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The interaction of nanostructured antimicrobials with biological systems: Cellular uptake, trafficking and potential toxicity

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ABSTRACT

Nanomaterials have been found increasing applications in the food sector. Nanostructured antimicrobials can be incorporated either to food matrix of food packaging or to provide extended safety and quality. However, the interactions and effects of nanomaterials with biological systems are still poorly understood. Nanoparticles can enter the organism by oral, dermal and inhalation routes and distributed to different tissues by the circulatory system. Increasing evidence indicate that targeting to specific tissues, cellular uptake and intracellular fate of nanoparticles are strongly influenced by size, shape and surface properties. The specific characteristics of nanomaterials are also determinant for their toxicity in higher organisms. The dose, exposure time and administration route are important aspects influencing toxicity of nanoparticles as well. Both *in vitro* and *in vivo* evaluation studies on different types of nanostructures have providing information to support a better understanding about the interactions of nanoscale materials with biological systems.

1. Introduction

Nanotechnology has opened an array of novel applications in food industry, including the development of nanomaterials to improve food processing, safety and quality. Nanometric materials present significant increase in surface area to volume ratio, resulting in the appearance of effects related to the large number of atoms on the surface and high specific area. This results nanomaterials have different physical, chemical and biological properties as compared with similar materials in a bulk state [1,2].

Many foods contain natural self-assembled nanostructures, and therefore can be studied in the field of nanotechnology [4]. In addition, different types of engineered nanomaterials (ENMs) have been proposed to introduce new functionalities into foods. Nanostructures like nanoemulsions, nanoliposomes, nanoparticles and nanofibers can be developed for delivery of vitamins, pigments, flavors and enzymes. Active/smart packaging nanocomposites and nanosensors for monitoring food quality are valuable nanomaterials as well [5,6]. Therefore, there are many current and emerging applications of the nanotechnology in the food sector. Among several promising applications, a number of nanostructures have been described as effective for delivery of antimicrobial substances, either by incorporation into food packaging or direct addition to food products [7].

Nanobiotechnology, or also bionanotechnology, refer to terms that represent the intersection between biology and nanotechnology. It could be associated with the description of structural and functional aspects of biomolecules in nanometric scale environments. In fact, biological systems have served as inspiration for nanotechnology, for development of nanodevices, nanoparticles or molecular nanostructures. One of the premises of nanobiotechnology is the study of the interaction of nanomaterials with biological systems [3]. These include implications in biological activities such as antimicrobial, and possible toxic effects.

In this article, the essential aspects of nanoparticle interactions with biological systems are addressed. The uptake and distribution of nanoparticles in the organism and their internalization mechanisms in eukaryotic cells are discussed. The trafficking and possible fates of nanoparticles once they reach the intracellular milieu is addressed. Finally, the potential toxicological aspects of nanoparticles following interaction with biological systems and the importance of investigation on this subject is highlighted.

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Table 1
Nanostructured antimicrobial agents intended to control foodborne bacteria[a].

Nanostructured antimicrobial	Inhibitory activity
Cellulose nanocomposite with Ag nanoparticles	Mesophilic aerobic, psychrotrophics, yeasts and molds in melon cuts
Polyvinylpyrrolidone nanocomposite with Ag nanoparticles	Psychrotrophics in asparagus
Coating of stainless steel with TiO_2 nanoparticles	*E. coli* in meat exudates
Carbon nanotubes	Damage to cell membranes in *E. coli*
Poly(lactic acid) coating with ZnO nanoparticles and nisin	*S. enterica* in liquid egg
Poly(vinyl alcohol) nanocomposite with Ag nanoparticles and cellulose nanocrystals	*E. coli*, *S. aureus*
Poly (lactic acid) nanocomposite with nanoclay	*L. monocytogenes*, *S. thyphimurium*, *S. aureus*, *E. coli* O157:H7
Methylcellulose nanocomposite with ZnO nanoparticles and pediocin	*S. aureus*, *L. monocytogenes*
Solid lipid nanoparticles with nisin	*L. monocytogenes*, *L. plantarum*
Liposomes encapsulating nisin	*L. monocytogenes* in milk
Liposomes encapsulating lactoferrin	*S. aureus*, *L. innocua*, *B. cereus*
Chitosan/poly-γ-glutamic acid nanoparticles encapsulating lysozyme	*B. subtilis*, *E. coli*

[a] Compiled from references [7,9].

2. Antimicrobial nanomaterials in food

The antimicrobial activity is one of the most relevant biological activities that has been associated with nanoparticles. The nanoparticles present in food or food contact materials have been opportunely classified according to their composition as either inorganic or organic [8]. This concept can be applied to the specific case of antimicrobial nanomaterials, where the inorganic nanoparticles correspond to metal and metal oxides like Ag, Au, ZnO and TiO_2, while organic nanoparticles are liposomes, nanoemulsions or polymeric nanoparticles encapsulating food-grade antimicrobials such as essential oils, bacteriocins, terpenes, among others [9]. In addition, antimicrobial substances have been incorporated into nanostructures with the premise of improving food protection, either as food additives or nanostructured packaging materials [7,10]. A summary of prospective applications of nanostructured antimicrobials for the inhibition of relevant foodborne microorganisms is presented in Table 1.

2.1. Efficacy testing

The efficacy testing of antimicrobial nanomaterials is often performed by agar diffusion or broth dilution methods that allow the evaluation of inhibition zones of microbial growth and changes in optical density during incubation, respectively [11]. These methods are simple, inexpensive, can be employed to a large number of samples and results are easy to interpret. Standard methods are recommended for quantitative determinations of MIC, MBC and time-kill curves. The most recognized standards are provided by the Clinical and Laboratory Standards Institute [12] and the European Committee on Antimicrobial Susceptibility Testing [13]. Methods based on fluorescent or colorimetric probes, associated with active microbial metabolism are also employed for efficacy testing of antimicrobial nanoparticles. However, the results by tetrazolium salts are prone to the interference of acidic pH and some nanomaterials that can induce inaccurate estimations [14].

2.2. Mode of action

Antimicrobial nanoparticles often exert their activities causing damage to microbial membranes, oxidative stress or protein denaturation. Nanoparticles can affect microbial cells by first adhering to the cell wall. Positively charged particles are attracted by the negative microbial cell membrane and result in the accumulation of the nanoparticles on the cell membrane, which may result in subsequent membrane damage. Interaction of nanoparticles with the microbial cell surface also influence on membrane permeability altering normal cellular transport and membrane enzyme activity. The generation of reactive oxygen species (ROS) is a common mechanism of microbial cell death, and can be induced by nanoparticles. The resulting free radicals have a bactericidal action by causing damage to intracellular structures, particularly to mitochondria and DNA, in addition to denaturation of ribosomes, which causes inhibition of protein synthesis [15,16].

The release of toxic ions has been also associated with antimicrobial activity of nanoparticles. Dissolution of some nanoparticles like Ag and ZnO under aqueous conditions form hydrated ions, such as Ag^+ and Zn^{2+} that can bind and inactivate intracellular enzymes. Besides, these ions interfere on cellular metabolism via modulation of signal transduction pathways in bacteria by interfering in phosphorylation. Ions released from nanoparticles can obstruct nucleic acids metabolism as well, by interacting with phosphorus residues in DNA, hindering replication and affecting transcription [15,17]. Some nanoparticles can exert direct toxic effect on microbial DNA metabolism. Damage to DNA has been reported by Ag and Au nanoparticles, obstructing transcription and inducing mutagenesis as well [17].

3. Antimicrobial nanomaterials in food and human exposure

The high volume production and utilization of ENMs such as Ag, TiO_2, ZnO and SiO_2 nanoparticles, may suggest that human exposure to these nanoparticles is possibly directly by food, water, healthcare products, drinking, and indirectly by release in the environment. Although these nanomaterials are used in many forms in food industry, those intentionally added as food additives are probable the main source of ingested exposure. The most common nanoparticles used directly in food products are TiO_2, SiO_2, Fe_2O_3, ZnO and Ag [18,19]. Considering the lack of knowledge about the environmental behavior and fate of ENMs, it is difficult to evaluate whether they may accumulate in the food chain. The basis of consumer exposure assessment of antimicrobial nanoparticles ingested through food products is similar to the assessment of conventional exposure to chemicals. The application of nanoparticles in food products or as additives may require additional data about the consumption of these foods, as this information is frequently absent in the conventional consumption databases [20]. Some available information on the most frequently ingested nanomaterials used in foods indicate values ranging 10–22, 1.25–3.59 and 5.3–6.4 μg/mg for Ag, TiO_2 and SiO_2 nanomaterials, respectively [18]. However, the consistent amount determination and characterization of nanoparticles in ingested food is relatively challenging because the lack of sensitive methods to detect nanoparticles in food matrices. Thus, it is difficult to measure nanoparticles in consumed foods, and an alternative approach could be the collection of reliable information from producers.

Although the beneficial effect of some antimicrobial nanomaterials used to control food pathogens seems evident, their interactions with higher organisms should be considered as well.

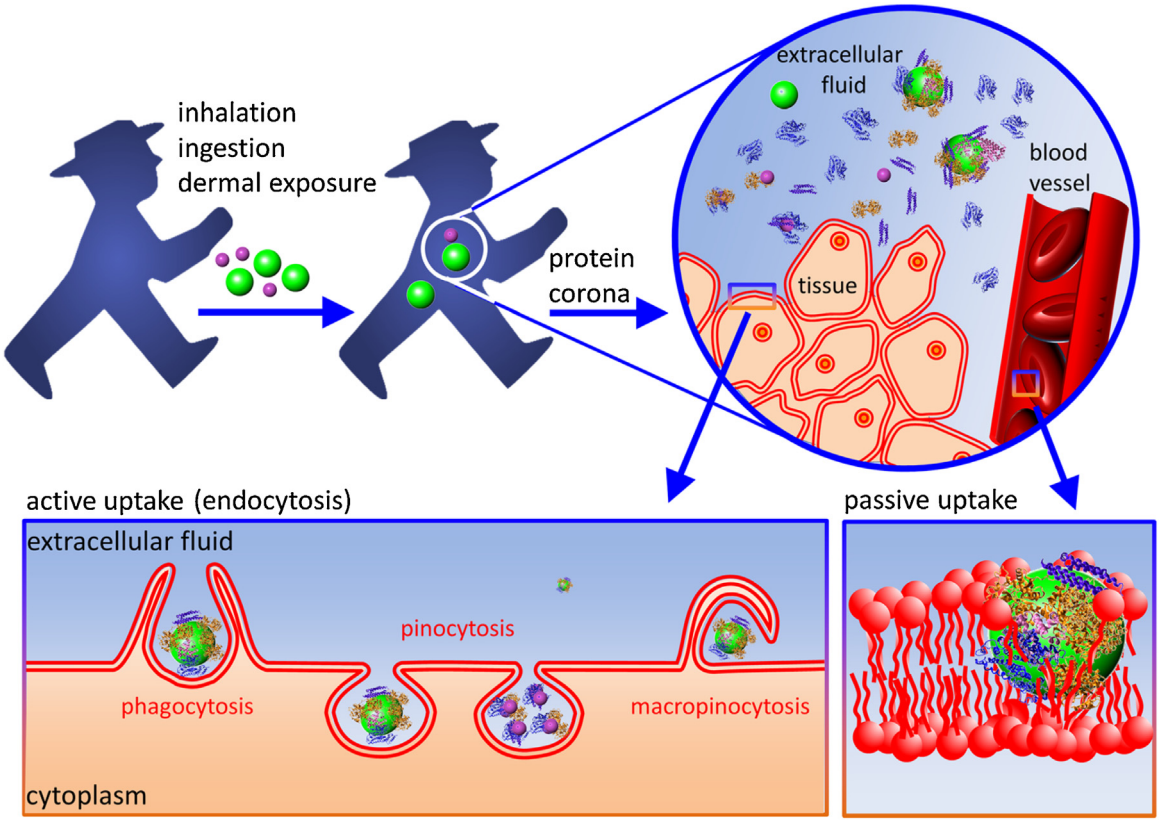

Fig. 1. Uptake of nanoparticles by higher organisms. Nanoparticles may be introduced in the human body by inhalation, ingestion or through the skin. In the extracellular fluid, nanoparticles are covered by proteins and other biomolecules. The protein corona regulates the nanoparticle interactions with the cells. Cellular internalization may involve mechanisms of active (receptor-mediated) or passive transport across the cell membrane. Reproduced from reference [22], under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licences/by/4.0/).

This is particularly relevant when the nanoparticles are delivered through food or food contacting materials. After oral exposure, the absorption, distribution, metabolism, and excretion (ADME) profiles are mostly influenced by the characteristics of nanoparticles in foods. However, restricted information is available about the relationship between the behavior of nanoparticles in the food matrix and their physicochemical properties. Increasing evidence suggest that interactions between nanoparticles and food components may have significant influence in the absorption of each other after oral exposure [8,21]. The ADME characteristics and interactions with biological systems after exposure to antimicrobial nanoparticles are discussed in the following sections.

4. Nanoparticle interactions with biological systems

Due to their unique properties, nanomaterials may have specific and differentiated interactions with biological systems as compared with their counterparts in the bulk state, and this is the reason why the study of their interaction and diffusion in biological systems is so relevant. Despite the studies on nanotoxicology have been increased in recent years, there is still limited knowledge about the subject. It is difficult to make generalizations about the bioavailability, biodistribution, degradation, elimination and effects of nanostructures in living organisms. It is also difficult to generalize molecular processes involved in cellular internalization, trafficking and processing of nanoparticles. Therefore, the knowledge about how cells cope and interact with these nanoscale materials is an essential part of nanotechnology.

In general, the interaction of nanoparticles with higher organisms begins with the absorption of the nanomaterial by inhalation, ingestion or dermal exposure, as shown in Fig. 1. Once the nanoparticle is absorbed by the organism, the interaction with proteins and other biomolecules occurs in the extracellular fluids. After the nanoparticle reaches the blood system, there is a competition between different biological molecules to adsorb on the surface of the nanoparticles [22,23]. In the blood system, the absorption of plasma proteins, called the 'protein corona' formed around the nanoparticles is the most studied process modulating targeted-drug delivery. Proteins that adsorb with high affinity form the 'hard' corona, which consists of tightly bound proteins that are not easily desorbed, and proteins that adsorb with low affinity form the 'soft' corona, which consists of in poorly bound proteins [24].

The effect of protein corona on the toxicity of metallic nanoparticles has been demonstrated. Gold nanoparticles modified with hydrophobic groups showed hemolytic activity proportional to the logP of the added group. However, it was observed that when these nanoparticles are incubated with plasma proteins, the hemolytic activity is blocked [25]. In the blood system, the protein corona formed around the nanoparticles will determine their fate, i.e., the speed of elimination, how they will interacts with the cell, what tissues they will be able to penetrate. Cell internalization may involve a mechanism mediated by receptor or passive transport by the membrane, thus it will be influenced by the specific characteristics of the nanostructure (Fig. 1).

4.1. Absorption of nanoparticles

4.1.1. Ingestion and gastrointestinal absorption

Considering the use of antimicrobial nanomaterials in food applications, the most evident contact route would be upon ingestion. Several properties of the gastrointestinal tract may influence the absorption of antimicrobial nanoparticles. These include a

highly variable pH range during the gastrointestinal transit (2–3 in the stomach to 6–7 in the intestine), surface-active molecules (bile salts, phospholipids, proteins), electrolytes, digestive enzymes, gut microbiota and mechanical forces [8]. These factors may alter the properties and aggregation state of nanomaterials and therefore their fate in the gastrointestinal system.

The oral absorption of nanoparticles could be initiated in the mouth. Nanoparticles can be absorbed by the highly permeable buccal and sublingual mucosae and directly pass to the systemic circulation and penetrate into tissues through fine capillaries [26]. Administration of some nanomedicines by the oral route indicates that it could be a fast and efficient way to provide daily intake of bioactive substances [19]. This concept could be expanded to antimicrobial nanoparticles present in food.

The uptake of nanoparticles in the gastrointestinal tract depends on diffusion through mucus, interaction with the epithelium, and translocation process. The mucus layer could be designated as the first barrier faced by the ingested nanoparticle to enter the organism, whereas the epithelium is considered as the second barrier. Mucus consists mainly of mucin proteins, which are highly glycosylated extracellular proteins with gel-forming characteristics. The particle charge plays a crucial role in the nanoparticle diffusion rate, as the electrostatic repulsion from negatively charged moieties of mucins favors the dispersion of positively charged particles. In general, smaller particles diffuse faster through mucus than larger particles [27]. The very small size of the nanoparticles may facilitates their transport through the intestinal mucosa and absorption by enterocytes. Nanoparticles with size ranging from 50 to 300 nm, positive zeta potential, and hydophobic surface are preferentially captured in comparison with others [28,29].

The capability of nanoparticles to adhere to the mucosa can represent an effective approach to increase their residence time and enhance absorption because it can facilitate transport across the epithelium. The interaction is generally achieved with natural or synthetic polymers, which can interact with mucin through hydrogen bonding, hydrophobic or electrostatic interactions. As mucin is negatively charged, the use of positively charged polymers such as chitosan provides an effective interaction [30]. In addition, poly(ethylene glycol) has been used as a mucoadhesive agent, since it may create interactions due to their capability to diffuse within the mucus network and establish hydrogen bonding [28].

In general, the epithelium represents the highest resistance against the passage of nanoparticles. Epithelial cells possess an apical surface where they face an internal or external surface and a basal site, facing the underlying tissue. The epithelium of the gastrointestinal tract is composed of different types of cells and permeation through some cell types is easier than through others. The structural basis of the barrier is often formed by only one cell type, namely the keratinocytes for the oral cavity and the esophagus, gastric epithelial cells for the stomach and enterocytes for the small and large intestine [19].

Increased absorption of nanoparticles by enterocytes is due to the modulation of tight junctions, receptor-mediated endocytosis and transcytosis, phagocytosis via specialized M cells and other mucosa-associated lymphoid tissues, and lymphatic absorption through the chylomicron uptake mechanism of enterocytes [31]. Although these mechanisms of nanoparticles penetration through the enteric mucosa have been established, two of them have been predominantly used: (a) the paracellular route that is slow and passive and (b) the transcellular process that shows a rate dependency on lipophilicity [28,29]. Routes of active transport involve transporting proteins or the opening of tight junctions. The cells of gastrointestinal epithelium are firmly connected by tight junctions, and the permeability of these junctions could be altered by specific biopolymers, which can expand the junctions and serve as ports for particles entry. In the transcellular route, nanoparticles are taken up by the apical side of the epithelium, transported within the Peyer patches through M cells, and are subsequently released at the basolateral side.

Most studies on the gastrointestinal fate of food-grade nanoparticles often ignore their interactions with components of food matrices and gastrointestinal tract. Considering the variety of types and levels of food components, and the diversity of food processing operations, the characteristics of nanoparticles can be significantly changed when they are dispersed in food products, which could be decisive for their subsequent gastrointestinal absorption [8,21]. For example, the interaction of nanoparticles with proteins has been well established [24] and the absence of fat seems responsible for the effective control of *Listeria monocytogenes* by nisin-loaded nanoliposomes in skimmed milk [32].

4.1.2. Inhalation and dermal exposure

Although the inhalation would not be the main route for interaction with food-derived nanoparticles, it could be considered when nanostructures are present in fine powder formulations. The respiratory tract has several unique anatomical and physiological features. There are approximately 300 million alveoli in the lungs, with a surface area greater than 100 m^2, and an alveolar epithelium as thin as 0.1 μm (for comparison, the intestinal epithelium is about 20–30 μm). This large surface area, combined with an extremely thin barrier between the pulmonary lumen and capillaries and a high rate of blood perfusion that provides direct access to the central circulation, creates conditions that are suitable for efficient mass transfer [33].

The dermal absorption of nanoparticles assumes a direct contact with the nanostructured material. The knowledge about nanoparticle absorption via the dermal route is essentially derived from studies on the administration of topical nanostructured drugs to facilitate local therapies. The administration of nanoparticles to the skin is carried out in three main sites: surface of the *stratum corneum* through intracellular and intercellular penetration, furrows, and hair follicle openings [34]. There is little evidence that nanoparticles larger than 100 nm cross the skin barrier into the dermal compartment, and it is generally accepted that the topical supply directs the nanoparticles to the deeper layers of the skin, but does not reach the viable epidermis. However, a greater penetration of particles occurs when the keratin barrier is compromised, such as in aged or diseased skin [35].

4.2. Distribution of nanoparticles

Once a nanoparticle reaches the circulatory system, it can be distributed systemically through the body by the vascular and lymphatic systems. Several interactions may occur with blood components, such as red and white blood cells, platelets, plasma proteins and coagulating factors. The distribution of a nanoparticle in a specific tissue correlates with the relative amount of cardiac output that passes through that tissue. As a result, tissues and organs with elevated blood flow (brain, liver, heart, intestines, lungs, kidneys, spleen, etc.) may be exposed to higher amounts of a nanoparticle, as long as the nanoparticle can penetrate the particular tissue of the vasculature. Therefore, a physiological parameter (cardiac output) can act as a filter for the nanomaterial distribution [36,37]. Very small nanomaterials, in the order of 1–20 nm, have long circulatory residence times and a slower extravasation of the vasculature to the interstitial spaces. This may cause a slower achievement of the maximum volume of distribution, or even an altered volume of distribution when administered intravenously. In the case of nanostructured drug delivery, local injections require nanoparticle engineering of slightly larger sizes, in the order of 30–100 nm [36].

A widespread distribution of nanoparticles has been detected inside organs of experimental animals, where smallest particles were found to be more concentrated in the brain, spleen, liver, and bone marrow as compared to larger particles; diverse nanoparticles, such as TiO_2 and Ag, were detected inside human red blood cells [38]. It was found that quantum dots and gold nanoclusters with less than 10 nm in diameter accumulate in the plasma membrane before gradually entering the intracellular region. In stark contrast, the large polystyrene nanoparticles (100 nm) were directly internalized without a prior detectable accumulation in the plasma membrane. Small nanoparticles have fewer ligand-to-receptor interactions than larger ones. Therefore, several small nanoparticles need to interact simultaneously with neighboring receptors to activate the wrapping by the membrane. In contrast, large individual nanoparticles can act as a linking agent to group receptors and induce uptake [39]. A size-dependent uptake in different cell lines has been observed for Au, iron oxide, polystyrene and mesoporous silica nanoparticles, with the maximum cellular uptake at a nanoparticle core size in the range of 30–50 nm, which suggests that there is an optimal size for active uptake [22,40].

Besides their sizes, nanoparticles can concentrate on specific organs or cells depending on surface characteristics. Although the passive targeting is based on a specific physiological parameter to act as a distributive filter, several examples of active targeting are described [36]. The surface of the nanomaterials can be linked to a biological marker, such as an antibody, an aptamer, or internalization peptides [41]. The bioconjugation of nanoparticles with cell penetration peptides, such as RGD, TAT and Pntn, may facilitate the direct transfer of particles across the cell membranes by a mechanism that is not yet fully understood [42].

ADME and toxicokinetics studies of antimicrobial nanoparticles are often dedicated to Ag or TiO_2. In general, Ag nanoparticles are less available than ionic Ag after oral administration, although both forms are deposited in diverse tissues, mostly in the stomach and intestines following in decreasing order by the liver, testes, kidneys, brain, lungs, blood, bladder, and heart [43]. Oral administration of 20 mg/kg Ag nanoparticles and Ag^+ to rats revealed that tissue distribution of Ag in liver, kidneys, and lungs was higher when Ag ions were administered compared to Ag nanoparticles. Orally administered Ag nanoparticles were mostly excreted through feces, suggesting low bioavailability [44]. In another study, Ag nanoparticles were predominantly found in feces, while their blood and urine concentrations were very low after oral exposure to rats. These nanoparticles were also detected in feces after 24 h intravenous administration, suggesting bile secretion of nanoparticles [45]. This agrees with the fact that when bile duct is ligated, the fecal excretion of orally absorbed Ag is reduced and accumulation in the liver is observed [43]. Toxicokinetics of TiO_2 nanoparticles has been investigated after exposition by inhalation, and the measured levels in blood, tissues and excreta suggest the translocation to the systemic circulation. Large amounts were recovered in feces compared to urine, suggesting that the inhaled nanoparticles were eliminated mainly by mucocilliary clearance and ingested [46]. Although TiO_2 nanoparticles have very limited bioavailability after oral exposure, Ti levels are detected in the liver and mesenteric lymph nodes after oral administration, suggesting that gastrointestinal absorption is possible [43]. Toxicokinetics of ZnO nanoparticles after 90-day oral administration in rats revealed increased plasma concentrations, indicating that repeated oral exposure to up to 125 μg/kg ZnO nanoparticles could accumulate in the systemic circulation [47].

Although kinetics studies of nanoparticles distribution after oral exposure are available, additional research is necessary on the ADME aspects of food-related nanoparticles to elucidate the mechanisms of these phenomena. The increasing information about the kinetics of nanoparticle distribution in the body will be valuable to specify target tissues where nanoparticles may have adverse effects, which is particularly important for the risk assessment [20]. Special attention should be devoted to the ability of nanoparticles to transpose the barriers, such as the placental barrier, gastrointestinal barrier, blood/milk barrier, and blood/brain barrier [38].

4.3. Nanoparticle-cell interactions

The plasma membrane is the main cellular interface, through which the interior of the cell communicates with the external environment. The initial cell contact with any extracellular material involves the interaction with membrane structural components, a phospholipid bilayer containing proteins, cholesterol and lipopolysaccharides. Thus, the assessment of membrane integrity is one of the first tests to evaluate the interaction of nanomaterials with eukaryotic cells. Many protocols for evaluation of membrane integrity are available, including the use of fluorescent probes and vital dyes [48]. In most cases, it is possible to measure the activity of lactate dehydrogenase, a typical intracellular enzyme released from cells with damaged membrane, or test the integrity of the membrane with a dye uptake assay, such as trypan blue exclusion [49].

Nanoparticles can access the cytoplasm of eukaryotic cells, due to damages caused to the membrane, or being internalized by the cells. To discern between these possibilities, it is important to evaluate the effects observed under normal conditions and compare them with results obtained at 4 °C, or in the presence of energy metabolism inhibitors. Ammonium chloride combined with 2-deoxy glucose have been used for this purpose. *In vitro* experiments at 4 °C, as well as inhibitors of energy metabolism, decrease the production of ATP, which inhibits the endocytosis, responsible for the internalization of nanoparticles [50]. In addition to inhibitors of energy metabolism, a general endocytosis inhibitor can be used. Dynasore is widely used, providing a rapid inhibition of GTPase dynamin-dependent endocytosis, which is effective in several pathways. In addition to GTPase inhibition, dynasore affects cholesterol in the plasma membrane, disturbs lipid raft organization and remodels F-actin in a dynamin-independent manner [51]. Comparing the results of experiments conducted in the presence or absence of inhibitors, it can be possible to obtain the first evidences about the mechanisms of particle internalization by the cells.

4.3.1. Passive diffusion

Smaller, non-polar molecules, such as oxygen, can pass through the membrane by simple Fick diffusion. On the other hand, larger and polar molecules (including ions) need transport mediated by protein carriers. In general, large macromolecules are exchanged between the extracellular environment and the cellular cytosol, through internalization (endocytosis) and exclusion (exocytosis) processes mediated by specific receptors [52,53]. Nanoparticles can also cross the cell membrane without any specific receptor-mediated interaction. The lipid bilayer can be deformed due to the adhesion of the nanoparticle on its surface, which leads to a total engulfment of the nanoparticle and its final uptake. This uptake of nanoparticles on the membranes can be driven only by general physicochemical interactions [54,55].

The passive nanoparticle engulfment process is governed by three energy contributions: adhesion energy in the contact area between the nanoparticle and the membrane (E_{adh}), membrane bending modulus (κ) and surface tension of the membrane (σ). The adhesion energy drives the nanoparticle into the membrane, while the resistance of the membrane to deformation, characterized by bending and elastic moduli, opposes the engulfment process [56]. Assuming a tensionless membrane model, a nanoparticle with a radius equal to or greater than the critical radius (r_c) will suffer a complete engulfment once the contact has occurred. Thus, the key descriptors governing passive nanoparticle internalization are

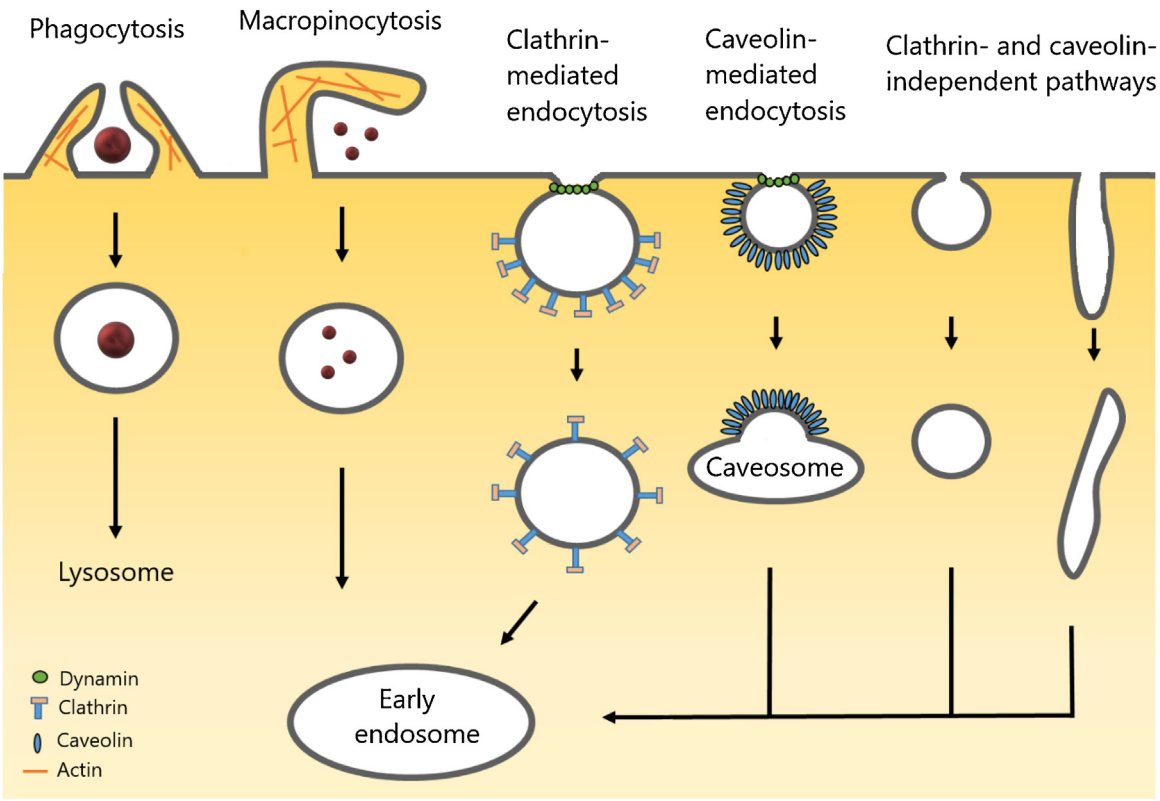

Fig. 2. Schematic representation of endocytosis pathways. Nanoparticles and other substances can be internalized by phagocytosis, micropinocytosis and diverse endocytosis pathways. The particles are the enclosed within the early endosomes, phagosomes or macropinosomes for further intracellular processing.

the nanoparticle and cell size, nanoparticle-membrane adhesion energy, and membrane elastic moduli and tension [55].

4.3.2. Endocytosis of nanoparticles

Endocytosis is an important mechanism of cellular communication, involving the flow of vesicles transporting a wide range of cargo molecules from the plasma membrane of eukaryotic cells into the cytoplasm. Endocytosis can be divided into phagocytosis (only for some specialized cells, such as macrophages) and pinocytosis (virtually present in all eukaryotic cells), which in turn, can be subdivided into clathrin-mediated endocytosis, caveolin-mediated endocytosis and macropinocytosis [52,53]. Among the different endocytic pathways described in eukaryotic cells, the major route for internalization of many cargoes is clathrin-mediated endocytosis [57]. An overview of the main endocytosis pathways is presented in Fig. 2. In addition, as vesicle trafficking inside cells is mediated by cytoskeletal fibers, they are important in determining which cytoskeletal fibers are relevant to the biological role of a given nanomaterial [58].

The study of endocytosis in living cells is typically performed using pharmacological inhibitors, mutant cells expressing non-functional proteins involved in the endocytosis pathways, or using siRNA methodology to reduce the expression of key proteins of the endocytosis pathways [59]. The use of chemical inhibitors has been widely used in the characterization of the internalization route of nanomaterials. The inhibition of endocytosis can be approached by selective and non-selective methods for each type of endocytosis, through the use of such chemical inhibitors. The protocols that use inhibitors can be advantageous, since they are inexpensive and fast. As examples, it can be mentioned the use of chlorpromazine, which acts in the inhibition of clathrin-mediated endocytosis, amiloride acting on macropinocytosis, and genistein inhibiting caveolin-mediated endocytosis [60]. However, a combination of methods is recommended when addressing the mechanisms of endocytosis, since several chemical inhibitors have additional effects to those originally considered to be specific.

Several studies have shown that the format, size, surface properties, such as porosity and charge, and composition of nanoparticles directly influence the interaction with eukaryotic cells and the endocytosis process [40]. For example, 15 nm, 45 nm and rod-shaped gold nanoparticles enter into cells through a receptor-mediated endocytosis pathway, while the star-shaped nanoparticles adopt not only clathrin-mediated, but also caveolin-mediated endocytosis pathways. However, the 80 nm nanoparticles mainly enter into the cells by macropinocytosis pathway [61]. The effect of nanoparticle size on the internalization pathway in non-phagocytic B16 cells was observed using fluorescent latex beads ranging from 50 nm to 1000 nm. The internalization of nanoparticles < 200 nm involve clathrin-coated pits, while increasing nanoparticle size, a shift towards caveolae-mediated internalization became apparent, which turned out to be the predominant entry route for 500 nm particles [22].

The uptake of Ag nanoparticles (5–100 nm) decreased significantly after the pre-treatment of B16 cells with chlorpromazine hydrochloride, which can specifically inhibit the clathrin-mediated endocytosis. The internalization efficiency of smaller nanoparticles (5, 20, 50 nm) was markedly reduced by methyl-β-cyclodextrin, a specific inhibitor of caveolin-mediated endocytosis, whereas 5-(*N*-ethyl-*N*-isopropyl) amiloride as an inhibitor of macropinocytosis inhibited the uptake of larger sizes of silver nanoparticles (50 nm and 100 nm) [62]. The results suggest that both the efficiency of cellular uptake and the type of endocytosis are influenced by the size of nanoparticles.

Several pathways have been considered to mediate the process of cellular uptake of gold nanoparticles, such as phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-

mediated endocytosis, and direct penetration. Specific inhibitors for endocytosis pathways were tested to study mechanism of gold nanoparticles uptake in lung alveolar basal epithelial cell line A549, human bronchial epithelial cells 16HBE and mesenchymal stem cells (MSC) cells. The clathrin-mediated endocytosis was the main pathway and lipid raft-mediated pathway (which is mediated by dynamin) was the secondary route during internalization of gold nanoparticles by the tested cells [63].

Considering the limited specificity of some inhibitors, it is necessary to standardize their use for each cell type. Initially, the toxic concentration of the inhibitor should be determined to warrant the observed effect occurs due to the inhibition of the route of interest and not by the toxicity of the inhibitor. As a general recommendation, standard viability tests (for example with MTT) should have a maximum of 15% reduction in viability, ideally using concentrations that do not present any toxicity [50]. In addition, it is important to consider the exposure time of the cells to the inhibitors. Long exposures should be avoided, as the cells can metabolize the inhibitors, which lose their effect. Then, it is necessary to standardize the inhibitor concentration using specific markers for each pathway that depends on the inhibitor, the cell type and the time of exposure. The most commonly used markers are transferrin for clathrin-mediated endocytosis, cholera toxin for caveolin-mediated endocytosis, and dextran for macropinocytosis. The markers are generally commercially available already associated with a fluorophore [64]. Finally, it is important to consider that cells generally use more than one pathway for the internalization of molecules or nanostructures. In the presence of an inhibitor, the cell can have a compensatory effect, using another endocytic route to supply the internalization.

4.4. Nanoparticle trafficking

After cellular internalization, regardless of the route used, the particle is initially enclosed in a membrane-bound vesicle (early endosome), without direct access to the cytosol or cytoplasmic organelles. Endosomes are formed at the plasma membrane and are classified into three types: early endosomes, late endosomes and recycling endosomes. Early endosome transports the cargo to the desired cellular target. Part of the cargo is recycled to the plasma membrane via recycling endosomes. Early endosomes transform into late endosomes via maturation and differentiation process [65]. The late endosomes will then integrate with lysosomes to form endo-lysosomal vesicles and hydrolytic enzymes contained within these vesicles can degrade the entrapped nanoparticles [40]. Thus, if the endosome leakage does not occur, the enclosed materials can be degraded in lysosomes, redirected to the external environment through recycling endosomes, directed to other organelles, or undergo exocytosis. The intracellular fate of nanoparticles as carriers of bioactive compounds is illustrated in the Fig. 3.

After internalization, most nanoparticles are directed to the lysosomes, in which they are degraded. Chlatrin-mediated internalization often drives nanoparticles to lysosomal degradation [36,57]. A detailed study using coumarin-loaded PLGA nanoparticles showed that cell internalization occurred through caveolin and clathrin-dependent endocytosis and Rab34-mediated macropinocytosis. The nanoparticles were transported to early endosomes, late endosomes, and finally to lysosomes. In addition, the PLGA nanoparticles were sent out of the cells by GLUT4 transport vesicles, classic secretory vesicles and melanosomes. The PLGA nanoparticles were also observed in autophagosomes, indicating that the nanoparticles can be delivered by the autophagy pathway [66]. In another study, nanoparticles of biodegradable polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) showed cell-specific uptake patterns when tested in two different epithelial cell lines (HeLa and SKOV-3). A classical endocytosis pathway and some caveolin-dependent internalization was observed in HeLa, whereas an independent way from traditional routes was observed in SKOV-3 cells. Furthermore, the final fate of nanoparticles was determined, showing that in both cell lines, nanoparticles ended up in lysosomes, where they are finally degraded, thereby releasing their contents [67]. Nanoparticles of biodegradable 3-hydroxybutyrate-co-3-hydroxyhexanoate (PHBHHx) were mainly internalized via clathrin and caveolin endocytosis, but two new pathways were observed: the micropinocytosis early endosome (EEs)-micropinocytosis-lysosome pathway and the EEs-liposome-lysosome pathway. The nanoparticles were delivered to cells by endocytosis recycling vesicles and GLUT4 exocytosis vesicles. Similar to other nanostructures, PHBHHx nanoparticles also induced intracellular autophagy and were then degraded via endolysosomal pathways [68].

The intracellular circulation of Au nanoparticles has been investigated as well. In RAW264.7 macrophage cells, Au nanoparticles can be directed to lysosomes, which move toward the nuclei with a perinuclear distribution in a time-dependent manner [69]. After internalization into A549 cells, Au nanoparticles are transported to lysosomes and then translocated to the mitochondria. The mitochondrial structures are damaged by nanoparticle storage and also affect their abilities to be excluded. In comparison, Au nanoparticles showed a quite different intracellular trafficking in 16HBE and mesenchymal stem cells [63]. In both cells, Au nanoparticles are located at endosomes/lysosomes, remaining there for some time for 16HBE while about 60% are excluded for mesenchymal cells. After internalization into lysosomes, Au nanoparticles cause significant changes in lysosomal membrane permeation in A549 cells, but not in 16HBE and mesenchymal stem cells, which provides a chance for Au nanoparticles to be released into cytoplasm. Finally, Au nanoparticles can target mitochondria of cancer cells and induce apoptosis, whereas they cause few effects on normal or mesenchymal stem cell [63]. Ag nanoparticles ranging from 5 to 100 nm were directly observed after internalization in B16 cell line, mainly within membrane-bound structures, such as intracellular vesicles and late endosomes [62].

The influence of surface charge on cellular uptake and intracellular trafficking of chitosan nanoparticles was investigated through a systematic study using eight different cell lineages, including fibroblastic, epithelial, endothelial, and blood cells. Intracellular trafficking results indicate that some positively charged nanoparticles can escape from lysosomes after being internalized and exhibit perinuclear localization, while the neutrally and negatively charged nanoparticles are preferentially co-localized with lysosomes [30].

The study of the intracellular traffic of nanoparticles after internalization is important to determine its potential effect into the cellular environment, in particular to determine the effectiveness of drug-loaded nanoparticles. Despite the intracellular traffic is strongly influenced by the internalization pathway, it is not always possible to predict the intracellular fate of nanoparticles. It is possible that the nanoparticle escapes from the endosomes to the cytosol or that its intracellular accumulation leads to degradation and/or exocytosis. The intracellular fate of nanoparticles can be fundamental for their biological or therapeutic role [70]. The mechanisms proposed for endosomal escape includes membrane fusion between the endosomal membrane and nanoparticle structure, osmotic rupture by the proton sponge mechanism, swelling of pH responsive nanostructure, and membrane destabilization by nanoparticle disassembly [71].

The nanoparticles can cause undesired effects after being internalized and pass to the endosome-lysosome system, since they can be degraded, releasing constituents that can, for example, generate reactive oxygen species (ROS). Damage to the lysosomal membranes can release lytic enzymes, such as proteases and lipases, which once in the cytosol, can attack the mitochondrial membrane,

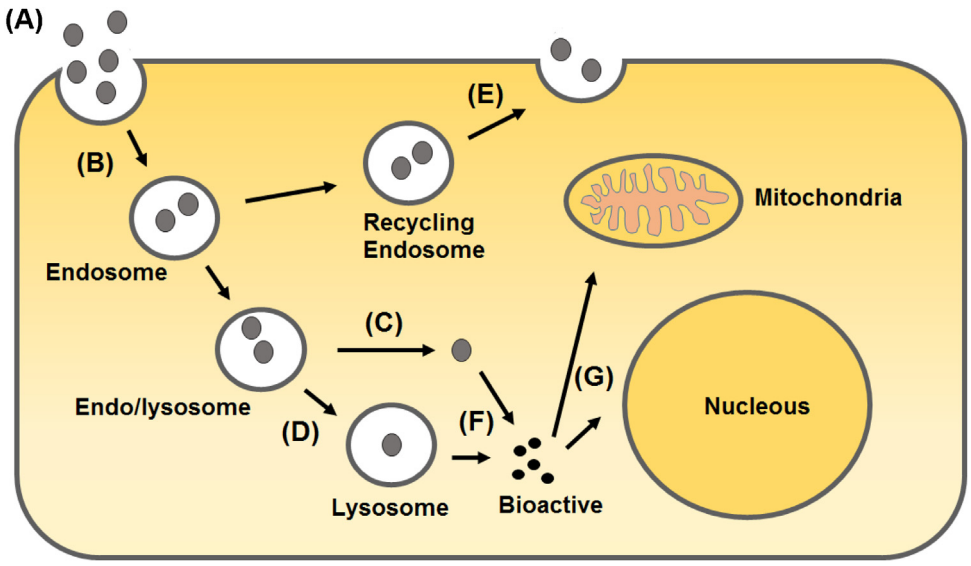

Fig. 3. The intracellular trafficking and cytosolic administration of bioactive substances through nanoparticle carriers. (A) Cellular nanoparticle interaction, (B) Internalization of the nanoparticles by endocytosis, (C) Endosomal escape of nanoparticles or (D) Lysosomal degradation of nanoparticles, (E) Exocytosis of nanoparticles, (F) Bioactive compounds released into the cytoplasm, (G) Cytoplasmic transport of bioactive compounds to the organelles.

causing injuries to DNA, proteins, and membranes until the rupture of this organelle [40,72]. By damaging the mitochondria, more ROS are produced, entering a cyclic process, in which ROS is produced, inducing more damage to the mitochondria, generating even more ROS. This process, in most cases, can lead to the mutagenicity of the genetic material (DNA) and cell death.

5. Toxicity of nanomaterials

A great number of studies dealing with toxicity of nanoparticles is currently available, although most studies investigate only one size and type of nanoparticles. Some examples about the effects and potential toxicity of nanomaterials on different biological systems *in vitro* and *in vivo* are summarized in Table 2.

Although metal and metal oxide antimicrobial nanoparticles are frequently considered as biocompatible, without significant toxic effect *in vivo* and *in vitro*, they are also associated with pro-inflammatory responses and induction of oxidative stress in diverse studies [74,77]. The induction of cell death by various metal oxide nanoparticles has been attributed to their dissolution in culture media. Dissolution is the release of ions from the nanoparticle surface with consequent surface changes. Free ions may present a different biological impact from the same atom when it is present in the nanoparticle lattice, including possible toxic effects [78]. Although further investigation is needed before definitive conclusions can be made, several studies indicate that toxic effects are caused by ions resulting from nanoparticle dissolution outside the cell [79,80]. It should be noted that ROS generation and release of toxic ions from metal and metal oxide nanoparticles are common mechanisms of antimicrobial activity as well [17], and therefore the potential toxicity of the nanomaterial could be associated with the effective dose reaching the organism and the influence of nanoparticle properties on their specific interactions with microbial or higher eukaryotic cells.

After an adequate nanoparticle characterization is achieved, the conventional approach is often to perform an *in vitro* evaluation of the nanomaterial, using bacteria or eukaryotic cell cultures, to understand its interaction with the biological setting. Then, dose-response tests performed *in vivo* are important to measure the adverse effect caused in a population, in a fixed time interval and amount of sample. Afterwards, the exposure evaluation is determined, considering the possible contact routes of the nanomaterial with the body (oral, dermal or by inhalation), the nanomaterial concentration and the exposure time. Finally, the probability of health risk for the organism and/or environment is calculated based on results of *in vitro*, *in vivo* and exposure assessment [20,81]. It has been suggested that a combination of methods to evaluate the toxicological effects of nanomaterials is often appropriate since there is no perfect scheme to predict the behavior of these materials in the human population and in the environment [82].

5.1. Nanoparticle properties and toxicity

Since nanomaterials are complex systems and their specific properties may have influence in the toxicological aspects, it is particularly important to determine their physicochemical properties, such as size, shape, surface area, composition, purity, dispersion, solubility and surface charges [9,83]. A series of methodologies have been established to achieve nanoparticle characterization, including electron microscopy, atomic force microscopy, dynamic light scattering, X-ray based methods, thermal analyses, infrared spectroscopy, electron diffraction, among others [73,84]. Electron and scanning probe microscopes are the most popular devices for the imaging of nanoparticles. Depending on the technique, resolutions lower than the nanometer range can be achieved. The scanning transmission electron microscopy technique provides a direct way to visualize the atomistic structure and composition of nanostructures at a sub-angstrom resolution [85,86]. Analytical tools can be coupled to electron microscopes for additional elemental composition analysis. The analytical methods should be sensitive enough to measure low concentrations, as small particles usually represent only a minor part of the total mass [73].

An interesting account about the relationship between nanoparticle properties and potential toxicity was provided by Leroueil et al. [87]. Poly(amidoamine) PAMAM dendrimers, and other amine-containing polymers, strongly interact with lipid bilayers and cell plasma membranes to induce substantial membrane permeability and, if sufficiently concentrated, cell lysis. However, nanoengineered generation 5 (G5) PAMAM is a particularly successful implementation of these materials for development of targeted chemotherapeutic drugs without apparent side effects [88]. Studies on structured lipid bilayers with PAMAM showed nanoscale hole

Table 2
Examples of nanoparticle toxicity and other effects[a].

Nanostructure	Toxicity study
SiO_2 nanoparticles	Inhibition of total oxygen uptake in activate sludge during wastewater treatment
SiO_2 nanoparticles	DNA damage in *Drosophila melanogaster*
Ag nanoparticles	Generation of ROS, protein carbonylation and up-regulation of proteins involved in SUMOylation of colon cancer cells
Ag nanoparticles	Inhibitory effect on photosynthetic activity of algae, diatoms and cyanobacteria; toxicity in green algae *Chlamydomonas reinhardtii*
TiO_2 nanoparticles	Increase of ROS production, membrane crystalline inclusions, disruption of thylakoid and plasma membranes
TiO_2 nanoparticles	Higher toxicity in rats inhalation study with aerosols
Carbon nanotubes	Cytotoxicity on HaCaT, HeLa, A549 and HEK293 T cells
Fullerenes C_{60}	Antibacterial activity in water suspensions
Alumina nanoparticles	Phytotoxicity of alumina nanoparticles
Quantum dots	Cytotoxicity on HEK293 cells and primary rat hepatocytes
ZnO, SiO_2, TiO_2, Fe_2O_3, CeO_2ZrO_2, $Ca_3(PO_4)_2$	*In vitro* cytotoxicity of metal oxide nanoparticles on human mesothelioma MSTO-211H and rodent 3T3 fibroblast cells
Nanoclay	Potential hepato and nephrotoxicity in mice
Cloisite Na^+ and Cloisite 93A	Significant HepG2 cell death
Cloisite Na^+ and Cloisite 30B	Toxic effects with decrease in lung epithelial cell viability

[a] Compiled from references [73–76].

formation in the bilayer membranes. The G7 dendrimer (8.1 nm, 512 surface groups) initiates the formation of about 20 nm holes in the plateau regions of the lipid bilayers and expands the size of existing holes, while the G5 dendrimer (5.4 nm, 128 surface groups) does not initiate the creation of new holes in the bilayer but does expand the size of existing holes and defects. The inhibition of nanoparticle uptake into cells at low temperatures (about 4–6 °C) has generally been considered to be evidence for the inhibition of an ATP-driven endocytosis process. In this case, cell uptake of G5 dendrimer and LDH leakage ceased, whereas both LDH leakage and G7 dendrimer uptake decreased but still clearly occurred at low temperature. Since the only parameter changed in these experiments was the size and charge density of the nanoparticle, this suggested that membrane disruption, in the form of hole or pore formation, was responsible for the continued LDH leakage and uptake at low temperature [87].

5.2. *In vitro testing*

After characterization of the nanoparticles, a cell viability assay (also called cytotoxicity assays) is often performed as the first step of a toxicological study. Cytotoxicity is an important indicator for biological evaluation *in vitro* and can be defined as the adverse effects observed from reactions with structures or processes that are essential for the maintenance of cells. Some of the most popular cytotoxicity tests, such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red and lactate dehydrogenase (LDH) assays, and the most common cell lines used for cell viability and cytotoxicity testing *in vitro* have been employed for studying nanoparticles as well [89,90].

However, some studies demonstrated that the classic cytotoxicity assays may be unsuitable to investigate the toxicity of some nanomaterials. Nanoparticles can interfere with reagents inducing false-negative or false-positive results, such as changing membrane permeability and/or inducing changes in redox processes, which are essential properties to determine cell viability using MTT reduction and neutral red uptake tests. In addition, some nanomaterials can bind LDH, influencing enzyme activity and thus resulting in biased conclusions about the effective cytotoxic effect [91,92]. Thus, adequate controls should be performed to warrant that the working concentrations of the nanomaterial under study cause no interference with a specific assay.

The induction of oxidative stress has been described as an important molecular mechanism of toxicity of several nanoparticles. It is well known that DNA damage and oxidative stress are closely associated and ROS are highly reactive molecules that can alter intracellular homeostasis and react harmfully with cellular macromolecules, including lipids, proteins and DNA. The most commonly used ROS assessment assay involves the use of dichlorofluorescein diacetate (DCFDA). The method is based on the deacetylation of DCFDA by endogenous esterases to generate dichlorofluorescein (DCFH), which then reacts with ROS to form the DCF fluorophore. This assay has been widely used for evaluation of potential toxicity of nanoparticles [93]. Some conflicting results are previously reported due to interference of different experimental approaches, nanoparticle properties and sample concentrations. This highlight the importance of proper method standardization for applying DCFH to detect ROS generation by nanoparticles [94].

Evaluation of DNA damage is also a valuable indicator of toxicity. DNA disruptions can be detected by the comet assay and the micronucleous test, which are reliable, fast and relatively simple methods to evaluate the potential genotoxicity of nanoparticles as well. TEM studies revealed the distribution of silver nanoparticles to cytoplasm and nucleus of human mesenchymal stem cells. Cytotoxic effects were observed at concentrations of 10 μg/mL, and both comet assay and chromosomal aberration test showed DNA damage just after 1 h at 0.1 μg/mL [95]. In another study, both comet assay and micronucleous test were used to show evidence of DNA damage in HEK293 cells exposed to yttrium oxide (Y_2O_3) nanoparticles [96].

The main benefits of cytotoxic assays using cell cultures are the control of the environmental (temperature, pH, osmotic pressure, O_2 and CO_2 tension) and physiological conditions (maintenance of nutrient concentrations, homogeneity of the cell line, reproducible tests). However, the definition of nanoparticles dose for an *in vitro* testing system is more dynamic, more complicated, and less comparable across particle types than it is for soluble chemicals. The rates of particles settling, diffusion and agglomeration differ depending on their size, density, and surface chemistry. These properties are expected to significantly affect cellular dosage, but are largely overlooked in the toxicity studies of nanomaterials [97,98]. Experimental evidence indicates that transport of 25–50 nm and 250–500 nm CeO_2 particles to cells is quite different, depending in the former case on diffusion and the latter case on gravitational settling. This differential transport affects cellular uptake rates and possibly toxicity. Extending this analysis to include differences in transport rates revealed that differences in nanoparticle transport to cells from settling and diffusion could significantly affect relative toxicity [97]. The particle dynamics in liquid media is well studied and mathematical approaches for describing both diffusion and gravitational settling have been developed [99]. Computational models for liquid-based *in vitro* systems suggest that the dose-rates

Table 3
Examples of *in vivo* toxicity evaluation of antimicrobial nanoparticles after long-term oral exposure.

Nanomaterial	Test system	Dose range[a]	Main outcome	Reference
PVP-coated Ag	Sprague-Dawley rats	90 mg/kg for 28 d	Persistent retention of silver in brain and testes	[111]
ZnO	Swiss albino mice	300 mg/kg for 14 d	Pathological lesions in the liver	[112]
ZnO	Sprague-Dawley rats	125 mg/kg for 90 d	Persistent increased levels in systemic circulation	[47]
TiO_2	Sprague-Dawley rats	50 mg/kg for 90 d	Lung, kidney and heart injuries	[113]
TiO_2 (E171)	Wistar rats	10 mg/kg for 100 d	Inflammatory development with preneoplasic lesions in the colon	[114]
TiO_2	ICR mice	5 mg/kg for 9 months	Dysfunction of gastric secretion, inflammation, atrophy	[115]

[a] Dose range values are on basis of body weight per day.

and target cell doses are not equal for all particles. They can vary significantly, in direct contrast to the assumption of dose-equivalency implicit in the use of mass-based media concentrations as metrics of exposure for dose-response assessment [100]. Thus, additional research for refining the methodologies for nanoparticle toxicity assessment *in vitro* is still necessary.

5.3. In vivo testing

In vivo studies can offer valuable information on the integrated biological effects of nanoparticles, such as the identification of the accumulation site and toxicological profiles within a specific organ, and provide a molecular basis for tissue stress. There is a current tendency to move from *in vivo* studies using traditional animal models to *in vitro* assays, *in vivo* assays using lower organisms, and computational models for toxicity assessments [101]. The *in vitro* models are faster, less expensive, without ethical problems, but do not allow all the possible studies carried out *in vivo*. As an example, a broad toxicity study on the effect of Ag nanoparticles in the rat model was described by Wen et al. [102]. In that study, several parameters such as serum biochemistry, histopathological examination of diverse tissues, genotoxicity evaluation and chromosome aberrations in bone marrow cells were considered. It was possible to observe that intravenous administration of Ag nanoparticles induced marked increase of alanine aminotransferase, blood urea nitrogen, total bilirubin and creatinine. Cell degeneration and necrosis in the liver and kidneys observed in the Ag nanoparticles group was consistent with the bio-distribution and biochemical results, and due to the high Ag concentrations that accumulated in these organs. Finally, both Ag nanoparticles and Ag^+ produced chromosome damages to the bone marrow cells, which were mainly in the form of chromosome or chromatid breakages [102].

Many investigations about acute, subacute, and subchronic toxicity, after oral exposure in rodents indicated that acute toxicity might occur at high doses, depending on size, chemical composition and coating of the nanoparticles [20]. The lack of sufficient understanding about the toxicity of nanoparticles and the inconsistencies between studies are often associated with the variability in protocols used in cell culture and animal studies and the difficulties for analytical detection of nanoparticles in complex biological systems like food matrices and mammalian tissues [8]. In addition, the experimental animals are frequently exposed to high levels under simulated conditions.

However, the use of higher animals, especially mammals, in scientific research has been the subject of many ethical discussions, due to the number of individuals required and the suffering caused during the experiments [103]. In addition, there is the high cost of maintaining these laboratory animals. An increased attention has been devoted to alternative models for *in vivo* studies, including terrestrial invertebrates such as nematodes and insects to freshwater and marine life including planarians, crustaceans, molluscs. The most frequently used models are the transparent nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the moth *Galleria mellonella*, and the water flea *Daphnia* spp. larvae [104].

The free-living nematode *C. elegans* is considered a valuable tool in molecular biology because its fully sequenced genome and some molecular mechanisms are conserved in humans, in addition to a relatively short lifespan (approximately three weeks), its small transparent body and easy maintenance and economic [105]. Nematodes are sensitive to many different types of stress and can respond by changing their growth rate, reproductive rate, breeding size, life cycle and other properties. *C. elegans* has four organ systems, which are the same as those in vertebrates, including the neural, digestive, immune, and reproductive systems. Potential disadvantages in toxicology studies with *C. elegans* could be related with metabolic differences compared to vertebrates and the lack of circulatory system, which means that nanoparticles cannot be administered intravenously. Despite these limitations, *C. elegans* is recognized as a relevant *in vivo* model to access the toxicity risk of nanomaterials [106].

The zebrafish (*Danio rerio*) is an established model of vertebrates for the study of development and disease and is increasingly used for preclinical studies and toxicological applications due to a variety of favorable characteristics. Zebrafish require relatively cheap housing, and they are small in size, which reduces housing requirements and the amount of agent required for testing. The genomes of zebrafish and human share about 70% similarity. There is also a very good conservation of the main processes of development and physiological, with systems of key organs, such as the digestive, nervous and cardiovascular systems, similar to humans [107,108]. This strongly supports the broad equivalence in response to pharmacological agents between the two species.

Fish embryos have been also used to investigate the developmental toxicity of nanoparticles. The effect of Ag nanoparticles on embryos of catfish (*Clarias gariepinus*) was evaluated in water up to 144 h post fertilization [109]. The mortality rate, malformations, and DNA fragmentation in embryos exposed to Ag nanoparticles increased in a dose- and embryonic stage-dependent manner. Various morphological malformations and histopathological lesions including severely distorted and wrinkled notochord were observed. The results indicate that the toxicity of Ag nanoparticles in *C. gariepinus* embryos is caused by oxidative stress and genotoxicity.

5.4. Long-term toxicity

Descriptive information about nanoparticle toxicity after long-term oral exposure indicated that diverse organs and systems could be injured. Increased oxidative stress and stimulation of inflammatory response are possible effects of repeated nanoparticle exposure in the liver, heart, lungs, and brain [18,110]. Accumulation in the systemic circulation may have possible consequences in the cardiovascular system, including pro-thrombotic effects and adverse effects on the cardiac function, for example, on heart rate and myocardial infarction [20]. Some examples of long-term toxicity evaluation of antimicrobial nanoparticles after oral exposure in mammals are presented in Table 3.

The diffusion of nanoparticles through biological barriers such as the blood/brain barrier, the placenta and the blood/milk barrier,

may be observed after exposure for long periods [116,117]. Despite additional examination is required, this could cause neurotoxic and embryotoxic effects because of nanoparticle exposure. Although the teratogenicity of nanoparticles on the fetus is of great concern, alterations in the reproductive index and offspring development need to be investigated as some nanoparticles were described to move across the placenta-blood barrier in rats.

6. Concluding remarks

The increased use of nanomaterials in diverse fields is a current reality. The food sector also incorporate nanotechnology with the promise of improving food quality and safety. Despite intensive research and advances were reached in the field of nanobiotechnology during the last decade, a careful and detailed characterization of nanoparticle properties and intensive validation of assay methods for toxicity assessment are still required. Additional studies in real foods are necessary to ensure the effectiveness and safety of nanomaterials. Due to the complexity of the food matrices, it is important that such studies could simulate the conditions of food processing, storage and transit through the gastrointestinal tract. There are limited information about the toxicity of nanoparticles in relation to their specific interactions with food matrices and what interactions of these particles in the digestive tract play a key role in the risk assessment to the consumer. The main points are related to their biological reactivity, the ability to cross biological barriers and accumulate in target organs, their biopersistence and how they can induce a dose-response biological effect. Internationally granted protocols for the toxicity tests are necessary to attain more standardized data on the wide variety of nanoparticles.

Declaration of Competing Interest

None to declare.

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