were 1.90 μg/kg bw/day, 0.26 μg/kg bw/day and 0.10 μg/kg bw/day for the age groups of 2–6 years old, 7–69 years old and ≥ 70 years old, respectively. Also Yin et al. [55] estimated that the mean daily intake of TiO_2 particles (including nanosized particles) from seafood and surimi products ranged from 0.02 to 3.09 μg/kg bw/day, with individuals aged 20–30 years old showing the highest exposure levels.

The progress observed during the last years in nanotechnology contributed for the increasing interest of its application in the food industry [43]. The use of nanotechnologies can be considered in every phase of food production, including the food processing and food preservation. Several applications in food industry have been reported in various dimensions such as the characteristics of foods (texture, taste, color, strength), the food additives encapsulation, the new flavors and sensations, the control of aroma release, and the stability/shelf-life of products [10]. NPs can be organic (lipid and protein NPs), inorganic (silver, titanium dioxide, or zinc oxide), or including carbon-based NPs (carbon nanofibers and carbon nanotubes) [12]. NPs can enhance some characteristics of foods such as texture or color and can also be incorporated in food packaging. Another important contribute to this area is the possibility of incorporating nanomaterials (e.g. titanium dioxide, silver nanoparticles, carbon nanotubes) that allow to mitigate food losses due to different microbial infections. [46, 52]. Additionally, the development of nanoprobes for detection of chemical (mycotoxins, pesticides, antibiotics, plasticizers, melamine) and microbiological contaminants (food spoilage) are nowadays established areas of research as pointed out by [39]. Nutraceuticals and functional antimicrobial ingredients used as encapsulated play a crucial role in the preservation and bioavailability of bioactive ingredients, food processing and storage, and also in the transport through the gastrointestinal tract. Nanoemulsions are used in flavored oils, salad dressing, personalized beverages, sweeteners, and other processed foods, to create lipid-soluble compounds that are bioactive for targeted delivery of lutein, β-carotene, vitamins A and D, and omega-3-fatty acids [38, 43].

The progress observed in nanotechnology has contributed to the transformation of food science and the food industry with increased investment and market share. As briefly explained, the broad applications of nanotechnology will contribute to a new digital improvements namely in the domains of food reliability, food safety, and shelf-life performance [26, 43]. However, there is a consensus regarding the challenges ahead in this field. The interactions of nanomaterials with the food systems need to be further estimated, thus contributing for harmonized actions at a global scale in a combined effort of food regulators, authorities, and industry [21, 26, 37, 43].

16.3 The Importance of Human Digestion for the Toxicity Assessment of Nanomaterials

Considering that ingestion is a route of human exposure to NMs, it is of utmost importance to assess the possible influence of digestion on these particles. The physiological response to specific nanomaterials in foods is understood in full when framed by the human digestive processes in more detail [4].

16.3.1 The Fate of Nanomaterials During Human Digestion

During digestion, the chemical environments are modified within the three main compartments (mouth, stomach and intestine) regarding pH, enzymes, and inorganic compounds. The transit through the gastrointestinal (GI) system may lead to several modification in the NP including dissolution, agglomeration and deagglomeration; all these may affect the intestinal absorption that is different if in presence of dissolved ions or nanoparticles, and depends on their size, shape and physicochemical properties [44].

The first step of digestion happens in the mouth and involves the mixture of food with salivary fluid, containing about 99.5% water with 0.3% of electrolytes and proteins, including amylase [35]. Salivary pH values vary between fasted to fed state, from 6.2–7.4 to 7.4–7.6, respectively [50]. The transient time in mouth compartment is short. Nevertheless, there may be an impact in some types of NMs such as silver NPs for which the aggregation of 52% of nanoparticles was reported [25] and for carbohydrate NPs that may be digested by amylase [33].

After bolus formation in the oral phase, it is processed in the stomach to a semi-solid chime, by action of the gastric juice constituted by hydrochloric acid, various electrolytes, enzymes (pepsin and gastric lipase), intrinsic factor, mucus, and hormones [27]. Stomach pH vary during digestion from 1.5 to 2.0 in the fasted state to 3.0–7.0 in the fed state. The key gastric proteolytic enzyme (pepsin) and gastric lipase are activated via acid hydrolysis [42]. The physic-chemical properties of gastric environment, mainly high strong acid conditions and high ionic strength, promote modifications in NMs. For lipid NPs, the aggregation status is modified due to changes in their surface properties, such as surface charge and steric coating. The triglycerides, common components of lipid NPs, start to digest when there is enough secretion of gastric lipase in the stomach [54]. Studies in gastric fluid showed that TiO2 NPs tended to agglomerate in the presence of gastric fluid with an effect more apparent and significant in the nanoparticle range [24]. Similar findings were reported for Ag NPs that in gastric fluid agglomerated by forming clusters with proteins [28], a process enhanced by the presence of pepsin [3].

The small intestine, where the highest percentage of chemical breakdown and absorption occur by secretions of the liver, gall bladder, pancreas, and intestinal epithelia, receives the gastric chime that is neutralized by bicarbonate raising the pH from 2 to 6.2. The degradation of food starts in the duodenum that receives about 1.2–1.5 L/day of pancreatic juice [23]. Simultaneously and gradually over the course of 3–4 h (depending of the meal ingested), the pancreatic juice, composed by a mixture of enzymes, proenzymes, protease inhibitors, sodium bicarbonate and other electrolytes, is secreted. The characteristics of NMs

when reaching the intestine encompass the influence of the different gastrointestinal tract environments, are determinant for their ability to absorption by intestinal epithelium and accordingly to the potential toxicity [33]. Small molecules are mainly absorbed at the small intestine passively through diffusion or actively through several transporter systems in the gut wall [23]. Although NPs suffer a process of agglomeration during the gastric phase, in the presence of the small intestine environment, characterized by a basic pH, presence of bile and pancreatic enzymes, the clusters are disintegrated [28]. Similar observations were reported for Ag NPs that retained their original size in intestinal fluid [53].

These findings are of utmost importance since it is known that the toxicity of a NM is the outcome of its intrinsic physicochemical properties such as size, shape, surface properties, and chemical composition, that will influence their cellular uptake [41]. Regarding the availability in the intestine and the absorption of NMs, it is crucial to understand properly the definitions of bioavailability and bioaccessibility (Fig. 16.1). Bioavailability is defined as the part of ingested compound that reaches the systematic circulation and is ultimately utilized [51] and oral bioavailability is resultant of three processes including: i) bioaccessibility (release of the compound from its matrix into digestive juice in the gastrointestinal tract); ii) intestinal transport, across the intestinal epithelium into the vena Portae; and iii) metabolism (degradation of the compound in the liver and intestine) [51]. Oral bioavailability includes bioaccessibility, which is defined as the quantity of a compound that is released from its matrix in the gastrointestinal tract, becoming available for absorption. Digestion is the chemical disintegration of food particles into absorbable molecules and, absorption refers to the transport of nutrients, water and electrolytes from the lumen of the small intestine into the cell, and then into the blood [51].

16.3.2 In Vitro Digestion Models

Several gastrointestinal models have been developed to better understand the effects of human digestion of nutrients, contaminants, additives, and other food components, as nanomaterials. As above mentioned, the gastrointestinal tract is a complex system with several physical and biochemical processes (*i.e.* hormonal response, gastric emptying, enzymes and fluids secretion, motility) that are dependent on the individual physiology and on the food consumed [36]. Although studies developed in humans are considered the "gold standard" for addressing diet related issues, *in vitro* methods have many advantages namely being more rapid, less expensive, and not presenting ethical constrictions. These characteristics make possible the analysis of several with a higher degree of standardization, reproducibility and in controlled conditions [9, 35].

These models may differ between each other regarding many parameters: the number of compartments and number of phases considered, the digestion fluids composition, the source of enzymes, the ratio between food and enzymes or digestive fluids, and the compartment staying time. The models may comprise a dynamic variation of these parameters along the digestion simulation, a semi-dynamic variation where only some parameters change through time, and static conditions that are maintained throughout the process.

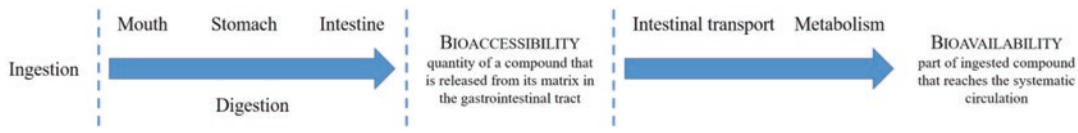

Fig. 16.1 Schematic representation of bioaccessibility and bioavailability concepts

One of the best-known dynamic models is the TNO Gastro-Intestinal Model (TIM), a multi-compartmental dynamic model from the early 1990s, that comprises the simulation of human digestion in three compartments (stomach, small intestine, large intestine). During this, the exposure conditions of meals regarding absorption of nutrients and water and secretion of digestive fluids, are modified intending to simulate the gastrointestinal tract [49]. The system is computer-controlled and the different parameters are combined in a protocol, thus allowing for reproducibility [49].

Other multi-compartmental models were also developed presenting different characteristics. The DIDGI® system was developed at INRA, focused on the stomach and the small intestine, and monitors the disintegration and the kinetics of hydrolysis of the food. The Simulator or the Human Intestinal Microbial Ecosystem (SHIME®), developed at Ghent University, is dedicated to the study of the gastrointestinal microbial ecology and physiology in healthy populational groups (adults, babies, elderlies) and also for individuals with specific disease conditions (e.g. Inflammatory Bowel Disease, pathogen infection). The Engineered Stomach and small INtestinal system (ESIN), developed at University of Auvergne (Clermont-Ferrand, France), is also dedicated to the simulation of human stomach and small intestine environment. The gastric compartment is patented and is able to reproduce the dynamic gastric emptying of liquids and solids during human digestion. The SIMGI® (SIMulator of the GastroIntestinal tract) from the Institute of Food Science Research CIAL (CSIC-UAM, Madrid, Spain) is also a computer-controlled model that simulates the human digestion process in the stomach and in the small intestine, but is also able to reproduce the microbiota responsible for metabolic bioconversions in the large intestine [11, 15]. Other dynamic models developed so far include mono-compartmental systems: Dynamic Gastric Model (DGM, Institute of Food Research, Norwich, UK), Human Gastric Simulator (HGS, University of California, Davis, USA) and the Artificial Colon (ARCOL, University of Clermont Auvergne, Clermont-Ferrand, France) [15].

Recently, with the aim of solving an existing gap between static and dynamic digestion models in what regards the human GIT physiological variations, a standardized semi-dynamic model was developed. This model is based on a previous static version, but it mimics closely the dynamic nature of gastric secretions and emptying. This model was foreseen for a broad use, comprising a wide range of foods [36].

Despite the innovation presented by dynamic and semi-dynamic digestion models, the static models are still recognized as simple, easy-to-use and reproducible [17, 32, 35]. These models that consider a constant ratio of food to enzymes and electrolytes and a constant pH for each digestive phase, are characterized as simple models and due to these characteristics have been used in several scientific fields [6]. Within the INFOGEST network [14] and considering the need for the harmonization of digestion conditions, an international consensus on fundamental digestion parameters for the static *in vitro* simulation of adult digestion was obtained and published in 2014 [35]. This method was further optimized, namely the assay for determination of pepsin enzymatic activity, and validated through an interlaboratory trial [17]. The method INFOGEST 2.0 that includes all the improvements was recently described by Brodkorb et al. [6]. The scope of application is very broad: evaluation of release of nutrients and/or food contaminants or assessment of endpoints resulting from digestion of foods by analyzing the digestion products. To perform the whole protocol, approximately 7 days should be considered, where ~5 d are needed to determine the activities of enzymes [6].

16.3.3 Application of Digestion Models to Nanomaterials

During digestion, the chemical environments are modified within the three main compartments (mouth, stomach, and intestine) regarding pH, enzymes, and inorganic compounds. These changes, as above mentioned, may induce nanoparticle modifications including dissolution,

agglomeration and deagglomeration, thus affecting the intestinal uptake [44].

During the recent years, several *in vitro* digestion models have been applied to study the potential impact of human digestion in nanomaterials properties and health effects. Table 16.1 provides examples of studies that used *in vitro* digestion models as well as the main characteristics of the models applied.

As it can be concluded from the information presented in Table 16.1, the available studies used *in vitro* digestion models with different characteristics not only regarding the digestive fluids' composition and pH in each compartment, but also regarding the static or dynamic conditions of the models.

The use of *in vitro* digestion models allowed obtaining results of the interactions of ENMs with food and GIT components and understanding their influence on nanomaterials' fate and transport, biokinetics and toxicological profile. Regarding Fe_2O_3 NP, differences in particle size, charge, and morphology were found among digested samples from the different compartments (mouth, stomach, and small intestine) [13]. This study demonstrated the influence of food matrix and the gastrointestinal environment in Fe_2O_3 NP biological properties [13]. For TiO_2 NP, the influence in mean particle diameter was also observed with increasing particle size when pH decreases (stomach), suggesting that the particles may have suffered an agglomeration or structural rearrangement under more acidic pH conditions [31]. The effect of TiO_2 NP in digestion of lipids using oil-in-water emulsions was assessed, and a reduced impact on the gastrointestinal fate and digestion of lipids was observed [31].

The use of *in vitro* digestion models has a major importance in studies where the transport through the intestinal barrier is evaluated. [1] using the model proposed by [51] concluded that the transport of silver as either total Ag or Ag NPs was limited (<0.1%), and the surface chemistry of Ag NPs and their digestion influence their dissolution properties, uptake/association with the Caco-2/HT29-MTX mono-layer. For Al NPs, [45] found no nano-specific cellular effects, either with or without *in vitro* digestion. The artificial digestion did not cause a complete aggregation of Al in the intestinal fluid, as observed for other NPs. Nano-specific toxicity caused by Al-containing nanoparticles was not observed [45]. Bettencourt et al. [4], using the harmonized protocol proposed by Brodkorb et al. [6], reported for TiO2 NPs that the primary size (Feret min, max and mean) and particle morphology (aspect ratio) of the anatase NM-102, rutile NM-103 and anatase/rutile NM-105 were not changed after the digestion. For the anatase/rutile NM-105, and when compared to the undigested NM, a more marked adverse outcome was shown after exposure to the digestion product [4]. The use of SIMGI® dynamic system, with more gastrointestinal compartments, made possible to go beyond the assessment of NPs effects on small intestine and assess the possible effects on microbiota. [11] reported that Ag NPs experienced several modifications in gastrointestinal fluids resulting in an exposure of intestine to forms that were structurally different from the original forms, even though not disturbing the composition and metabolic activity of human intestinal microbiota [11].

The inclusion of *in vitro* digestion models to have a more complete and accurate toxicological profile has been an achievement in the last years. These studies allowed taking a step forward in the knowledge of nanomaterials' toxicity. However, it should be emphasized the importance of using harmonized protocols so that the comparison of results obtained under these studies may be possible.

16.4 Importance of *In Vitro* Digestion for Risk Assessment

Human health risk assessment of chemicals present in foods, a fundamental scientific component of risk analysis, corresponds to a complex process of evaluating the potential incidence of an adverse health effect to humans, as a consequence of various exposure conditions. It is composed of four different and interconnected steps, that include: 1) hazard identification and 2) characterization (together usually considered as hazard assess-

Table 16.1 Main characteristics of the *in vitro* digestion models recently used for nanomaterials

Reference	NM	Type of model	Compartments	Incubation (time, temperature)	Digestive fluids	pH	Aim
[13]	Iron oxide (Fe_2O_3)	Static	Mouth Stomach Intestine	2 min, 37 °C 2 hours, 37 °C 2 hours, 37 °C	Mucin, amylase, salts HCl, pepsin, salts Bile salts, lipase, salts	6.8 NR 7.0	To simulate GI digestion of nano-enabled food models, in three compartments, with characterization of ENM modifications across the GI.
[31]	Titanium Dioxide (TiO_2)	Static	Mouth Stomach Intestine	2 min, 37 °C 2 hours, 37 °C 2 hours, 37 °C	Mucin, amylase, salts HCl, pepsin, salts Bile salts, lipase, salts	6.8 2.5 7.0	To study the impact of the TiO_2 particles on the properties of the lipid droplets in their passage through the GIT, and also on lipid digestion.
[11]	Silver (ag)	Dynamic SIMGI®	Stomach Small intestine Ascending colon Transverse colon Descending colon	70 min; 37 °C 120 min, 37 °C Time (NR), 37 °C Time (NR), 37 °C Time (NR), 37 °C	Pepsin in 0.5 mL/min of gastric juice Pancreatic juice (5 mL/min)	5.6 to 2.0 7.0 5.6 6.3 6.8	To assess modifications in microbiota composition, microbial metabolic activity, and in ag NPs size, shape, stability, and aggregation in the SIMGI® (stomach, small intestine, ascending colon, transverse colon, and descending colon).
[1]	Silver (ag)	Static	Mouth Stomach Intestine	5 min, 37 °C 2 hours, 37 °C 2 hours, 37 °C	Saliva fluid (salts, amylase, mucin) Gastric fluid (salts, proteins, pepsin, mucin) Duodenal fluid (salts, proteins, pancreatin, lipase) + bile fluid (salts, proteins, bile)	7.0 5.0 7.0	To assess the impact *of in vitro* digestion on the uptake/association and transport of the ag NPs in/or through the intestine with a Caco-2 and HT29-MTX co-culture transwell model.
[45]	Aluminum (Al)	Static	Mouth Stomach Intestine	5 min, 37 °C 2 hours, 37 °C 2 hours, 37 °C	Mucin, amylase, salts, urea Mucin, pepsin, salts, HCl Salts, pancreatin, trypsin, bile, urea	NR 2.0 7.5	To study the molecular effects of artificially digested Al species on intestinal cells.

(continued)

Table 16.1 (continued)

Reference	NM	Type of model	Compartments	Incubation (time, temperature)	Digestive fluids	pH	Aim
[4]	Titanium Dioxide (TiO_2)	Static	Mouth Stomach Intestine	2 min, 37 °C 2 hours, 37 °C 2 hours, 37 °C	Amylase, salts HCl, pepsin, salts Bile salts, pancreatin, salts	NR 3.0 7.0	To characterize the physicochemical properties of three types of TiO_2 after the simulated digestion process, through comparison with the primary NMs' properties. To assess the impact of the digestive process on the NMs' cytotoxicity in two human-derived intestinal cell lines.

NR = Not referred; ENM = Engineered NanoMaterials; GIT = Gastrointestinal tract

ment) examining if, and the conditions by which, a certain chemical has the potential to induce a particular adverse health effect, as well as, the relationship between the level of exposure and the related adverse health effect (usually recognized as dose-response relationship); 3) exposure assessment, determining the frequency, magnitude and duration of the ingestion of a given chemical compound; and, 4) risk characterization, which integrates the results from the previous steps, estimating the associated degree of concern [20].

Under the context of foods and the associated risk assessment of chemical compounds potentially present in foods, the concept of bioaccessibility assumes particular importance, considering that the ingested amounts of a certain chemical present in foods does not always reflect the amount of that compound available to the body and, consequently, to produce its toxic effects on target organs [22]. Consequently, *in vitro* methods that contributes to study the effects of digestion on ingested compounds constitute an important layer adding crucial information.

The amount of a specific compound that reaches the intestine after ingestion corresponds to the highest amount of that compound that could be absorbed, and consequently, reach its target organ and, therefore, producing toxicity. Thus, the determination of the bioaccessibility of a compound contributes with important information since it corresponds to the maximum oral bioavailability, and therefore an appropriate approach to estimate the internal exposure. This maximum oral bioavailability could be easily calculated by multiplying the estimated daily intake of that specific compound by its bioaccessibility value [2, 30, 51]. Regarding the nanomaterials, despite EFSA highlighted in their guidance for the risk assessment of nanomaterials used in the food chain that it is important to follow the fate of nanomaterials in the GIT, to determine whether they reach the intestinal cells in nanometric form or if they break down during the digestive process, few studies reporting that approach are available [16, 25, 34]. Additionally, just recently a study was published aiming at developing an *in vitro* method to follow the fate of silver nanoparticles in the gastrointestinal tract. This study highlighted the importance of considering the fate of nanomaterials in the gastrointestinal tract to accomplish an accurate risk assessment of nanomaterials [28].

Despite the inherent uncertainty associated to *in vitro* approaches, *in vitro* digestion models combined with intestinal cells (e.g. Caco-2 cells) could be of particular utility e.g. in addressing mechanistic questions, mimicking as much as possible the physiological conditions, before progressing to animal studies, innately involving higher costs and efforts.

16.5 Future Perspectives

Considering the growing inclusion of nanomaterials in foods due to the enhancement of physicochemical properties, as already described, it is fundamental an accurate risk assessment of these compounds. Although several studies became available in the last years, some gaps worth to be addressed still exist. Regarding the use of *in vitro* digestion models, the inclusion of a food matrix in the model is an aspect frequently reported as fundamental. The biologically active molecules present in food might alter the signaling pathways and consequently change the effects of NPs in a biological system [5, 8, 31, 34]. The use of a standardized food model based on dietary pattern and the adjustment of analytical conditions to real exposure scenarios are also aspects emphasized by [29, 56], respectively, to be addressed.

A more complete knowledge on the toxicological profile of nanomaterials is a major contribute not only for implementation of preventive measures aiming to protect human health, but also for the development of safer-by-design nanomaterials.

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