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# Food Nanoparticles and Intestinal Inflammation: A Real Risk?

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Additional information is available at the end of the chapter

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## 1. Introduction

Nanotechnology is a rapidly evolving field of research and industrial innovation with many potentially promising applications in agriculture, healthcare, engineering, processing, packaging or delivery of drugs or food supplements. Engineered nanomaterials (ENMs) already became part of our daily life as food packaging agents, drug delivery systems, therapeutics, biosensors, etc. In 2011, according to the Woodrow Wilson Nanotechnology Consumer Products Inventory, Ag nanoparticles (Ag-NPs) were the most commonly consumed ENMs, followed by TiO<sub>2</sub>, SiO<sub>2</sub>, ZnO, Au, Pt, etc (<http://www.nanotechproject.org>). By the most recent definition of European Parliament and Council [1] 'nanomaterial' (NM) is any material that is characterized to have at least one dimension  $\leq 100$  nm, or that comprises of separate functional parts either internal or on the surface, which have one or more dimensions  $\leq 100$  nm, including structures, e.g. agglomerates or aggregates, which may be larger than 100 nm, but which retain the typical properties of nanoscale.

In many countries ENMs are already used as food supplements and in food packaging: (i) nanoclays as diffusion barriers [2]; (ii) Ag-NPs as antimicrobial agent [3,4]; (iii) silicates and aluminosilicates (E554, E556, E559) as anti-caking and anti-clumping agents and in tooth-pastes, cheeses, sugars, powdered milks, etc [5]; (iv) TiO<sub>2</sub> (E171) for whitening and brightening, e.g. in sauces and dressings, in certain powdered foods [6], etc. According to FAO/WHO report [7] the ENMs have several current or projected applications in the agro-food sector: nanostructured food ingredients; nanodelivery systems; organic and inorganic

nanosized additives; nanocoatings on food contact surfaces; surface functionalized NMs; nanofiltration; nanosized agrochemicals; nanosensors; water decontamination, ...

With an increasing number of ENMs present in consumer and industrial products, the risk of human exposure increases and this may become a threat to human health and the environment [8]. Individual ENMs may lead to one or more endpoints, which are not unique to NMs, but which need to be taken into account, e.g. cytotoxicity, stimulation of an inflammatory response, generation of reactive oxygen species (ROS) and/or genotoxicity. Although the exact mechanism underlying NPs toxicity is yet to be elucidated, studies have suggested that oxidative stress and lipid peroxidation regulate the NP-induced DNA damage, cell membrane disruption and cell death [9-12]. It has been suggested that ROS, in turn, modulate intracellular calcium concentrations, activate transcription factors, induce cytokine production [13], as well as lead to increased inflammation [14,15]. Small sized metallic NPs, e.g. Ag-NPs, TiO<sub>2</sub>, Co-NPs may also cause DNA damage [16-20]. *In vitro* studies with different types of NPs (metal/metal oxide, TiO<sub>2</sub>, carbon nanotubes, silica) on various cell lines have demonstrated oxidative stress-related inflammatory reactions. It is believed that this response is largely driven by the specific surface area of the NPs and/or their chemical composition [21-25]. Typically, the biological activity of particles increases with the particle size decrease [26-29]. Moreover, depending on their chemistry, NPs show different cellular uptake, subcellular localization and ability to induce the ROS production [30]. On the contrary, there are also cases reported of NPs having anti-inflammatory properties, such as certain Ce oxide [31] and Ag-NPs [32]. Nanocrystalline Ag has been demonstrated to have antimicrobial and anti-inflammatory properties and was found to reduce colonic inflammation following oral administration in a rat model of ulcerative colitis, suggesting that nano-silver may have therapeutic potential for treatment of this condition [32].

To sum up, based on the information currently available, no generic assumptions can be made regarding the toxicity upon exposure to NMs, their endpoints and the implications of different organs and tissues.

## 2. Behavior and fate of ENMs in the GIT

The gastrointestinal tract (GIT) is a complex barrier-exchange system and is one of the most important routes for macromolecules to enter the body, as well as a key actor of the immune system. The epithelium of the small and large intestines is in close contact with ingested materials, which are absorbed by the villi. To date, studies on exposure, absorption and bioavailability are mainly focused on the inhalation and dermal routes, and little is known about the toxicokinetic and toxicodynamic processes following oral exposure, particularly in relation to ingestion of ENMs that are present in food.

ENMs can reach the GIT either after mucociliary clearance from the respiratory tract after being inhaled [33], or can be ingested directly in food, water, drugs, drug delivery devices, etc [8,34]. The dietary consumption of NPs in developed countries is estimated around 10<sup>12</sup> particles/person per day, consisting mainly of TiO<sub>2</sub> and mixed silicates [35].

It has been shown that several characteristics, such as (i) the particle size [36], (ii) surface charge [37], (iii) attachment of ligands [38,39], (iv) coating with surfactants [40], as well as (v) the administration time and dose [41] affect the fate and extent of ENMs absorption in GIT. The published literature on the safety of oral exposure to food-related ENMs currently provides insufficient reliable data to allow a clear safety assessment of ENMs [42] that is connected primarily with inadequate characterization of ENMs [43]. For instance, it has been demonstrated that smaller particles cross the colonic mucus layer faster than larger ones [37]. The NPs kinetics in the GIT also depends strongly on their charge, *i.e.* positively charged latex particles remain trapped in the negatively charged mucus, while negatively charged ones diffuse across the mucus layer and their interaction with epithelial cells becomes possible [41].

NPs that pass the mucus barrier may be translocated through the intestinal epithelium, which will depend not only on physicochemical characteristics of NPs [36-41], but also on the physiological state of the GIT [44]. The translocation of NPs potentially used as food components through the GIT remains to be explored [45]. Much of the current knowledge concerning the potential toxicity of NPs has been gained from *in vitro* or *in silico* test systems. Following ingestion, translocation of particles across the GIT can occur via different pathways:

1. Endocytosis through 'regular' epithelial cells (NPs < 50 - 100 nm) [46].
2. Transcytosis via microfold (M) cell uptake at the surface of intestinal lymphoid tissue (NPs of 20 - 100 nm and small microparticles *i.e.* 100 - 500 nm) [47]. M cells are specialized phagocytic enterocytes that are localized in intestinal lymphatic tissue – Peyer's Patches (PP). This transcytotic pathway occurs via vesicle formation at the apical (*i.e.* luminal) cell membrane that engulfs some extracellular material, which then moves across the cell, escaping therefore to fusion with lysosomes, fuses with the basolateral membrane (*i.e.* serosal) and releases the material at the opposite side of the intestinal barrier. The mechanism is size-dependent - the smaller the particle, the easier is the passage through the epithelium [48-50].
3. Persorption, where 'old' enterocytes are extruded from the villus into the gut lumen, leaving 'holes' in the epithelium, which allow translocation of even large particles, such as starch and pollen [51-53].
4. Another possible route by which NPs can gain access to the gastrointestinal tissue is the paracellular route across tight junctions (TJs) of the epithelial cell layer. TJs are remarkably efficient at preventing paracellular permeation, although their integrity can be affected by diseases, *e.g.* inflammation, and/or by metabolites (*e.g.* glucose), calcium chelators (*e.g.* citrate) [54] and even particle endocytosis [55].

All above-mentioned routes could be involved in NPs translocation. There are a number of published reports stating the involvement of different types of endocytosis in the process of NPs internalization: clathrin-mediated pathway, caveolin-mediated endocytosis and macropinocytosis for TiO<sub>2</sub> [56], size-dependent endocytosis for Au-NPs [57]; endocytotic pathways were described for SiO<sub>2</sub> [58,59] and Ag-NPs [60], etc.

Several studies demonstrated that the phenomenon of persorption is also true for NPs, e.g. in the case of colloidal Au-NPs [36]. Small and large NPs gain potentially access to this route, nevertheless its quantitative relevance remains low, as it seems to be very inefficient compared to the active uptake of particles by M-cells. For instance, it was indicated that one lymphoid follicle dome of the rabbit PP could transport about  $10^5$  microparticles of 460 nm diameter in 45 min [61]. It could be assumed that for smaller particles this would be even more efficient.

Particulate uptake may occur not only via the M-cells of the lymphoid follicle-associated epithelium (FAE) in PP [49,62], but also via the normal intestinal enterocytes [46]. A number of reports on intestinal uptake of micro- and nanoparticles state that the uptake of inert particles occurs trans-cellularly through normal enterocytes and via M-cells [61,63-65], as well as, to a lesser extent, through paracellular pathway [66].

### 3. Appropriate *in vitro* model of the intestinal barrier

There are several recognized parameters currently used for *in vitro* cytotoxicity assessment of ENMs, such as cell viability, stress and inflammatory responses, genotoxicity, etc [67]. However, it should be noted that due to specific physicochemical properties of ENMs, currently existing *in vitro* toxicity assays may have limited use and the methods should be carefully designed in order to discard the influence of nano-sized materials on the assay itself [28]. The risk assessment is further impaired by the lack of standardized test systems that fulfil these criteria. According to the new European Chemicals Legislation (REACH), new test systems for toxicity screening of ENMs should be developed, e.g. cell culture systems that will better reflect *in vivo* toxicity parameters [68].

Human colon adenocarcinoma (Caco-2) cells reproducibly display a number of properties characteristic to differentiated enterocytes and are the most popular cell culture system for studying intestinal passage and transport [69,70]. Cultured Caco-2 cells differentiate spontaneously into polarized monolayers [71] that possess an apical brush border and express functional TJs, biotransformation enzymes and efflux pumps [72]. Caco-2 cells grow as a monolayer and fully differentiate also on semi-permeable membranes of bicameral inserts. This permits to separate the apical (AP) compartment from the basolateral (BL) one, reflecting the intestinal lumen and the serosal side, respectively [65]. Transport of molecules and ions from the AP to the BL side and vice versa requires the passage either through the cells (transcellular route) or between the cells through TJs (paracellular route).

The gut lining epithelium is for the most part impermeable to microorganisms and microparticles, except for the lymphoid FAE found in PP [49,73,74]. M cells are responsible for transport of antigens, bacteria, viruses, as well as micro- and NPs to the antigen presenting cells within and under the epithelial barrier as the first step in developing immune responses [75]. There is only an incomplete and inadequate understanding of the development and function of FAE, as well as of the genes and proteins responsible for their specialized functions. One potential approach to study such complex and specialized tissues is to use cell

culture systems more precisely reproducing the features of the *in vivo* tissue. Kernéis *et al.* [76] demonstrated that co-culturing of Caco-2 cells with murine PP lymphocytes appears to convert Caco-2 cells into M-like cells, including enhanced transport of particles across the epithelium monolayer. The induction of this phenotype did not require direct cell contact, as it was also achieved via physically separated co-culturing of Caco-2 and human Burkitt's lymphoma (Raji B) cells in bicameral culture inserts [77]. Although it is not clear whether this model faithfully reproduces all of the features of *in vivo* M cell function, nevertheless studies have confirmed that Caco-2 cells co-cultivated with Raji B cells *in vitro* express several genes specifically expressed in FAE *in vivo* [78].

In an improved *in vitro* co-culture model in bicameral system Caco-2 cells were exposed to lymphocytes from the BL chamber. In a so-called 'inverted' model (Figure 1) the lymphocytes were shown to migrate into the monolayer and induce the conversion of the enterocyte phenotype into the M-cells one [76,79]. Recently, des Rieux *et al.* [65] characterized the inverted model and compared it with previously developed one [77]. According to these results, in the inverted model, the M cell conversion rate was estimated to range between 15 - 30% (for comparison it was <10% in the human FAE [80]). The comparison of the *in vitro* models revealed that the inverted model appears to be physiologically and functionally more reproducible and efficient than the normally oriented one [65]. Thus this improved model could be used to better characterize and understand the biological effects, absorption and transportation mechanisms of NPs in intestinal cells.

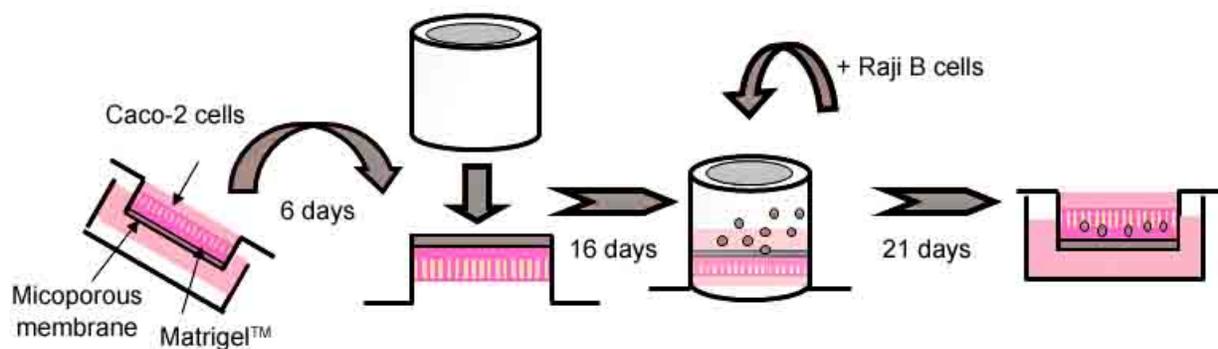


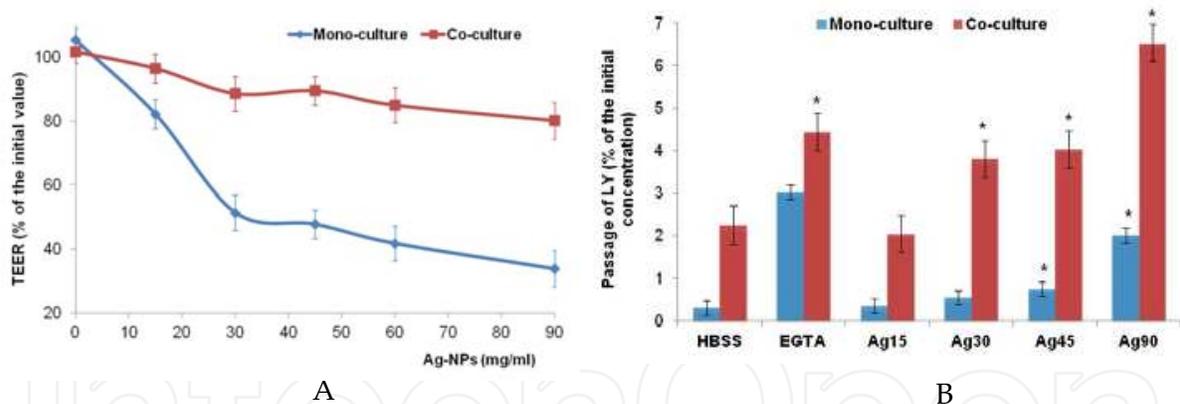
Figure 1. Co-culture model of Caco-2 and Raji B cells [63].

#### 4. Epithelial barrier integrity and inflammatory response under the influence of NPs

During their differentiation epithelial cells develop junctional structures between the neighboring cells and form a tight protective barrier that restricts the absorption to some nutrients and substances while, in the meantime, provides a physical barrier impairing the permeation of pro-inflammatory molecules, e.g. pathogens, toxins, antigens and xenobiotics from

the luminal environment into the mucosal tissues and circulatory system. This barrier comprises several structures [81], where the TJs are the most apical components of the junctional complex and are the main gatekeepers of the epithelial paracellular passage. TJ barrier disruption and increased paracellular permeability, followed by permeation of luminal pro-inflammatory molecules can activate the mucosal immune system, resulting in chronic inflammation and tissue damage [75]. Intestinal TJ barrier is evidenced to have a critical role in the pathogenesis of intestinal and systemic diseases [82-84]. Under physio-pathological conditions, pro-inflammatory cytokines, antigens and pathogens contribute to barrier impairment [85,86]. Considering the TJs integrity impairments under inflamed conditions, it could be assumed that NPs that lead to stress and/or inflammatory responses could also influence the TJs integrity.

Several methodological approaches allow measuring the barrier function in cell cultures, e.g. the evaluation of the transepithelial electrical resistance (TEER) and the passage of marker molecules, such as Lucifer Yellow (LY) [87]. Our results revealed that under the influence of Ag-NPs < 20 nm, a disruption of the barrier integrity occurs. In figure 2A the TEER values of both mono- and co-cultures of Caco-2 cells after 3h of incubation with different concentrations of Ag-NPs are shown. TEER values decreased as Ag-NPs concentration increased, even though the reduction was less obvious in co-culture conditions – a model that is closer to the physiological conditions of FAE.

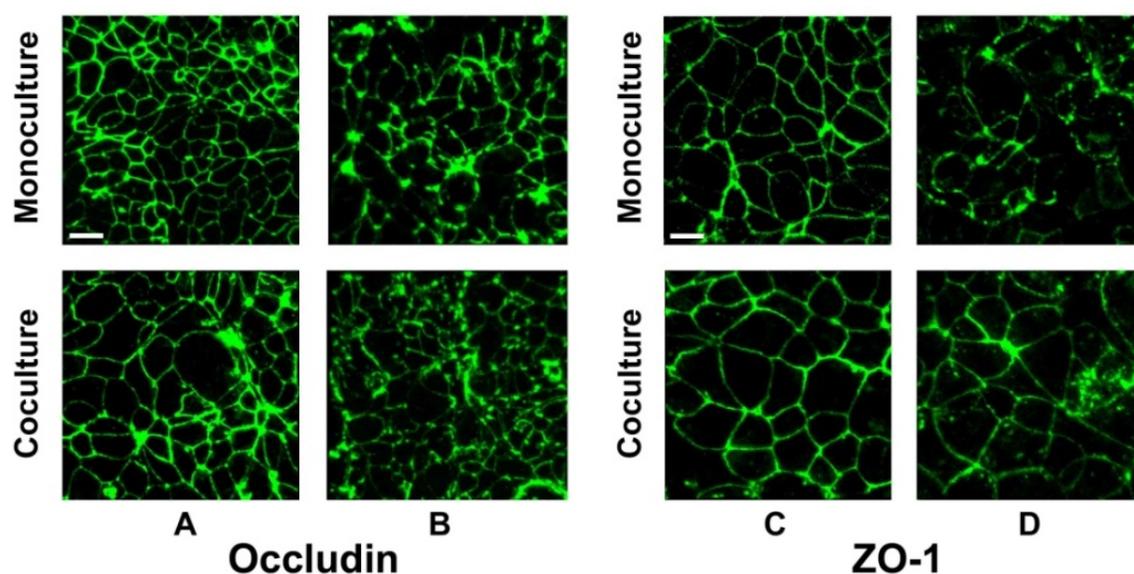


**Figure 2.** TEER values (A) and LY passage (B) of mono- and co-cultures of Caco-2 cells upon incubation with Ag-NPs (NM-300K, JRC repository, Ispra, IT) at 15 – 90  $\mu$ g/ml. Experiments were conducted on mono- and co-cultures (*i.e.* Caco-2 cells with Raji B lymphocytes) cultivated for 21 days in polycarbonate bicameral inserts with 3  $\mu$ m pore size (Transwell™, Corning Costar, NY) to reach a full differentiation and, for co-cultures, partial conversion into M like cells. TEER values were measured via Millicell-ERS volt-ohm meter (World Precision Instruments, Sarasota, FL) at the beginning and after 3h incubation period with Ag-NPs. The transport of LY was observed during 3h period with a 30 min sampling time from the BL compartment. Both the changes in TEER values ( $P < 0.0001$ ) and the LY passage ( $P < 0.003$ ) were calculated as a percentage from the initial value. Data represent the means  $\pm$  SEM of 4 independent experiments. \*Samples significantly different from the control (results were considered significant at  $P < 0,05$ ).

The passage of LY was evaluated by the amount of LY that passed from AP to BL compartment (Figure 2B). The presence of Ag-NPs increased the level of LY in the BL compartment that was dependent on the NP concentration. These results are in correlation with the NP-

induced reduction of TEER values. Interestingly, in contrast to TEER results, the co-cultures had more elevated rate of LY passage than the corresponding mono-cultures.

To have an idea about the molecular mechanisms of the Ag-NPs-induced barrier integrity disruption, an immunostaining with confocal microscopy analysis of two TJs proteins occludin and ZO-1 was realized. As illustrated on Figure 3, in Ag-NP-treated cells the continuity of both occludin and ZO-1 was disrupted with the control comparison and the aggregation of both proteins was observed. It should further be noted that mono-cultures were more susceptible to the influence of Ag-NPs than co-cultures and the alterations in proteins distributions were more visible in mono-cultures. The immunostaining results in turn confirmed the TEER data, where a more obvious reduction was estimated in the case of mono-cultures (Figure 2).



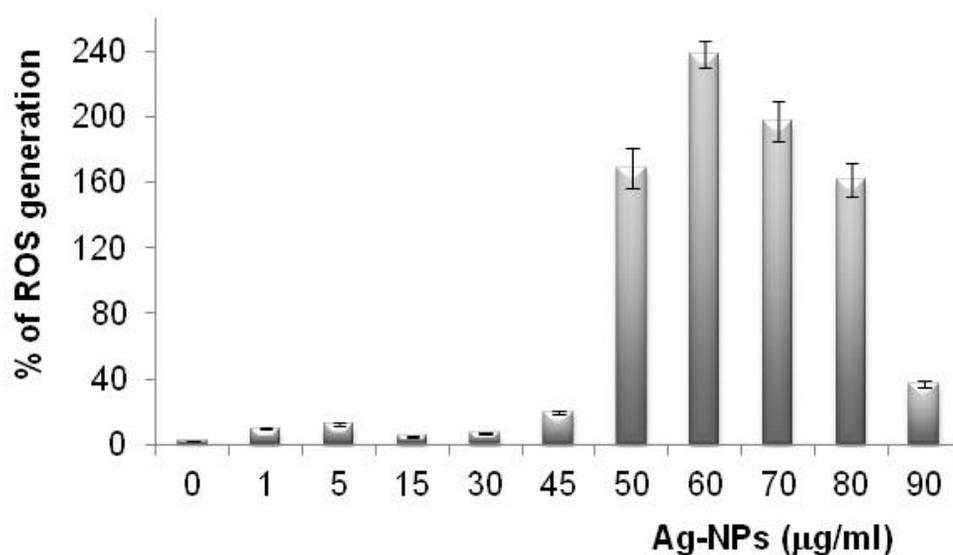
**Figure 3.** Subcellular localization of the occludin and ZO-1 TJs scaffolding proteins. Mono- and co-cultures of Caco-2 cells grown on bicameral inserts were treated with Ag-NPs (45  $\mu\text{g}/\text{ml}$ ) for 3h and then processed for immunostaining (B and D). Untreated cells were used as controls (A and C). In order to visualize the occludin and ZO-1 mouse anti-Occludin and mouse anti-ZO-1 (both from Invitrogen) were used as primary antibodies, as well as Alexa Fluor 488 goat anti-mouse (Invitrogen) as the secondary antibody. Images were collected by confocal laser scanning microscope; scale bars are 15 and 25  $\mu\text{m}$  for occludin and ZO-1 staining, respectively.

The observed changes were reversible at low Ag-NPs concentrations (up to 30  $\mu\text{g}/\text{ml}$ ): the TEER values and TJs proteins distributions were recovered until the control level. Other NPs were also reported to possess the ability to open the TJs. For instance, the chitosan NPs were capable to open transiently and reversibly the epithelial TJs [88].

In contrast to Ag-NPs, we observed no change neither in TEER value and LY passage rate, nor TJs proteins distributions upon incubation of cell mono- and co-cultures with amorphous  $\text{SiO}_2$  < 25 nm (NM-200, JRC repository, Ispra, IT) (results not shown). These findings provide additional evidence that the major input in the NPs-mediated barrier integrity disruption seems to belong to the charge of the NPs. Particularly, it has been previously report-

ed that neutral and low concentrations of anionic NPs have no effect on blood-brain barrier integrity, in contrast to anionic NPs at high concentrations and cationic NPs [89]. A number of recent *in vitro* and *in vivo* studies highlight the importance of NPs surface charge for cellular uptake and biodistribution [90-92], indicating that for the majority of NPs the positive surface charge enhances cellular internalization [92-94]. The latter is likely linked to the adsorption of different bio-molecules at the surface of NPs, dependent on surface charge, as well as on chemical characteristics of NPs [95].

Another underlying condition in the TJs disruption is likely to be the cellular oxidative stress possibly induced by NPs [96]. Our results have shown that the fluorescence intensity of an oxidative stress indicator dichlorofluorescein was increased upon exposure of cells to Ag-NPs within a 3h time period (Figure 4). The ROS generation induction was dependent on NPs concentration reaching from about 1,5 to 3-fold increase, as compared with the untreated cells. Thus one mechanism of toxicity of Ag-NPs could likely be mediated by oxidative stress, already reported to be involved in the modulation of TJs integrity [97].



**Figure 4.** Effect of Ag-NPs (5 – 90 µg/ml) on intracellular ROS generation in Caco-2 cells. The ROS generation was investigated using the dichlorofluorescein (DCFH) assay. After being oxidized by intracellular oxidants, DCFH becomes DCF and emits fluorescence, quantification of which is a reliable estimation of overall oxygen species generation. The intracellular ROS level is presented as a percentage of the corresponding initial value after incubation together with NPs during 3h at 37°C. Data represent means  $\pm$  SEM of 3 experiments with 3 different samples per condition,  $P < 0.0001$ .

Altogether, the results reveal that some NPs, e.g. chitosan or Ag-NPs may enhance the epithelial barrier permeability and could therefore serve as an effective carrier for oral drug delivery [44]. However, it should be noted that the epithelial permeability increase in turn might favor the systemic absorption of ENMs, toxins and other xenobiotics, and would likely cause immune activation.

## 5. Potential toxicity of ENMs in the case of altered intestinal physiology

It has been reported that the exposure to some NPs is associated with the occurrence of autoimmune diseases, such as systemic lupus erythematosus, scleroderma, and rheumatoid arthritis [35]. Diseases, such as diabetes, may also lead to an increased absorption of particles in the GIT [41]. Furthermore, inflammation may lead to the uptake and translocation of particles of up to 20 nm [98]. Thus, an issue to be considered in relation to ENMs ingestion is a possible increase in their intestinal absorption in the case of systemic exposures, such as in Inflammatory Bowel Disease (IBD) and/or Crohn's disease (CD), which represent chronic disorders characterized by recurrent and serious inflammation of the GIT [99]. Crohn's disease affects primarily people in developed countries, where the highest incidence rates and prevalence for CD and ulcerative colitis (UC) have been reported from northern Europe, the United Kingdom and North America [100] with a frequency of 1 in 1,000 people in the Western world [5]. However, reports of increasing incidence and prevalence from other areas of the world, e.g. southern or central Europe, Asia, Africa, and Latin America state the progressive nature and worldwide rise of these diseases [100].

An abnormal intestinal barrier function plays a pivotal role in IBD [101]. Increased intestinal permeability has been reproducibly described in patients with CD, which is likely a predisposing factor to the pathogenesis and impaired epithelial resistance [102,103]. A barrier dysfunction has been reported in the colonic mucosa of patients with Irritable Bowel Syndrome (IBS), which results from increased paracellular permeability, presumably by an altered expression of ZO-1 [104]. Moreover, stress is believed to contribute to induction of IBS and recurrence of intestinal inflammation and can increase the paracellular permeability [105]. It should be noted that mediators of inflammation, such as ROS, endotoxins (lipopolysaccharides) and cytokines are able to provoke the disruption of TJs and thereby increase the paracellular permeability [97]. Significant changes in epithelial TJs structure and function were also observed in UC [106,107]. Thus the altered intestinal permeability could certainly be a result of disease progression, but there is evidence that it might also be the primary causative event.

Recently it was suggested that there could be an association between high levels of dietary NPs uptake and CD. Experimental results indicate that the accumulation of insoluble NPs in humans may be responsible for the compromised gastrointestinal functioning, as described in the case of CD and UC [5]. Microscopy studies have also shown that macrophages located in lymphoid tissue can uptake NPs, e.g. spherical anatase (TiO<sub>2</sub>) with size of 100-200 nm from food additives, aluminosilicates of 100-400 nm typical of natural clay, and environmental silicates of 100-700 nm [108]. According to another study, some insoluble NPs, such as TiO<sub>2</sub>, ZnO and SiO<sub>2</sub>, upon their absorption and passage across the GIT, come into contact with and adsorb calcium ions and lipopolysaccharides. The resulting NPs–calcium–lipopolysaccharide conjugates activate both peripheral blood mononuclear cells and intestinal phagocytes, which are usually resistant to stimulation [109].

Despite the insufficiency of data linking the NPs consumption to the initiation of CD and UC, it seems that particles of 0.1 – 1.0 µm may be adjuvant triggers for the exacerbation of

these diseases [110]. Micro and NPs have been constantly found in organs, e.g. in colon tissue and blood of patients affected by cancer, CD, and UC, while in healthy subjects NPs were absent [111]. Some evidence suggests that dietary NPs may exacerbate inflammation in CD [6]. More precisely, some members of the population may have a genetic predisposition where they are more affected by the intake of NPs, and therefore develop CD [9]. It has been also reported that micro- and NPs in colon tissues may lead to cancer and CD progression [111]. By contrast, a diet low in calcium and exogenous micro- and NPs has been shown to alleviate the symptoms of CD [5]. This analysis is still controversial, with some proposing that an abnormal response to dietary NPs may be the cause of this disease, and not an excess intake [6].

Although there is a clear association between particle exposure/uptake and CD, little is known of the exact role of the phagocytosing cells in the intestinal epithelium and particularly of the pathophysiological role of M cells. It has been shown that M cells are lost from the epithelium in the case of CD. Other studies found that endocytotic capacity of M cells is induced under various immunological conditions, e.g. a greater uptake of particles of 0.1 – 10  $\mu\text{m}$  has been demonstrated in the inflamed colonic mucosa of rats compared to non-ulcerated tissue [109,112].

Thus more vulnerable members of the population, *i.e.* those with pre-existing digestive disorders, may potentially be more affected by the presence of ENMs, although, in contrast, ENMs may offer many potential routes to therapies for the same diseases. The diseases associated with gastrointestinal uptake of NPs, such as CD and UC have no cure and often require surgical intervention. Treatments are aimed at maintaining the disease in remission and mainly consist of anti-inflammatory drugs and specially formulated liquid meals [5]. If dietary NPs are conclusively shown to cause these chronic diseases, their use in food should be avoided or strictly regulated.

## 6. Potential health risks/benefits of nanotechnology-based food materials

The absorption, distribution, metabolism and excretion (ADME) parameters are likely to be influenced by the aggregation, agglomeration, dispersability, size, solubility, and surface area, charge and physico-chemistry of NPs [113]. Amongst these parameters the size, chemical composition and surface treatment appear to be the most critical ones for nanotoxicity issues [114]. Chemical composition, beside the chemical nature of the NP itself, also includes the surface coating of the NPs [115]. Coatings can be used to stabilize the NPs in solution, to prevent clustering or to add functionality to the NPs, depending on its intended use. Surface coatings can influence the reactivity of the NPs in various media, including water, biological fluids and laboratory test media [116,117]. From this point of view the interaction of NPs with food components is another aspect that may need consideration and about which little information is currently available. The possible interaction of food components may alter the physicochemical properties of ENMs that in turn may influence their passage through the GIT and their ADME properties.

ENMs, with their very large surface areas, may adsorb bio-molecules on their surface upon contact with food and/or biological fluids to form a bio-molecular “corona” [96,118]. Depending on the nature of the corona, the behavior of the NPs may differ, and there could be the potential for novel toxicities non-characteristic neither for the non-coated NPs, nor for the adsorbed biological material. These bio-molecules include proteins, lipids, sugars, different secondary metabolites and it is those interactions that may actually determine how ENMs will interact with living systems. Thus, the foregoing information on the food should be considered carefully, taking into account its major ingredients or components, which have physiological properties likely to influence the absorption/translocation of ENMs in the GIT.

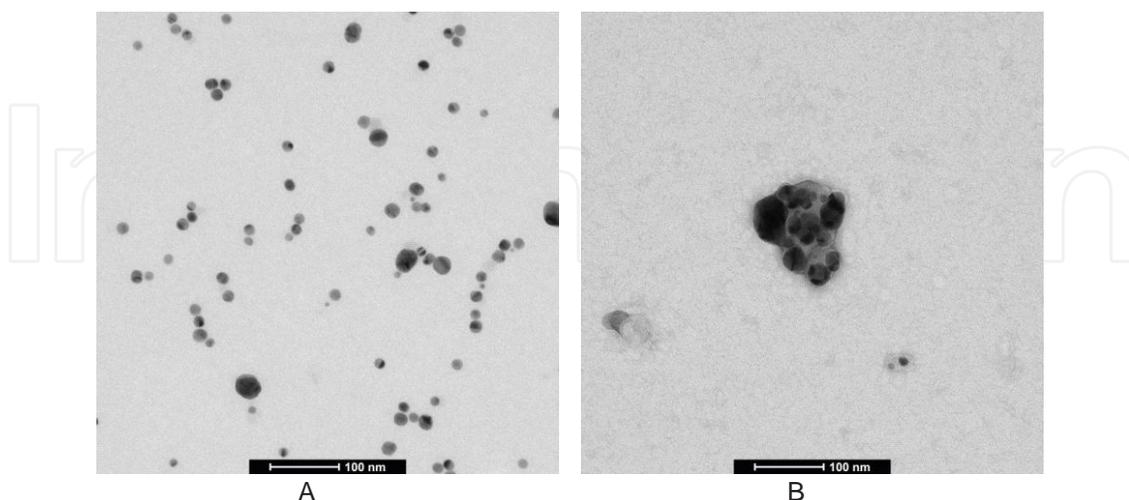
Several studies have demonstrated that various food components provide beneficial anti-inflammatory and anti-mutagenic effects in the GIT. Although the information regarding these effects on intestinal TJ barrier integrity is limited, some results are available e.g. for glutamine [119,120] and fatty acids [121-123]. A growing number of data suggest the potential protective effect of phenolic compounds on the epithelial barrier function and their anti-inflammatory properties [124,125]. In particular, certain flavonoids that represent a part of human daily nutrition, e.g. epigallocatechin gallate, genistein, myricetin, quercetin and kaempferol are reported to exhibit promotive and protective effects on intestinal TJ barrier [124,126].

We have observed (unshown results) that quercetin attenuates the cytotoxic effect of Ag-NPs on Caco-2 cells, as well as allows recovering of the epithelial barrier function, which was evidenced by the recovery up to the initial value of the TEER and the LY passage rate in both mono- and co-cultures. The immunostaining analysis of occludin and ZO-1 also revealed the recovery of the protein distributions in the presence of quercetin, which additionally suggests the protective effect of the latter upon the harmful effects of Ag-NPs. In a similar study it was reported that positively charged Ni-NPs can efficiently enhance the permeation and uptake of quercetin into cancer cells, which can have important biomedical and chemotherapeutic applications [127].

A number of published reports indicate the potential application of antioxidants [10,128-130] and anti-inflammatory drugs [6,131] that are able to treat the adverse health effects caused by NPs. For instance, berberine, an alkaloid with a potential biomedical application, has been shown to attenuate TJ barrier defects induced by TNF- $\alpha$ , known to disrupt TJ integrity in IBD [132]. It has been reported that rats that underwent instillation of NPs into the lungs together with an antioxidant, *i.e.* n-acetylcysteine, showed an inflammation decrease up to 60% in comparison to those exposed to NPs alone [10].

To have an idea about the state of Ag-NPs in the presence of quercetin, NPs were characterized by transmission electron microscopy (TEM) (Figure 5). It could be seen that in the presence of quercetin a “capping” of Ag-NPs occurs, which confirms already existing data on Ag-NPs stabilization with reducing agents. Surface-active molecules, such as terpenoids and/or reducing sugars are believed to stabilize the NPs in the solutions, *i.e.* they are believed to react with the silver ions (Ag<sup>+</sup>) and stabilize the Ag-NPs [133,134]. Flavonoids have been suggested to be responsible for the reduction of Ag<sup>+</sup> to Ag-NPs [135]. Fatty acids such

as stearic, palmitic and lauric acids are used as agents for the formation and stabilization of Ag-NPs [136].



**Figure 5.** TEM analysis of Ag-NPs < 20 nm (NM-300K) alone (A) and in the presence of quercetin (B). The average size of Ag-NPs was about 20 nm, scale bar: 100 nm. NPs were characterized by transmission electron microscopy (TEM) (Technai Spirit TEM, FEI Company, Eindhoven, NL) by Dr. J. Mast at the Electron Microscopy Unit of the Veterinary and Agrochemical Research Centre VAR-CODA-CERVA, Uccle, BE.

Another major phenolic compound present in human diet is resveratrol, which possesses many beneficial health effects [137-141]. Considering abundance and health-promoting effects of resveratrol, we have also investigated its potential protective activity against the Ag-NP-induced cytotoxicity. The results indicated no protective effect of resveratrol and moreover, at a concentration of 100  $\mu$ M, non-toxic by itself, it increased the toxic effect of Ag-NPs, illustrating a synergistic effect.

To conclude, it could be assumed that phenolic compounds, depending on the nature and concentration, may exhibit different effects on cells in the presence of NPs. This is not surprising, as it is known that these substances, depending on concentration, may exhibit both beneficial and toxic effects [141].

## 7. Future perspectives

Nanotechnology offers a wide range of opportunities for the development of innovative products and applications in agriculture, food production, processing, preservation and packaging. However, the present state of knowledge still contains many gaps preventing risk assessors from establishing the safety for many of the possible food related applications of nanotechnology [142]. Currently the routine assessment of ENMs *in situ* in the food or feed matrix is not possible, as well as equally impossible to determine physico-chemical state of ENMs, which increases the uncertainty in the exposure assessment. Complex matrices present in the food complicate the detection and characterization of

ENMs in final food/feed products, which itself contain a wide range of natural structures in the nano-size scale. The information on the potential of ENMs to cross the epithelial barriers, such as the GIT, blood-brain, placenta and blood-milk barriers are also important for hazard identification. It is also clear that the evaluation of the pro-inflammatory potential of ENMs is another issue of current importance, as the inflammation itself is associated with a number of high frequency diseases, e.g. cancer, diabetes, bowel diseases, etc.

From the above discussion and the research presented in this review, the need for more toxicology research on manufactured ENMs is clear. In addition to standard tests, there is a need to develop appropriate and rapid screening methods to be able to control the exposure level, as well as improved models that will permit to assess the toxicity and allow better understanding of the mechanisms that are involved. Employment of developed and well characterized *in vitro* cell culture systems may be relevant for evaluation of gut and immune responses to ENMs and to adapt conditions to specific health conditions or to consumer groups with special needs, such as in the case of bowel diseases. Further studies are necessary to assess whether the characteristic daily intake of ENMs may exacerbate or trigger disease symptoms in subjects with increased susceptibility, such as inflamed state of the GIT in the case of IBD, CD, UC, or even be its cause.

Another aspect deserving thorough investigation is the possible interaction of ENMs with food/feed components, which in turn could influence the overall behavior and effect of not only ENMs, but also the bioavailability of food components.

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