Mucus and microbiota as emerging players in gut nanotoxicology: the example of dietary silver and titanium dioxide nanoparticles

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Abstract

Given the growing use of nanotechnology in many common consumer products, including foods, evaluation of the consequences of chronic exposure to nanoparticles in humans has become a major public health issue. The oral route of exposure has been poorly explored, despite the presence of a fraction of nano-sized particles in certain food additives/supplements and the incorporation of such particles into packaging in contact with foods. After their ingestion, these nanoparticles pass through the digestive tract, where they may undergo physicochemical transformations, with consequences for the luminal environment, before crossing the epithelial barrier to reach the systemic compartment. In this review, we consider two examples, nano-silver and nano-titanium dioxide. Despite the specific features of these particles and the differences between them, both display a close relationship between physicochemical reactivity and

bioavailability/biopersistence in the gastrointestinal tract. Few studies have focused on the interactions of nanoparticles of silver or titanium dioxide with the microbiota and mucus. However, the microbiota and mucus play key roles in intestinal homeostasis and host health, and are undoubtedly involved in controlling the distribution of nanoparticles in the systemic compartment.

Keywords

dietary nanoparticle, silver, titanium dioxide, gut, mucus, microbiota

1. Introduction

Nanomaterials are increasingly used in many sectors of industry (the automobile, electronics, environmental, hygiene, biomedical and agro-food industries) and in many common or innovative consumer products (e.g. textiles, catalysts, detectors, imaging, coatings, packaging, sunscreen, nutraceuticals). Their metrological forms differ according to the target application (nanoparticles, nanoclays, nanotubes or nanofibers), but their utility lies in their nanometric dimensions and their high specific surface area (up to several hundred m^2/g of product), resulting in unique properties (mechanical, physicochemical or biological) differentiating them from their larger counterparts (Nel *et al.* 2006). In October 2011, the European Commission defined a "nanomaterial" as "a natural, manufactured or accidentally formed material containing free particles in the form of aggregates or agglomerates, in which at least 50% of the particles, in terms of numerical distribution by size, have one or more external dimensions between 1 nm and 100 nm." However, in accordance with European recommendations (2011/696/UE: JOUE no. L275-21.10.2011), in certain specific cases (safety, public health, environmental protection, competitiveness), this threshold of 50% can be expanded to a range from 1% to 50%.

Given the growing use of nanotechnology products in our everyday environment, including the addition of inorganic nanoparticles (e.g. metal oxides, silver, amorphous silica) to food, evaluations of the consequences of chronic exposure in humans have become a real public health issue. Few studies have investigated the oral route of exposure and related toxicological hazards to consumer health (Chaudhry *et al.* 2008). However, some nanometric inorganic particles are found in food additives/supplements or incorporated into packaging in contact with food, to add texture or improve sensorial, gustatory, nutritional, visual, mechanical or antimicrobial properties

(Chaudhry *et al.* 2008). Once ingested, these nanoparticles pass through the gastrointestinal tract, where they may be subject to morphological and physicochemical transformations and may affect the luminal environment, before eventually crossing the epithelial barrier to reach the systemic compartment. In this review, we present two examples, nano-silver and nano-titanium dioxide (TiO_2), which differ in terms of their physicochemical properties, solubility and uses, and, thus, in their potential impact on host physiology. We focus, in particular, on two key elements of the biological barrier function in the intestine: the microbiota and the mucus. The intestinal microbiota is defined as all the microorganisms resident in the digestive tract, and the mucus layer is a viscoelastic gel that covers and protects the epithelium. The mucus and/or microbiota may affect the fate and potential effects of nanoparticles in the rest of the body (Figure 1). We also deal with the physicochemical processes governing the biotransformation of these nanoparticles on contact with intestinal fluids. Various aspects of the two examples presented here, nano-silver and nano-TiO_2, may be studied to address the issue of their toxicity following ingestion by humans, and to improve our understanding of the risks inherent to the incorporation of nanoparticles into our diet.

2. Dietary exposure to silver nanoparticles

2.1. Silver nanoparticles in food

Over the last decade or so, with the advent of nanomaterials, silver nanoparticles (or "nano-silver") have been shown to be useful for many medical and technological applications (Tolaymat *et al.* 2006, Ahamed *et al.* 2010). The term "nano-silver" was coined in the wake of regulations still in force today, taking into account only the chemical composition of the generic substance. In reality, this umbrella term covers a wide range of nanoparticles differing in size,

morphology or surface functional groups. The non-exhaustive list of products available, based on the French market as a representative example, reveals a large number of applications making use of aggregated, agglomerated or colloidal forms of nano-silver (AFSSET 2010, ANSES 2015). The surface of the nanoparticles of interest is often covered with ions (citrate), molecules (cysteine) or polymers (polyvinylpyrrolidone (PVP)). These nanoparticles have been incorporated into everyday products, principally for antibacterial and antifungal purposes (Lansdown 2002). It has been estimated that about 320 metric tons of nano-silver are produced and used annually worldwide (Nowack *et al.* 2011). In Europe, production levels are moderate, at about 10 metric tons per year, although this estimate is based on highly variable data (Piccinno *et al.* 2012).

It is difficult, if not impossible, to generate a precise list of all the products containing silver nanoparticles available on the market, and present at intermediate stages from production to distribution, because these products are sold under different trade names and are freely available for purchase online. According to the Woodrow Wilson International Center for Scholars in the US (2013), consumer products containing nano-silver have been the nanoproducts most widely available on the market since 2011, and the number of such products is steadily increasing (EFSA 2014, ANSES 2015). In France, it is now mandatory to declare all nanoparticle substances manufactured, imported or distributed in the country (https://www.r-nano.fr/), but this has not made it possible to improve the exhaustiveness and reliability of the inventory in an entirely satisfactory manner, so as to ensure the traceability of nano-silver throughout its life cycle (French Administration, 2014).

Few data are currently available concerning the silver content of foodstuffs. However, it has been established that wheat flour and bran contain 0.4 and 1.0 mg silver/kg dry weight, respectively (US EPA, 1980). The silver contents of shellfish and meat have been estimated at 0.1-10.0 mg/kg dry weight and 0.004-0.024 mg/kg wet weight, respectively. High silver contents, of the order of several hundreds of milligrams per kilogram, have also been reported for some mushrooms (US EPA, 1980). Indeed, since the middle of the 20th century, silver has been entering prepared foods, principally through contact with packaging and storage sites (Kehoe *et al.* 1940). Silver is also used in its elemental form as a food additive (E174 in a powder form), to color the surface of some products, such as cakes and sweets. In humans, daily silver intake varies from 0.4 to 27 µg per individual (Hadrup and Lam 2014), corresponding to a range of exposure extending from 0.007 to 0.5 µg/kg body weight (bw)/day. However, higher intakes, of up to 90 µg/day have occasionally been reported (Wijnhoven *et al.* 2009). In 2016, EFSA re-evaluated the Europe-wide risks of such high levels of consumption, by re-assessing the food additive E174. In particular, the categories of foods concerned and the levels of exposure in target populations were reported (EFSA, 2016). For example, the mean amount of silver ingested was found to be between 1.60 and 3.47 µg/kg bw/day in children and between 1.29 and 2.65 µg/kg bw/day in adults. E174 accounts for about 30% of all possible sources of exposure to silver, through the ingestion of water and milk in children, and shellfish and crustaceans in adults. Experts have also stressed the need to characterize the nanodimensional fraction of particles present in the commercial powder, together with silver ion release. Silver may not only be present in a nanoparticle form in food, as additives such as E174, but it may also be used in other applications linked to food. For example, nano-silver may be added to supplements and

food packaging and to the internal coating of refrigerators (Chaudhry *et al.* 2008). Many studies have shown that nano-silver is unstable, resulting in the leaching of silver ions from silver nanoparticles after oxidation upon exposure to air, water or saline solutions (Hlidek *et al.* 2009; Saulou *et al.* 2009; Körner *et al.* 2010; Zanna *et al.* 2010).

In 2014, the European Commission highlighted the scarcity, or even total absence, of information about the consequences of silver nanoparticle ingestion and long-term exposure in the general population (Epstein *et al.* 2014), particularly for the intestinal barrier. Nevertheless, in addition to the exposure of the population to nano-silver in the workplace (inhalation, via the skin), exposure by ingestion cannot be ruled out, given the many dietary sources of silver now available on the market. This oral exposure may occur either directly, through the consumption of products containing silver nanoparticles, or indirectly, through the ingestion of foods contaminated with silver released by products with which they have been in contact (packaging, refrigerator coatings, storage containers or other equipment and coatings) or through concentration in the food chain. The labels of food supplements containing nano-silver tend to make claims such as “purification and conservation of unknown targets”, “boosts the immune system” and “useful against serious diseases”. In the absence of evaluation by regulatory agencies, all such claims must be considered unfounded (Epstein *et al.* 2014).

2.2. Influence of intestinal fluids on the fate of silver nanoparticles and their ability to cross the intestinal barrier: in vitro *approach*

The fate and bioavailability of ingested silver nanoparticles depend, at least partly, on their properties, such as their size and surface chemistry, which influence their physical and chemical reactivity during their transit through the gastrointestinal tract. Mwilu *et al.* (2013) investigated

the interactions between synthetic gastric fluid and silver nanoparticles of different sizes, with and without coating agents. Changes in the chemical composition, morphology and size of the nanoparticles were observed after 15 minutes of contact. The nanoparticles tended to agglomerate through the release of silver ions, leading to the formation of a precipitation of silver chloride on their surface. The smallest particles (< 10 nm) had higher aggregation rates than larger particles (75 nm). Similarly, Böhmert *et al.* (2014) described the physicochemical characterization of silver nanoparticles after passage through an *in vitro* system mimicking the digestion process (i.e., contact between nanoparticles and synthetic saliva and artificial gastric and intestinal juices). The cytotoxicity of the nanoparticles was then evaluated in cultured Caco-2 human epithelial cells. In the study conditions used, contact with intestinal fluids led to an increase in particle size linked to changes in the conformation of stabilizing agents and/or protein adsorption (formation of a "corona") rather than aggregate formation. These morphological and physicochemical changes had no deleterious effects on Caco-2 cells. In particular, no cytotoxicity of the nanoparticles to proliferating or differentiated cells was observed. In another study (Bouwmeester *et al.* 2011), Caco-2 epithelial cells were co-cultured *in vitro* with M cells (microfold cells) from the dome of aggregated lymphoid tissues forming Peyer's patches in the small intestine (i.e., the immune sensors of the gut). These M cells specialize in the capture of inorganic and organic antigens for presentation to immune cells located in the Peyer's patches for the induction of immune tolerance or defense against pathogens. After four hours of exposure, the translocation of silver nanoparticles, essentially in the form of silver ions, was observed, but the mechanisms involved were not determined. This translocation had no effect on the integrity of the epithelial barrier in this co-culture model. By contrast, a transcriptomic

analysis carried out after four hours of contact between cells and nano-silver demonstrated the overexpression of certain genes involved in stress responses (oxidative stress, endoplasmic reticulum stress, apoptosis), although this response to silver did not appear to be specific to either its nanoparticle form or its metallic nature (i.e., same general response to stress observed for silver ions and marine toxins) (Bouwmeester *et al.* 2011).

2.3. Oral exposure and fate of silver nanoparticles in the intestine: from animal models to humans

Few studies have investigated the fate of ingested nano-silver in the gut *in vivo*. In this review, we describe only the results obtained for certain mammals (rodents, pigs), although many other studies have provided concordant results concerning the toxicological impact of nano-silver on other animal species, from aquatic environments in particular (Asharani *et al.* 2008, Bilberga *et al.* 2010, Scown *et al.* 2010, García-Alonso *et al.* 2011, Kwok *et al.* 2012). Most of these studies aimed to evaluate the potentially deleterious effects of silver nanoparticles on the body after oral exposure. However, Fondevila *et al.* (2009) evaluated the possibility of using nano-silver to replace antibiotics as a growth factor in animal feed.

No clear consensus concerning the bioavailability and toxicity of nano-silver in the intestine emerges from these studies, probably at least partly due to the differences in experimental conditions between studies. Indeed, the studies carried out to date have used silver nanoparticles of different sizes (10 to 320 nm), prepared in different ways (presence or absence of stabilizing agents, such as PVP or carboxymethyl cellulose). They have also differed in terms of:

(i) The animal models used (rats, mice, pigs);

(ii) The duration of exposure (14 to 90 days, with *ad libitum* administration or single vs. repeated daily oral gavage);

(iii) The doses administered (between 0.25 and 1000 mg/kg bw; note that a NOAEL (no observable adverse effect level) of 30 mg/kg bw was determined for rats (Kim *et al.* 2010));

(iv) The control conditions (inclusion or absence of tests with ionic silver in the form of silver nitrate or silver acetate).

These differences render extrapolations and the proposition of generic mechanisms of action difficult. Nevertheless, most of the studies carried out have given convergent results concerning the distribution of nano-silver, as follows:

(i) Size- and dose-dependent accumulation in certain target organs (kidneys, liver, spleen, brain, lungs, testicles) (Kim *et al.* 2008, Kim *et al.* 2010, Park *et al.* 2010, Loeschner *et al.* 2011, van der Zande *et al.* 2012, Lee *et al.* 2013);

(ii) Differences in the distribution of silver between males and females, particularly in the kidney (Kim *et al.* 2008, Kim *et al.* 2010);

(iii) Persistence for several weeks after the end of oral exposure, particularly in the brain and testicles (van der Zande *et al.* 2012, Lee *et al.* 2013);

(iv) Considerable similarity in the responses induced by nano-silver and ionic silver (Loeschner *et al.* 2011, van der Zande *et al.* 2012).

In the intestine, the silver ingested by rats, whether as nanoparticles or in ionic form, is found in the ileal tissue (and, to a lesser extent, in the stomach), principally in the form of nanometric nodules associated with the elements sulfur and selenium, in the lysosomes of macrophages in

the lamina propria, in the basal layer of the epithelium and in the submucosa (Loeschner *et al.* 2011). Levels of excretion in the feces seem to be higher for nano-silver than for ionic silver. An exacerbated inflammatory response in the presence of silver nanoparticles has been described in mice (Park *et al.* 2010). It has also been shown that the administration of nano-silver triggers the formation of lesions of the intestinal glands and microvillosities of the epithelial cells of the small intestine in mice, partly accounting for the observed weight loss in these animals, due to a decrease in the surface area available for absorption (Shahare and Yashpal 2013).

To date, only one study has been carried out on humans. This study concerned 60 healthy volunteers in the US, who were studied after the daily ingestion, over a period of 14 days, of a commercial nano-silver product (doses tested: 10 and 32 µg/mL, corresponding to 150 µg/day and 480 µg/day, respectively) (Munger *et al.* 2014). In the conditions studied, no abnormalities were observed in the clinical features of the study subjects, although the authors stressed the need to evaluate the effects of longer term exposure.

3. Dietary exposure to TiO_2 nanoparticles

3.1. Nanoparticles of titanium dioxide in food

Titanium dioxide is a white pigment that is widely used for its brightness and very high refractive index in paints, coatings, plastics, papers, inks, medicines, pharmaceuticals, food products, cosmetics and toothpastes. It accounts for 70% of world pigment production, with 5000 metric tons produced per year, expected to rise to 60000 metric tons by 2025. In the food industry, it is used in an ultrafine form as a white coloring (referred to as food-grade additive E171 in EU) for confectionery, sauces, cakes and pastries. In the US, the Food and Drug Administration approved the use of food-grade TiO_2 in 1966 with the stipulation that TiO_2

content should not exceed 1% of the food by weight (Joint FAO/WHO Expert Committee on Food Additives, 2006). In Europe, the current EU Directive 94/36/EC authorizes the use of E171 in foodstuffs without the establishment of an acceptable daily intake by the Joint FAO/WHO Expert Committee on Food Additives, because TiO_2 absorption was considered to be very low (EFSA Panel on Food Additives and Nutrient Sources Added to Food, 2005). The particles used as coloring agents are principally around 200-300 nm in size, but nanoparticles (<100 nm) are nevertheless present in the powder during the manufacturing process, accounting for 17 to 36% of the particles present, depending on the commercial supplier of E171 (Weir *et al.* 2012, Yang *et al.* 2014), and are easy to extract from a food matrix such as chewing gum (Chen *et al.* 2013). The levels of nano-TiO_2 ingestion in food are higher than those for other nanoparticles, and children under the age of 10 years have particularly high levels of exposure, with a TiO_2 consumption of between 1 and 3 mg/kg bw/day (Weir *et al.* 2012). In addition, the daily use of toothpaste (which contains TiO_2, either as the additive E171 or as an ingredient called CL77891 in the INCI nomenclature used for cosmetic products) increases oral exposure to TiO_2 (Lomer *et al.* 2004). The synthesis of nanocomposites of silver and TiO_2 has also been proposed for antimicrobial packaging applications (Yu *et al.* 2011). Current knowledge concerning the detection of nano-TiO_2 in food, the fate of such nanoparticles in the body and their potentially deleterious effects was summarized in a recent review (Bettini and Houdeau 2014). This previous review dealt with advances in our understanding of the mechanisms by which nanoparticles cross biological barriers, including the intestinal epithelium. We, therefore, chose to focus here on aspects relating to the physicochemical processes of nano-TiO_2 transformation in the luminal compartment of the intestine.

3.2. Influence of intestinal fluids on the fate of TiO_2 nanoparticles: in vitro *approach*

It has recently been shown, with reconstituted medium (or "juices") mimicking the gastric (pepsin in HCl, pH 2) and intestinal (gastric juice supplemented with pancreatin and bile extract, pH 7) compartments, that TiO_2 nanoparticles undergo changes in size and surface charge in these compartments, due to agglomeration and the adsorption of proteins to the surface (formation of a protein "corona") (Brun *et al.* 2014). Similar conclusions have been drawn before, despite the use of reconstituted juices of different compositions, with different characteristics (pepsin in HCl, pH 1.2 for the gastric environment; trypsin, KH_2PO_4 in NaOH, pH 6.8 for the intestinal environment) (Wang *et al.* 2013). Cho *et al.* (2013) showed that, unlike ZnO nanoparticles, TiO_2 nanoparticles dissolved poorly in a model gastric environment (pH 1.5) and in intestinal conditions (pH 7.4). The composition of the artificial juices used in this study was not specified. According to the authors, the lower solubility of TiO_2 nanoparticles than of ZnO nanoparticles may contribute to their lower levels of uptake into the systemic compartment after oral administration (<5%) (Bettini and Houdeau 2014). As for nano-silver, the physicochemical reactivity of TiO_2 nanoparticles is closely related to their biopersistence in the gastrointestinal tract. None of these studies took into account the presence of the microbiota in the intestinal lumen, which probably modifies the physicochemical properties of the nanoparticles and their bioavailability for absorption.

4. Interactions between nanoparticles, mucus and microbiota in the gut: *in vitro* and *in vivo* approaches

Most of the published data for the translocation of nanoparticles concern the epithelial barrier, and were obtained through *in vitro* studies on Caco-2 intestinal cells (Gerloff *et al.* 2012, Gerloff

et al. 2013, Böhmert *et al.* 2014, Böhmert *et al.* 2015) or TR146 buccal cells (Tay *et al.* 2014). The conditions in these tests effectively mimic an integral biological membrane potentially endowed with the capacity to absorb nanoparticles, but they are not very representative of the real environment in the gut. Indeed, although there is increasing evidence to suggest that the microbiota and mucus are major players in digestive health (Johansson *et al.* 2013, Donaldson *et al.* 2016), these two factors have often been neglected in food toxicology and are rarely considered, either separately or together, in the context of nanoparticles. Pietroiusti *et al.* (2016) recently reviewed the gut microbiota/microbiome-mediated effects of engineered nanomaterials and pointed out that such evaluations were clearly in their infancy. Possible toxicity-driven clinical implications (colitis, obesity, immunological dysfunctions) were also highlighted, although beneficial therapeutic applications (dietary supplements, treatment of infections) could not be ruled out (Pietroiusti *et al.* 2016). In their review, Fröhlich and Fröhlich (2016) confirmed the lack of studies investigating the potential effects of food nanoparticles on the oro-gastrointestinal microbiota of the host. The authors also highlighted the role of mucus as a selective barrier preventing both bacteria and nanoparticles from penetrating intestinal cells.

4.1. Nanoparticles and the intestinal microbiota

The intestinal microbiota plays an essential role in digestive health. The gastrointestinal tract houses 10^{14} microorganisms, consisting predominantly of bacteria from two major phyla, the Bacteroidetes and the Firmicutes, associated with 500 or even 1000 different species (Gill *et al.* 2006, Tap *et al.* 2009, Marchesi 2011). In addition to the endogenous population, other microorganisms, such as lactic acid bacteria, may be supplied transiently by diet. The microbiota thus constitutes a considerable reservoir of enzymes and metabolites. It contributes actively to

the maintenance of host homeostasis, by carrying out several key functions in protection, maturation and metabolite production. The intestinal microbiota acts as a barrier against pathogens, preventing their implantation. It ferments the substrates available in the colon, producing a large range of metabolites, including short-chain fatty acids, an essential energy source for the epithelial barrier. It also participates in xenobiotic metabolism and in the production of essential vitamins present in insufficient quantities in food (Gill *et al.* 2006). Finally, it is essential for the education and maturation of the immune system and the intestinal epithelium (Olszak *et al.* 2012, Tomas *et al.* 2013).

Studies of the relationships between nanoparticle ingestion, modulation of the microbiota and impact on host health are scarce (Table 1). Merrifield *et al.* (2013) showed, in adult zebrafish, that exposure to silver nanoparticles (500 mg/kg food) for 14 days had no effect on the richness and diversity of the microbiota. However, a similar treatment with copper nanoparticles (500 mg/kg food) modified the composition of the microbiota, with a loss of *Cetobacterium somerae*, a bacterial species that produces vitamin B12. The oral administration of PVP-stabilized 14-nm silver nanoparticles for 28 days in rats (4.5 mg or 9.0 mg/kg bw/day, once- or twice-daily oral gavage) did not alter the amount of Firmicutes or Bacteroidetes in the cecum (Hadrup *et al.* 2012). Similarly the oral administration of silver nanoparticles of two different sizes (20 and 110 nm) and with two different coatings (PVP and citrate) for 28 days in mice (10 mg/kg bw/day) did not change the membership, structure and diversity of the gut microbiome (Wilding *et al.* 2015). By contrast, another study (Williams *et al.* 2015) on rats fed by twice-daily oral gavage for 13 weeks with silver nanoparticles of various sizes (10, 75 and 110 nm) and at various doses (9, 18 and 36 mg/kg bw/day) reported a general increase in the levels of Gram-

negative bacteria in the ileum. Treatment with 10-nm particles led to a decrease in levels of Firmicutes, particularly for members of the genus *Lactobacillus*. However, this effect did not appear to be specific to the nanoparticle form of silver, because a similar effect was noted with silver acetate (Williams *et al.* 2015). In another *in vivo* study on mice (Lecloux *et al.* 2015), 28 days of exposure to silver (46, 460 or 4600 µg/L) or silica (5, 50 or 500 mg/L) nanoparticles resulted in a significant decrease in bacterial richness. At the phylum level, a significant and dose-dependent increase in Firmicutes, accompanied by a decrease in Bacteroidetes, was observed for nano-silver. Differences in the size and concentration of silver nanoparticles, and in the mode of administration (duration, frequency and animal model), intestinal region of interest, species-specific microbiota composition and method used for microbiota analysis, may account for these discrepancies.

Most of the published results for nano-silver concern the composition of the microbiota rather than its metabolic activity (e.g., the production of short-chain fatty acids). However, in the study by Das *et al.* (2014) on a synthetic stool mixture of 33 different isolates (derived from a healthy human donor) subjected to anaerobic batch fermentation, physiological and cellular responses (respiration, fatty acid levels) were characterized as specific signatures of the nano-silver-mediated changes in the bacterial communities. The detailed mechanisms underlying the antibacterial action of silver nanoparticles against the gut microbiota have recently been reviewed, focusing on the model bacterium *Escherichia coli*, and comparisons of this bacterium with other gram-positive and gram-negative bacteria (Fröhlich and Fröhlich 2016).

The effects of TiO_2 nanoparticles on the composition of the intestinal microbiota are largely unknown, including possible differences in the ability of bacteria to trap and take up

nanoparticles, with consequences for their own growth and metabolic activity. In a recent *in vitro* study (Taylor *et al.* 2015), the exposure of a gut microbial community from a healthy donor to three different types of metal oxide nanoparticles (3 mg/L TiO_2, 0.01 µg/L ZnO or 0.01 µg/L CeO_2) in a model colon for five days in the dark induced changes in the phenotypic traits of the gut community, including short-chain fatty acid production (particularly for butyric acid), cell hydrophobicity, sugar content of extracellular polymers, cell size and electrophoretic mobility (an indicator of cell surface charge). Most published studies on interactions between bacteria and TiO_2 have focused essentially on *E. coli* (Liu *et al.* 2010, Kumar *et al.* 2011, Zhukova *et al.* 2012) and the antibacterial properties of nano-TiO_2. These properties are generally associated with the photocatalytic effects of the compound, although damage to the external lipopolysaccharides of *E. coli* has also been reported, together with a decrease in membrane fluidity, after treatment with TiO_2 alone, in the absence of UV irradiation (Liu *et al.* 2010). In *E. coli* cultured in the dark, TiO_2 nanoparticles can induce both oxidative stress (i.e., generation of reactive oxygen species and a decrease in glutathione level accompanied by an increase in lipid peroxidation) and DNA damage (Kumar *et al.* 2011). Zhukova *et al.* (2012) showed, also in *E. coli* cultured in the dark, that electrostatic attraction could potentially occur between TiO_2 nanoparticles and bacteria, due to their opposite surface charges.

4.2. Nanoparticles and the intestinal mucus

Mucus is a translucent viscoelastic gel that lines and protects the intestinal epithelium, separating it from the lumen contents, in many animals, including mammals, insects and fish. It is secreted continuously along the whole intestine by specialized goblet cells in the epithelium, and is present in larger amounts in the colon than elsewhere. Mucus is a highly hydrated gel, consisting

principally of water (95%), but it also contains mucins (highly glycosylated proteins with a molecular weight of 0.5 to 20 MDa) (Bansil and Turner 2006), ions, lipids, antimicrobial molecules and exfoliated epithelial cells. Mucus functions as a dynamic barrier that is permeable to gases, water and nutrients, but impermeable to most microorganisms. It therefore acts as a veritable selective filter, providing protection against pathogen entry. Mucus was long considered to act as a "simple" physical barrier, but it is now known to have other key functions essential for the preservation of intestinal homeostasis (Johansson *et al.* 2011, Juge 2012, Ouwerkerk *et al.* 2013), including (i) lubrication of the epithelium, facilitating the progress of material along the digestive tract, (ii) maintenance of a stable microenvironment at the epithelial surface, (iii) protection of the epithelium through the presence of immune system molecules and (iv) provision of an ecological niche for the bacteria of the intestinal microbiota (see below).

Mucus covers the intestinal epithelium to different extents along the intestinal tract. In the small intestine, the mucus fills the space between the villi and covers them, but is not attached to the epithelium (Ermund *et al.* 2013). In the colon, the mucus is organized into two layers: an inner, stratified mucus layer that adheres firmly to the epithelial cells and is approximately 50 μm thick; and an outer, non-attached layer that is usually approximately 100 μm thick in mice (Johansson *et al.* 2011). These mucus layers are organized around the highly glycosylated Muc2 mucin, forming a large, net-like polymer continuously secreted by the goblet cells. The inner mucus layer is dense and impenetrable to bacteria, thereby ensuring that the epithelial cell surface remains free from bacteria. The inner mucus layer is subsequently converted into the outer layer, mostly through host protease activities, because this transition process also occurs in the mucus of germ-free mice (Johansson *et al.* 2008). There is increasing evidence to suggest that intimate

mucus/microbiota crosstalk occurs in the gut, but this crosstalk has never been explored in the context of oral exposure to nanoparticles. Indeed, the outer colonic mucus layer is the natural habitat of the commensal bacteria (Johansson *et al.* 2011). A proteolytic increase in MUC2 mucin pore sizes allows bacteria to penetrate into the net-like structure of the mucin and to gain access to the diverse mucin-bound carbohydrates that they can use as an energy source (Backhed *et al.* 2005; Sonnenburg *et al.* 2005; El Kaoutari *et al.* 2013) or as preferential binding sites through bacterial adhesins (Juge 2012). It has even been suggested that the enormous repertoire of potential ligands and/or nutritive sources could explain the regio-specific colonization of bacteria in the gut (Robbe *et al.* 2004). The composition of the microbiota has recently been shown to shape the mucus phenotype in the colon of conventional mice (Jakobsson *et al.* 2015). Bacterial lipopolysaccharides, peptidoglycan and short-chain fatty acids are probably also involved, as they can stimulate mucus production (Burger-van Paassen *et al.* 2009, Petersson *et al.* 2011).

Few studies have focused on the relationships between nanoparticles and mucus, particularly for nano-silver and nano-TiO_2 (Table 2). Only potential therapeutic applications have received sustained attention (Ensign *et al.* 2012). Behrens *et al.* (2002) established, *in vitro*, with cultured cells with (HT29-MTX Cl.E12) and without (Caco-2) mucus secretion, that mucus acted as a barrier to nanoparticles with different surface physicochemical properties (hydrophobicity, electronegativity). It has also been shown, *ex vivo* on excised porcine buccal mucosa, that 200-nm negatively charged carboxyl polystyrene particles form aggregates and are entrapped within the mucus (Roblegg *et al.* 2012), whereas non-functionalized neutral particles of the same size permeate the mucus layer and penetrate into the epithelial tissue (Teubl *et al.* 2013). For nano-

silver, the first *in vitro* study in the field, performed in a Caco-2/HT29-MTX mucus-producing co-culture, revealed that the mucus layer was able to trap 200-nm silver nanoparticles, thereby reducing their interaction with the cellular membrane and resulting in lower levels of toxicity, oxidative stress, IL-8 release and proteomic alterations than for 20-nm silver nanoparticles and silver nitrate (Georgantzopoulou *et al.* 2016). To date, the only study of oral exposure and its consequences performed *in vivo* (Jeong *et al.* 2010) demonstrated that 28 days of exposure to silver nanoparticles (60 nm) in rats promoted the secretion of mucus in the ileum and rectum and changes in mucin composition (amounts of neutral and acidic mucins and proportions of sulfated and sialylated mucins).

Similarly, few studies have reported results for nano-TiO_2, and most reports to date have concerned the use of cell models *in vitro* (Chen *et al.* 2011, Brun *et al.* 2014). TiO_2 nanoparticles have been shown to stimulate mucin secretion by human ChaGo-K1 bronchial epithelial cells in the airways (Chen *et al.* 2011). In the intestine, different capacities for the absorption and transport of TiO_2 nanoparticles have been described (Brun *et al.* 2014), depending on whether the epithelial cells are cultured alone or with goblet or M cells. Caco-2 cells in monoculture displayed only low levels of intracellular nano-TiO_2 accumulation after 24 hours of contact, whereas the same treatment in the presence of goblet cells (Caco-2/HT29-MTX mucus-producing co-culture) led to 50 times higher levels of accumulation (Brun *et al.* 2014). The co-culture of Caco-2 cells with RajiB cells (which reproduces an epithelium consisting of M cells) resulted in 100 times higher levels of accumulation (Brun *et al.* 2014). This suggests that the goblet cells are half as efficient as M cells for nanoparticle uptake from the gut lumen, but it also shows that these mucus-producing cells are an important pathway for nanoparticle translocation

through the gut. *Ex vivo* studies focusing on the contribution of the oral cavity in risk assessments for nano-TiO_2 uptake have recently been performed on the porcine buccal mucosa (Teubl *et al.* 2015a, Teubl *et al.* 2015b). In particular, it was shown that TiO_2 nanoparticles, regardless of their size and hydrophilicity/hydrophobicity, were able to permeate the mucus layer and penetrate into the underlying tissue. As highlighted by Fröhlich and Roblegg (2012) and illustrated here, the existing *in vitro* cell models now require adaptation or improvement, not only to integrate mucus more effectively as a key interface in the fate of nanoparticles, but also to make it possible to apply "impaired" intestinal barrier conditions. These authors also highlighted the difficulties involved in the evaluation *in vivo* of the effects of ingested nanoparticles in the gut, due to differences between species (rodents vs. humans) and probable variability between individuals, not only in terms of the composition and thickness of mucus, but also in terms of the properties of the microbiota, physiological characteristics and environmental factors.

4. Conclusion

Despite the rapidly increasing use of nanoparticles in food, evaluations of the consequences of chronic oral exposure are scarce. Agencies involved in risk assessment remain vigilant, but the introduction of nanotechnologies in the food industry raises many questions, particularly given that nanoparticles, with their high surface reactivity, may be able to cross certain biological barriers. The diversity of models and experimental conditions (type, metrological form, size distribution, and dose of nanoparticles; *in vitro*, *ex vivo* and *in vivo* approaches; animal models and modes of administration/exposure; control conditions) makes it even more difficult to analyze the results and to define generic rules governing the impact of nanoparticles on animal

and human health. Our findings for nano-TiO_2 and nano-silver, despite the differences between these particles (desired properties, high capacity of nano-silver to dissolve and to be released in an ionic form), indicate that, for both types of particle, there is a close relationship between physicochemical reactivity (changes in size, charge, surface hydrophilicity, ionization) and bioavailability/biopersistence in the gastrointestinal tract. Concerning the selective barrier function of the intestine, we demonstrate that the mucus and microbiota are tightly connected emerging players in gut nanotoxicology that should be investigated further in future studies. The structure of the food matrix and its deconstruction throughout the digestion process may also be key parameters in the kinetics of nanoparticle availability/spread throughout the body, although these aspects are rarely considered when defining experimental strategies. Another element that should be taken into account in future studies is the influence of the mode of exposure, mostly in healthy individuals. However, it seems likely that the deleterious effects of ingested nanoparticles might be potentiated or worsened by disruption of intestinal homeostasis, under pathophysiological conditions (impaired intestinal barrier function, gut inflammation) for example. The questions raised here highlight the broad range of issues still to be explored to improve risk assessment for the exposure of humans to nanoparticles.

The authors have no conflict of interest to declare.

Acknowledgments

We would like to thank Claire Saulou-Bérion (INRA, UMR782 Génie et Microbiologie des Procédés Alimentaires, AgroParisTech, Thiverval-Grignon, France) for fruitful discussions and critical reading of the manuscript.

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Table 1. Effect of nano-silver and nano-TiO_2 on bacteria of the intestinal microbiota: *in vivo* and *in vitro* approaches.

Dietary nanoparticle	Experimental conditions	Main results	References
Nano-silver	Zebrafish: 14-day exposure (60 nm; 500 mg/kg food)	No effect on the richness and diversity of the microbiota	Merrifield *et al.* (2013)
	Rats: 28-day exposure (14 nm, PVP-stabilized; 4.5 mg or 9.0 mg/kg bw/day)	No effect on the amount of Firmicutes or Bacteroidetes in the cecum	Hadrup *et al.* (2012)
	Mice: 28-day exposure (20 and 110 nm, PVP and citrate coatings; 10 mg/kg bw/day)	No changes in the membership, structure and diversity of the gut microbiome	Wilding *et al.* (2015)
	Rats: 13-week gavage (10, 75 and 110 nm; 9, 18 and 36 mg/kg bw/day)	Increase in Gram-negative bacteria in the ileum 10-nm particles: decrease in Firmicutes (*Lactobacillus*)	Williams *et al.* (2015)
	Mice: 28-day exposure (46, 460 or 4600 µg/L)	Reduction of bacterial richness Dose-dependent increase in Firmicutes and decrease in Bacteroidetes	Lecloux *et al.* (2015)

	In vitro: synthetic stool mixture, anaerobic batch fermentation	Changes in bacterial communities Physiological and cellular alterations (respiration, fatty acids)	Das *et al.* (2014)
Nano-TiO_2	*In vitro*: gut microbial community, 5-day model colon	Changes in short-chain fatty acids, cell hydrophobicity, sugar content of the extracellular polymers, cell size and electrophoretic mobility	Taylor *et al.* (2015)
	In vitro: culture of *E. coli* (no UV irradiation)	Damage to LPS Decrease in membrane fluidity	Liu *et al.* (2010)
	In vitro: culture of *E. coli* (in the dark)	Oxidative stress DNA damage	Kumar *et al.* (2011)
	In vitro: culture of *E. coli* (in the dark)	Electrostatic attraction bacteria/nanoparticles	Zhukova *et al.* (2012)

Table 2. Effect of nano-silver and nano-TiO_2 on buccal and intestinal mucus: *in vivo*, *ex vivo* and *in vitro* approaches.

Dietary nanoparticle	Experimental conditions	Main results	References
Nano-silver	*In vitro*: Caco-2/HT29-MTX cell co-culture, 2-h (oxidative stress) or 24-h contact (other parameters)	Mucus-mediated trapping of 200-nm sized nanoparticles: lower levels of toxicity, oxidative stress, IL-8 release and proteomic alterations than for 20-nm silver nanoparticles and $AgNO_3$	Georgantzopoulou *et al.* (2016)
	Rats: 28-day exposure (60 nm; 30, 300 and 1000 mg/kg bw/day)	Secretion of mucus in the ileum and rectum Changes in mucin composition	Jeong *et al.* (2010)
Nano-TiO_2	*In vitro*: Caco-2/HT29-MTX cell co-culture, 24-h contact	Intracellular accumulation (x50 vs. Caco-2 cells in monoculture)	Brun *et al.* (2014)
	Ex vivo: porcine buccal mucosa, 4-h incubation	Permeation of the mucus layer	Teubl *et al.* (2015a, 2015b)

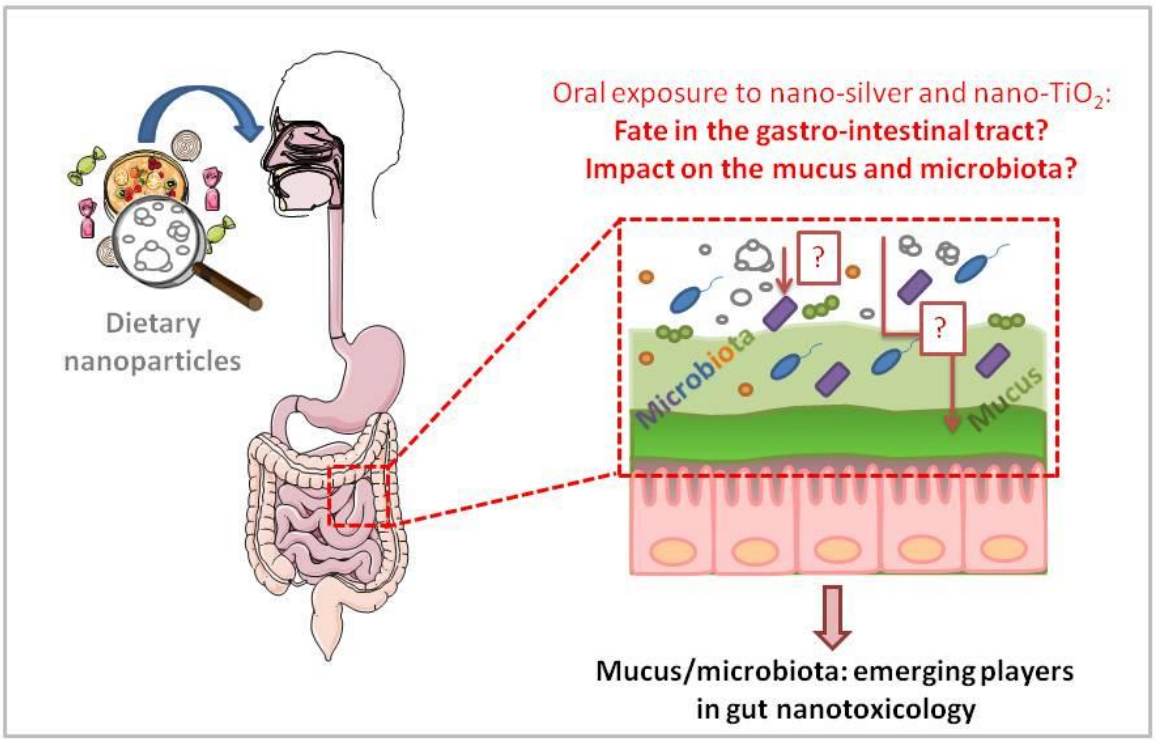

Figure 1. Schematic representation of the possible interactions between dietary nanoparticles and the mucus/microbiota within the gut, and of the toxicological consequences.