FINAL REPORT

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Possible protective effects of food phenolic compounds on the potentially deleterious interaction of nanoparticles with the human intestinal barrier: *in vitro* studies

Grant holder: Dr. **Alina Martirosyan**

Project leader: Prof. Dr. Yves-Jacques SCHNEIDER

Host Organization: Catholic University of Louvain, Institute of Life Sciences, Laboratory of Cellular, Nutritional and Toxicological Biochemistry, Croix du Sud 4-5, B 1348 Louvain-la-Neuve, Belgium.

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Executive Summary

Engineered nanomaterials (ENMs) are already a part of our daily life in the form of cosmetics, food packaging, drug delivery systems, therapeutics, biosensors, etc. According to the Woodrow Wilson Inventory, nowadays silver nanoparticles (Ag-NPs) are the most commonly used nanomaterial in consumer products (http://www.nanotechproject.org). With an increasing number of consumer and industrial products containing Ag-NPs, mostly used for antimicrobial and pharmaceutical applications, the risk of human exposure increases, which could have toxicological implications.

The objective of the project "Possible protective effects of food phenolic compounds on the potentially deleterious interaction of nanoparticles with the human intestinal barrier: *in vitro* studies" was to better understand the likely biological impact of food-related NPs, and mainly of Ag-NPs, on the human gastrointestinal tract (GIT) via the advanced cell-based models. The project highlighted several aspects of the cytotoxicity of Ag-NPs on the human GIT *in vitro*, as well as the potential protective effect of food matrix component, *i.e.* phenolic compounds, against the harmful effect of Ag-NPs.

The project was funded by the Belgian Science Policy Office (BELSPO) (Post-doc fellowships to non-EU researchers), together with the Marie Curie Actions of the European Commission (FP7-PEOPLE- COFUND-2008).

Background to the project

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. 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-NPs were the most commonly consumed ENMs, followed by TiO_2, SiO_2, ZnO, Au, Pt, etc (http://www.nanotechproject.org).

In many countries ENMs are already used as food supplements and in food packaging: (i) nanoclays as diffusion barriers [FSAI 2008]; (ii) Ag-NPs as antimicrobial agent [Sanguansri & Augustin 2006, Chaudhry et al. 2008]; (iii) silicates and aluminosilicates (E554, E556, E559) as anti-caking and anti-clumping agents and in toothpastes, cheeses, sugars, powdered milks, etc (Lomer *et al*. 2002); (iv) TiO_2 (E171) for whitening and brightening, e.g. in sauces and dressings, in certain powdered foods (Lomer *et al*. 2004), etc.

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 (Oberdörster *et al*. 2005). 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 (Oberdörster 2004, Donaldson & Stone 2003, Reeves *et al*. 2008).

Human exposure to ENMs present in food or food contact materials occurs through ingestion. The whole cascade of events including absorption, distribution, metabolism and excretion/elimination (ADME) occurs following ingestion and determines the internal exposure and toxicity of these substances. However, due to the

interactions of NMs with surrounding matrix and unexpected effects resulting from this, little is known regarding their behaviour and fate in the GIT.

From this point of view the interaction of ENMs 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, their ADME properties.

Phenolic compounds (PCs) represent a group of plant secondary metabolites that widely occur e.g. in fruits, vegetables, wine, tea, chocolate and other cocoa products and are an important part of human diet. They are mostly derivatives and/or isomers of flavones, isoflavones, flavonols, catechins, stilbenes and phenolic acids. Recently, growing interests on phenolic compounds have focused on their biological activities linking to human health benefits, such as antioxidant, cardioprotective, anticancer, antiinflammatory, antiaging and antimicrobial properties. Experimental data indicate that a significant proportion of these biological actions can be attributed to their intrinsic antioxidant capabilities, mainly ascribed to their free radical scavenging and metal chelating properties, as well as their effects on cell signaling pathways and on gene expression (Soobrattee *et al*. 2005, Dell Agli *et al*. 2005).

Considering all above-mentioned, the important objectives of the project therefore were to study the potential toxicity of food related ENMs, and based on the widespread use mainly of Ag-NPs on the GIT, on one hand, as such and, on the other hand, in the presence of food matrix compounds, *i.e.* PCs that are already known as protective against ROS production and intestinal inflammation. Due to the existing controversial date on the involvement of silver ions (Ag^+) in the toxicity of Ag-NPs, within the frames of this project, the potential role of Ag^+ in the acute toxicity of Ag-NPs on GIT was also evaluated. The evaluation and understanding of the behavior and effects of NPs alone and in the presence of PCs in advanced cell-based models can give indications on the behavior and fate of NPs in physiological conditions in GIT and permit the prediction of their health-threatening effects upon ingestion with food.

Progress towards the objectives

Given the wide range of applications of Ag-NPs in food and pharmaceutical industry, it becomes mandatory to study its toxicity, on one hand, as such and, on the other hand, in the presence of food matrix components, *i.e.* PCs, which represent the major part of human daily diet.

Objective 1. Unravel the potential acute toxicity of Ag-NPs (NM-300K, < 20 nm, JRC repository, Ispra, It) via the advanced cell-based models of the GIT: (i) the toxicity mechanisms following the interaction of NPs with cells were elucidated; (ii) efforts were done to estimate the absorption and transport of NPs through the epithelial monolayer; (iii) NP-induced oxidative stress and inflammatory responses were elucidated

In the current study the concentrations of Ag-NPs were chosen based on the available daily consumption dose of silver that may reach up to 90 μg (Wijnhoven *et al.* 2009, WHO 2003). The Ag-NPs were shown to possess cytotoxic effect on Caco-2 cells depending on concentration (Figure 1A). Our results *in vitro* seemingly confirm the *in vivo* results, estimating the Ag-NPs are cytotoxic starting from concentration of 30 μg/ml (Kim *et al.* 2010).

Our results have shown that the fluorescence intensity of oxidative stress indicator DCFH-DA was increased upon 3h exposure of cells to Ag-NPs, resulting in about 1.5 to 3-fold increase of intracellular ROS level as compared with the untreated cells (Figure 1B). Thus one predominant mechanism of toxicity of Ag-NPs is likely to be mediated by oxidative stress.

The inflammatory chemokine interleukin 8 (IL-8) levels were shown to decrease in a dose-dependent manner under the influence of Ag-NPs in the apical compartment of the bicameral insert, while practically no changes were observed in the basolateral compartment (data not shown). The IL-8 decrease was observed in both mono- and co-cultures with more obvious increase in the co-cultures. There were no changes observed in either

compartment for the IL-6 levels neither in mono-, nor in co-cultures.

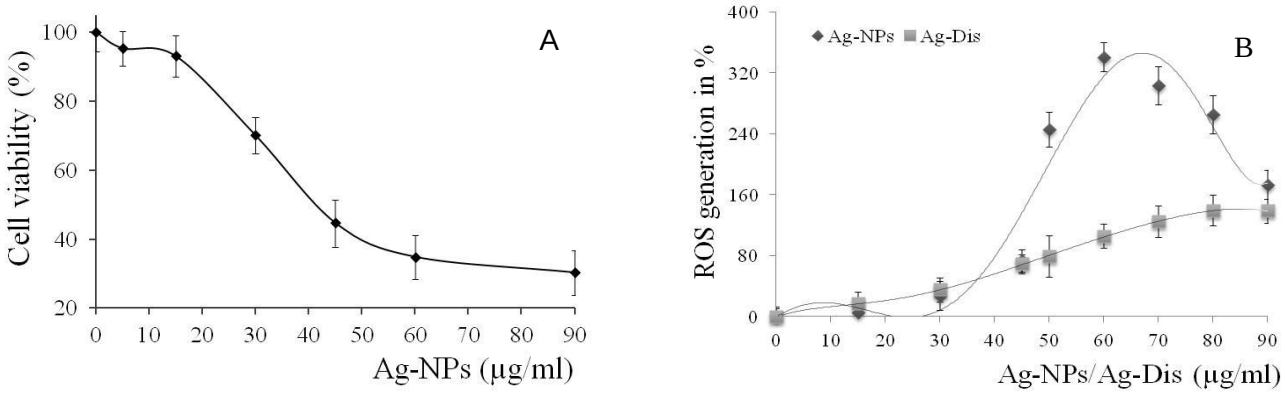


Figure 1. Effect of Ag-NPs on metabolic activity (MTT test) (A) and ROS generation induction (DCFH-DA assay) (B) of mono-cultures of Caco-2 cells, incubated for 3h at 37^0C with Ag-NPs (15 – 90 µg/ml). Data represent means ± SEM (N=2, n=3, P<0.001).

Nitric oxide (NO) concentration changes were observed in both compartments of the Transwell inserts depending on Ag-NP concentration. Changes were more significant in the basolateral sides and again, in correlation with the IL-8 data, NO level changes were more expressed in co-cultures when compared to mono-cultures.

Objective 2. Evaluate the complication of cell-cell interactions and thus the intestinal barrier function upon exposure to Ag-NPs. Main attention for the evaluation of the barrier integrity was given to the tight junctions (TJs) – the most apical components of the junctional complex and main gatekeepers of the epithelial paracellular passage.

The gastrointestinal epithelium allows the absorption of nutrients, while being responsible also for the intestinal barrier function. One of the major gatekeepers of the intestinal barrier is represented by epithelial TJs (Ward *et al.* 2000) that link neighboring epithelial cells together and thus determine the paracellular permeability. In the current study, the influence of Ag-NPs on the TJs proteins occludin and associated with it *zonula occludens* 1 (ZO-1) was shown. In the presence of Ag-NPs the occluding and ZO-1 distributions were altered (Figure 2).

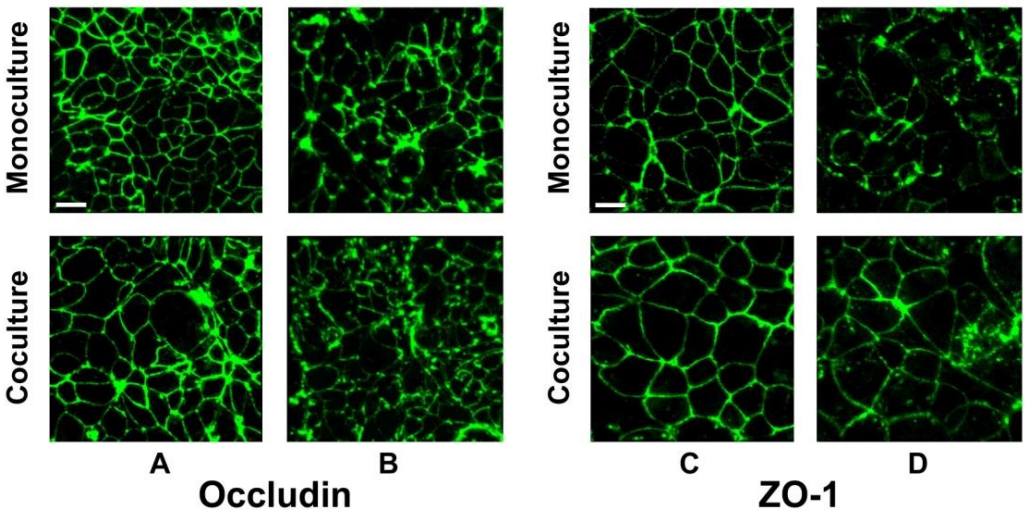
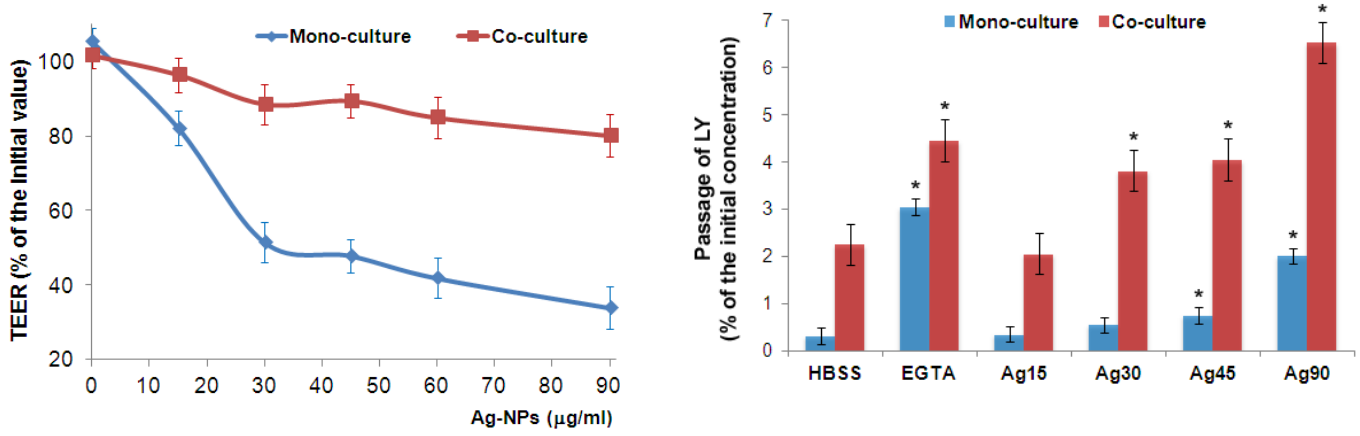


Figure 2. Subcellular localization of the occludin and ZO-1 TJs scaffolding proteins. Mono- and co-cultures of Caco-2 cells grown on bicameral inserts were treated with Ag-NPs (45 µg/ml) for 3h and then processed for immunostaining (B and D). Untreated cells were used as controls (A and C). In order 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 microscopy; scale bars are 15 and 25 µm for occludin and ZO-1 staining, respectively.

The labeling of TJs proteins was less intense upon treatment with NPs, junctions appearing to be dashed and degraded. The delocalization of occludin and ZO-1 led to a decrease in intercellular binding capacity and thus triggered an increase of epithelial permeability. The latter was evidenced by the NP-induced decrease of TEER value (Figure 3A) and increased passage of Lucifer Yellow (LY) (Figure 3B) (Martirosyan *et al*. 2012). The barrier integrity disruption seems to be characteristic endpoint of Ag-NPs toxicity, as the Ag-NPs has been shown also to increase the blood-brain barrier permeability (Trickler *et al.* 2010).



A (P<0.0001) B (P<0.003)

Figure 3. 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 µg/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 µm pore size (TranswellTM, 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 and the LY passage were calculated as a percentage from the initial value. Data represent the means ± SEM of 4 independent experiments. *Samples significantly different from the control (results were considered significant at P<0.05).

Objective 3. Evaluate the role of silver ions (Ag^+) in the toxicity of Ag-NPs on GIT.

Observed adverse toxic effects of Ag-NPs could be associated with the release of Ag^+ (Asghari *et al.* 2012, van der Zande *et al.* 2012). Contradictory data in the literature stimulate us to study the contribution of Ag^+ in the toxicity of Ag-NPs. Our results revealed that the claimed 10% release of Ag^+ from Ag-NPs solution (van der Zande *et al.* 2012, Geranio *et al.* 2009) leads to a cell viability decrease. This effect was about two times weaker than that of Ag-NPs, suggesting a partial contribution of released Ag^+ in the overall toxicity of Ag-NPs. However, it should be taken into account that in the presence of complex biological matrices the dissolution kinetics of Ag-NPs could be much faster, e.g. in the presence of hydrogen peroxide (Geranio *et al.* 2009, Ho *et al.* 2010) and at acidic pH. Thus the interaction of Ag-NPs with cells/cell metabolites may cause more rapid Ag^+ release than predicted/estimated for the oxidation of Ag-NPs suspensions (Navarro *et al.* 2008, Ho *et al.* 2010).

MTT assay revealed a dose-dependent decrease of Caco-2 cells metabolic activity upon 3h incubation with Ag^+ (Figure 3) (Martirosyan *et al*. 2013). Ag^+ at concentrations, corresponding to 10% of the total silver amount, as reported by van der Zande *et al*. (2012), which, although maximal, is relevant to what could be released from Ag-NPs, was toxic for Caco-2 cells. Nevertheless, even in these extreme conditions, Ag^+ appeared less toxic than

the Ag-NPs. It worth noting that Ag^+ at the lowest used concentration of 1.5 µg/ml increased significantly the metabolic activity of Caco-2 cells, in contrast to Ag-NPs, which tended to decrease the cells metabolic activity.

The analysis of released Ag^+ capability to induce oxidative stress revealed that upon 3h incubation of Caco-2 cells with $AgNO_3$, the intracellular fluorescence of DCFH-DA was increased depending on the Ag^+ concentration, indicating the ROS formation induction (Figure 4) (Martirosyan *et al*. 2013). In line with the cytotoxicity data, the oxidative stress induced by Ag^+ was much lower compared with that induced by Ag-NPs. It is therefore evident that increased ROS production as a consequence of Ag^+ release has a partial contribution in the induction of oxidative stress via Ag-NPs.

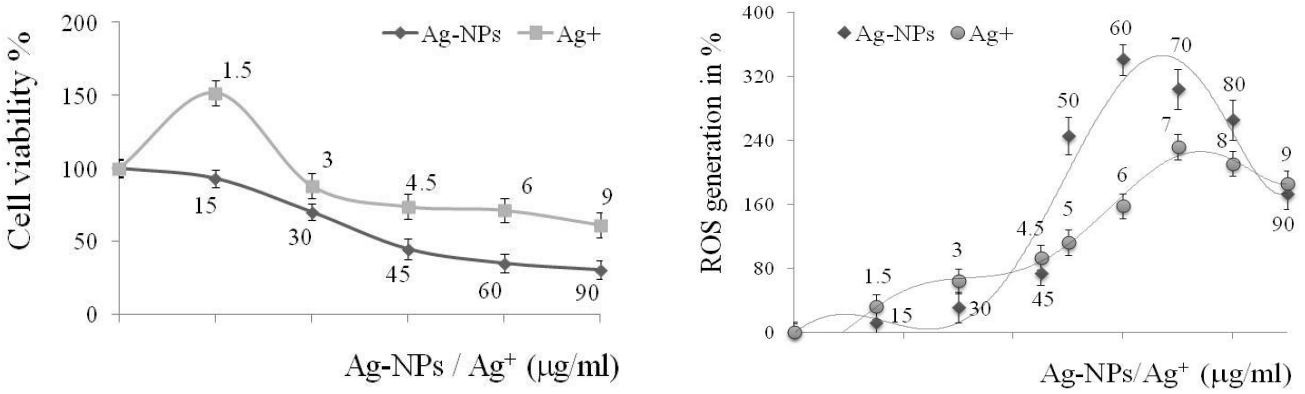


Figure 4. Cell viability (left) and ROS generation induction (right) under the influence of Ag-NPs (15 – 90 µg/ml) and Ag^+ (1.5 – 9 µg/ml).

Objective 4. Unravel the toxicity of Ag-NPs and Ag^+ in the presence of PCs.

The above-mentioned parameters of NP-cell interaction were evaluated in the presence of PCs, *i.e.* quercetin (Q), kaempferol (K), resveratrol (R). For the first time the preventive effect of two flavonoids Q and K against the cytotoxic effect of nanosilver, as well as prevention of NP-induced epithelial barrier impairments were shown (Figure 5) (Martirosyan *et al*. 2013).

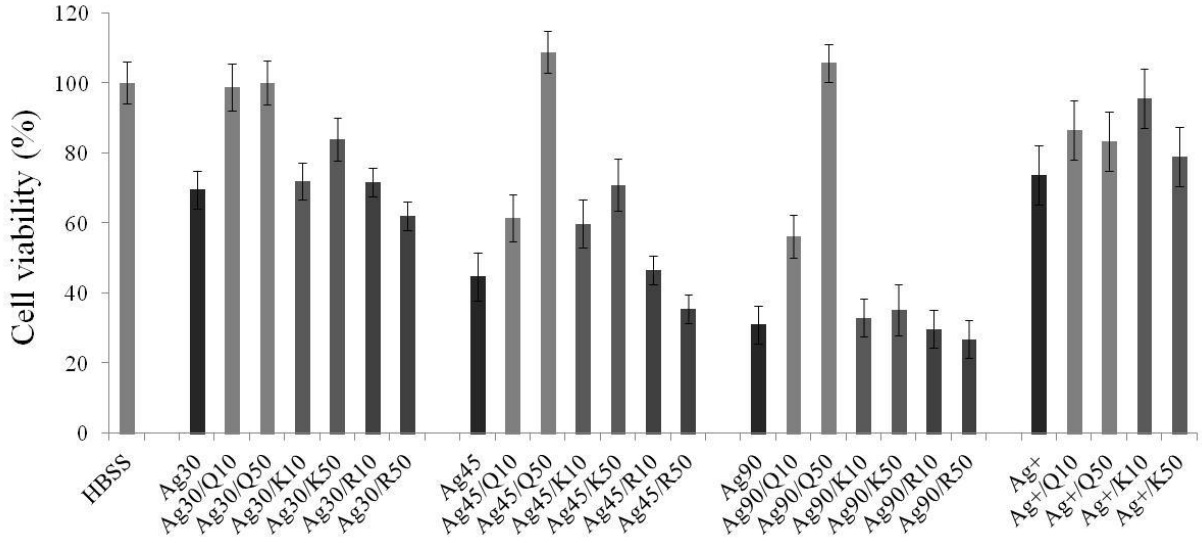


Figure 5. Cell viability of Caco-2 cells under the influence of Ag-NPs (15 – 90 µg/ml) and Ag^+ (4.5 µg/ml) in the presence of PCs – Q, K and R at concentrations of 10 and 50 µM.

For the first time, in this study the potential protective effect of PCs Q and K on Caco-2 cells against the cytotoxic effect of Ag-NPs was shown, while no protective effect was observed with R (Figure 4). Q and K not

only attenuated the cytotoxic effect of Ag-NPs, but also reduced the NP-induced increase of the intracellular ROS generation until the control level and even lower (data not shown). The protective effect of Q and K was also observed in the case of Ag^+ (not shown). The protective effect of flavonoids against the Ag-NPs/Ag^+ toxicity could be due to their potent antioxidative properties (Tatsimo *et al.* 2012, Middleton *et al.* 2000; Silva *et al.* 2006). Although in our study a protective effect of Q and K against the Ag-NPs induced ROS generation was shown, it does not prove that this effect is due only to their antioxidant properties. This conclusion follows from the fact that R, which is also a potent antioxidant (Gülçin 2010), had no protective effect against the cytotoxic effect of Ag-NPs. Moreover, in the presence of Q and K (less obvious) during the 3h incubation period the apical solution containing Ag-NPs changed the color from yellow to red-brown, suggesting the possible aggregation of Ag-NPs (Sotiriou & Pratsinis 2010). This effect of flavonoids may be due to their stabilizing effect/capping of Ag-NPs. Such effect was reported for Q, where the formed Ag-NPs size and shape was dependent on the initial NP concentration (Lukman *et al.* 2011). The stabilization of Ag-NPs with PCs in turn may decrease the level of Ag^+ release and hence the toxicity of Ag-NPs.

A growing number of data suggest the potential protective effect of flavonoids on the epithelial barrier function (Suzuki & Hara 2011). According to our data, Q and K led to a significant improvement of the occludin and ZO-1 distributions, disrupted by Ag-NPs (data not shown), certified also by restored TEER values and LY passage rates of cell monolayers until the control level (Martirosyan *et al*. 2013). These findings are in agreement with the existing literature data, where Q and K revealed to have an intestinal barrier-enhancing function (Amasheh *et al.* 2008; Suzuki & Hara 2011). It was reported that these PCs promote the cytoskeletal association of occludin, *zonula occludens* and claudins (Suzuki & Hara 2009; Suzuki *et al.* 2011, Amasheh *et al.* 2008). The mechanism of the barrier function enhancement by Q and K is not well understood and is believed to be mediated by different kinases and phosphatases, leading to the phosphorylation induction of TJs proteins, e.g. occludin, ZO-2, claudin-1 that favors the assembly of the TJs (Suzuki & Hara 2011, Suzuki *et al.* 2011).

Methodologies employed

Nanoparticles used and their dispersion: Ag-NPs (NM-300K, particle size: 15 nm; D90 < 20nm) was purchased from JRC repository, Ispra, It. The Ag-NPs are supplied monodispersed with capping agent (monolayer): aqueous dispersion of silver with stabilizing agents, 4% each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene (20) Sorbitan mono-Laurat (Tween 20).

Cell lines and cell-based models: Human colon carcinoma Caco-2 cells are the most popular cell culture system to study the intestinal passage and transport mechanisms (Artursson & Karlson 1991, Sergent *et al.* 2011, Martirosyan *et al*. 2012). To study the cell response to NPs exposure 2D (well-plate) and 3D (Tanswell insert) cell-based models were applied. The latters are modular, adaptable biomedical systems that range in complexity from a single cell type (monotypic), representing the minimum unit of the differentiated GI tissue *in vivo* to complex co-culture models that recapitulate both the 3D architecture and the multicellular complexity of the GIT. In an improved *in vitro* co-culture model in bicameral system Caco-2 cells were exposed to lymphocytes that migrate into the monolayer and induce the conversion of the enterocyte phenotype into the M-cells one (Figure 6) [des Rieux *et al.* 2007].

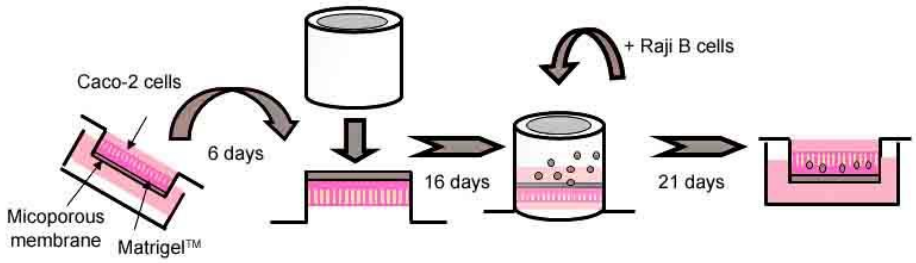


Figure 6. Co-culture model of Caco-2 and Raji B cells [des Rieux *et al.* 2007].

Thus this improved model was used to better characterize and understand the biological effects, absorption and transportation mechanisms of NPs in intestinal cells, providing more reliable and more predictive correlations between *in vitro* studies and *in vivo* outcomes.

Assessment of impact of NPs: A range of approaches of toxicology and molecular biology were applied in order to assess the toxicity and the molecular mechanisms of action of Ag-NPs:

- MTT-test to assess the cytotoxicity;
- DCFH-DA (2′,7′-dichorofluorescein diacetate) assay to evaluate the ability of NPs to cause oxidative stress;
- ELISA assays to evaluate the changes in the cytokine/chemokine levels (IL-6, IL-8);
- Nitrate/Nitrite Colorimetric Assay Kit to assess the nitrix oxide (NO) generation.

Determination of the cell monolayer 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 LY. Within the project the laser scanning confocal microscopy (CLSM) has been routinely used to visualize the state of the tight junctions proteins occludin and Zonula Occludens 1 (ZO-1) in order to study the barrier integrity of the GIT.

QualityNano Transnational Access Grant was awarded for a short-term research (one month) in the Center of Bionano Interactions (CBNI) at University College Dublin.
In the frames of this grant several approaches, such as High-Throughput Screening, luminescent-based methods (evaluation of the cellular glutathione levels and lipid peroxidation) were applied to better understand the cell response to Ag-NPs.
Several methods of NP characterization (TEM, Dynamic Light Scattering (DLS), differential centrifugation, Nanoparticle Tracking Analysis (NTA)) were used in order to characterize the Ag-NPs in HBSS, as well as complete growth medium (DMEM with 10% fetal bovine serum) alone and in the presence of PCs Q and K.

Data analysis: Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student's t-test by means of the JMP9 program of the SAS Institute. Results were expressed as means ± SEM and $p<0.05$ was considered statistically significant.

Dissemination of research results

The results obtained within the frames of the project were disseminated as:

Book review chapter

- Martirosyan A, Polet M, Bazes A, Sergent T, Schneider Y-J. (2012) Food Nanoparticles and Intestinal Inflammation: A Real Risk? In: ***Inflammatory Bowel Disease***. Imre Szabo (Ed.) ISBN: 978-953-51-0879-5, ***InTech***, Ch.8, p.259-282. doi: 10.5772/52887.

Peer-reviewed article

- Martirosyan A, Bazes A, Schneider Y-J. In vitro toxicity assessment of silver nanoparticles in the presence of phenolic compounds – preventive agents against the harmful effect? (2013) ***Nanotoxicology*** (accepted to publication). doi:10.3109/17435390.2013.812258

Presentations at professional conferences and meetings

1. Martirosyan A, Polet M, Schneider Y-J. In vitro safety assessment of nanosilver with improved cell culture systems. ESACT (European Society for Animal Cell Technology) Meeting, June 23-26, 2013, Lille, France, p.195, REF-D063.
2. Martirosyan A, Schneider Y-J. In vitro evaluation of the toxicity of silver nanoparticles and silver ions. XIVth BELACT Meeting Advanced models of (stem) cells in toxicology, pharmacology, safety assessment, and cell therapy. December 14, 2012, p.25-26.

3. Martirosyan A, Bazes A, Schneider Y-J. Variation in silver nanoparticles toxicity in the presence of phenolic compounds. International Conference on Safe production and use of nanomaterials, Nanosafe 2012, November 13-15, 2012 – Grenoble, France, O3b-6
4. Martirosyan A, Polet M, Bazes A, Schneider Y-J. Modulation of the effect of nanosilver on intestinal tight junctions integrity by quercetin, 4th EMBO meeting advancing the life sciences, 22-25 Sept 2012, Nice, France, B184, p.148-149.
5. Martirosyan A, Polet M, Bazes A, Schneider Y-J. Changes in the molecular organization of tight junctions under the influence of silver nanoparticles and quercetin. EMBO Conference Series "Mophogenesis and Dynamics of Multicellular Systems" 7-9 Sept 2012, Heidelberg, Germany, p.123.
6. Martirosyan A, Polet M, Bazes A, Schneider Y-J. Quercetin - preventive agent against the harmful effect of silver nanoparticles?6th symposium of GCNAS (Groupe de Contact FNRS Nutrition, Alimentation et Santé), 1 June, 2012, UCL, Louvain-la-Neuve, Belgium.
7. Martirosyan A, Bazes A, Schneider Y-J. Variation in silver nanoparticles toxicity caused by quercetin and resveratrol. IVTIP Spring Meeting 2012 "Safety assessment of nanomaterials: current status and challenges ahead" April 19, 2012, Bilbao, Spain.

Master theses

- Bormans Mathieu (2012) Evaluation de la toxicité de nanoparticules de silice sur un modèle in vitro de barrière intestinale. Mémoire en vue de l'obtention du diplôme de Master en Bioingénieur - orientation agronomie. Promoteur Yves-Jacques Schneider. Année académique 2011 – 2012.
- Claes Séverine (2013) In process for the defense in Autumn 2013

Presentations to students and colleagues

Reports to funding organization

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