Assessing the effects of silver nanoparticles on monolayers of differentiated Caco-2 cells, as a model of intestinal barrier

Laura Vila\textsuperscript{1}, Alba García-Rodríguez\textsuperscript{1}, Constanza Cortés\textsuperscript{1}, Ricard Marcos\textsuperscript{1,2,§}, Alba Hernández\textsuperscript{1,2,§}

\textsuperscript{1}Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra, Spain; \textsuperscript{2}CIBER Epidemiología y Salud Pública, ISCIII, Spain.

§Corresponding authors at: Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Edifici Cn, Campus de Bellaterra, 08193 Cerdanyola del Vallès (Barcelona), Spain.

E-mail: alba.hernandez@uab.es (A. Hernández)
ricard.marcos@uab.es (R. Marcos)

Running title: Silver nanoparticles and Caco-2 monolayers
ABSTRACT

Since ingestion is one of the main routes of entry of nanoparticles (NPs) in our organism, simple and fast in vitro models of the intestinal barrier can be helpful to evaluate NPs risk. The human colon adenocarcinoma Caco-2 cell line has been extensively used due to its ability to differentiate, forming a well-structured cell monolayer. In this study, we have used these differentiated cells as a model of intestinal barrier to evaluate a wide set of effects caused by exposure to silver nanoparticles (AgNPs) with an average size of 7.74 nm. Different parameters such as toxicity, monolayer integrity and permeability (assessed by changes in cells’ morphology and gene expression pattern), internalization (uptake), translocation, and induction of DNA damage (DNA breaks and oxidative DNA damage) were evaluated. No significant effects were observed on the monolayer’s integrity/permeability after exposure to silver nanoparticles, although cellular uptake was demonstrated by using confocal microscopy. Despite the observed uptake, no translocation of AgNPs to the basolateral chamber was demonstrated with any of the different experimental approaches used. The genotoxic effects evaluated using the comet assay indicate that, although AgNPs were not able to induce direct DNA breaks, its exposure induced a significant increase in the oxidative DNA damage levels, at non-toxic concentrations.

Key words: Silver nanoparticles, differentiated Caco-2 cells, monolayer integrity, uptake, translocation, genotoxicity.
1. Introduction

Engineered nanomaterials (ENMs) are increasingly used in many consumer products such as sporting goods, cosmetics, electronics, and medicine (drug delivery), as well as in food industry (packaging, nutrient delivery systems, and food additives). This implies its increasing presence in the environment, making human exposure possible (Kühnel and Nickel, 2014).

Among nanoparticles (NPs), metal-based NPs are the most known and produced, due to their advantageous physicochemical features. Since their discovery, the main NPs produced worldwide are titanium dioxide (TiO$_2$NPs) and silicon dioxide (SiO$_2$NPs). These NPs are broadly applied in food industries (Yang et al., 2014; Niu et al., 2016; Lee et al., 2017). However, silver nanoparticles (AgNPs) are gaining interest and, currently, there is an increasing number of consumer and industrial goods containing AgNPs. Since food supplements and pharmaceutical products use AgNPs for antimicrobial purposes, increased environmental and human exposure occurs, concretely via ingestion, with important toxicological implications (Wijnhoven et al., 2009; Martirosyan et al., 2014).

Two proposed modes of action of AgNPs are generally accepted. i) The release of silver ions (Ag$^+$), and ii) the induction of reactive oxygen species (ROS) (Wijnhoven et al., 2009). Both mechanisms could determine the harmful genotoxic effects associated with AgNPs exposure (Duran et al., 2016; Wang et al., 2017). Currently, few data exist on the toxicokinetic and toxicodynamic processes taking place upon oral exposure to AgNPs, particularly those concerning their use as food additives/complements (van der Zande et al., 2012; Bergin et al., 2016). In terms of oral exposure to NPs, different dose-dependent animal toxic effects have been reported including death, weight loss, hypo-activity, alterations in neurotransmitter, liver enzymes and blood values, enlarged hearts and immunological effects (Hadrup and Lam, 2014). In addition, the effects of ingested AgNPs on mammalian gut microbiota could be a factor causing different
health adverse effects (Fröhlich and Fröhlich, 2016). Nevertheless, controversy and disparity of data are usually reported. For instance, although Shahare and Yashpal speculate on the capability of 20 nm AgNPs to interact with the protective intestinal barrier in a mice model causing structural changes and microvilli destruction (Shahare and Yashpal, 2013), others described that nanocrystalline silver administrated orally was able to decrease ulcerative colitis in rat models by suppressing the protein expression of TNF-α, MMP-9 and IL-1β (Bhol and Schechter, 2007).

As an alternative to the complexity of the in vivo approaches, in vitro models of the intestinal barrier have been proposed. Among them, the human colon adenocarcinoma Caco-2 cell line has been extensively used due to its capacity of undergoing spontaneous differentiation, leading to the formation of a cell monolayer. This system mimics the mature small intestine enterocytes, both morphologically and functionally (Carr et al., 2012). The use of Caco-2 cells monolayers (CCM), as a standard permeability-screening assay for prediction of drug intestinal permeability, has grown in many areas of nutrition, pharmacology and toxicology research (Shah et al., 2006). Moreover, it has been demonstrated that permeability through CCM correlates well with in vivo absorption in humans (Artursson et al., 2001). Due to the aforementioned advantages, the CCM system has also gained popularity in the nanotoxicology field, since it allows the prediction of in vivo absorption of ENMs by studying the passive transcellular and paracellular transport (Shah et al., 2006; Carr et al., 2012; Bannunah et al., 2014). Nevertheless, it must be indicated that this model is a bit far away of the in vivo complexity of the intestinal barrier, where there are other cells besides the enterocytes, like mucus secretory and goblet cells (Garcia-Rodriguez et al., 2018).

Because humans are environmentally exposed to AgNPs primarily via ingestion (Wijnhoven et al., 2009), efforts should focus on increasing our knowledge of their interaction with the intestinal barrier. Therefore, the goal of our work has been to explore these potential effects using the CCM as an alternative in vitro model. In our study, the transcellular and paracellular transport, as well as the cellular uptake of
AgNPs with an average size of 7.74 nm were assessed with different qualitative and quantitative techniques such as confocal microscopy, transmission electron microscopy complemented with energy dispersive X-ray (TEM+EDX) and ICP-MS, respectively. In addition, the induction of oxidative DNA damage by the comet assay, and changes in gene expression by qPCR were also assessed. The selected genes are involved in Caco-2 function, coding for the brush border enzymes sucrose isomaltase (SI) and solute carrier (SLC15A1), and tight junction components such as occludin (OCLN) and claudin (CLDN2) genes.
2. Materials and methods

2.1. Nanomaterial dispersion and characterization

Silver nanoparticles (AgNPs, NM300K) were obtained from Fraunhofer (Fraunhofer IME, Germany). To disperse them, AgNPs were pre-wetted in 0.5% absolute ethanol and suspended in 0.05% bovine serum albumin (BSA) in MilliQ water. AgNPs were sonicated in the dispersion medium for 16 min to obtain a dispersed stock of 2.56 mg/mL, according to the Nanogenotox protocol (Nanogenotox, 2011). Although AgNPs were well characterized under the NanoReg EU project framework (http://www.nanoreg-materials.eu/Documentation/) further characterization was carried out. Transmission electron microscopy (TEM) was used to determine the nanoparticle’s dried size and morphology on a JOEL JEM-1400 instrument (Jeol LTD, Tokyo, Japan). Over 100 TEM images in random fields of view were processed with Image J software to calculate their diameter. Moreover, the hydrodynamic size and Z-potential were evaluated by using dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) methodologies, in a Malvern ZetasizerNano-ZS zen3600 device (Malvern, UK). In this study, a concentration of 100 µg/mL of AgNPs, dissolved in DMEM cell culture media, was analyzed at 0 and 24 h after sonication.

2.2. Cell culture and AgNPs exposure

The human colon adenocarcinoma cell line Caco-2 was kindly provided by Dr. Isabella Angelis, from the Istituto Superiore di Sanità (ISS, Italia). Caco-2 cells were maintained in Dulbecco's modified Eagle's High Glucose medium without pyruvate (DMEM w/o, Life Technologies, NY) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA) (PAA Laboratories GmbH, Pashing, Austria) and 2.5 mg/mL plasmocin (Invivo Gen, San Diego, CA). Cells were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37 ºC. To differentiate Caco-2 cells, 170,000 cells/transwell were grown for 21 days in 12-well culture plates onto polyethylene terephthalate transwells (PET) with 1 µm mean pore size and a diameter
of 1.12 cm (Merck KGaA, Darmstadt, Germany). Cell culture medium was changed every 2 days. To study the different NPs-induced biological effects, both un- and differentiated Caco-2 were exposed to different concentrations of AgNPs for 24 h.

2.3. Cell viability analysis

Differentiated and undifferentiated Caco-2 cells were treated for 24 h with different doses of AgNPs ranging from 1-100 µg/mL, dispersed in complete culture medium. After 24 h of treatment, cells were washed three times with 0.5 mL of PBS (1%). Then, cells were incubated 3 min at 37 °C with 0.25 mL of trypsin (1.5%) to detach and individualize them. Finally, cells were diluted (1/10) in ISOTON, washed trice to eliminate dead cells, and counted with a ZTM Series coulter-counter (Beckman Coulter Inc., CA) (Vila et al., 2017). ISOTON is an isotonic solution used to keep Caco-2 cells in stable condition while the machine counts the cells. Viability values for each concentration were calculated from averaging three independent survivals curves after relativizing to an untreated control. The IC50, defined as the concentration of nanomaterials (NM) that reduces the cell viability by 50%, was calculated from averaging three independent survival curves.

2.4. Monolayer integrity and permeability evaluation

Measurements of the trans-epithelial electrical resistance (TEER) were performed to evaluate the integrity of Caco-2 cells monolayers. The TEER of each monolayer was measured through the monolayer formation 7, 14 and 21 days after seeding. To evaluate the potential effects of AgNPs in the monolayer stability, TEER was measured prior to AgNPs treatment, and after 24 h. All measurements were performed with an epithelial voltmeter (Millicell-ERS volt-ohm meter). Monolayers with TEER > 350 Ω·cm² were used for further assays. The TEER values were calculated as TEER = Ω (cell inserts) - Ω (cell-free inserts) x 1.12 cm². For permeability studies, the paracellular flow of Lucifer yellow (LY) was determined. After 24 h of AgNPs treatment, Lucifer yellow was added to the apical compartment at a final concentration of 0.4 mg/mL.
Monolayers were then incubated for 2 h at 37 °C. Fluorescence of the basolateral chamber medium was then measured at 405 nm excitation and 535 nm emissions with a prompt fluorimeter plate reader (Victor III, Perkin Elmer) to determine the amount of LY able to cross the monolayer. Three independent experiments, with three replicates each one, were carried out for each experimental dose. PET transwells without cells were used as positive control of LY flux.

2.5. Silver nanoparticles uptake and internalization

Confocal microscopy was used to visualize and locate AgNPs in the different cellular compartments of differentiated Caco-2 cells. To this end, differentiated Caco-2 cells were treated with 50 µg/mL of AgNPs for 24 h. Cells were then stained with Hoechst 33342 and Cell Mask at 1/500 and 2/500, respectively, during 10 min. AgNPs were visualized in the green channel, taking advantage of their own reflective capability. Images of the stained samples were acquired with the confocal laser scanning microscope Leica TCS SP5. Confocal images were processed with Huygens essential 4.4.0p6 (Scientific Volume Imaging, Netherlands) and Imaris 7.2.1 (Bitplane, AG). We have previously reported the advantage of confocal microscopy in nanotoxicology (Vila et al., 2017).

2.6. Silver nanoparticles translocation through monolayer

To determine the translocation of AgNPs from the apical to the basolateral compartment, different techniques were used. Transmission Electron Microscopy with Energy-dispersive X-ray spectroscopy (TEM-EDX) (Hitachi H 7000), Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) 7500ce (Agilent Technologies) and Fluorescent Confocal Microscopy (LEICA TSC SP5) techniques were used to detect and quantify AgNPs able to cross the differentiated Caco-2 cells monolayer. Briefly, after 24 h of treatment, the total medium (1.5 mL) of each basal compartment was resuspended and collected in 1.5 mL microtubes. Samples were treated with proteinase K to eliminate crystallized proteins and centrifuged in a speed vacuum at 37
ºC for 2 h to concentrate the AgNPs. This concentrate NPs suspension was used in the above-mentioned techniques. For ICP-MS samples they were digested in HNO₃ at 150 ºC for 30 min. For TEM analysis we follow our previous established protocols (Vila et al., 2017)

2.7. Genotoxic and oxidative DNA damage induction. Comet assay

The alkaline comet assay was used to assess the induction of DNA damage of differentiated Caco-2 cells. Samples used to specifically measure the oxidative DNA damage were also treated with formamidopyrimidine DNA glycosylase (FPG enzyme). Briefly, differentiated Caco-2 cells were treated for 24 h with increasing concentrations of AgNPs. Thirty min of 5 mM potassium bromate (KBrO₃), and 2.5 mM of methylmethanesulfonate (MMS) were used as positive controls of oxidative and genotoxic damage, respectively; untreated cells were used as negative control. All treatment conditions were performed in duplicates. After the aforementioned treatment, all samples were washed twice with PBS, trypsinized (1% trypsin) and centrifuged at 1000 rpm for 8 min. The pellet was resuspended in PBS to obtain around 17,500 cells/25 µL and placed at 4 ºC to avoid DNA repair. Cells were mixed with 0.75% of low melting agarose at 37 ºC and, dropped in triplicates (7 µL/drop) onto two different Gelbond® films (GF) and placed overnight in pH 10 lysis buffer at 4 ºC. After that, the GFs were washed twice (1 x 5 min, and 1 x 50 min) in pH 8.0 enzyme buffer at 4 ºC, and incubated for 30 min at 37 ºC with enzyme buffer. FPG enzyme (1/10.000) was added to one of the GF incubations to expose the oxidized purines from correspondently damaged DNA. FPG was a gift of Prof A. Collins (University of Oslo, Norway). Both GFs were then incubated with electrophoresis buffer (alkaline buffer) for 35 min at 4 ºC, followed by 20 min of electrophoresis at 20 V and 300 mA. Finally, GFs were rinsed twice in ice-cold PBS for 5 min, once in distilled water for 1 min, and then fixed in absolute ethanol for at least 2 h. GFs were then air-dried overnight at room temperature and stained with SYBR Gold (Invitrogen) for 20 min. Each GF film was cut
to fit into an acrylic slide (52.5 x 75 x 3 mm) and a coverslip of 52.5 x 75 mm was placed on top of the samples, effectively sealing them. Gels were analyzed using an epifluorescent Olympus BX50 microscope, and damage was quantified measuring the percentage of DNA in the tail by using the Komet 5.5 Image Analysis Software. One hundred randomly selected comet images were analyzed per sample.

2.8. RNA extraction and real-time RT-PCR

Total RNA from differentiated Caco-2 cells was extracted using TRIzol® Reagent (Invitrogen, USA) following the manufacturer's instructions. RNase-free DNase I (DNA-free TM kit; Ambion, UK) was used to remove residual DNA contamination. The first-strand cDNA synthesis kit (Roche, Basel, Switzerland) was used to obtain cDNA, using 100 ng of total RNA. The resulting cDNA was analyzed by real-time PCR with a LightCycler-480 to evaluate the relative expression of genes coding for the brush border enzymes sucrose isomaltase (SI) and solute carrier (SLC15A1), and tight junction components such as occludin (OCLN) and claudin (CLDN2) genes. β-actin was used as housekeeping control. Each PCR reaction was carried out in a total volume of 20 µL, containing 5 µL cDNA, 10 µL of 2x LightCycler 480 SYBR Green I Mater (Roche, Germany), 3 µL of distilled H₂O, and 1 µL of each primer at a final concentration of 500 nM. The cycling parameters were the following: a first step of 95 ºC for 5 min, then 45 cycles of 95 ºC for 10 s, 61 ºC for 15 s, and 72 ºC for 25 s. Cycle time (Ct) values were calculated with the Light Cycler 480 software package and then normalized with β-actin Ct values.

The primers used were: SI: direct 5’-TGGTGGCACTGTTATCCGAC-3’ and reverse 5’-GACCACCACGGACATGTAGG-3’; SLC15A1: direct 5’-CTTCGATGCTGTGCTGTACC-3’ and reverse 5’-GGCCAAGTGTCCATCTCTCT-3’; OCLN: direct 5’-ACAGACTACAACTGGCGG-3’ and reverse 5’-GCAGCAGCCATGTACTCTCTC-3’; CLDN2: direct 5’-TACTCACCACGTGCCCTGA-3’
and reverse 5'-GAGAGCTCCTTGTAAGCA-3'; β-Actin: direct 5'-
GCATGGAGTCCTGTGGCATC-3' and reverse 5'-CCACACGGAGTACTTGCGCT- 3').

2.9. Statistical analysis

Results were analyzed by using one-way ANOVA with Tukey's post-test, or an
unpaired Student's t-test, using the GraphPad Prism version 5.00 for Windows
(GraphPad Software, San Diego California USA, www.graphpad.com). Differences
between means in all cases were considered statistically significant at P<0.05.
3. Results

3.1. Characterization of silver nanoparticles

Figure 1 shows TEM morphologies and the mean size distribution of AgNPs calculated by measuring over 100 particles in random fields of view. This size was 7.74±2.48 nm (mean ± standard deviation) (Fig. 1A and 1B). This value was lower than the primary particle size (16.7 nm) reported by the supplier. The average hydrodynamic radius and zeta potential of AgNPs were measured in cell culture medium (DMEM) at a concentration of 100 μg/mL. The observed values, right after sonication and after 24 h, were 80.81±3.40 nm and -6.84±0.20 mV, and 94.47±0.34 nm and -8.32±1.00 mV, respectively (Fig. 1C). These measurements indicate some degree of agglomeration when NPs are dispersed in cell culture medium.

3.2. Cell viability after AgNPs exposure

In order to find the optimal working concentrations to test AgNPs non-toxic effects on our system, a viability assay was performed scoring surviving cells by using a cell counter device. The results are shown in Figure 2. As observed, differentiated Caco-2 cell viability was not significantly affected by AgNPs compared to the untreated control at the screened doses, showing a decrease of only 20% of their viability, after being exposed for 24 h to 100 μg/mL. Consequently, concentrations under 50 μg/mL were chosen to test further effects of AgNPs, giving their low cytotoxicity. An interesting observation is that these viability results strongly differ from those obtained with undifferentiated Caco-2 cells, where an IC_{50} value of 12.23 μg/mL was obtained (Figure 2). This would indicate that differentiated Caco-2 cells become resistant to the toxic effects of AgNPs, with regards to the original undifferentiated cell line. This could be associated with a lower uptake in differentiated cells and, consequently, with a higher intracellular accumulation of AgNPs in undifferentiated cells.

3.3. Monolayer integrity
After 21 days of culture, differentiated Caco-2 formed a well-structured monolayer (CCM). Figure 3 shows different TEM images depicting the monolayer’s ultrastructure and its different components. As observed, cells have acquired several morphological markers of differentiation, such as the appearance of microvilli and tight junctions. To determine the potential effects of AgNPs on the integrity of this differentiated CCM, TEER and LY assays were carried out. Initially, TEER values during differentiation were analyzed. Results on Figure 4 indicate that, as expected, TEER progressively increased from week 1 to week 2, reaching a plateau in week 3 (Figure 4A). Interestingly, AgNPs exposure did not induce significant changes in this parameter when using sub-toxic concentrations (1, 10, 25 and 50 µg/mL), suggesting that membrane integrity remains unaffected under these concentrations (Figure 4B).

To evaluate the integrity of the CCM in a different way, a Lucifer yellow assay was carried out. As Lucifer yellow transport is paracellular, it can measure tight-junction integrity and paracellular permeability. As observed in Figure 5, the different concentrations of AgNPs tested did not lead to significant changes for Lucifer yellow able to cross the monolayer and, consequently, to be detected in the basolateral medium. This would support the data obtained with TEER measurements. When the LY flux was measured in empty (without cells) PET transwells, an average of about 50% was detected in the basolateral chamber. Taken together, these results indicate that sub-toxic concentrations of AgNPs do not alter Caco-2 monolayer, maintaining the isolation of the apical and basolateral compartments.

3.4. Cellular uptake of AgNPs

Cell uptake is an important parameter to be evaluated in order to interpret the potential biological effects of AgNPs. In a first instance, TEM was used as a first approach to visualize internalized AgNPs, but no clear presence of these particles was detected inside the cells, probably due to the granulated nature of the Caco-2 cytoplasm (Figure 6). The complementary use of energy dispersive X-rays
microanalysis (EDX), to discriminate suspicious dots, granted negative results (data not shown). To solve this problem, we tried a novel approach using confocal microscopy, since this method is capable of detecting metallic NPs due to their ability to reflect polarized light. As observed in Figure 7, this method resulted very fruitful since a large amount of AgNPs were detected inside the cells. The use of specific dyes allowed us to differentiate those NPs enclosed in the cytoplasm and in the nuclear compartment; as well as those attached to cellular or nuclear membranes. These results confirm that AgNPs are internalized by the CCM and that confocal microscopy is a suitable method, not only to detect AgNPs cell uptake but also to localize the exact fate of the studied NPs.

3.5. Silver nanoparticles translocation through Caco-2 cells monolayer

In addition to the demonstrated uptake of AgNPs by Caco-2 cells, it is important to know if AgNPs are able to cross the epithelial barrier. To evaluate the capability of AgNPs to reach the basolateral compartment, different techniques including TEM, TEM+EDX, ICP-MS, and confocal microscopy were used. As shown in Figure 8, no observed peaks corresponding to Ag were obtained in the TEM assay when complemented with EDX analysis (Figure 8B). Similar negative results were obtained using ICP-MS. In this case, a concentration-related increase was observed in the apical medium, accordingly to the applied dose (Figure 8D). Although an apparent concentration-related effect was also observed in the basolateral media, values were always below 0.01 µg (Figure 8C), which is the quantification limit of the technique. Thus, all those values under the limit of quantification (LOQ) are approximations and non-real absolute values. Given the clear positive results obtained when evaluating AgNPs uptake by confocal microscopy, this technique was also used to evaluate translocation. As shown in Figure 8F, no translocation was observed when analyzing the samples with this technique, even though its usefulness in assessing the translocation of other metallic NPs, such as TiO$_2$, was proved (Figure 8G) as
demonstrated in the frame of the EU project NanoReg (NanoReg, 2017). The positive findings obtained with TiO$_2$NPs support the goodness of the used methodology to detect NPs translocation through Caco-2 monolayer. Taken as a whole, these results imply that even though AgNPs are internalized, they are not able to cross the differentiated CCM to reach the basolateral compartment, at least at significant concentrations.

**3.6. Genotoxic and oxidative DNA damage induction**

The levels of genotoxic and oxidative DNA damage induced by AgNPs were assessed using the alkaline comet assay. The effects of four concentrations (1, 10, 25 and 50 µg/mL) were evaluated after 24 h of exposure using the percentage of DNA in the tail as a biomarker. The obtained results showed no increases of DNA breaks, in contrast to the results obtained when treating with the positive control, MMS 0.5 mM (Figure 9A). However, when the FPG enzyme was added to induce single-strand breaks, after oxidized-bases excision, a small but significant concentration-dependent effect was observed, suggesting that a significant induction of DNA oxidative damage occurs after AgNPs treatment (Figure 9B).

**3.7. Gene expression changes**

To detect the potential effects at a molecular level on the barrier constituted by differentiated Caco-2 cells, changes in the expression of genes specifically associated with the enteric epithelium function/morphology were evaluated. Four genes were selected, two related to the brush border function (SI and SLC15A1), and two related to the tight junction components (OCLN and CLDN2). Results displayed in Figure 10 show that 24 h of exposure to different concentrations of AgNPs were able to induce concentration-dependent increases in the expression of the four selected genes. Although 2-3 fold upregulation was obtained with concentrations higher than 10 µg/mL, these increases did not attain statistical significance.
4. Discussion

Caco-2 cells, originally established from a human epithelial colorectal adenocarcinoma, are able to spontaneously differentiate and polarize into enterocyte-like cells and have been widely used by the pharmaceutical industry (Shah et al., 2006). Some of the most representative subcellular structures of enterocytes, such as microvilli and tight junctions between cells, drug carriers and efflux proteins, appear after polarization and differentiation of Caco-2 cells, particularly when they are cultured in a two-dimensional system with semipermeable inserts (Sambuy et al., 2005). All these characteristics make this model very suitable to be used for determining the potential translocation, through the intestinal barrier, of ingested nanomaterials (Faust et al., 2014).

One interesting finding of our study is that the cytotoxicity of AgNPs in differentiated cells is significantly lower than in undifferentiated cells. The obtained IC$_{50}$ for undifferentiated cells (12.23 µg/mL) was much lower than the value obtained in differentiated cells (129.37 µg/mL). This suggests that the state of differentiation is able to modulate the sensitivity of Caco-2 cells to different toxic agents, including NPs. After differentiation, Caco-2 cells acquire several epithelial characteristics such as cell polarity, the formation of microvilli, the presence of tight junctions, expression of digestive enzymes in the brush border, and secretion and assembly of a matrix. All this could be partly responsible for the protection against the cytotoxic effects of NMs observed in undifferentiated cells. Furthermore, cell density and cellular confluence could also influence the sensitivity of cells to NMs toxicity, making the more confluent cells more resistant to the effects of NMs (Gerloff et al., 2013). According to this, the internalized amount of AgNPs can change significantly depending on the status, affecting cell viability. Unfortunately, we have not quantitative measures of the cellular content of AgNPs to confirm this proposal. Similar results have been obtained for other nanomaterials like TiO$_2$NPs (Song et al., 2015), where authors assumed that microvilli
may impede the attachment of NPs to cells and their consequent internalization, hence preventing cytotoxicity. At higher confluence conditions, the number of cells per cm$^2$ is increased and therefore the amount of NMs per cell also decreases. Taken together, these data point out the importance of differentiation status to assess the toxic effect of NMs. In this way, the use of undifferentiated cells in the estimation of toxicity could lead to an overestimation of the real toxicity of NMs when applied to differentiated cells. At this point, it should be indicated that AgNPs dietary uptake is estimated to be 70 to 90 µg/day (Wijnhoven et al., 2009). To extrapolate this dose to in vitro concentrations we must take into account that most of the nutrient absorption process occurs in the first section of the small intestine. This section has approximately 900 cm$^2$ of absorbing surface area (Muir and Hopfer, 1985; Kararli, 1995). Therefore, an estimated daily ingesting of 70–90 mg/day of AgNPs supposes a daily exposure of the small intestine to approximately 100 µg/cm$^2$. Assuming an enlargement factor of approximately 13 times, due to the presence of microvilli, and considering that our in vitro barrier is grown in a PET transwell with a surface area of 1.12 cm$^2$ (Helander et al., 2014), we should have at least 8.61 µg of AgNPs per PET transwell. This means that our range of selected doses can be considered as a realistic one. Importantly, in vitro simulation of digestion indicated that AgNPs only partially aggregate as a result of the digestive process, without significant changes in their biological properties (Böhmert et al., 2014). Nevertheless, AgNPs can dissolve in culture medium and released ions can differentially affect Caco-2 cells, depending of their differentiation status. AgNPs present an important solubility and after 24 h in cell culture medium; about 10% of the initial concentration is present on the ionic form. Nevertheless, this value does not undergo important changes in the following 72 h (Vila et al., 2017).

Cell barrier integrity is an essential prerequisite to avoid undesired transport/absorption of NPs. In our model, the obtained TEER values of the differentiated monolayer were considered adequate for this type of experiment (589±37 Ω·cm$^2$), and similar to those reported by different authors (Piret et al., 2012; Tu et al., 2016). In fact,
our threshold value was more stringent than the ones proposed by Piret et al. (400 Ω·cm²) and Tu et al. (450 Ω·cm²). In addition, the permeability of the CCM, measured by the passive crossing through the paracellular space, is another way to evaluate monolayer integrity (Liu and Chiu, 2013). In our study, an incubation of 24 h with AgNPs did not alter the integrity or permeability of the intestinal epithelium, regardless of the concentration. Although these negative findings agree with those obtained by other members of the NANoREG consortium (unpublished data), they differ with those reported by Martirosyan et al. (2014) who found alterations in TEER and LY values after exposure to 30-90 µg/mL of AgNPs. These differences could be attributed to the use of co-cultures of Caco-2 and Raji-B cells by Martirosyan and colleagues. In their co-culture conditions, some Caco-2 cells differentiated into M cells, weakening the barrier function of the monolayer and, consequently, inducing changes in the integrity parameters when exposed to silver nanoparticles. Indeed, they also reported toxicity in their study, with an IC₅₀ of 40 µg/mL.

Gene expression profile of Caco-2 cells maintained in transwell, and cultured for 21 days, shows a coordinated decrease in the expression of genes involved in cell cycle progression, DNA synthesis, RNA processing and protein translation, but an increase in the expression of genes involved in the metabolism of xenobiotics and in the deposition of extracellular matrix. In addition, other genes specifically linked to the enterocytic phenotype, such as the brush border proteins DPP4 and VIL1, the CDH1 adherent binding protein and the CLAUDIN 7 tight junction binding protein also undergo an increase in expression (Mariadason et al., 2002). According to that, we analyzed changes in the expression of brush border transporter genes (SLC15A1 and Sf) and cell junction (CLDN and OCLN) genes to detect possible effects of AgNPs on differentiated Caco-2 after 24 h of exposure, as potential markers of barrier disturbance. Although increases in mRNA levels were observed for all four selected genes, the observed increases did not attain statistical significance, due to the great variability observed in the gene expression levels. This lack of effect would support the
previously indicated data of TEER and LY, pointing out that AgNPs exposure does not induce changes at structural or at functional levels on the Caco-2 monolayer. Moving to the protein level, changes in the distribution of occludin and zonula occludens-1 proteins were reported after AgNPs exposure by using immunostaining methods (Martirosyan et al., 2014). Nevertheless, it must be noted that immunostaining and subsequent confocal measurements only give semi-quantitative measurements of expression changes. In spite of that, these positive findings could indicate that protein levels are a better biomarkers than gene expression to detect this type of AgNPs effects. According to that, a recent proteomic study reported a considerable number of proteins differentially expressed after treatment with AgNPs in differentiated CCM. Those were related to protein folding, synthesis or modification of proteins as well as cellular assembly and organization (Oberemm et al., 2016).

In addition to the potential disruption of the monolayer structure, it is important to evaluate if NMs are able to internalize into Caco-2 monolayer or, more importantly, if they are able to cross this barrier. By using confocal microscopy, we have been able to demonstrate AgNPs uptake by differentiated CCM. This points out the usefulness of this method to detect internalized metallic NMs, especially when the use of differential staining allows to distinguish if NMs are located on the surface of the epithelia or if they are internalized in the cells’ cytoplasm or in their nuclei (Tariq et al., 2016). Interestingly, the important uptake of AgNPs was not associated with toxicity or monolayer disruption. Furthermore, in spite of the great uptake, no AgNPs translocation was observed through the CCM, regardless of the multiple techniques used (EDX coupled to TEM, confocal microscopy, and ICP-MS). This suggests that there is no significant translocation of AgNPs through the monolayer of differentiated Caco-2 cells, at least after 24 h of exposure. Similar results have been recently published where no translocation of AgNPs was observed by using ion beam microscopy (Lichtenstein et al., 2017).
One of the common mechanisms of toxicity mediated by NPs is the induction of oxidative stress (Oberdörster et al., 2005; Ayres et al., 2008). Indeed, this has been demonstrated with AgNPs, which induce high levels of reactive oxygen species (ROS) within cells (Kawata et al., 2009; Eom et al., 2010). Among the potential targets of ROS, oxidative attack to DNA is probably the most important. Several studies have shown that exposure to AgNPs induce genotoxicity in cultured cells expressed as increases in the levels of DNA breaks detectable by the comet assay (Liu et al., 2010; Piao et al., 2011), adducts in DNA, and increases in the frequency of micronuclei (AshaRani et al., 2009; Foldbjerg et al., 2012). In the present study, increases in the levels of oxidative DNA damage were observed by exposing differentiated CCM to AgNPs for 24 h. This effect was dose-dependent, as observed using the FPG-modified comet assay. Few studies have been designed to detect oxidative stress in Caco-2 cells induced by AgNPs, but none of them used the comet assay as approach. This is, therefore, a remarkable characteristic of our study. The other studies use indirect approaches like measuring intracellular levels of ROS by using the DCFH assay or measuring the expression of genes related to specific pathways like Nrf/HO. These studies have been carried out using both undifferentiated (Aueviriyavit et al., 2014; Song et al., 2014), or differentiated cells (Böhmer et al., 2015; Georgantzopoulou et al., 2016). Interestingly, negative effects were observed in co-cultures with mucus secretory cells (HT29), where the presence of mucus acts as a mechanical barrier reducing their interaction with the cellular membrane (Georgantzopoulou et al., 2016). The use of mixed cultures combining Caco-2 and mucus secretion cells is a future challenge looking for more realistic models of the intestinal barrier.

Summing up our results, we can conclude that when differentiated Caco-2 cells form a well-structured monolayer, they became resistant to the toxic effects of AgNPs, in contrast with what has been observed in undifferentiated cells. This structure also became resistant avoiding the paracellular traffic of AgNPs and maintaining its integrity. Nevertheless, differentiated cells can uptake important amounts of AgNPs, as
demonstrated by using confocal microscopy. This result supports the advantages of using confocal microscopy to determine the intracellular fate of metallic NPs like AgNPs. In spite of the demonstrated uptake, we failed to observe translocation of AgNPs through the monolayer, independently of the numerous methodological approaches used. Finally, the use of the comet assay complemented with the use of FPG enzymes that recognize oxidized DNA bases and excise them, has confirmed that in differentiated Caco-2 cells AgNPs increase the induction of ROS, which are able to target DNA. Although oxidative damage itself can be considered as a risk factor, more emphasis is needed to understand the possible translocation of AgNPs through the membrane. To this end, more complex models, including goblet cells secreting mucus, should be used to better evaluate their interaction with these in vitro models simulating the intestinal barrier. It could be also interesting to find models of intestinal barrier that allow us to perform long-term studies to mimic a most real exposure scenario.
Acknowledgements

This investigation has been partially supported by the Ministry of Economy and Competition (SAF2015-63519-R), and the EC FP7 NANoREG (Grant Agreement NMP4-LA-2013-310584). A. Garcia-Rodriguez and L. Vila were funded by postgraduate fellowships from the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, respectively.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.
References


cells: intestinal uptake and onward movement. Prog. Histochem. Cytochem. 46, 185-252.


FIGURE LEGENDS

**Figure 1.** (A) TEM images of AgNPs in dry form. (B) Size distribution of AgNPs was calculated over 100 randomly selected particles. (C) NPs average size measured by TEM and DLS. NPs charge was obtained by pre-wetting with 0.5% ethanol and steric stabilization was achieved using sterile-filtered 0.05% w/v BSA. Data are represented as mean ± SD.

**Figure 2.** Cell viability of differentiated and undifferentiated Caco-2 cells treated with a range of 0-100 µg/mL of AgNPs for 24 h. Data are presented as percentage of viable cells compared to untreated controls. Results were analyzed with an unpaired Student's t-test. *P<0.05, **P<0.01, ***P<0.001.

**Figure 3.** TEM images of the differentiated Caco-2 cells monolayer. The individual cells (C), their microvilli (MV) and tight junctions (TJ) can be appreciated on the transwell support matrix (T).

**Figure 4.** TEER values evaluated during Caco-2 cells differentiation and monolayer formation (A) and after 24 h of AgNPs treatment at different concentrations (B). In this last figure, values just before and after exposure are indicated. Data represented as mean ± SEM. Results were analyzed according to a one-way ANOVA with a Tukey post-test. ***P<0.001.

**Figure 5.** Percentage of LY in the basolateral chamber after 24 h of treatment with concentrations of AgNPs ranging from 1 to 50 µg/mL. Data represented as mean ± SEM. Non statistically significant differences were observed (one-way ANOVA). The percentage of LY in the basolateral chamber of empty (without cells) PET transwells was about 50%.

**Figure 6.** Detailed TEM images from untreated differentiated Caco-2 cells monolayer. As portrayed, the granulated cytoplasm can complicate NPs identification.

**Figure 7.** Confocal images from differentiated Caco-2 cells treated with 50 µg/mL of AgNPs (A, B, C). In A, boxes at right and bottom correspond to lateral views. Cellular uptake was evaluated 24 h after treatment. Nucleuses are stained in blue, cell membranes in red, and AgNPs in green. Arrows indicate the localization of AgNPs in cells.

**Figure 8.** Translocation studies of AgNPs through Caco-2 monolayer using different techniques. Basolateral medium concentrates were analyzed 24 h after AgNPs exposure. TEM image (A). TEM+EDX analysis (B). ICP-MS quantitation of total Ag in the basolateral (C) and apical chambers (D) indicating as dotted line the limit of
quantification (LOQ). Confocal microscopy images of negative control (E) and samples treated with AgNPs (F) and TiO$_{2}$NPs, as positive control (G).

**Figure 9.** Analysis of genotoxic (A) and oxidative DNA damage (B) after 24 h of AgNPs exposure, at different concentrations, using the comet assay. As positive control, 30 min of MMS (0.5 mM) or KBrO$_{3}$ (5 mM) were used to assess genotoxic and oxidative damage, respectively. Data represented as mean ± SEM. Results were analyzed according to a one-way ANOVA with a Tukey post-test. ***$P<0.001$.

**Figure 10.** Relative mRNA expression measured by real-time RT-PCR of $SI$, $SLC15A1$, $CLDN2$ and $OCLN$ genes after 24 h of exposure to AgNPs, compared to the control. Three biological replicates were used per dose. Data represented as mean ± SEM.
Figure 1

A

B

C

<table>
<thead>
<tr>
<th>NP</th>
<th>TEM Mean size (nm)</th>
<th>DLS Average diameter (nm)</th>
<th>DLS PDI</th>
<th>LDV Zeta potential ζ (mV)</th>
<th>LDV Electrophoretic mobility (µm cm V⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNPs (0h)</td>
<td>7.74 ± 2.48</td>
<td>80.81 ± 3.4</td>
<td>0.369 ± 0.054</td>
<td>-6.84 ± 0.2</td>
<td>-0.54 ± 0.01</td>
</tr>
<tr>
<td>AgNPs (24h)</td>
<td>/</td>
<td>94.47 ± 0.34</td>
<td>0.394 ± 0.009</td>
<td>-8.32 ± 1.00</td>
<td>-0.65 ± 0.07</td>
</tr>
</tbody>
</table>
Figure 2

![Graph showing relative survival rate (%)](image)

- **Differentiated Caco-2 cells**
- **Undifferentiated Caco-2 cells**

- * indicates a statistically significant difference compared to undifferentiated Caco-2 cells.
- ** indicates a very significant difference.
- *** indicates an extremely significant difference.

**Relative survival rate (%)**

**AgNPs (µg/mL)**

0 20 40 60 80 100 120
Figure 3
Figure 4

A

![Graph A]

B

![Graph B]
Figure 5

![Graph showing the percentage of LY in the BL chamber for different treatment levels (µg/mL).]
Figure 6
Figure 8

(A) Basolateral medium

(B) Apical medium

(C, D) Treatment of AgNPs (µg/mL)

E, F, G: Images of treated samples
Figure 9

A  Genotoxic DNA damage

B  Oxidative DNA damage
Figure 10

- **SI**
  - mRNA expression (% of CT) vs. Treatment of AgNPs (µg/mL)

- **SCL15A1**
  - mRNA expression (% of CT) vs. Treatment of AgNPs (µg/mL)

- **CLDN2**
  - mRNA expression (% of CT) vs. Treatment of AgNPs (µg/mL)

- **OCLN**
  - mRNA expression (% of CT) vs. Treatment of AgNPs (µg/mL)
Highlights

- An *in vitro* model of intestinal barrier constituted by differentiated Caco-2 cells has been used.
- A wide set of biological effects induced by AgNPs were measured.
- AgNPs uptake depends on the differentiation state.
- No morphological/functional changes in the barrier were observed.
- Confocal microscopy detects uptake, but not translocation.
- Exposed cells present increased levels of oxidized damage in DNA.