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PhD thesis in Science

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Inflammatory effects of silver nanoparticles in an *in vitro* model of the intestinal barrier

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Il ne reste plus qu'à tourner la page ...

Abstract

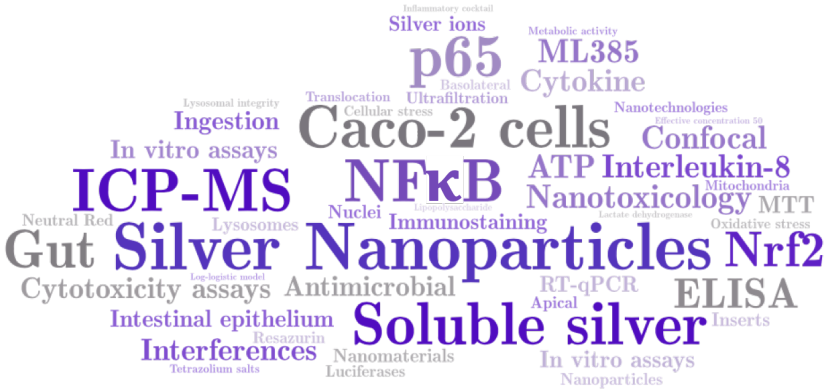
Antimicrobial properties of silver and the advances of nanotechnology have led to the development of silver nanoparticles (AgNPs) that can be found in more and more food-related applications varying from coatings, sprays. Some unauthorised food supplements are also sold to treat infections or boost immune defences. All these applications could result in ingestion of AgNPs that will undoubtedly raise with the increasing incorporation of AgNPs in consumer products. Nevertheless, only few studies among existing literature have focused on the gut, despite the major role of this organ in immunity. In addition, this organ is exposed to the highest concentrations of ingested components, compared to internal tissues. However, numerous studies have observed a toxicity of AgNPs in different *in vitro* cellular models. Regarding inflammation, studies are not unambiguous. Most *in vivo* studies suggested anti-inflammatory properties of silver, presenting beneficial properties for wound and burn treatments. Nevertheless, *in vitro* studies are less unanimous.

The general purpose of this study was to investigate the inflammatory properties of AgNPs on the intestinal barrier, using an *in vitro* model of the small intestine achieved by Caco-2 cell cultures.

This study confirmed the need of appropriate controls in the study of nanomaterials toxicity. In this context, *in vitro* models could provide a toolbox for comprehensive toxicity assessment. Furthermore, in our model, AgNPs were unable to induce the inflammatory NF-κB pathway, even inhibiting its activation by an inflammatory cocktail. A part of this inhibition is due to silver nanoparticles them-

selves. In addition, AgNPs, induced a polarised secretion of the pro-inflammatory chemokine interleukin-8, which might be mediated by an oxidative stress response pathway i.e. the Nrf2 signalling pathway. This effect is mainly related to silver ions present in AgNPs suspensions. In conclusion, AgNPs seem to affect inflammatory responses in Caco-2 cells.

Keywords


Inflammatory cocktail
Silver ions
Metabolic activity
p65
ML385
Basolateral
Cytokine
Lysosomal integrity
Translocation
Ultrafiltration
Cellular stress
Nanotechnologies
Effective concentration 50
Ingestion
Caco-2 cells
Confocal
In vitro assays
ATP
Interleukin-8
ICP-MS
NFκB
Mitochondria
Nanotoxicology
MTT
Lipoproteinlipase
Neutral Red
Lysosomes
Nuclei
Immunostaining
Lactate dehydrogenase
Oxidative stress
Gut
Silver Nanoparticles
Nrf2
Log-logistic model
Cytotoxicity assays
Antimicrobial
RT-qPCR
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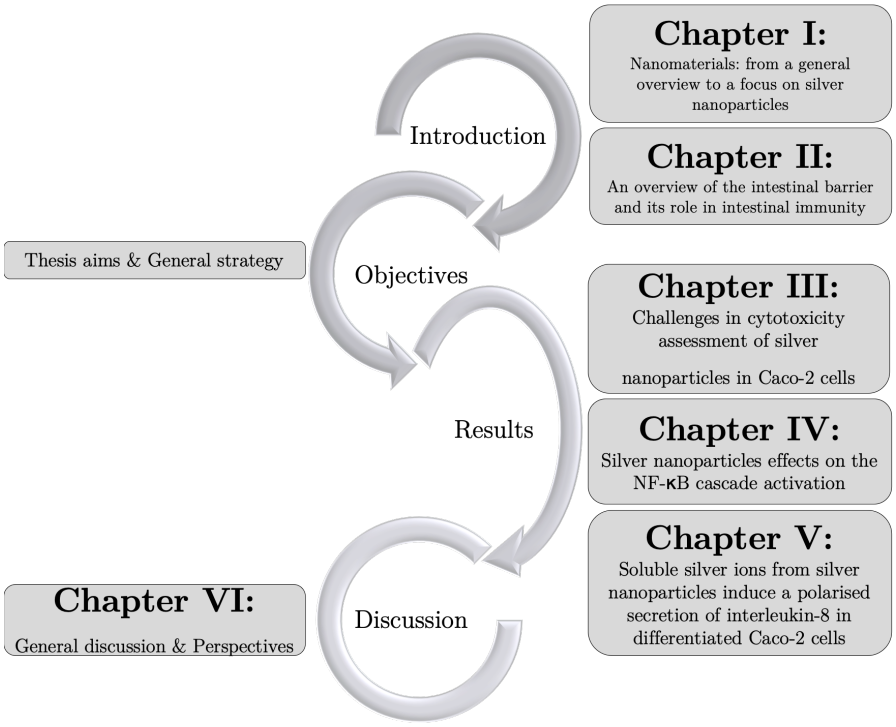
In vitro assays
Tetrazolium salts
Luciferases
Nanoparticles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Thesis structure

The introduction of this thesis is divided in two parts: Chapter 1 reviews generalities about nanomaterials with a focus on silver nanoparticles (AgNPs), it has been written jointly with Laurie Laloux, a PhD student working in parallel on the topics of AgNPs. Chapter 2 describes the intestinal barrier and two major pathways involved in the response of cells to external stresses. A book chapter, written jointly with Laurie Laloux about these two topics can be found in Appendix B. The aims of this thesis are then presented, followed by three chapters of results. Chapter 3 reports two challenges in the cytotoxicity assessment of AgNPs while Chapter 4 and Chapter 5 concerns their effects on NF-κB signalling pathway activation and interleukin-8 secretion, respectively. Chapter 6 comprises a general discussion of results and concludes with perspectives to extend this study.

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Part 1

Introduction

I

Nanomaterials: from a general overview to a focus on silver nanoparticles

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List of abbreviations

Ag^+ silver ions

AgNPs silver nanoparticles

CLP classification, labelling and packaging of substances

EC European Commission

ENMs engineered nanomaterials

EU European Union

GIT gastro-intestinal tract

ISO International Organisation for Standardisation

NMs nanomaterials

NPs nanoparticles

OECD Organisation for Economic Cooperation and Development

REACH registration, evaluation, authorisation and restriction of chemicals

ROS reactive oxygen species

TiO_2 titanium dioxide

1.1 General overview of nanomaterials

1.1.1 Introduction

We are constantly exposed to nanomaterials (NMs) and even more since the emergence of nanotechnology. According to StatNano, a renowned portal compiling the data and the statistics related to nanotechnology, more than 166,000 articles on this topic were published in 2018, representing almost 10% of the total scientific publications [1]. Although nanotechnology has become an important part of society recently, its origins go back 60 years ago [2]. The physicist Richard Feynman was the first to introduce the concept of nanoscience in his lecture "*There's plenty room at the bottom*" during the meeting of the American Physical Society in December 1959 [3]. Fifteen years later, the term nanotechnology was formulated for the first time by Norio Taniguchi [4]. The 1980s marks afterwards the beginning of the nanotechnology era with the observation of individual atoms due to the development of the scanning tunnelling microscope by Gerd Binnig and Heinrich Rohrer. During the 1990s, the first nanomaterials were discovered and since then the number of applications using NMs is only growing up to such an extent that nanotechnology could be considered by some as the most prominent technology of the 21st century [5–9].

The prefix "nano" comes from the Greek word $\nu\alpha\nu o$s, meaning dwarf and is usually employed to describe new research fields including *e.g.* nanoscience, nanotechnology, nanomedicine or nanotoxicology [5]. Since 2007, international organisations such as the Organisation for Economic Cooperation and Development (OECD) and the International Organisation for Standardisation (ISO) defined some terms with the prefix "nano". Several definitions of the term nanomaterial were also proposed by authorities of numerous countries including Canada, Australia, Denmark, United Kingdom and United States of America. This large number of definitions is ambiguous and confusing for industry, regulators and

consumers as a same structure could be recognised as a nanomaterial by one definition but not by another [10–12].

Considering this, a single international definition suitable for all regulations and policies would be profitable. Therefore, the European Parliament requested in 2009 a comprehensive science-based definition of nanomaterials that could be applied to all European Union (EU) legislations and that should be in accordance with definitions given by international organisations [10, 13, 14]. As a result, the European Commission (EC) recommended in 2011 the following definition (2011/696/EU): a nanomaterial can be described as a *"natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm"* [15]. This extremely detailed definition takes into account simultaneously the different origins, states and nanoscale dimensions of nanomaterials as it is summarised in Figure 1.1.

Regarding the origin, the definition applies to both natural NMs and NMs derived from anthropogenic activities (both incidental and manufactured). The first ones can be released in the atmosphere during natural events such as forest fires, dust storms or volcanic eruptions. Natural NMs are also present in organisms, one example among many others is the casein micelles of milk. For their part, NMs produced by human activities are split in the recommendation in two subcategories: on one hand, NMs released accidentally during industrial processes such as welding, ore refining or combustion and, on the other hand, engineered nanomaterials (ENMs) that are intentionally manufactured for their interesting and desired properties. For instance, ENMs composed of carbon, titanium dioxide (TiO_2) or silver are already produced and incorporated in cosmetics, textiles, appliances or medical devices as detailed in section 1.1.3 of the present chapter [5, 11, 16, 17].

The recommendation suggested by the European Commission applies to unbound particles defined by ISO as *"a minute piece of matter with defined physical boundaries"*. It corresponds to the smallest indivisible entity. Sometimes, these particles can form agglomerates or aggregates depending on the environmental conditions. Aggregates and agglomerates are also defined in the ISO norm. The difference between the two terms comes from the strength of the bonds. In agglomerates, particles are weakly held together by van der Waals or electrostatic forces so that they dynamically evolve with the media properties like pH or ionic strength. On the contrary, aggregates cannot be easily broken down as particles are maintained together by strong forces like covalent or metallic bonds. The EC recommendation also includes agglomerated and aggregated particles as they could display the same properties as unbound particles [14, 15, 18–21].

Finally, as mentioned in the definition of the European Commission, NMs may have one, two or three external dimensions in the nanoscale. Materials with their three external dimensions in the nanometer range correspond to nanoparticles (NPs) while particles with only one or two external dimensions in the nanoscale are respectively defined by ISO as nanoplates and nanofibers [9, 11, 14, 22].

In the present chapter, the description of the exposure pathways and the potential adverse health effects of nanomaterials will be provided after an overview of the general properties and applications of NMs. European safety regulation regarding ENMs will also be reviewed before focusing on silver nanoparticles (AgNPs), the nanomaterial found in the higher number of consumer products. The end of the chapter will point out AgNPs synthesis and their applications in the food industry, as well as their antimicrobial properties and their adverse health effects.

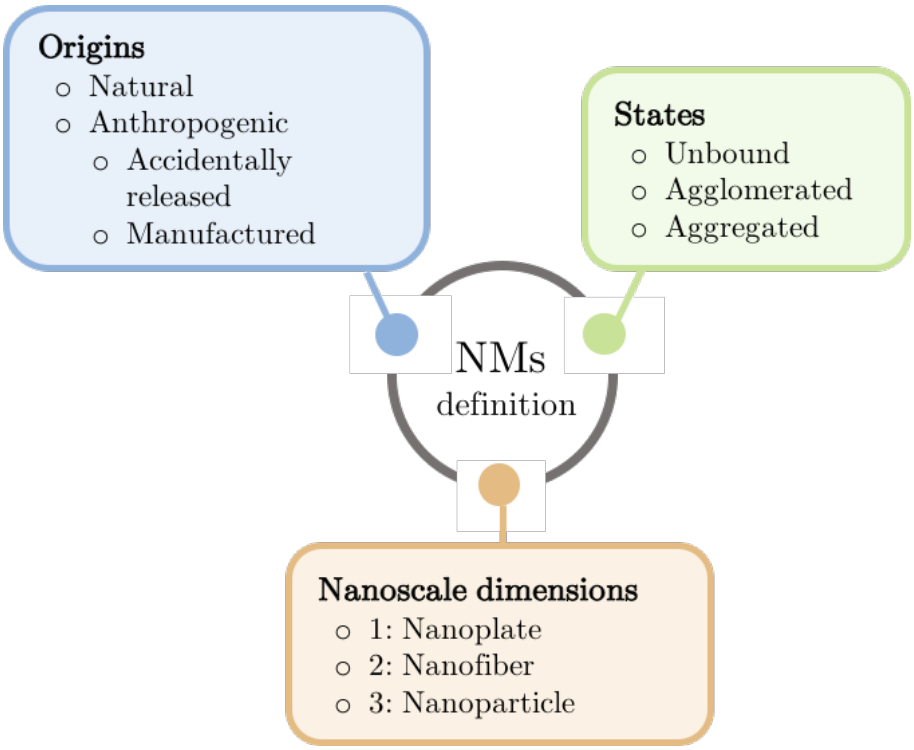

Figure 1.1: NMs definition according to the recommendation of the European Commission. The European definition takes into account the different origins, states and nanoscale dimensions of nanomaterials.

1.1.2 General properties

The rise of nanotechnology is explained by the large scope of opportunities it offers in term of material characteristics improvement. NMs often behave differently than bulk material with the same composition so that they can enhance some features (*e.g.* resistance, conductivity, lightness) or even give new purposes to materials [10, 11, 19]. Due to their very small dimensions, NMs can acquire special properties (*e.g.* optical, thermal, magnetic, chemical) that could be beneficial for a wide range of applications [2, 9, 23, 24]. Even specific physical properties such as melting point or colour undergo modifications by switching to the nanoscale.

For instance, silver and gold nanoparticles exhibit respectively yellow and red colours unlike metallic grey and golden bulk materials [9, 25]. Another example is the drastic reduction of gold nanoparticles melting point (from approximately 300°C for 2 nm NPs to 850°C for 5 nm NPs), to be compared to that of the bulk material (1 063°C) [2, 26, 27].

These alterations can be explained by the small size of NMs. Indeed, when particle size decreases, the surface area relative to volume grows up [5, 21, 28] as it is illustrated by the following formula of the surface-to-volume ratio for a spherical particle with radius "r" [10, 29]:

$$\frac{Surface\ area}{Volume} = \frac{4\pi r^2}{\frac{4}{3}\pi r^3} = \frac{3}{r}.$$

Atoms are stable and well coordinated inside a material while atoms at the surface are often energetically unstable so that biological and chemical reactions generally take place at the surface of materials. In nanomaterials, the number of atoms at the surface become prominent, which explains the substantially magnification of NMs reactivity compared to coarser particles for which most of the atoms are inside the particle. As an illustration, gold nanoparticles are highly reactive so that they are used as catalysts while the bulk form is considered as a noble metal extremely resistant to oxidation and corrosion [2, 5, 10, 19, 28–30].

However, although these unique properties are beneficial for a large number of applications, they could also be responsible of adverse effects for human health [24]. Indeed, due to their small size and the subsequent increase of reactivity, they are able to cross biological barriers of the human body and interact with biological molecules easier than coarser particles, as it will be explained in section 1.1.5 [9, 10, 31, 32].

1.1.3 Applications

Owing to their particular physico-chemical properties previously described, NMs have been introduced on the market. Human exposure to NMs might occur either directly from a contaminated environment or after a release from consumer products or drugs [33, 34]. Different consumer products inventories have been created to identify NMs in their composition. Even if based on data from manufacturer's claims, these inventories are a popular indicator of the prevalence of nanotechnologies in everyday life [35]. Figure 1.2 reports the number of consumer products containing NMs in each category of applications according to an analysis of the "*Project on Emerging Nanotechnologies*" inventory by Vance *et al.* (2015) [35, 36].

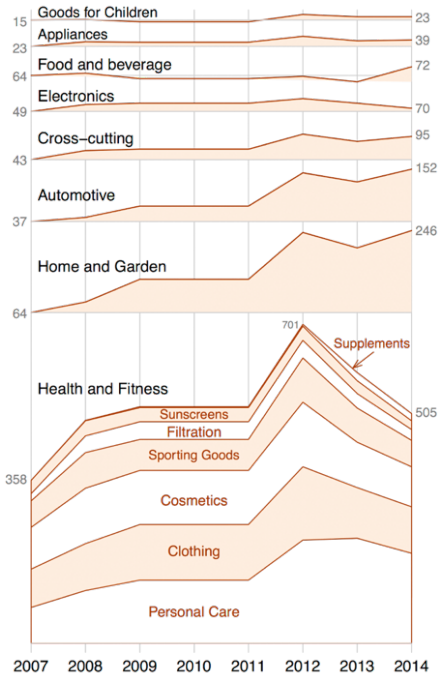

Figure 1.2: Number of consumer products containing NMs in each product category. Health and fitness category is divided in subcategories. Figure coming from Vance *et al.* (2015) [35].

The number of consumer products containing NMs reported in the inventory has globally increased over time. Among the largest category of "health and fitness" products, personal care products are the most represented. This subcategory comprises toothpastes, lotions, hair products and skin creams. Hansen *et al.* (2016) have drawn similar conclusions from the analysis of the Danish Nanodatabase [37, 38].

Asides these applications in consumer goods, specific properties of NMs are also desirable for medical applications. In medicine and pharmacology, nanotechnology has been developed for diagnosis and therapeutic purposes [39,40]. Based on their small size, NPs might more readily interfere with targeted cellular components. For example, nanometric liposome-like structures have been developed to deliver anti-cancerous drugs like paclitaxel inside mitochondria [41]. Besides, drug carriers NMs have been highly investigated to enhance the stability and absorption of drugs in the gastro-intestinal tract (GIT) [42–45]. For instance, lectin molecules were conjugated with polymeric nanoparticles in order to increase interactions with the surface of epithelial cells and/or the mucus [46, 47].

The use of nanotechnologies in food and beverages category is less represented in available inventories, reporting only consumer products. However, they are actually studied, developed and, in some cases, used throughout all the supply chain, from farm to fork applications [48–50].

Agriculture has taken advantage of nanotechnology to fight against plant diseases and pests. Indeed, some conventional pesticides are encapsulated to improve their delivery while some nanomaterials have shown insecticidal properties [6]. Nanotechnology has also led to the development of nanosensors towards a "precision farming", to control plant nutrients or detect plant diseases or contaminants [51,52]. Nanotechnology based devices are in fact currently used for detection of pathogens, contaminants, sugars and proteins in USA and Australia [6].

Water purification is also achieved with the help of some nanoparticles, such as silver or iron oxide coated on filtration devices, owing to their ability to react with organic impurities and microorganisms [6].

Numerous foods contain naturally nanostructured components, some of them being formed during the production. The majority are natural, such as proteins or emulsions. Furthermore, the formation of NMs during digestion process is recognised like phosphate calcium NPs formation in the small intestine [53]. Other NMs are also intentionally used as food additive or found as a subpopulation in the conventional food additive. For instance, amorphous silica, used under the name E551 as an additive for its anti-caking properties and humidity absorbent in food products presented in powders, can be found in nanoparticular form [54, 55]. It can also be the case for another common food additive, named E171, composed of TiO_2 and used for food colouring purposes [56]. Due to its use in candies, white icing and powdered sugar toppings, children could be more exposed to TiO_2 NMs than the average adult [57].

Besides, some kinds of NMs are intentionally added in food to encapsulate ingredients or bioactive substances to improve their bioavailability. For example, organic nanomaterials, generally lipid-based structures such as micelles or liposomes, are mainly developed for the encapsulation of additives. They can trap ingredients like preservatives, antioxidants and vitamins to protect them from degradation [50].

Due to the lack of information from manufacturers, the amount of dietary exposure to NMs is difficult to estimate. Indeed, the measure of NMs quantity in consumer products is a great analytical challenge, because of their low concentration in products with a complex composition, not compatible with current analysis methods [58]. However, some studies have been published about daily exposure to NMs [59]. Weir *et al.* (2012) estimated an exposure between 0.07 and 1.08 0.02mg.kg

body weight^{-1}.day^{-1} of nano-TiO_2 and Dekkers *et al.* (2011) estimated a worst case intake of 1.8 mg silica NMs/kg body weight/day for an average adult [54, 56, 57]. The dietary exposure to AgNPs is even more difficult to estimate, as this nanomaterial is not directly added in the food, and human exposure comes from migration in food. However, for people ingesting food supplements based on colloidal silver, the Danish Environmental Protection Agency estimated an exposure of 0.02 mg.kg body weight^{-1}.day^{-1} [57, 60].

The different materials composing NMs found in consumer goods are reported in Figure 1.3, compiled from three different inventories *i.e.* the American Project on Emerging Nanotechnologies with worldwide products, the Danish Nanodatabase reporting European products and the Iranian Statnano database. The presence of NMs was advertised by the manufacturer in around half of the consumer goods listed in the Project of Emerging Nanotechnologies inventory [35].

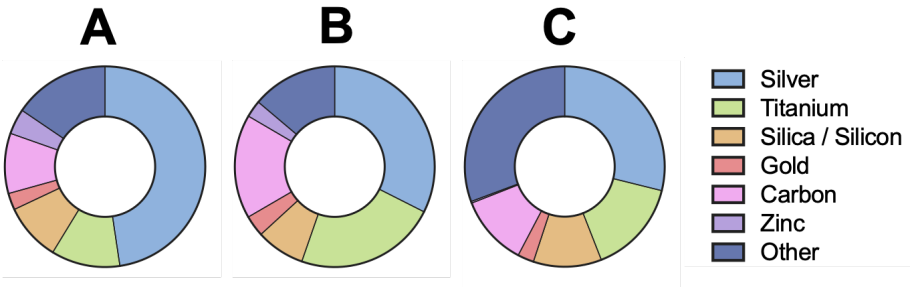

Figure 1.3: Types of NMs retrieved in consumer products based on three inventories: Project on Emerging Nanotechnologies inventory (A), Nanodatabase (B), and Statnano (C) [36, 38, 61].

Metallic-based NMs are the most represented in consumer goods [62]. They have been used for decades, even if the terminology "nano" was not employed [55]. According to Figure 1.3, silver is the most represented in consumer products, mainly because of its antimicrobial properties de-

scribed in section 1.2.3 of the present chapter. Applications of AgNPs will be discussed in section 1.9. Titanium NPs, mainly in the form TiO_2, are the second most encountered NMs in consumer goods, followed by carbon and silica NPs. Optical properties of TiO_2 are harnessed in sunscreens for their UV properties, or in toothpastes or paintings as whitening agents [5, 63]. Carbon nanotubes are added as flame retardants in electronics and textile industries [62]. Finally, silica particles are used for their anti-caking properties in healthcare and home products such as toothpastes, detergent and cosmetics [58].

According to Hansen *et al.* (2007) [64], NMs can be incorporated in different forms in consumer goods. First, the NMs can be at the surface of the material, their composition being either different or similar to the bulk material [64]. For example, the surface of some make-up or kitchen instruments are covered with AgNPs [62, 65]. Furthermore, some NMs can be suspended either in a bulk solid, like carbon nanotubes mixed inside polymers to confer some interesting properties [64], or in a liquid phase. For instance, AgNPs in a colloidal suspension constitute some unauthorised food supplements [66]. The last category consists of nanoparticles suspended in the air (or in a gas), free from any matrix. NPs present in antimicrobial sprays are an example of this last category of airborne NPs [64].

Exposure to NMs could occur after use of consumer goods, food or drugs, or after migration from packaging in these products. In addition, occupational exposure, through manufacturing of these consumer goods or through industrial processes might occur in workplaces producing paints, coatings, cosmetics, catalysts and other composites [62]. Wastewater contamination, after sewage, could also lead to indirect exposure, even if the physico-chemical properties of NMs might be affected over time in the environment [67].

1.1.4 Exposure pathways

Figure 1.4 represents the possible routes for NMs entrance in the human body. Exposure through NMs can occur through three major pathways *i.e.* inhalation, ingestion and dermal contact [5, 40, 68]. Some other minor routes are also recognised in literature such as ocular or genital contact [68]. Epithelia, *i.e.* the skin, intestinal, nasal and pulmonary epithelia, are the first tissues to encounter NMs, forming an important barrier against them [5].

According to Hansen *et al.* (2016), the dermal route seems the most prominent route for the majority of consumer products reported in the Nanodatabase [37, 38]. Studies have shown only a limited entrance of NMs through intact skin. However, this entrance of topically-applied NMs is increased in case of damaged skin or mechanically-stressed skin [5, 68, 69].

Nevertheless, the inhalation exposure seems to be the most studied, as it occurs after occupational exposure to airborne particles during the manufacturing of ENMs [68]. After inhalation, particles larger than 10 μm are more prone to deposition in the upper airways such as the nasopharyngeal region while smaller can reach alveoli [5]. However, nanoparticles smaller than 10 nm tend to be captured by mucus in the nasopharyngeal region [68]. Soluble nanoparticles can dissolve in cellular fluids but non-soluble particles undergo a much slower clearance process. In the upper airways, this elimination is mediated by the muco-ciliary escalator, provoked by the movement of bronchial cells that moves the mucus trapping NMs towards the pharynx where they can reach the gastro-intestinal tract [5]. In lower airways, the particles clearance is mainly mediated by macrophage phagocytosis [5].

Oral exposure to NMs occurs from intentional ingestion of food supplements, medicines, food incorporating or contaminated with NMs. NMs could also be ingested, albeit to a lesser extent, from unintentional "hand-to-mouth" transfer, in particular for children goods, or after muco-ciliary escalator clearance for inhaled particles [68]. Because of the

huge surface exposed by the GIT, intestinal epithelium forms a major interface in contact with NMs [34]. NMs seem mainly absorbed in Peyer's patches that are immune structures from the gut-associated lymphoid tissue and specialised for the sampling of luminal antigens. In addition to this physiological entrance, cytotoxicity of NMs might increase their entrance as suggested by Hillyer & Albrecht (2001) who showed that gold NMs were taken up because of gaps created by enterocytes death [70].

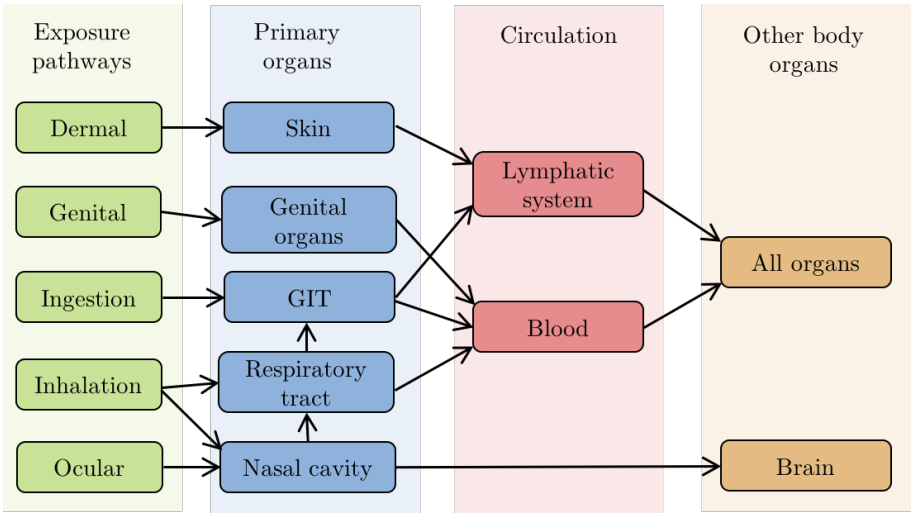

Figure 1.4: Human exposure pathways to NMs, primary organs affected by NMs uptake, and the subsequent distribution into circulatory system and other body organs.

Ocular contact can occur following exposure to airborne NMs, cosmetics placed near eyes or following transfer from hands [68]. The presence of NMs in the nasal cavity after inhalation or ocular contact can provide access to the brain, through olfactory nerves, as observed after inhalation of manganese oxide NPs [71] or carbon nanotubes [72] by rats. Uptake of NMs by cornea or conjunctival tissue has also been observed in literature and might be used to improve drug delivery systems [73,74]. The genital road is far less studied in literature, but it might occur due to the presence of NMs in increasing number of hygiene products [75].

1.1.5 Potential adverse health effects

Through these various exposure pathways, NMs are first in contact with epithelial, *e.g.* pulmonary or intestinal, cells. Their access to the rest of the body is limited by the barrier role of these epithelia. However, owing to their small size, some NMs are able to cross these barriers by either paracellular or transcellular pathways and have directly access to the lymphatic and circulatory system. Once in the general circulation, NMs might reach almost all body organs including brain [5, 71, 72] and placenta [76, 77] and exert some effects at cellular and subcellular levels. The study of potential adverse effects has led to the emergence of a new branch of toxicology, named "nanotoxicology" by Donaldson *et al.* (2004) [24, 78].

The toxicity of conventional chemicals depends on their nature but also on the dose, duration of exposition and route of exposure [79]. But due to their particular properties and as reported in Figure 1.5, NMs toxicity is also impacted by other parameters, giving rise to a different toxicity than the same material in bulk form [24, 39, 48, 80, 81]. According to Gatoo *et al.* (2014), the major ones are size, shape and surface charge [28]. In particular, the size is a critical parameter. Indeed, the similar size range between NMs and biomolecules such as proteins, lipids or DNA facilitates their interaction, which could alter the biological molecules functions [82, 83]. Furthermore, as previously explained in section 1.1.2, the size of NMs is also responsible for a huge increase in surface area, raising their reactivity and biological activity. In some cases, it has positive consequences sought for nanomedicine applications (*e.g.* capacity to deliver therapeutics or ability to penetrate cell barriers) but it can also cause negative effects and be a source of toxicity in other cases [24]. Other parameters like hydrophilicity or solubility play a role in their toxic effects [40, 84]. In consequence, nanotoxicology studies in literature are difficult to compare because of the huge number of parameters involved in the toxicity of NMs [80].

In addition, the interaction between NMs and the surrounding biological medium can alter their properties by the formation of a corona, which can impact their biological interactions with cells or subcellular compartments, as well as modulate their cellular uptake [80, 85]. As a result, nanotoxicology studies should be accompanied by a complete physico-chemical characterisation of the tested NMs, in their pristine state, but also in the surrounding biological medium.

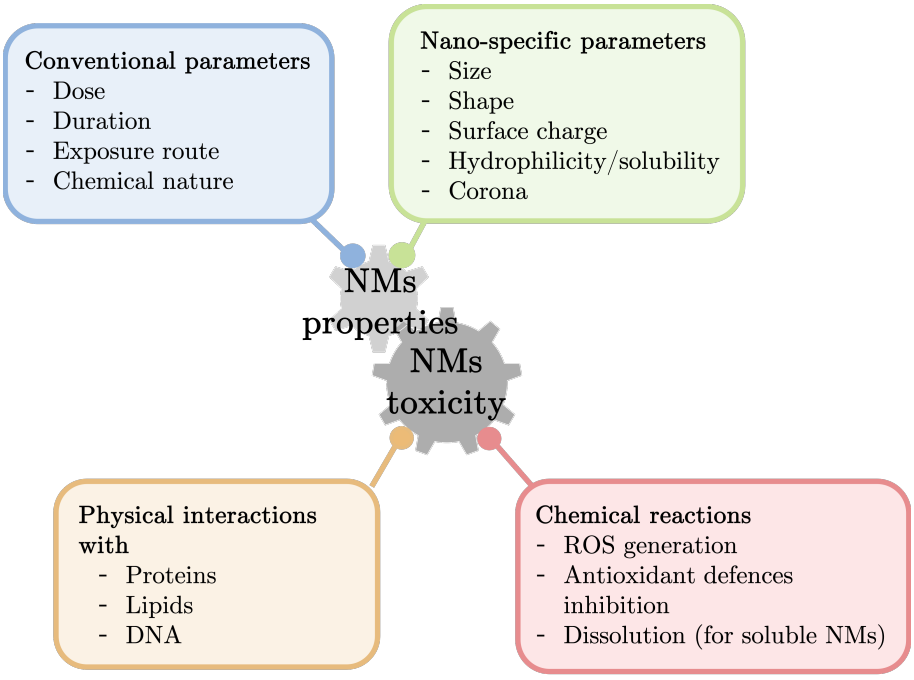

Figure 1.5: Conventional and NMs-specific parameters influencing NMs toxicity with consequences of NMs that interact, in the body, either physically or chemically with biological molecules.

The first step explaining the toxicity of NMs is their entrance in cells. Due to the small pore size of ion channel (between 0.3 and 12 Å), it is totally unlikely that NMs enter in cells through this pathway [83]. However, ions released from the dissolution of some kinds of NMs might

enter through this pathway. The entrance of nanoparticles into cells occurs mainly by endocytosis. With the help of specific inhibitors, different types of endocytosis were observed for NMs, *e.g.* caveolae endocytosis, pinocytosis or clathrin mediated endocytosis [83, 84, 86]. The type of endocytosis is affected by the physico-chemical properties of NMs such as shape, size or surface chemistry but also by cell type [84, 87, 88]. Passive entrance in cells was also observed for carbon nanotubes [89] or silicon dioxide NPs [90]. In addition, some nanoparticles can interact with the lipid bilayer membrane, making it more flexible and subject to passive penetration of NMs [83, 91].

Once entered in cells by endocytosis, NMs can either escape the endosome vesicle by rupturing its membrane or gain access to lysosomes [83, 88]. For metallic particles, Sabella *et al.* (2014) suggested a common mechanism called "lysosome-enhanced Trojan horse effect" intensifying the toxicity of NMs. After their internalisation in lysosomes, the acidic conditions inside lysosomes induce the release of toxic metallic ions in cells [85]. For instance, lysosomes promote the release of Zn^{2+} from ZnO NPs, which generates cytokines secretion and cytotoxic effects, and disturbs the zinc homeostasis [84].

Inside cells, NMs can exert their toxicity through physical and chemical interactions with living matter components [80, 84]. Physical mechanisms involve direct interaction between NMs and subcellular components, such as membrane lipids or proteins. The formation of a corona with proteins modifies both the biological response caused by NMs but also the structure and the function of the proteins, which can affect cell signalling [92]. Furthermore, interaction with enzymes can modify their conformation or affect their folding [23, 93]. Protein degradation seems also enhanced when they are coated at the surface of NMs [78]. In addition, NMs interact with DNA through direct intercalation or physical interaction with the double helix [80], inducing DNA damages and genotoxicity [94]. Entrance in nuclei might occur through passive diffu-

sion across nuclear pores, whose typical diameter varies between 6 and 9 nm [88].

Oxidative stress seems to be a common mechanism for NMs toxicity described in literature. Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the capacity of cellular antioxidant defence systems. ROS are byproducts of cellular metabolism. During the ATP synthesis, the reduction of oxygen to water is not complete, as a few amounts of oxygen transforms into superoxide radical. This radical is converted into highly reactive hydroxyl radical and other ROS.

NMs might affect both causes of oxidative stress. On one hand, NMs induce the generation of ROS through various mechanisms: (i) chemical reduction at the surface of NMs. For instance, AgNPs, while being oxidised at the surface into silver ions, reduce in turn oxygen into superoxide radical; (ii) catalysis of Fenton reaction by metallic compounds released by transition metal NMs such as iron or silver; (iii) alteration of mitochondrial function by some NMs able to enter and produce physical damages and (iv) secretion of ROS by immune cells following inflammation induced by NMs. On the other hand, NMs interact with antioxidant defences produced by cells to counter ROS, exacerbating the sensitivity of cells to these ROS. Oxidative stress induced by NMs can thus be attributed to the formation of ROS combined with a disruption of cellular antioxidant defences [95].

Oxidative stress has deleterious consequences for cells, such as lipid peroxidation that affects cellular and subcellular membrane structure, DNA damage, dysregulation of cell signalling and subsequent cytotoxicity or cancer initiation. Different kind of damages to protein can also occur, affecting protein function or structure, such as oxidation of cysteine residues, formation of intra- or inter-molecular disulphide linkages or dithyrosine formation [31, 39, 48, 79, 96–103]. Chemical mechanisms of

toxicity also involve the dissolution of some kinds of NMs, releasing toxic ions as it is the case for zinc oxide, silver, copper or cadmium particles, which could affect oxidative stress and subsequent oxidative damages to biological molecules [39, 78, 84, 104].

1.1.6 European safety regulation

As detailed in section 1.1.2, NMs own unique properties (*e.g.* small size, high surface-to-volume ratio, high reactivity) rendering them promising for a large range of applications. However, these characteristics can also be responsible of deleterious effects on health, as it has been explained in section 1.1.5. Therefore, it is imperative to have legislation that regulates the use of NMs to minimise potential hazards caused by their use [11, 105].

No legislation completely devoted to NMs has been put in place so far [106]. In the EU, as all chemicals, they have to follow the registration, evaluation, authorisation and restriction of chemicals (REACH) and classification, labelling and packaging of substances (CLP) regulations. REACH entered into force in 2007 and stated that chemical substances introduced in the EU market in more than one ton per year must be registered by companies in a central database [107], accompanied by a risk assessment of the product [105, 108, 109]. In 2009, the CLP regulation came into effect to correctly label and package hazardous substances and protect workers and consumers [107, 108, 110]. Despite the substance definition covered nanomaterials, REACH did not mention any definition of the term nanomaterial so that it was difficult to differentiate NMs from other materials [8, 106, 111]. Annexes concerning nanomaterials have been finally revised after years of discussion to clarify registration duties for NMs. These new annexes have been applied since 1^{st} January 2020 [112].

Besides this European legislation, some member states decided to

put in place national registries such as France, Sweden or Belgium [107].

In cosmetics and biocidal products, a risk assessment should be provided to the European commission before being placed on the market, and the term [nano] should be added besides the chemical name in the list of ingredients [105, 107, 113–117].

Several regulations already cover the use of nanomaterials in the food sector. The presence of NMs must also be mentioned in the list of ingredients, followed by the term [nano] [105, 107, 115, 118]. In addition, a food additive containing NMs is also considered as a new additive and has to be submitted to the authority before being placed on the market [119, 120]. Similarly, food consisting of manufactured nanomaterials must be considered as a novel food and tested for safety. Finally, only some substances in nanoform are authorised in the plastic material in contact with food [105, 115, 121].

1.2 A focus on silver nanoparticles

1.2.1 Silver nanoparticles in the society

The use of AgNPs can be traced back to ancient times, owing to their recognised optical properties [122, 123]. Because of their small size, AgNPs do not exhibit the same spectral absorption than bulk material. It is due to the surface plasmon resonance phenomenon, coming from the interaction between light and collective oscillation of conduction electrons from metallic particles such as gold or silver [124, 125]. It gives birth to iridescence and dichroic effect explaining their use as pigments in lustre and glass technology [123]. This phenomenon is also responsible of the yellow colour of AgNPs suspensions, while bulk silver is metallic grey [9].

Since Antiquity, metallic NPs composed of *e.g.* gold, copper and silver have been used for glass staining [123], such as the Lycurgus cup dated to 400 A.D. (Figure 1.6). Other vessels have been conserved but are far less impressive than the Lycurgus cup [122, 126].

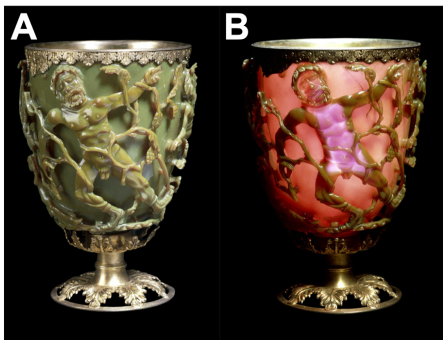

Figure 1.6: Lycurgus cup in (A) reflected light and (B) transmitted light ©Trustee of the British Museum.

The presence of nanostructured silver particles was also detected in medieval yellow glasses in a Portuguese church [127] and in decorations of some Mesopotamian medieval ceramics [128].

Biocidal properties of silver, described in the following section 1.2.3, have been recognised for ages. Indeed, during Antiquity, silver vessels were known to be efficient for water conservation as water never went bad in that kind of vessels [65, 129, 130]. Even if not deliberately in the form of NPs, different preparations of silver were already used before common era for treatment of burns or ulcers, as silver plates by Macedonians, silver nitrates by Romans or various preparations by Hippocrates [130]. Therapeutic properties of silver were also recognised by Indian [131]. The synthesis of silver colloids, containing silver in the nanoparticle form, was already reported as soon as 1889 for the cure of various diseases until the emergence of antibiotics during the World War II [66, 131].

1.2.2 Synthesis

Different methods have been developed to synthesize AgNPs, either by "bottom-up" methods, based on the formation of nanoparticles from aggregation of silver atoms [132], or less used "top down" approaches by ablation of particles from a bulk silver material [133–135]. Current

methods for AgNPs synthesis can be divided in three broad categories reported in Figure 1.7: chemical, physical or biological approaches. Synthesis methods were first focused on chemical approaches. Physical and biological approaches were then developed as safer and greener methods [136]. These methods impact physico-chemical characteristics of AgNPs *e.g.* size, morphology, shape, or stability [75, 133, 136, 137].

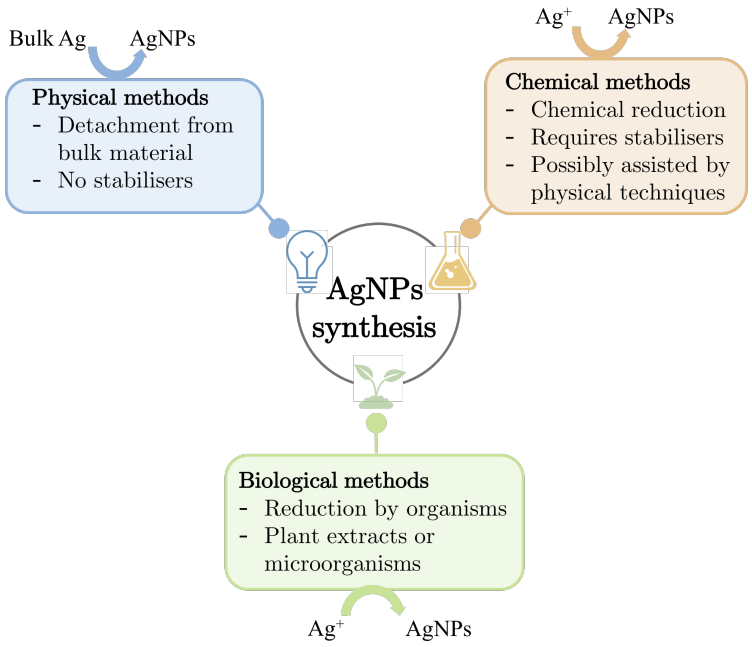

Figure 1.7: Methods developed to synthesize AgNPs by physical, chemical or biological methods.

The most straightforward route is the chemical reduction of silver ions with different types of organic and inorganic reducing agents such as sodium borohydride, citrate or ascorbate, or hydrogen gas [75, 137]. However, the chemical approach often requires stabilisers to control the size and shape of AgNPs formed and avoid their agglomeration [133]. Stabilisers are adsorbed or covalently bound to the particle to provide repulsive forces to resist to aggregation with other particles. Numerous

coatings have been used for this purpose, ranging from carboxylic acids to polymers and surfactants [138]. Capping agents are stabilisers added during the synthesis with some functionalities (thiols, acids, amines, or alcohols) that interact with particles during synthesis to stabilise their growth [133]. In some cases, the capping agent also acts as the reducing agent, such as sodium borohydride or citrate [67, 137]. Stabilisers can also be added at the end of the synthesis to avoid aggregation or agglomeration [135]. This chemical reduction can be assisted by physical techniques such as separation in two phases, sono-electrochemistry or photoinduced reduction [133].

Physical methods are typically "top down" approaches using heat, electricity or laser strengths to detach particles from a bulk material [133]. These methods have the advantage over chemical techniques that they usually do not require capping agents to stabilise nanoparticles. The main techniques are laser ablation and evaporation followed by condensation in a furnace tube [137]. Furthermore, Tien *et al.* (2008) [139] developed a synthesis with arc-discharge between electrodes composed of silver rods that release negatively charged AgNPs in water suspension without any stabiliser.

Finally, biological methods have emerged. In this approach, the chemical reduction of a silver salt is achieved by different kind of organisms or biological extracts, ranging from simple prokaryotes like bacteria to eukaryotic fungi and yeasts or even plant extracts [133, 134]. Various synthesis based on plants have been reported in literature and were reviewed by Ahmed *et al.* (2016) [135]. Biological methods were developed to be more "eco-friendly", as reducing agents and/or stabilising agents are biological molecules *e.g.* polysaccharides, tannins or phenolics, avoiding the use of chemical reagents [135, 137]. As a result, they are often termed "green synthesis" [134]. Nanoparticles produced by biological methods are generally more stable over time than chemically produced [135, 137].

1.2.3 Antimicrobial properties

As mentioned in section 1.2.1, silver has been employed since a long time for their antimicrobial properties but its use declined with the emergence of antibiotics. However, pathogenic bacteria developed some resistance mechanisms protecting them against these antibiotics. Pharmaceutical companies are thus looking for new solutions to deal with these pathogens. The emergence of nanotechnology has brought new opportunities and in particular, AgNPs are increasingly considered as an effective candidate to fight pathogenic microbes [140–142]. Indeed, AgNPs exhibit activity against a broad spectrum of microbes including bacteria, viruses, fungi, algae [65, 132, 140, 143], and they can even decrease bacterial biofilms [95, 144].

Regarding bacteria, the exact mode of action of AgNPs remains open to question but some mechanisms have been proposed based on morphological and structural alterations observed in several bacterial strains [95, 140]. These mechanisms detailed below are graphically summarised in Figure 1.8:

- The toxicity might be partially explained by AgNPs anchoring to the bacterial envelope as observed with transmission electron microscope by Shrivastava *et al.* (2007) [95, 145]. As bacterial envelope is negatively charged due to carboxyl, phosphate and amino groups, positively charged AgNPs could adhere to bacteria due to electrostatic interactions [143, 144, 146]. However, negatively charged AgNPs are also able to bind to cell membrane [141]. This could be attributed to the interaction of AgNPs with sulphur-rich proteins contained in the bacterial envelope [140,141,143,147–149].
- These interactions might alter membrane functions. Indeed, the respiratory chain contains sulphur-rich proteins that could be targeted by AgNPs, which disturbs the respiration process and leads to the loss of energy production [140, 148–150]. Besides, the presence of AgNPs could increase membrane permeability and cause

membrane perforations [95, 132, 141, 143, 148, 149, 151, 152]. Cytoplasmic components might then leak by these pits, which finally results in the bacterial cell death [95, 144, 149, 152].

– After adhesion and damaging bacterial envelope, AgNPs could also infiltrate bacterial cell. Once inside, AgNPs could interact with different biomolecules. Besides sulphur-rich proteins, AgNPs has also a tendency to interact with phosphorus containing components such as DNA as observed by Yang *et al.* (2009). This reaction with DNA could lead to mutation but also to the DNA replication disruption [140, 144, 148, 151–153]. Biomolecules alteration by AgNPs might also modulate the cell signalling pathways and lead to bacterial dysfunction [144]. For example, Shrivastava *et al.* (2007) demonstrated that AgNPs modified the profile of tyrosine phosphorylated proteins, which inhibits enzymes involved in bacterial growth [145]. In their study, Shrivastava *et al.* (2007) also emphasised the lower toxicity observed in gram-positive bacteria, compared to gram-negative [145]. This could be explained by the difference in the envelope composition [154]. The cell envelope of gram-negative bacteria is composed of two membranes separated by a thin layer (about 7-8 nm) of peptidoglycan. The outer membrane contains lipopolysaccharides, a component composed of lipids and polysaccharides but lacking of strength and rigidity. In addition, gram-positive bacteria possess only one membrane protected by a thick layer (about 20-80 nm) of peptidoglycan, forming a rigid structure more difficult to penetrate [145].

– Another mechanism that might explain the antibacterial activity of AgNPs is the generation of ROS in cells [95, 144, 150, 152]. AgNPs could indeed act as a catalyst in the ROS formation, but it could also be a consequence of the respiratory chain disruption or the inhibition of ROS scavenging pathways [95, 142–144, 150, 152]. Resulting oxidative stress damages biomolecules like DNA or proteins, and leads to lipid peroxidation and the bacterial membrane

impairment [143, 144, 152].

– Finally, it is recognised that AgNPs are able to release silver ions (Ag^+) in aqueous solution following their oxidative dissolution [155–157]:

$$4Ag_{(s)} + O_{2(aq)} + 4H^+ \longrightarrow 4Ag^+ + 2H_2O_{(aq)}.$$

These silver ions are either attached to the NPs surface or released in solution [157]. As ionic silver is well-known to have antimicrobial properties, it is usually suggested that they might be involved in the antibacterial effect of AgNPs [95, 143]. The mechanism of action of Ag^+ ions could be similar to that of AgNPs. Like AgNPs, silver ions can also damage bacterial envelope and alter the respiratory chain [142–144, 154]. Ag^+ can also increase the ROS level inside the bacteria leading to oxidative stress. Finally, they can disrupt and bind to phosphate- and sulphur-rich components such as DNA and proteins leading to signalling pathways perturbation [95, 144, 147, 148, 152, 154].

The part of toxicity attributable to silver ions still remains controversial. Owing to the study of Xiu *et al.* (2012), AgNPs synthesized and tested in anaerobic conditions did not induce toxicity. As the absence of oxygen prevents NPs oxidation, authors stated that the antibacterial effect of AgNPs observed in aerobic conditions was exclusively due to silver ions release [155]. On their side, El Badawy *et al.* (2011) observed bactericidal effects of AgNPs even after ultrafiltration and elimination of ionic silver, suggesting that Ag^+ is not the only cause of toxicity of NPs [146]. Sotiriou & Pratsinis (2010) declared that the implication of ionic silver might actually depend on the NPs size. They demonstrated that the antibacterial activity was mainly due to the release of silver ions for small nanoparticles while for larger AgNPs (more than 10 nm) the toxic effect was also due to the particles themselves [158]. Smaller size AgNPs can easier dissolve into silver ions so that the activity of the ions

might be preponderant in this case [95, 150].

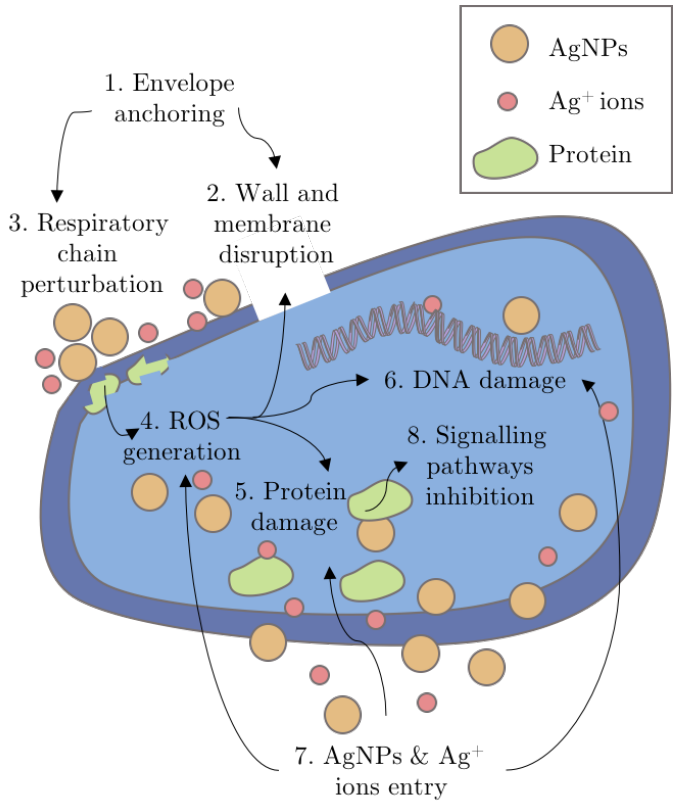

Figure 1.8: Potential AgNPs mechanisms of action to disturb bacteria. (1) AgNPs could anchor to bacterial envelope, which (2) damages the cell membrane and (3) disrupts the respiratory chain. This leads to the (4) production of ROS, which alters in turn lipids, (5) proteins and (6) DNA. (7) AgNPs can also enter in bacteria and bind to (6) DNA and (5) proteins, which (8) disturbs the signalling pathways and lead to the bacterial cell death.

The NPs size has a huge impact of the antibacterial effect of AgNPs, smaller particles showing generally a higher effect than larger ones [147, 159]. Other parameters can also affect the activity of AgNPs [95]. For instance, Pal *et al.* (2007) demonstrated that the NPs shape mod-

ified the toxicity as truncated triangular nanoplates exhibited a higher antibacterial effect than spherical and rod shaped particles [140,147,151]. The surface coating and the charge of the nanoparticles have also an impact on their bactericidal activity as it was observed by El Badawy *et al.* (2011) [146].

The ability of AgNPs to alter several targets in bacteria may suggest the difficulty for bacteria to counteract their mechanisms of toxicity [151]. However, a recent study displayed that gram-negative bacteria might acquire a resistance due to repeated exposure of AgNPs. This resistance was conferred by the production of the flagellum protein flagellin, which allowed the AgNPs aggregation and their antibacterial activity inhibition. This mechanism appeared to be specific to AgNPs while no resistance was observed in the presence of ionic silver [160]. Caution must therefore be taken to avoid the apparition of AgNPs resistance due to extensive use of NPs for a wide range of applications [147].

1.2.4 Applications

As previously discussed in section 1.1.3, AgNPs are found in the highest number of consumer products containing NMs. In addition, it is also one of the most commercially produced NPs with around 320 tons produced each year [75]. AgNPs are even emerging as one of the fastest growing products from the industry of nanotechnology [161]. This business success can be attributed principally to their antimicrobial properties, but also to their conductivity [75], catalytic [162], thermal [136] and optical properties such as their UV protection [163] or plasmonic resonance [162].

Three different databases reporting consumer products containing NPs *i.e.* (i) the American Project on Emerging Nanotechnologies [36], (ii) the Danish Nanodatabase [38] and (iii) the Statnano database [61] were analysed to estimate the sectors in which consumer products con-

taining AgNPs are present (Figure 1.9). Different examples of AgNPs applications are reported in Figure 1.10.

Fauss (2008) has analysed the consumer products from the "Project on Nanotechnologies" claimed for containing AgNPs [164]. The use of nanotechnology is often reported even if the concentration and how the nanoparticle is integrated are rarely mentioned in the product description [164]. According to Fauss (2008), the overall average size of AgNPs employed in consumer products was 24 nm [164].

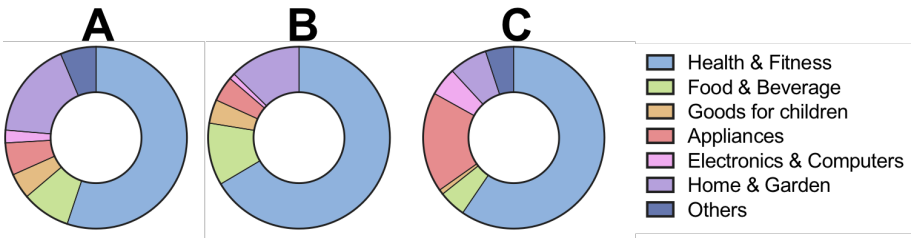

Figure 1.9: Categories of consumer products containing AgNPs in their composition, based on three consumer products inventories: (A) Project on Emerging Nanotechnologies; (B) Nanodatabase; (C) Statnano [36, 38, 61].

AgNPs are present under different forms in these products, either liquid or solid. Liquid form of AgNPs can be used directly as colloidal suspensions, applied as a spray to form a coating over the product or be incorporated in a liquid product such as a shampoo or toothpaste. Solid forms of AgNPs are either used as a raw powder, incorporated in the coating or in textile fibres, or embedded in the product [164].

The concentration of AgNPs incorporated in consumer products varies largely, ranging from 6 to 10 000 ppm [94, 165–168]. Different studies underlined the migration and leaching of these AgNPs coming from textiles [167, 169], washing machines [170], or medical devices [171]. For example, Benn & Westerhoff (2008) measured a leaching of up to 650 μg

AgNPs from socks [169]. Their release in environment can contaminate aquatic ecosystems [65].

Owing to their antibacterial, anti-fungal and antiviral properties described in section 1.2.3, healthcare and fitness is the major category of products in which AgNPs are incorporated. This sector is expected to be the fastest growing sector for AgNPs applications [136]. This category comprises different kinds of products *e.g.* textile, cosmetics, medical and dental care. The antibacterial property of AgNPs is used to reduce transpiration odour in textiles, in particular socks or sport clothes. In these kind of products, silver is incorporated in cotton or silk fibres. [169, 172]. Vigneshwaran *et al.* (2007) have suggested that AgNPs could also protect from UV [163]. Furthermore, AgNPs are added in some hospital clothes to avoid the presence of germs [131]. Some cosmetics and personal care products *e.g.* facial soaps or skin creams also contain AgNPs as preservative agents [173, 174]. Indeed, silver represents 12% of NMs used in cosmetics [174].

AgNPs are also incorporated in medical products, making use of their biocidal properties *e.g.* in burn treatments or as coating of biomedical devices and instruments [65, 66, 131, 140]. Incorporation of AgNPs in various dental materials, such as resins, endodontic materials, implants or orthodontic adhesives has also been reported and might lead to their ingestion [175–177].

Besides, household appliances *e.g.* fridges, washing machines, ... take antimicrobial properties of AgNPs to their advantage, to reduce the formation of biofilms that could impair their operation [65, 163, 164]. Some devices for air and water disinfection contain also AgNPs [147]. In 2003, Samsung introduced an antibacterial technology in their washing machines, air conditioners and refrigerators [11]. Similar technologies are used for electronic applications, such as antimicrobial phones, keyboards or computer mice [36].

Children products comprise fabric products, plush toys or cleaning products that commonly come in contact with children [62]. Quadros *et al.* (2013) examined the release of silver from these products in different liquids in contact with these products. The release was evaluated after soaking product samples in the different liquids. This study revealed a relatively high release of silver, up to 38% of total silver mass contained in products in different liquids such as urine, sweat or saliva. They suggested the prominence of the dermal exposure to silver in these products [178].

Finally, AgNPs are also found in food and feed applications, as it will be described in the following section.

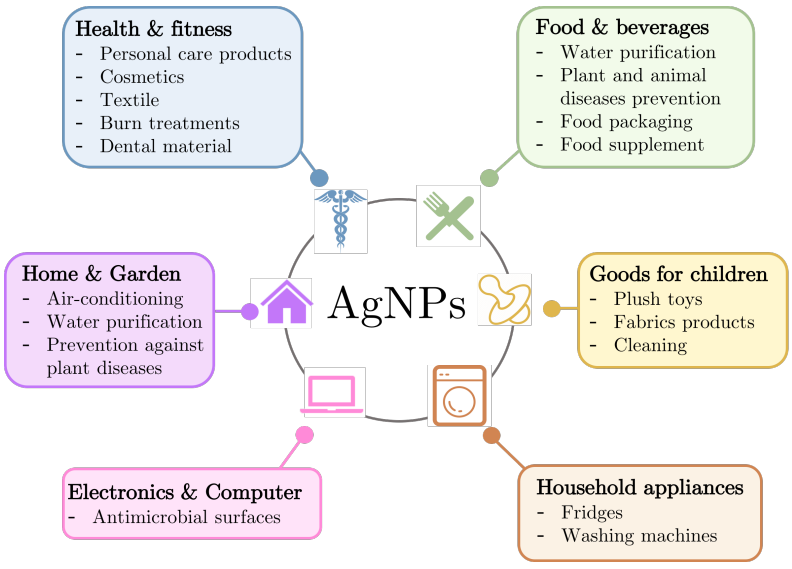

Figure 1.10: Examples of AgNPs applications in consumer products, according to categories identified by consumer products inventories.

1.2.5 Silver nanoparticles in the food industry

Besides health and fitness applications of AgNPs, their uses in the agri-food sector have gained popularity. Owing to their antimicrobial properties, they are found throughout all phases of food production *i.e.* water treatment, agriculture, feed, food and food packaging materials [65]. According to Peters *et al.* (2016), silver is the most frequently type of NMs used in biocidal and in food packaging material [179].

Water filtration devices have been developed taking advantage of action against microorganisms. AgNPs are coated on filters to remove microorganisms, decompose organic impurities and improve cattle and household water quality [147]. AgNPs also prevent the formation of biofilms on the filtration devices [180]. In addition, AgNPs are used in agriculture as prevention against plant diseases due to fungi or bacteria [52]. Silver is also recognised as a plant-growth stimulator [6].

Some animal husbandry applications contain AgNPs mainly for disinfection purposes [147]. AgNPs are applied on surfaces in which animal could be in contact, such as animal breeding facilities, transport chambers or living spaces [147]. AgNPs are also used as an additive for animal feeding of pigs and chicken, either to replace antibiotics or improve meat [147, 181–183]. Sawosz *et al.* (2013) have experienced the *in ovo* injection of AgNPs inside eggs as supplement during their development towards chicken embryos and have observed an improved chicken muscle growth when AgNPs were injected [183].

In contrast, AgNPs are not intentionally added in human nutrition, even if some silver can be found in the nanoparticular form in some E174 silver food colouring agent. Indeed, Verleysen *et al.* (2015) indicated the presence of AgNPs in silver pearls used for pastry decorations [184]. The most important application of AgNPs seems to be food packaging. AgNPs are incorporated in some plastics so that migration in the stored food can occur and guaranty the claimed antimicrobial properties.

Finally, aqueous dispersions of colloidal silver, containing AgNPs in suspension, are claimed for having benefits on the immune system [48,

65, 66, 185]. Even if these supplements are unauthorised on the market, they are readily available online [186].

1.2.6 Oral exposure to AgNPs

Thousands of products containing AgNPs are currently on the market, but this amount is based on claims provided by manufacturers, and is probably underestimated [36]. Even if each source contains only few amounts of AgNPs, the increasing use and applications might increase our exposure to AgNPs [65].

OECD, as well as the Scientific Committee of Consumer Safety, from the European Commission, both point out the need for more data to estimate the exposure and the subsequent safety assessment of AgNPs [187, 188]. In addition, analytical methods are limited and not able to discriminate particle from other forms in complex matrices. As a result, the majority of exposure data is restricted to modelling based on AgNPs production volumes [189]. The large range of products in which AgNPs are incorporated further complicates the exposure assessment and studies generally focus on the release of AgNPs by some types of products, such as textiles or children toys [188]. In addition, the lack of data concerning production, emission and market penetration rates is a major source of uncertainties [190].

ANSES measured silver content in the general French diet. Although 82% of the analysed products had an undetectable amount of silver, the highest silver content was found in mollusks and crustaceans (6,48mg/kg) followed by offal and then frozen desserts. Children are more probably exposed to silver by water and milk [191]. Unfortunately, it was not possible to discriminate the form of silver. Oral exposure to AgNPs could occur from three main sources: (i) ingestion of food supplements, (ii) leakage from consumer products and (iii) contamination by the environment. First, food supplements containing AgNPs are ingested for their claimed immune health effects [48, 65, 66, 185]. As these supplements are not authorised on the market, this complicates the evaluation of hu-

man exposure. A worst case scenario of 0.02mg.kg body weight^{-1}.day^{-1} was estimated by Larsen *et al.* [60]. However, to our knowledge, market penetration rates of these supplements have not been discussed in literature. Secondly, migration of AgNPs from food products such as packaging or storage boxes in food has been observed in numerous studies. For instance, the presence of silver was detected in boneless chicken breasts [192] or orange juice [193] stored in an AgNPs-embedded packaging material. The migration process seems more efficient at acidic pH [194–197]. A majority of the silver is found in the form of oxidised silver. However, the migration of pristine AgNPs was also evidenced [194, 196, 198, 199], suggesting that the oxidation of silver is not the cause of its migration from packages. Pang *et al.* predicted a silver exposure of 0.02mg.kg body weight^{-1}.day^{-1} for children, based on the presence of AgNPs in sippy cups or baby blankets [200]. Finally, AgNPs can enter in the environment during their whole life cycle, from the production to the recycling. Luoma *et al.* predicted a release in the US environment of around 128T/year [201]. In the environment, many changes could occur to nanoparticles, such as aggregation or, in the case of silver, oxidation and silver sulfide formation thanks to sulfide present in the environment could inactivate AgNPs [67, 201, 202]. However, new different nanoparticles could also be formed during the process of wastewater treatment [190]. A literature review by Gottschalk *et al.* modelled concentrations around 10^{-2} μg/L silver in surface waters, which seems to be negligible compared to other sources [190].

1.2.7 Adverse health effects

Due to their antimicrobial properties, AgNPs are promising for different sectors and their use is constantly growing. However, their peculiar properties could also be deleterious for human health. It is primordial to assess their potential adverse effects while their expanding applications increase the risk of human exposure [152, 203]. Ingestion is certainly the major exposure pathway for AgNPs because of their presence in

food products *e.g.* food packaging, food supplement and food additive E174. Besides, AgNPs can also enter through inhalation, be cleared out by the muco-ciliary escalator and dispatched to the gastro-intestinal tract. Dermal exposure is another important route following the use of wound dressings and antimicrobial textiles. Finally, AgNPs are also incorporated in medical devices and could enter the body, for instance, after the use of dental or bone cement [65, 157].

Absorption of ingested AgNPs has been observed in various *in vivo* studies, as well as clinical studies. Park *et al.* (2011) have studied the toxicokinetics of ingested AgNPs in Sprague-Dawley rats and observed poor absorption rates of AgNPs, with bioavailability of 4.2% in 10 mg.kg body weight^{-1} treated rats [204]. However, clinical cases of argyria and argyrosis have been reported after ingestion of colloidal silver, an unauthorised food supplement claimed for boosting the immune system, having antimicrobial properties and beneficial effects against cancer, diabetes and arthritis [205–207]. Argyria and argyrosis are irreversible bluish-grey pigmentation of respectively skin and eyes due to the accumulation of silver [152, 205, 207–209]. Indeed, once inside the body, ingested silver first binds to proteins because of its affinity for thiol groups. In skin and eyes, light photo-reduces these compounds into metallic silver, which in turn binds to sulphur and selenium to form low soluble granules that accumulate in tissues [157, 207, 210]. Argyria can either remain localised closed to the exposure area or be generalised depending on the silver dose and the exposure pathways [205, 209]. Argyria is generally innocuous but a case associated with polyneuropathy and myopathy was reported by Jung *et al.* (2017) [207]. Another case of generalised argyria associated with hepatotoxicity was published after the use of Acticoat to treat young patient burns. Acticoat is a wound dressing containing silver nanocrystals with an average size of 15 nm [65, 211].

Concerning the distribution, *in vivo* studies on mice and rats have been performed to better understand the localisation of AgNPs into the body. No matter the exposure pathway, AgNPs were detected in blood and various organs in addition to skin [208, 212]. After oral admin-

istration of AgNPs in rats, high levels of silver were recorded in the GIT [213, 214]. Its presence impacts the GIT since an increase of goblet cells and mucus, as well as epithelial cell microvilli damages were observed in rats and mice [212, 215, 216].

After absorption of AgNPs through the gut, silver can reach the systemic circulation [203]. Most studies generally found the highest silver levels in the liver and spleen [157, 210, 212–214]. Besides the liver is the first organ to receive portal vein blood after intestinal uptake, it also contains a lot of thiol-containing compounds for which silver has a high affinity [203, 213]. This accumulation could lead to hepatic enzymes and cholesterolaemia modifications [103, 217]. Silver was also present in other organs including testes, kidneys, brain, lungs, bladder and heart [210, 212, 213, 217]. In addition, AgNPs were found in foetal circulation [218] and were transferred to the offspring [219]. Few authors have reported damages other than accumulation in organs. However, a recent sub-chronic study of 12 weeks observed a decrease in body weight and a significant reduction of feed and water intake in rats. It was accompanied by liver and kidney enzymes damage as well as disruption of haematological and biochemical parameters. Their results suggest that effects of accumulation might be visible after long term exposure [220].

Relating to their metabolism, transformations of AgNPs occur once inside the body, such as oxidation into ionic silver and formation of sulfide and selenide compounds in which silver gets accumulated [157, 207, 210, 221]. Furthermore, interactions with metallothioneins have been reported. These Golgi proteins could be involved in the protection of cells against AgNPs-induced damages. They contain around 30% of cysteine residues that, due to their thiol groups, could bind to several metals. As a result, they are involved in metal regulation and detoxification [222]. Indeed, upregulation of metallothioneins, following incubation with AgNPs, has been observed in various cell types such as human astrocytes [223], human lung cells [224] or HeLa cells as soon as after 3h incubation [225] and could act as a cellular defence against AgNPs effects. In addition to these modifications of AgNPs, AgNPs could in turn

have consequences in cells. *In vitro* studies may be useful in providing information about these consequences [203]. Cellular effects of AgNPs have already been described for bacterial cells in section 1.2.3. However, animal cells differ from prokaryotic cells in different points. Bacteria have a cell wall in addition to the cell membrane, which is not the case for eukaryotic cells. On their side, eukaryotic cells are larger and they own a membrane-enclosed nucleus and organelles such as lysosomes or mitochondria [65, 154].

Numerous studies have already been performed with animal cells but till now, the complete mechanism of AgNPs toxicity is not fully understood [101, 210]. The lack of standard methods in nanotoxicology makes it difficult to compare the researches and draw conclusions [210, 226]. Indeed, toxic effects depend on the different properties of AgNPs, *i.e.* size, shape, charge, dissolution, surface modification, agglomeration and aggregation [101, 210, 218, 226]. Besides, biological models (*e.g.* cell type and species) and experimental design (*e.g.* duration of exposure and particle concentration) influence the observed AgNPs toxicity [203, 210, 226]. Nevertheless, it is beyond doubt that AgNPs disturb several cellular targets leading to toxic effects [226, 227]. Potential cellular mechanisms are summarised in Figure 1.11 and detailed below:

– Cell membranes might be the first target since AgNPs could bind to membrane proteins, which activates signalling pathways and alters membrane permeability [101, 228]. Cell entry of AgNPs was also observed in various studies, either by diffusion or mainly by several endocytotic mechanisms [101, 157, 225, 227–229]. They could be transported to lysosomes afterwards [225, 230], which can alter their integrity [231]. As emphasised in section 1.1.5, AgNPs might be readily dissolved into Ag^+ in endosomes and lysosomes due to the pH decrease in these organelles [157, 225]. Silver ions can then diffuse in whole cell as observed with fluorescent probe by De Matteis *et al.* (2015) [225]. AgNPs might act as a "Trojan horse" as released Ag^+ can alter cell [232]. As an il-

lustration, ionic silver might bind to DNA in nucleus due to its affinity for phosphate ions [228]. Silver ions might also preferentially attach to thiol groups and disturb protein structure and functions [101, 142, 228]. Enzymes involved in the defence against oxidative stress as well as glutathione might be especially targeted, which could lead to the increase of intracellular ROS and oxidative stress [142, 157, 203, 225, 228, 232–234].

– Oxidative stress is often considered as the main mechanism of AgNPs deleterious effects [101]. High ROS concentrations generated by the presence of AgNPs could damage cell membrane, organelles and nucleus since ROS are known to alter DNA and oxidise lipids and proteins [101, 103, 154, 217, 225, 226]. ROS are also able to modify signalling pathways, which finally leads to inflammation and apoptosis [101, 226, 235]. Mitochondria might be especially altered by oxidative stress, which explains the damage observed in the presence of AgNPs, *i.e.* disruption of mitochondrial membrane potential and inhibition of respiratory chain [103, 225, 226, 234, 236]. The perturbation of the electron transport chain might intensify the AgNPs toxic effects since it leads to a higher production of ROS and an interruption of ATP synthesis, which in turn jeopardises cell survival and growth [103, 203, 234, 236, 237]. Besides the indirect impact of oxidative stress, AgNPs might also directly alter the mitochondria since NPs deposition could be observed in this organelle [226, 237, 238]. However, this translocation must be investigated in more details since only few authors reported it [157, 234].

– Finally, silver ions present in solution could also contribute to the toxicity of AgNPs as they could impair the cell membrane permeability [225] or enter cells via a copper transporter (Ctr1) [239]. Nevertheless, the degree of contribution from Ag^+ in the observed toxicity is still under discussion [203, 218, 228] and seems to depend on the amount of Ag^+ present in AgNPs suspension [240].

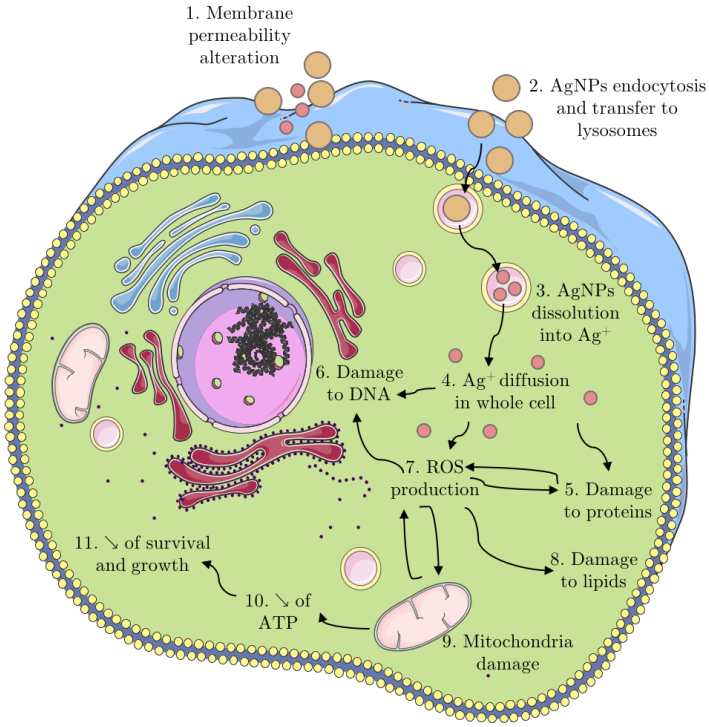

Figure 1.11: Potential cellular mechanisms of AgNPs toxicity. (1) AgNPs might attach to membrane proteins and alter permeability. They could also (2) enter cells by endocytosis and (3) be dissolved into Ag^+ in lysosomes. (4) Ag^+ might diffuse in whole cells, (5) damage proteins and (6) DNA and (7) lead to the ROS production. ROS can then alter (6) DNA, (5) proteins, (8) lipids as well as (9) mitochondria, which disturbs the respiratory chain and leads to (7) an increase of ROS and (10) a decrease of ATP production. Finally, (11) the lack of energy decreases the cell survival and growth. Image created with a scheme adapted from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.

Regarding their excretion, AgNPs were eliminated in faeces and urine in *in vivo* studies [212–214, 217]. Excretion by urine was also observed in burn humans treated with nanocrystalline silver [209, 211]. However, silver excretion is not total as argyria can be persistent [209]. Besides, although Van der Zande *et al.* (2012) observed a quasi-complete clear-

ance of silver in most organs, silver was still detected in brain and testes after 8 weeks post-exposure [213].

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II

An overview of the intestinal barrier and its role in intestinal immunity

Contents

List of abbreviations

AMPs antimicrobial peptides

ARE antioxidant response element

BTB Broad complex, Tramtrack and Bric-a-Brac

bZIP basic leucine zipper

CTR C-terminal region

GALT gut associated lymphoid tissue

GIT gastro-intestinal tract

IBDs inflammatory bowel diseases

IECs intestinal epithelial cells

IECs intestinal enterocyte cells

IgA immunoglobulin A

IL-8 interleukin-8

IVR intervening region

LPS lipopolysaccharide

Maf musculoaponeurotic fibrosarcoma

NLRs nucleotide binding oligomerisation domain like receptors

NLS Nuclear localisation Sequence

Nrf2 nuclear factor-erythroid 2-related factor

NTR N-terminal region

RHD Rel homology domain

ROS reactive oxygen species

TLRs Toll-like receptors

2.1 Short description of the small intestine

2.1.1 Organ and tissues presentation

Localised between the pyloric sphincter of the stomach and the ileocecal valve of the colon, the small intestine comprises the duodenum, jejunum and ileum as seen on figure 2.1. After gastric processing, the luminal content enters in the small intestine where the end of biochemical digestion occurs either in the lumen or at the surface of intestinal mucosa. In the large intestine or colon, the chyme only contains undigestible compounds, electrolytes and water [1, 2].

The small intestine constitutes the major organ for nutrient absorption. To cope with this role, it presents adaptations to increase contact surface with luminal content. Indeed, it is the longest segment of the gut, with a length between 5 and 7 meters in humans. Moreover, it is the place where "segmentation" starts. This muscular contraction leads to mixing and propulsion of the chyme, increasing the contact between nutrients and mucosa. Finally, it presents a multi-folded organisation at different levels *i.e.* circular folds, villosities and microvillosities as can be observed on figure 2.1. In addition, the small intestine tissue itself presents deep circular folds named *plicae circulares* in which the intestinal mucosa forms protrusions of around 1 mm in the lumen, delimiting villi and crypts [2,3]. Furthermore, the apical membrane of each cell comports around 3 000 projections in the lumen forming the brush border [1–3].

Besides its roles in digestion and absorption of nutrients, the small intestine also forms a selective barrier against luminal content and all the food antigens, microorganisms or various xenobiotics it could contain. Its role in immune regulation will be described in 2.3.2.

Nutrients are absorbed thanks to paracellular or transcellular routes. Paracellular transport is regulated by tight junctions, desmosomes and adherent junctions [4]. Transcellular passage is selectively mediated by passive or active transporters, depending on the nutrient [2].

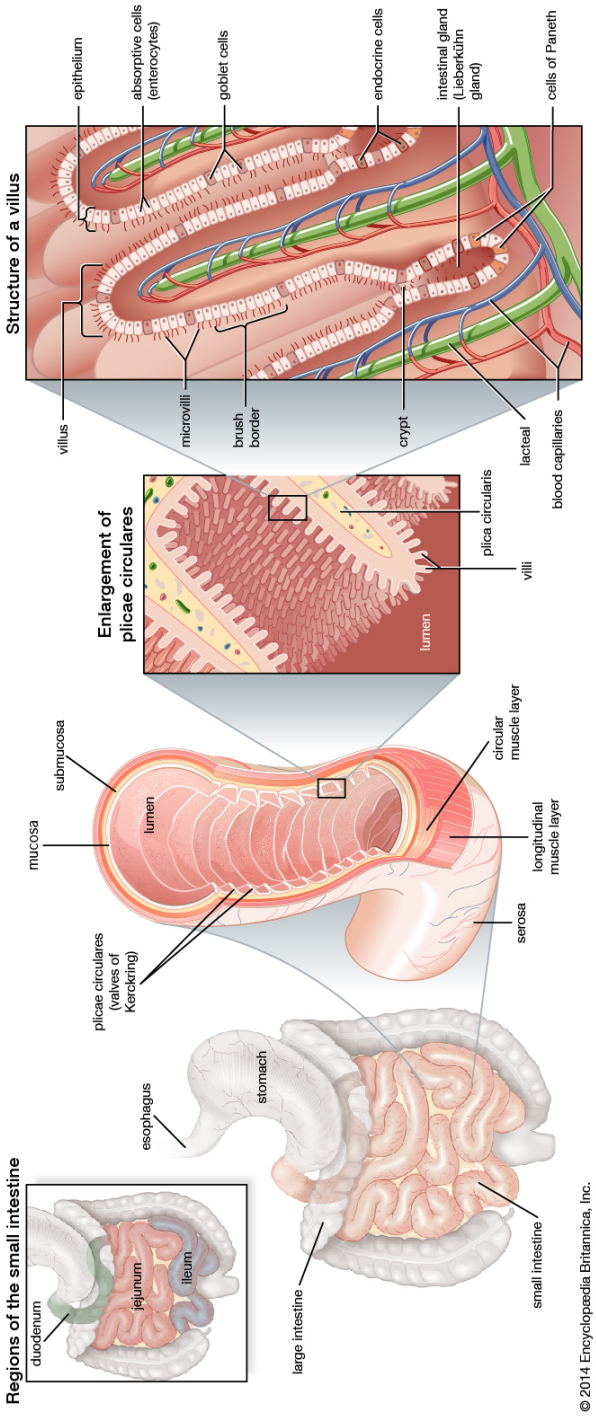

Figure 2.1: **Tissue organisation and folding at different levels in the small intestine** [5]

Four different tissue layers are encountered in the small intestine (figure 2.1), starting from the lumen [1–3, 6]:

- The mucosa is the inner layer of the small intestine, devoted to absorption of nutrients and secretion of mucus and digestive enzymes while forming a barrier against antigens and pathogens from the lumen. This tissue is composed of an epithelium, formed by intestinal epithelial cells (IECs) that will be described in 2.1.2. This epithelium covers a connective tissue, the *lamina propria*. A thin layer of smooth muscle, the *muscularis mucosae* separates the mucosa from the submucosa and is responsible of mucosa folding and local movements of the mucosa.

- The submucosa, formed of collagen fibers, forms a mechanical support for the mucosa and is traversed by blood and lymphatic vessels to feed intestinal tissue but also to transport absorbed elements and chylomicrons for the rest of the body. Submucosa contains nervous cells, which regulates the transmission of information between the digestive and the nervous system to modulate different gastro-intestinal activities.

- The *muscularis externa* mediates the peristaltic movement of the food bolus. It contains usually two layers, a circular inner layer and a longitudinal external layer.

- The serosa comports a loose conjunctive tissue and a smooth membrane formed by a thin layer of cells (the visceral peritoineum). These cells secrete fluid to reduce friction due to the peristaltic movements.

In this short review, we will focus on the epithelium covering the mucosa because it constitutes the first line barrier of the body encountered by ingested silver nanoparticles when they arrived in the small intestine.

2.1.2 Cell types lining the small intestine epithelium

The epithelium of the intestinal mucosa is the largest of the body, presenting a total active surface estimated between 32 [7] and 400 m^2 [8]. It forms a polarised monolayer almost impermeable thanks to intercellular tight junctions [9]. Although the major role of intestine is nutrient absorption, achieved by enterocytes, various cell types, with different roles in the mucosa, are present [10]. Intestinal epithelium is folded, forming villi that project in the lumen and crypts invaginated in the *lamina propria*. These compartments contain different proportion of cells. The intermediary zone is a "transit amplifying zone" in which proliferating cells from crypts progress towards villi where all cells are post-mitotic differentiated cells. At the top, cells become senescent and are detached intact in the lumen. This process of cell migration from crypts to villi allows the epithelium renewing in around 4-7 days [11]. All cells (except Paneth cells) are subject to this migration stream, their location indicating their level of maturation. The closer from villi extremity are the more differentiated with the shortest lifespan [12].

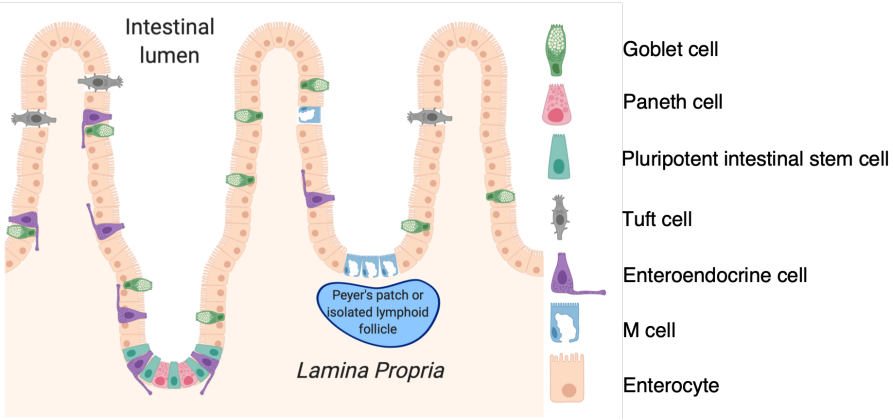

Figure 2.2: **Representation of small intestine epithelium and the different cell types** composing this epithelium as represented in the right legend. The figure was designed with Biorender.

Different cell types are encountered in the intestinal epithelium (Figure 2.2):

- **Goblet cells** (in green) are scattered throughout the epithelium and produce mucus, an aqueous gel containing highly glycosylated mucins, whose most abundant is mucin 2 (MUC2). In addition to mucins, goblet cells also secrete trefoil factor 3 able to crosslink mucins to provide structural integrity to mucus, and resistin-like-molecule-β, which promotes mucin 2 secretion and regulates T cell responses. This mucus forms a barrier against gut microflora and ingested microbial material [10].

- **Paneth cells** (in red) are immune cells specialised in the secretion of antimicrobial peptides (AMPs) towards the lumen. These AMPs are able to disrupt bacterial wall components or membrane of other microorganisms [13]. They are stored in apical cytoplasmic granules in Paneth cells, and are secreted after stimulation by bacterial products such as lipopolysaccharide (LPS). Growth factors for surrounding stem cells are as well secreted by Paneth cells, usually localised in the base of intestinal crypts. Unlike the other IECs, they migrate towards the bottom of crypts. 5 to 15 Paneth cells are found in the base of each crypt [6], almost exclusively in the small intestine [14].

- **Pluripotent intestinal stem cells** (in turquoise) present in crypts bottom proliferate and differentiate into the other types of cells. The proportion of stem cells in the epithelium variates among published papers between 0.4% and 60% [12]. This discrepancy could be due to the difficulty to have correct markers for intestinal stem cells. Moreover, properties of stem cells are really sensitive to experimental conditions. As there are around 250 cells in each crypt, 0.4% would correspond to one unique stem cell per crypt. Classical

models estimate around 4 to 6 intestinal stem cells per crypt [12]. Except Paneth cells, daughter cells progress in epithelium during the differentiation process towards the top of villosities where they will shed from the epithelium to allow a constant cell renewal [11].

- **Tuft cells** (in grey) present thick microvilli projections into the intestinal lumen, forming a "tuft". They constitute only a minor proportion of intestinal enterocyte cells (IECs) with approximatively 0.4%. These cells harbor taste-chemosensory machinery for example the receptor TRPM5 that transduces signals from bitter-, sweet-, and umami-tasting substances in lingual taste cells. Although their precise function in the intestinal mucosa is not completely elucidated, Gerbe *et al.* suggested a role in the regulation of type 2 immune responses following parasites infections [11]. Howitt *et al.* hypothesised the involvement of taste receptors in this chemosensory recognition of pathogens [15].

- **Enteroendocrine cells** (in purple in figure 2.2) are able to secrete intestinal hormones and neuropeptides to regulate intestinal mobility and digestive secretions such as motilin, somatostatin, cholecystokinin, neurotensin, vasoactive intestinal peptide, glucagon-like peptide, serotonin, histamine, substance P, calcitonin gene-related peptide and neuropeptide Y [16]. These cells represent around 1% of all IECs [17].

- **M cells** (in blue) are found in the follicle-associated epithelium and exert a role in immune defence as they capture antigens and particles from the intestinal lumen to present them to subjacent lymphoid tissue. To facilitate access and adhesion of external components, they do not display microvillosities (or smaller ones) and do secrete neither mucus, nor digestive enzymes, nor antibacterial

agents nor immunoglobulins. Antigens are released from M cells to be exposed to lymphocytes, macrophages and dendritic cells intricated in basolateral membrane of M cells. Only a limited amount of M cells is found in the epithelium with around one cell for every 10 million enterocytes [14].

- **Enterocytes** (in orange) are the major cell type found in this epithelium, constituting 80% of the IECs [16]. These polarised cells are specialised in absorption of nutrients at their apical pole, exhibiting microvillosities to increase the surface presented to the lumen [18]. At their basolateral side, they release nutrients towards blood or lymphatic capillaries. As it will be discussed in 2.3.1, enterocytes form a major barrier against luminal antigens thanks to their selective permeability achieved by, on one hand, tight junctions, to limit unselective entrance of low molecular weight hydrophilic components and, on the other hand, specific transporters in their brush border to absorb nutrients. Tight junctions form a selective barrier through the paracellular space to allow nutrients passage while avoiding toxic or microbial compounds entrance [19]. These dynamic structures are composed of a scaffold of membranous and cytoplasmic proteins. Membranous proteins such as claudins, occludins or junctional adhesion molecule present a part in the intercellular space while being connected to cytoplasmic proteins such as ZO-1 [19–21]. ZO-1 is bound to actin, linking tight junctions to the cytoskeleton [22]. In addition to these roles of absorption and barrier, it became evident that enterocytes also play a role in the immunological tolerance to ingested proteins [14]. Enterocytes are also secretory cells producing mucins that forms the glycocalyx, a supplementary barrier covering the enterocyte apical surface. Moreover, although to a lower extent than Paneth cells, they secrete two types of AMPs, β-defensins and HIP-HAP, which target bacterial walls [9].

2.2 *In vitro* models of the small intestine

Ex vivo models of the human guts present several limitations such as hyperpermeability, difficulty to form a monolayer and overproduction of mucus, restricting their use in toxicity studies [23, 24]. Different models have been developed to overcome this situation but we will focus on the most used as intestinal permeability model, *i.e.* the Caco-2 cell line [25]. Caco-2 cells form a continuous cell line isolated from a human colonic adenocarcinoma. These cells were first cultivated in 1975 by Fogh *et al.* [26]. Since then, they have been extensively used over the last years as a model of the intestinal barrier to investigate intestinal uptake and passage of several compounds, in particular drugs and xenobiotics [27, 28]. Some authors consider it as the golden standard [29]. In nanotoxicology, Caco-2 cells are the most widely used for nanomaterial translocation assessment [30]. Although their colonic origin, they behave much more like small intestinal enterocytes but the reasons behind this discrepancy are not fully elucidated [31]. During the culture process, they differentiate spontaneously at confluence in 18 to 21 days to form a polarised monolayer. This model exhibits structural and functional similarities with absorptive enterocytes from the small intestine such as tight junctions, microvilli at the apical membrane [32], expression of several membrane enzymes and transporters [28, 33] or formation of domes, typical structures of transporting epithelial cells [33].

Different models have been developed to take into account the presence of other cell types in the epithelium barrier.

- The presence of goblet cells can be mimicked by a coculture of Caco-2 and methotrexate-treated HT-29 cells [34]. Methotrexate was used to isolate mucus producing cells from the heterogeneous HT-29 cell line [35]. In this coculture, cells are covered with a 10μm layer of mucus, not as thick as physiological mucus but more resistant to washings than artificially added mucus [36].

- No cell line mimicking M cells exist. However, the conversion of

Caco-2 cells in a M cell phenotype has been shown in the presence of immune cells [37]. Cocultures of Caco-2 cells with Raji B cells, a continuous line of lymphoblastoid cells, have been developed and improved to investigate the impact of M cells [38, 39].

- Both models can be combined to investigate the involvement of mucus and M cells as the model developed by Bazes *et al.* [40]. However, increasing the number of cell types also augments the complexity of the model, and could complicate the interpretation of data.

2.3 Small intestine role in intestinal immunity

At the interface between the commensal microbiota and the underlying immune cells in the *lamina propria* [6, 9, 16, 41–43], IECs play a major role in the regulation of intestinal immunity and contribute largely to immune homeostasis. In addition to the formation of a selective barrier against luminal content, they communicate with immune cells and actively participate in the mechanism of oral tolerance.

2.3.1 Barrier function of the small intestine

The intestine should be considered as an external body surface exposed to a variety of foreign material [42]. Only a small amount of ingested antigens and microbial threats have survived to the gastric digestion and attacks of pancreatic secretions and bile salts [6] before arriving in the small intestine where they will pass only some hours due to a short transit of around 3-5 hours [6, 44]. The small intestine has developed different barriers to avoid uncontrolled response, described in figure 2.3. Each type of IECs contributes to the formation of this barrier [10]. The commensal microbiota forms the first physical barrier encountered by ingested components. Gut microflora resists to colonisation by external bacteria due to several mechanisms including substrate and enterocyte receptors competition and communication with antibiotic substances or

signalling molecules [6]. The microbiota is not evenly distributed through the gastro-intestinal tract (GIT) and rises with increasing distance from the stomach, being less dense in the small intestine compared to the colon [45].

The second barrier is the mucus layer covering the intestinal epithelium. It is produced mainly by goblet cells but also by enterocytes. Mucus consists in an aqueous gel formed by mucins crosslinked by trefoil peptides and surfactants [6, 10]. This layer is thinner than in the colon due to a lower presence of goblet cells in the small intestine epithelium [46].

Covering the microvilli, the glycocalyx, composed of glycolipids, glycoproteins and some transmembranous mucins, constitutes the third barrier [42, 46]. It forms a meshwork in the lumen. Some digestive enzymes are found in the glycocalyx to complete the digestion of nutrients [1]. As described in 2.1.2, the cells themselves form also a physical barrier, with the intercellular spaces sealed by tight junctions. In addition to a protection against luminal content, it minimises the loss of fluid from mucosa [42, 43]. In a steady state epithelium, tight junctions only allow the passage of up to *ca.* 500 daltons hydrophilic molecules, which are generally less immunogenic than bigger molecules [14]. Moreover, lysosomal digestion can occur for antigens that have escaped to all these barriers and been endocytosed [14].

Finally, Paneth cells but also enterocytes to a smaller extent, secrete AMPs in the lumen. Intestinal AMPs comprises defensins, cathelicidins, C-type lectins, ribonucleases and S100 proteins, which act by different mechanisms such as membrane disruption or enzymatic attack of cell wall components to inhibit microbial survival [13]. These products are retained by the mucus layer and model the composition of the commensal microbiota [46, 47]. Immunoglobulin A secretion also occurs due to communication with underlying immune cells. Enteroendocrine cells coordinate this barrier by modulating mucus secretion and intestinal motility [10], secreting hormones that regulate gut immunity by impacting cytokine production of other cells.

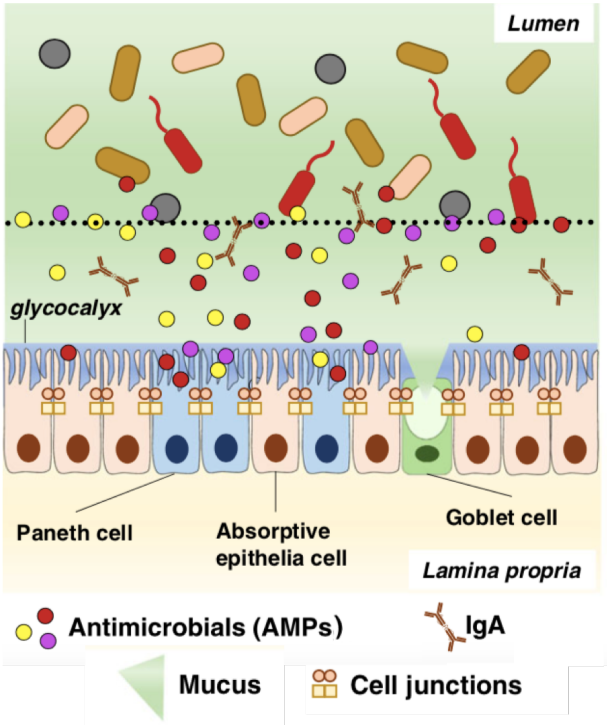

Figure 2.3: **The different barriers encountered by luminal content in the small intestine**. The dashed line represents the limit of the chemical barrier formed above IECs. Adapted from Okumura *et al.* [45]

2.3.2 Role of IECs in immunity

Although these remarkable protections developed by the IECs, it is likely that some intestinal bacteria, viruses or fungi can overpass these barriers and traverse the intestinal epithelium [41]. IECs play a role in the immune defence reaction, communicating with underlying immune cells to coordinate the appropriate response towards the threat. Intestinal immune cells are part of a major primary immune organ: the gut associated lymphoid tissue (GALT). This tissue contains about 60% of total body immunoglobulins [6] and forms in the mucosa, isolated or aggregated follicles (termed Peyer's patches). The epithelium above these follicles contains M cells and less mucus than the classic intestinal epithelium [6].

IECs are in close contact with luminal content and act as sentinels to recognise and communicate the presence of pathogens. For this purpose, they exhibit pathogen recognition receptors like Toll-like receptors (TLRs) or nucleotide binding oligomerisation domain like receptors (NLRs). TLRs are expressed in membranes, either plasma or on organites, while NLRs are intracellular [16]. Upon ligand recognition, these receptors could initiate the inflammatory response involving the NF-kB cascade described in 2.4.1 but also epithelial shedding, mucus production by goblet cells or neuropeptides secretion by enteroendocrine cells depending on the involved receptor [16].

In the presence of antigens, both IECs and immune cells communicate by secreting inflammatory mediators like cytokines or chemokines establishing a bidirectional crosstalk between these cells. Table 2.3.2 reports the cytokines and chemokines secreted by IECs and by immune cells, respectively. The latter can act on the epithelial physiology and affect the barrier or transport properties of the epithelium [48]. Even if IECs are able to produce only a small amount of cytokine per cell compared to immune cells, the huge surface area covered by IECs could lead to a quite important total amount [43].

Table 2.1: **Cyto/chemokines involved in the crosstalk between IECs and immune cells**. Adapted from Perdue [48].
Legend: GM-CSF, granulocyte monocyte-colony stimulating factor; IFN-γ, interferon-γ; IGF, insulin-like growth factor; IL, interleukin; MCP, monocytic chemoattractant protein; NGF, nerve growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor.

Secreted by	**Signaling to**	**Cyto/chemokine**
IECs	Immune cells	IL-1α, IL-1β, IL-1 receptor antagonist, IL-6, IL-8, MCP-1, TNF-α, IGF-II, IGF binding proteins I-V, GM-CSF, NGF, TGF-α, TGF-β_1−3
Immune cells	IECs	IL-1β, IL-2, IL-3, IL-4, IL-6, IL-13, IFN-γ, TNF-α, TGF-β_2, IGF

Among these different inflammatory mediators, IECs express interleukin-8 (IL-8) in response to various pro-inflammatory cytokines and LPS. IL-8 is involved in the chemoattraction of neutrophils at the inflammation site [49]. This chemokine is present in higher quantity in an inflamed mucosa [49] and can be used as a local marker for inflammation in enterocytes [50]. The presence of its receptor has been underlined at the apical pole of enterocytes, suggesting a role in autocrine signalling [51].

IECs are as well involved in acquired immune response by presenting antigenic components to underlying immune cells. M-cells are specialised in this function, but enterocytes also participate as they express MHC-I as well as MHC-II molecules whose expression is regulated by cytokines. As a result, these cells are able to present antigens to immune cells [14, 42].

Acquired immune response leads to the production of an immunoglobulin specifically associated with body secretions, *i.e.* immunoglobulin A (IgA), largely present in intestine. IECs exhibit receptors for polymeric IgA at their basolateral membrane to facilitate their transcytosis, after binding to the secretory component, also acting as a specific receptor, and luminal secretion to recognise selectively pathogens [41, 42]. Finally, IECs directly secrete some complement components of the innate immune response to neutralise microorganisms [42].

2.3.3 Oral tolerance towards luminal antigens

The sentinel IECs have to recognise potentially pathogenic microorganisms among a huge amount of dietary antigens and the commensal microflora and to maintain a complex balance between steady-state and inflammation. The role of barrier described in 2.3.1 is then crucial. In addition, different adaptations have been developed in IECs to avoid an excessive immune response towards commensal antigens. For instance, the expression of receptors responsible for pathogen recognition is restricted. Indeed, only a few of them are in contact with the lumen and

the majority is localised in the basolateral or at the surface of organites to detect only pathogens that have penetrated inside cells [41].

In addition, IECs express molecules inhibiting TLR signalling such as Toll-interacting protein. Moreover, the activation of some TLRs in particular TLR-4, which recognises LPS, induces the expression of PPARγ that inhibits NF-κB and attenuates inflammation [41]. Neish *et al.* [52] have even shown that bacteria abrogate polyubiquitination, which is a necessary step for NF-κB activation. As a result, this pathway is less activated by the microbiota.

2.4 Defensive pathways against cellular stresses

In case of cellular stresses, different pathways can be activated to coordinate an adequate response and here we will focus on NF-κB pathway, triggering inflammatory response and Nrf2 pathway involved in the defence against oxidative stress.

2.4.1 NF-κB pathway

Since its discovery in 1986 [53], as a nuclear factor - NF - involved in the transcription of the immunoglobulin kappa - κ - light chain in extracts of B-cell tumors, many thousands of studies and reviews have investigated the role of NF-κB in the orchestration of complex biological processes. Although conserved from *Drosophila* to humans, this pathway seems notably absent in yeasts or *C. elegans*, suggesting one of its primary role to control immune and inflammatory responses [54]. In addition to this role, NF-κB is also involved in responses to various cellular stresses and regulates processes such as cytokine production, apoptosis, cancer, cell adhesion or proliferation [55]. Rather than being only a central mediator in immune response, NF-κB represents a regulator of stress responses [56].

A simplified vision of NF-κB cascade is often presented as in figure 2.4. In resting cells, NF-κB transcription factor is maintained in the

cytoplasm thanks to the binding to its inhibitor, IκB. After phosophorylation and ubiquitinylation, IκB is degraded by the proteasome, releasing NF-κB transcription factor that translocates in nucleus to activate the expression of target genes. The phosphorylation of IκB is mediated by IKK complexes, which are activated by some stimuli, such as cytokine or bacterial compounds [57, 58].

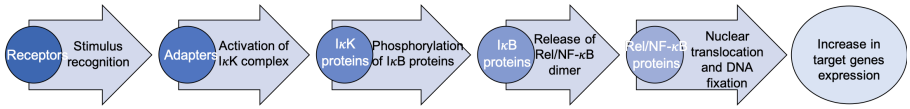

Figure 2.4: **Simplified version of NF-κB cascade** presenting the different actors families and main events resulting in increase of NF-κB-driven gene expression.

However, as we will discuss in point 2.4.1 this NF-κB cascade is quite more complex, with different isoforms of NF-κB, IκB and IKK proteins. The name of NF-κB pathway hides also different pathways categorised in literature as canonical and alternative depending on the actors involved. In some reviews, a third category named "atypical" is also recognised, such as in Perkins *et al.* [59].

The different actors involved will first be presented after what the way they orchestrate together will be described. Their importance in intestinal inflammation will finally be explained.

Actors

Rel/NF-κB proteins NF-κB central actors are Rel/NF-κB proteins, a family of transcription factors comporting 5 known members in mammals: p65 (also known as RelA), RelB, c-Rel, p50 (processed from p105 encoded by NF-kB1 gene) and p52 (obtained from p100 encoded by NF-kB2 gene). In drosophila, three proteins are part of this family: Relish,

Dorsal and Dif [54, 60] while in chicken v-rel is also part of this transcription factors family [60]. p50 (containing amino-acids 1-430 of p105) and p52 (containing amino acids 1-454 of p100) are derived respectively from the longer precursor proteins p100 and p105 by proteasomal either co- or post-translational splicing but also by mRNA splicing [61, 62]. As presented in figure 2.5, Rel/NF-κB proteins share in their N-terminal region the common Rel homology domain (RHD) of approximately 300 amino acids [60, 61], responsible for DNA binding and dimerisation [63]. RHD can be divided in three subdomains, a DNA binding part, a portion required for dimerisation and a Nuclear localisation Sequence (NLS) that allows the nuclear translocation of Rel/NF-kB proteins. In inactive state, NLS is kept hidden by IκB proteins [63].

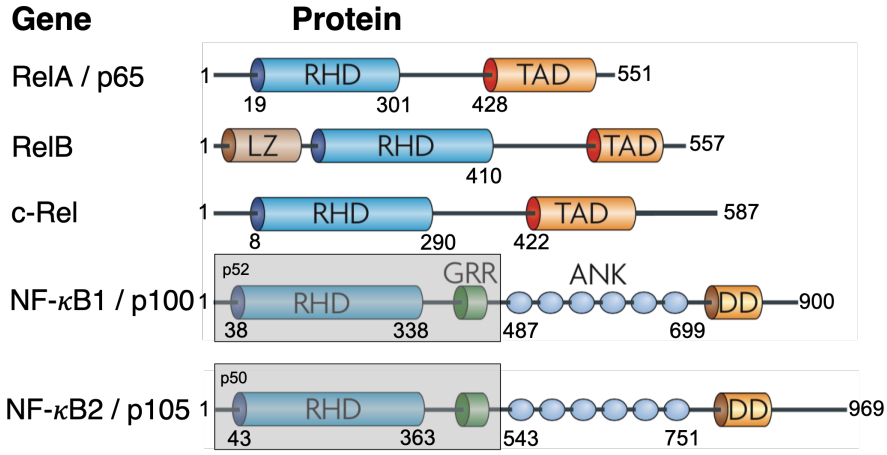

Figure 2.5: **Mammalian members of Rel/NF-κB proteins** (adapted from Gosh *et al.* and Perkins *et al.* [59, 64]). Grey squares represent p52 and p50 after proteolytic processing from respectively p100 and p105. The principal domains of each protein are reported. Numbers represent the amino-acids number in the human protein sequence, although some boundaries might differ between publications (RHD: Rel homology domain; LZ: leucine zipper; TAD: transactivation domain; GRR: glycine-rich region; ANK: ankyrin repeats; DD: death domain).

These structurally-related proteins are able to form homo- or het-

erodimers. Different combinations of Rel/NF-κB are possible, which confers a specificity in transcriptional responses [64]. As each Rel/NF-κB complex exhibits unique biological activities, each dimeric combination affects cell fate and function in various ways [62]. The classic NF-κB dimer is an heterodimer composed of p50 and p65 [65] and often named the "canonical NF-κB". Other dimers such as p65/p65, p65/c-Rel, p65/p52, c-Rel/c-Rel, p52/c-Rel, p50/p50, RelB/p50 and RelB/p52 are present in some cell types [55]. These dimers bind with a part of their RHD to a set of 10 base pairs DNA sites, collectively called κB sites found in promoter and enhancers of target genes [61]. Small variations in this sequence confer a preference for selected Rel/NF-κB dimers, increasing the ability of dimers to differentially regulate gene expression [60,61]. Although all members are able to bind to DNA, only p65, RelB and c-Rel possess a transactivation domain (TAD) that allows them to positively regulate the expression of target genes [63]. p100 and p105 (precursors of p50 and p52) possess ankyrin repeats similar to IκB that can bind to NF-kB dimers and sequester them in the cytosol (as described in 2.4.1). p100 and p105 are thus also IκB members. In addition, p100 and p105 contains glycin-rich region (GRR), required for the proteolytic formation of p50 [66] and p52 [67]. Finally, p100 and p105 present homologies to the death domain (DD), known to transduce apoptotic signals (Xiao 2001). After performing deletion experiments, Xiao *et al.* [68] have also suggested another role of DD in the regulation of p100 and p105 processing. The processing of p105 occurs in higher amount than p100, with a relatively small amount of p52 relative to its precursor in most cell types [69]. Because the proteolytic shortening of p100 and p105 occurs around the GRR, p50 and p52 only contain the domain RHD. Moreover, as p50 and p52 lack TAD, if they are not associated in heterodimer with TAD containing Rel/NF-κB protein (p65, RelB or c-Rel), they might repress the transcription of target genes [63]. Indeed, homodimers of p50 are known to repress NF-κB target genes when bound to DNA [62].

IκB proteins IκB proteins are characterised by the presence of multiple ankyrin domains [63], responsible for binding to Rel/NF-κB dimers by their RHD domain, masking the NLS region and sequestering them in the cytosol in the absence of stimulation of the cell [70]. However, the situation is actually not so simple. Indeed, in resting cells, the IκB protein does not hide completely the RHD of some dimers (for example IκBβ and the homodimer p65-p65) and their subcellular localisation varies constantly between cytosol and nucleus [70]. Even the canonical dimer p50-p65 has not an exclusive cytosolic localisation, as IκBα masks only the NLS of p65 while the NLS of p50 stays exposed [71]. However, IκBα also comports a nuclear export sequence so that the nuclear presence of the complex is really short in resting cells [59]. As a result, complexes shuttle between cytosol and nucleus despite an apparent cytosolic location. When the NF-κB signalling cascade is activated, the degradation of IκBα drastically alters this balance between both localisation and favors the nuclear one. In addition to the "multiple ankyrin domain", IκB proteins also share a "signal receiving domain" at the N-terminal part and a proline, glutamate, serine and threonine rich (PEST) sequence [70].

IκBα, IκBβ and IκBϵ are the most known IκB proteins and are involved in canonical NF-κB pathways. The canonical p50-p65 heterodimer is largely bound to IκBa [63].

Precursor proteins p100 and p105 contain ankyrin repeats domain and are thus also classified as IκB proteins. They are able to form dimers with Rel/NF-κB subunit thanks to their RHD domains, but also to bind to these dimers by their ankyrin repeats domain. The site of cleavage is determined by a glycine-rich region which prevents full degradation [72]. In general, p105 generates p50 during mRNA translation by the 26S proteasome although other mechanisms have also been proposed [59, 73]. p100 is more subject to post-translational degradations, activated by alternative pathways. Its association with RelB forms the dimer involved in alternative pathways.

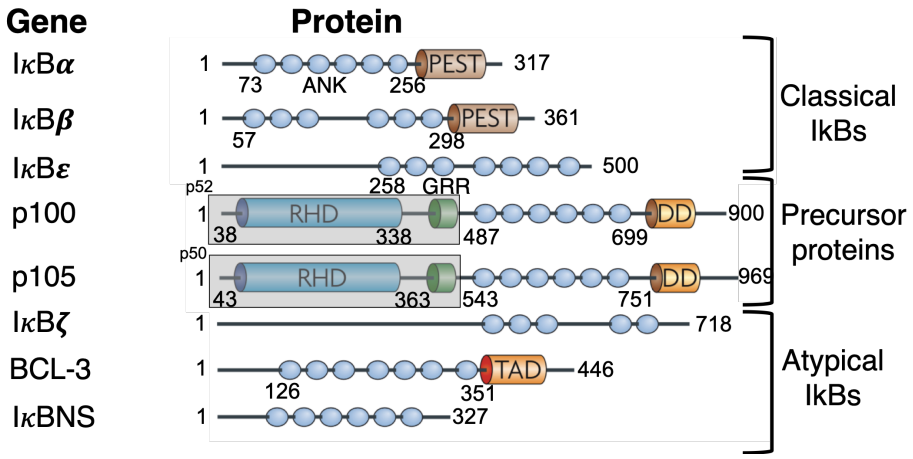

Figure 2.6: **Mammalian members of IκB proteins** (adapted from Gosh *et al.* and Perkins *et al.* [59, 64]). Grey squares represent p52 and p50 after proteolytic processing from respectively p100 and p105. The principal domains of each proteins are represented. Numbers represent the amino-acids number in the human protein sequence, although some boundaries might differ between publications (RHD: Rel homology domain; LZ: leucine zipper; TAD: transactivation domain; GRR: glycine-rich region; ANK: ankyrin repeats; DD: death domain).

Atypical IκBs have also been more recently discovered but are less understood and comports IκBζ (encoded by NFKBIZ), Bcl-3, and IκBNS (encoded by NFKBID) [63, 64, 70].

The stability of NF-κB dimers is affected by IκB proteins, associated with them in resting cells and therefore IκB has an impact on the type of dimers present in a given cell [64]. IκB proteins have specificity to different Rel/NF-κB dimers, classical IκBs tending to prefer dimers containing p65 or c-Rel whereas atypical IκBs have a preference for p50 or p52 homodimers [70]. In addition to the inhibitory role of classical IκB on NF-κB, atypical IκBs stabilise nuclear and DNA-bound dimers, positively regulating the transcription of target genes [64].

IκBζ, BCL-3 and IκBNS are atypical IκBs proteins. Their expression is upregulated following NF-κB activation and thus these proteins exerts their effect at the end of NF-κB response. Typically, they bind

to Rel/NF-κB dimers once they have already translocated in nuclei and interact with DNA. For example, their binding to the repressive p50 homodimers removes the inhibition of target gene transcription [63]. As a result, atypical IκB can modulate this interaction with DNA and positively or negatively regulate the expression of target genes [63, 64, 74]. Because of atypical IκBs role, IκBs proteins are no longer considered as NF-κB inhibitors but more as NF-κB cofactors, able to modulate NF-κB transcriptional responses [63].

IκB kinases proteins Canonical NF-κB pathways are regulated by IKK complex, composed of two IκB serine-specific kinases (IKKα and IKKβ), associated as homo-or heterodimer together with a regulatory subunit called NEMO (for NF-κB essential modulator or IKKg) [70]. This subunit is an important adaptor between IκB subunits for regulation. It appears that IKKα and IKKβ preferentially form heterodimers *in vivo* [63]. The activation of IKKs occurs by phosphorylation following transduction of signals coming from membrane receptors. IKK are the central meeting point of extracellular signals activating NF-κB [63].

In most canonical pathways, IKKβ is sufficient for IκBa phosphorylation, however, IKKα plays other regulatory roles. It contains a nuclear-localisation sequence and, as a serine kinase, it is thus able to phosphorylate nuclear substrates such as NF-κB itself or co-activators and co-repressors that NF-κB will find in nuclei [64, 75]. Moreover, IKKα could also regulate the surrounding chromatin structure of NF-κB target genes by the phosphorylation of some parts of histones [64, 75]. In alternative pathways, the processing of p100 and p105 depends for its part on IKKα.

Adaptors: TRAF and RIP proteins Signals from a huge variety of receptors have to converge towards one of the NF-κB pathways. For this purpose, all these pathways share components, *i.e.* adaptor proteins that play the role of signal nodes between receptors and the different NF-κB pathways. TNF receptor associated factor (TRAF) and Receptor-

interacting serine/threonine protein kinase (RIP) proteins are the major families of adaptor proteins that play similar roles leading to IKK activation. The combination of these adaptors shapes the NF-κB response to correspond to the stimulus and the cell type. In general, TRAFs proteins are involved in almost all NF-κB pathways (except DNA damage response pathways) while RIP proteins are required only for canonical pathways [75].

Seven members compose the family of TRAFs proteins. They all share a C-terminal domain TRAF, which mediates interprotein interactions, in particular binding to receptors and signalling mediators. Their interactions with other adaptors (such as MyD88) modulate and shape the appropriate cellular response to the stimulus. Six TRAFs (all but TRAF1) have a RING finger domain presenting an E3-ubiquitin-ligase activity. As a result, they catalyse the ubiquitylation of target proteins involved in e.g. the activation of IKK complex for canonical pathways. TRAFs could also inhibit NF-κB pathways. For example, TRAF3 has been shown to negatively affect NIK, the protein that triggers alternative pathways. TRAFs could thus affect positively or negatively NF-κB activation, directing the response towards the stimulus. Because TRAFs are not exclusive to NF-κB, they are major branch points with other pathways, such as AP-1. Several receptors use more than one TRAF, and TRAF proteins can function downstream of various receptors [63, 74, 75].

RIP family is composed of seven members sharing a conserved serine/threonine kinase domain. RIP proteins act either upstream or in common with TRAFs depending on the stimulus. They interact with NEMO and contribute to the activation of IKK complex through direct oligomerisation or ubiquitylation mechanisms. Some of them (such as RIP1) seem to be required for activation of canonical pathways by different receptors while others (such as RIP3) may repress NF-κB [63, 75].

Receptors NF-κB is activated in response to various physiological and physical stresses such as infection, ischemia, irradiation or oxidative stress. Chemical agents interfering with cell function, such as cy-

cloheximide, an inhibitor of protein synthesis or monensin that disrupts endoplasmic reticulum [56] can also activate NF-κB. The receptors for these various stresses are differentially expressed in tissues, leading to different cellular responses to the same stimulus. NF-κB receptors can be either membranous or cytosolic [76]. Among receptors, TLRs and C-type lectin receptors are involved in pathogen recognition [76]. TLRs are able to recognise various microbial molecules such as peptidoglycans, LPS, lipopeptides, or flagellin. Some of them are endosomal and recognise microbial nucleic acids. These receptors can form heterodimers together to further expand the variety of recognised molecules [76]. Some TLRs recognise bacterial and fungal structures (TLR-2, TLR-4, TLR-5, TLR-9) while other are in charge of viral products (TLR-3, TLR-7 and TLR-8) [14]. In enterocytes, TLRs are polarised and mediate different patterns of protein synthesis. Only a few of them are expressed in the apical enterocytic membrane [41]. For example, the activation of TLR-9 at the apical pole of enterocytes increases the accumulation of IκBa, suppressing NF-κB activation. On the opposite, TLR-9 stimulation at the basolateral pole results in IκBa degradation and NF-κB activation. This differential expression limits the inflammatory effect of gut commensal bacteria. Some TLRs such as TLR-4 recognising LPS are found only inside cells or at the basolateral side, and as a result are activated only if bacterial components have penetrated through the intestinal barrier [14]. Interleukin-1 receptors are related to TLRs as they share a common cytoplasmic domain, but are specialised in the recognition of endogeneous interleukin-1. Tumor necrosis factor and interleukin-1 receptors are well recognised membranous receptors triggering canonical pathways after interaction of inflammatory cytokines produced by NF-κB activated cells [75]. In the cytoplasm, NOD receptors and RIG recognise microbial components such as peptidoglycan or muramyl dipeptides found in bacterial cell walls [76].

Types of NF-κB pathways

The stimulation of NF-κB is induced after activation of different membranous or intracellular receptors. The signal is then transduced by cells to adequately respond to the stimulus. A variety of pathways have been recognised with different receptors, adaptors and actors involved in the pathway. However, they are classically regrouped in two categories. Canonical pathways (sometimes termed "classical") and alternative pathways are the two different types of NF-κB signalling pathways generally reported in literature, even if some reviews mention the atypical way as a third type of NF-κB independent of IKK. In the latter, stimuli such as hypoxia, hydrogen peroxide or UV light provokes the phosphorylation of IκBa without IKK participation [59].

NF-κB pathways were previously classified based on the membrane receptor that is activated by the stimulus. Bacterial products (such as LPS) and proinflammatory cytokines usually lead to the activation of canonical pathways while alternative pathways are activated by only a subset of TNF superfamily receptors (but not TNF-α) [63, 77]. After stimulus, both pathways lead to the release of Rel/NF-κB proteins that translocate in nuclei to regulate target genes expression. Hayden *et al.* [63] suggested that it would be more appropriate to categorise NF-κB pathways according to which IKK protein is involved. Indeed, canonical pathways depend on IKKβ and NEMO (even if IKKα could play its role in nucleus) while alternative pathways only require IKKα [63,77]. In general, canonical pathways are involved in rapid and reversible inflammatory response while alternative pathways mediate slower and irreversible developmental responses [78].

Canonical NF-κB pathways are induced by almost all NF-κB stimuli thanks to different families of receptors, all converging to the activation of IKKβ and NEMO. In particular, pro-inflammatory signals such as cytokines, pathogen-associated molecular patterns or some danger-associated molecular patterns tend to activate canonical pathways [78]. Adaptors proteins, transducing the signal between receptors and IKK

complex ubiquitinate the lysine 63 of NEMO, which do not lead to its degradation but rather facilitates interactions with some protein domains. This event recruits kinases to phosphorylate IKKβ. Once activated, IKK complex phosphorylates IκB at their death domain, resulting in polyubiquitination followed by proteasomal degradation of IκB. Rel/NfkB dimers (mostly heterodimers containing p65) are then released and translocate in nuclei where they could modulate the expression of target genes (figure 2.7) [70, 75].

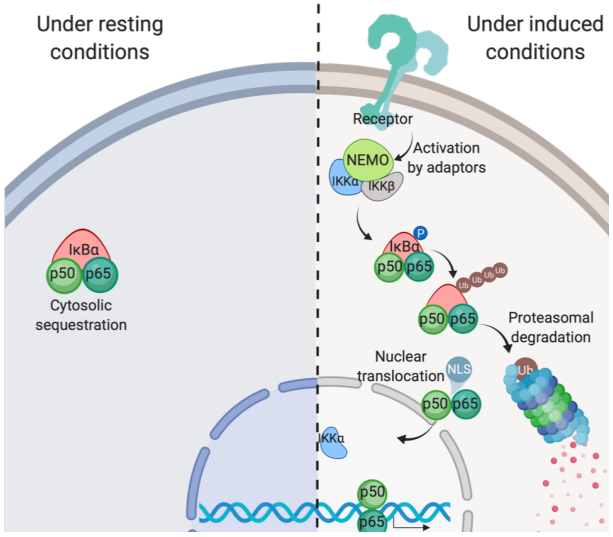

Figure 2.7: **NF-κB canonical pathways** with the most encountered actors reported in this figure. The appropriate stimulus activates IKK complex leading to the phosphorylation and subsequent proteasomal degradation of IκBα. This event releases the NLS of heterodimer p50-p65 that then translocates in nucleus to affect target genes expression. The figure was designed with Biorender.

Genotoxic stimuli (such as ionising radiations or some chemotherapeutic drugs) activate canonical NF-κB pathways by a different way. NEMO translocates in nucleus, is sumoylated, phosphorylated and finally mono-ubiquitinylated by ATM kinase, forming a complex with this kinase. The complex is exported from nucleus and activates by phospho-

rylation cytosolic IKK complex [59].

Alternative pathways are activated by a few subset of TNF receptor superfamily (but not by the receptor to TNFα) [59, 75] involved in secondary lymphoid organ development, B cell control and osteoclastogenesis [72]. Some viruses such as the Epstein-Barr virus are also able to trigger this signalling cascade [59]. Alternative pathways involve the Rel/NF-κB dimer composed of p100 and RelB. Both are linked by their RHD domain, but p100 hides the nuclear export sequence of RelB with its ankyrin repeats domain. Inducing stimuli (such as lymphotoxin-B, CD40 or B cell activating factor) results in the activation of NF-κB inducing kinase (NIK) which activates IKKα [77]. In turn, IKKα phosphorylates the p100 subunit, leading to its proteasomal processing (rather than the degradation observed in canonical pathway) to form p52. The resulting p52-RelB heterodimer is released, and translocates in nucleus to modulate gene expression.

These pathways have the characteristic to be independent of NEMO and IKKβ, but all described alternative inducers have also the property to co-induce the NF-κB canonical pathway [72]. Alternative pathways are generally slower than canonical ones generating two waves of cellular responses to stimuli according to cell type, the first one due to canonical pathways while the other is mediated by alternative pathways [72]. Alternative pathways lead to an increased expression of genes involved in the development and maintenance of lymphoid organs [79]. Other Rel/NF-κB proteins have been found in association with p100 such as p100/p65 and p100/c-Rel but they are associated, after shortening of p100, with IκB inhibitors and require activation of IKK complex. As a result, they could be seen as a crosspoint between alternative and classical pathways. They are produced of alternative pathways (to cleave p100) but are activated by the classical pathway, as IKKβ and NEMO are required.

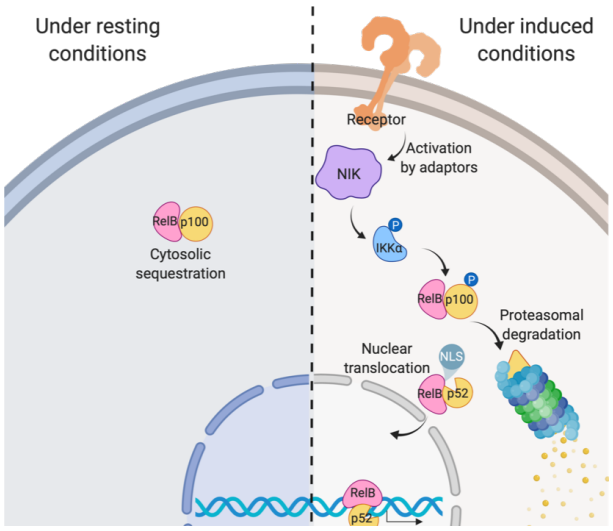

Figure 2.8: **NF-κB alternative pathways** with the most encountered actors reported in this figure. The appropriate stimulus activates NIK leading to the processing of p100 in p52. The resultant heterodimer p52-RelB then translocates in nucleus to affect the expression of target genes. The figure was designed with Biorender.

Regulation and feedback loop

NF-κB response should be transient to avoid a permanent inflammatory state and stressful responses. The expression of some of the actors described in 2.4.1 is under NF-κB control, such as IκBα, p105 and p100 [56]. Indeed, it is accepted that the synthesis of IκB proteins in response to NF-κB activation is the key event that allows the ending of NF-κB activation [64, 73]. IκB recently translated can enter the nucleus to separate Rel/NF-κB dimers from their DNA binding site [56]. They could also affect binding of cofactors to Rel/NF-κB dimers. Ubiquitination and proteasomal degradation have also been described for nuclear p65-heterodimers, probably mediated by p65 phosphorylation by IKKα [64]. In addition, Zambrano *et al.* [80] have described an oscillatory response of NF-κB-driven gene expression and demonstrated that this phenomenon was due to mRNA degradation.

Physiological effects of NF-κB

Since its discovery, DNA binding sites have been described in numerous gene promoters and enhancers [60]. Besides NF-κB members themselves (as described in 2.4.1), a majority of target genes are involved in the host immune response, such as cytokines, chemokines, MHC, receptors involved in immune recognition [56], which explains why NF-κB has primarily been studied for its function in the regulation of immune response. However, it fulfils a diversity of other biological roles such as apoptosis, cancer, senescence or tissue development [55, 81].

All target genes are not expressed after all NF-κB-activating stimuli, and cells have to shape an appropriate response regarding its role in organism. The diversity of responses is allowed by a remarkable diversity of the actors presented in 2.4.1. This diversity takes place at different levels.

First, the DNA binding site responds to a consensus sequence allowing a certain variability with different binding preferences for each Rel/NF-κB dimer. Moreover, dimers can be composed of different subunits, each with its own characteristics and interaction with the other pathway actors. Each IκBs or IKK also presents different characteristics and interact by a different way with the other actors, following canonical or alternative pathways. Receptors shape the adequate response by recruiting different kinds of adaptors. Interactions with other pathways could also occur at different levels but in particular with TRAF proteins. In addition, cofactors also modulate the response. Finally, all these actors are differentially expressed according to the tissue, to allow a specific response in accordance with the role of the cell in the organism, whether it is a macrophage with its preventive role for infections or an epithelial cell in contact with endogenous microbiota. The same stimulus will not activate each cell type to the same way, because some cells either lack the receptor or the different actors described in 2.4.1. All these parameters allow a fine tuning of the cellular responses to the initial stimulus [56, 62–64, 75].

Role of NF-κB in the gut

NF-κB pathway appears to play a central role in intestinal inflammation, as it mediates the response to pathogenic bacteria by recruitment of inflammatory cells. However, as described in 2.3.3, IECs present adaptations to avoid an excessive inflammatory response towards commensal bacteria such as a restricted presence of NF-κB receptors to bacteria. NF-κB is also involved in chronic inflammation and has a role in the pathogenesis of inflammatory bowel diseases (IBDs) *i.e.* Crohn's disease and ulcerative colitis. IBDs are indeed associated with a deregulation of this NF-κB pathway as the expression of many cytokines, enzymes and adhesion molecules involved in IBDs are modulated by this pathway [65,82,83]. As expected, NF-κB is overstimulated in the gut mucosa of IBDs patients, with for instance an overexpression of p65 [84, 85]. As a result, NF-κB is a common target for treatment of IBDs such as glucocorticoids [83], salicylates or proteasome inhibitors [65]. More specific treatments have also been developed like antisense oligonucleotide for p65 that alleviate IBDs effects. However, inhibition of IKK/NF-κB *in vivo* also leads to severe inflammation in mice. According to Pasparakis *et al.*, this could be due to the role of NF-κB in the control of the maintenance of the intestinal epithelial barrier, as NEMO deficient cells are more susceptible to apoptosis, disrupting the cell monolayer [85]. In addition to IBDs, NF-κB cascade seems also involved in the promotion of colorectal cancer [84].

2.4.2 Nrf2 pathway

Discovered in 1994 by Moi *et al.* [86], nuclear factor-erythroid 2-related factor (Nrf2) pathway plays an important role in preventing cellular stress, coordinating the cellular response to oxidative stress [87]. It involves the Nrf2 transcription factor, a member of the Cap'n'collar family of basic leucine zipper [88]. Contrary to other pathways such as NF-κB, Nrf2 recognises a chemical reactivity rather than a structure in inducing molecules [89].

Actors

The two major proteins involved in this pathway are Nrf2 and Keap1, reported in figure 2.9.

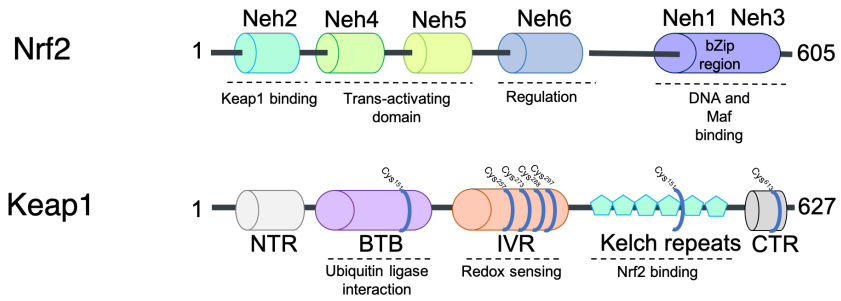

Figure 2.9: **Nrf2 and Keap1 proteins structure with functional domains**. Numbers represent the amino-acids number in the human protein sequence. Adapted from Magesh *et al.* [90].

Nrf2 protein is expressed in detoxifying organs such as liver or kidney but also in skin, lungs and the GIT [91]. This transcription factor binds to antioxidant response elements in the promoter of target genes to regulate their expression. Six functional domains can be found in Nrf2 termed Neh 1-6 and a basic leucine zipper (bZIP) region. This bZIP region is responsible for DNA binding to antioxidant response element (ARE) and heterodimerisation with musculoaponeurotic fibrosarcoma (Maf) proteins. Neh2 allows the binding to Keap1 protein and contains a NLS. Neh3, Neh4 and Neh5 are involved in the transcriptional activation. Neh6 has a role in the degradation of Nrf2 by ubiquitin ligase E3 in nucleus to return to resting state after oxidative stress [90].

Keap1 is the negative regulator of Nrf2. It contains 5 domains: the N-terminal region (NTR), the Broad complex, Tramtrack and Bric-a-Brac (BTB) domain, the intervening region (IVR), the Kelch domain and the C-terminal region (CTR). The BTB domain interacts with ubiquitin E3 ligase leading to Nrf2 degradation when Keap1 is bound. IVR senses oxidative and electrophilic stress through the multiple cystein residues

it contains. In the protein, 7 cysteins have been identified as playing a role in redox sensing and are reported in figure 2.9. These residues are highly reactive with ROS and electrophiles and subject to oxidation. The Kelch domain contains 6 Kelch repeats binding to Nrf2 by its Neh2 domain [90].

Nrf2 pathway activation

Under resting state, Nrf2 is sequestered by Keap1 homodimer through the binding of Neh2 with Kelch repeats domains. Keap1 induces the proteasomal degradation of Nrf2 by ubiquitin ligase recruitment thanks to BTB domain [87, 89, 90]. This negative regulation by Keap1 allows a minimal expression level of Nrf2 target genes [87]. Nrf2 pathway is activated by modification of thiol groups of cystein residues. It could occur by oxidation, but also by electrophiles molecules able to bind to cystein residues [90]. Cysteins modifications lead to the dissociation of Nrf2 and Keap1, releasing the NLS of Nrf2 that translocates in nucleus [90, 92]. Nuclear Nrf2 heterodimerises with Maf proteins and binds to ARE sequences. Heterodimerisation of Nrf2 with other bZIP transcription factors is required for transcriptional activation of target genes [93].

Physiological effects of Nrf2

In the nucleus, Nrf2 binds to ARE sequence found in the promoter of Nrf2 target genes. The main function of Nrf2 is to alleviate cellular damages due to oxidative stress [88]. The majority of target genes encodes thus for enzymes involved in redox balance and the elimination of reactive oxygen species (ROS), such as catalase [94], ferritin [95], superoxide dismutase [96] or glutathione peroxidases [97]. Phase-2 enzymes are also induced by Nrf2 to protect cells against metabolites generated by oxidative stress. They inactivate these toxic metabolites either by conjugation such as gluthation-S-transferases [94], or by reduction like NAD(P)H quinone oxidoreductase [87]. The expression of some efflux pumps like multi-resistance proteins and multi-drug resistance transporters is also

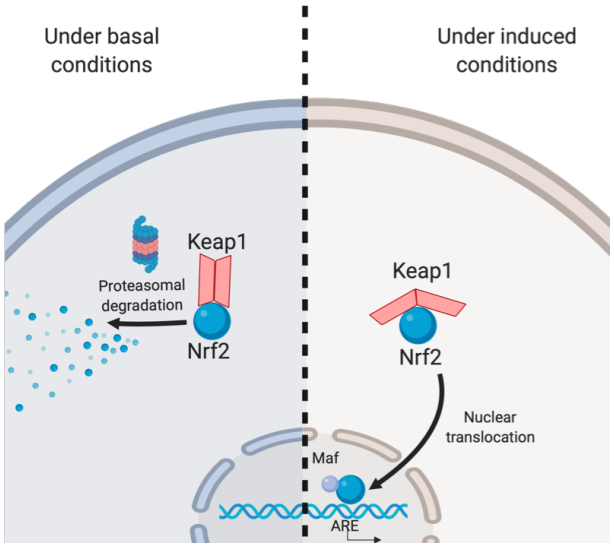

Figure 2.10: **Nrf2 pathway representation**. Under basal conditions, Nrf2 is sequestered by Keap1 in the cytosol, leading to its degradation by the proteasome. Oxidative stress affects Keap1 conformation, which then releases Nrf2 for translocation in nucleus and induction of ARE-driven genes. The figure was designed with Biorender.

increased by Nrf2, suggesting a role in detoxification of xenobiotics.

Role of Nrf2 in the gut

Redox homeostasis is essential in the gut as various processes such as development, innate immunity, or wound healing are redox sensitive [98]. Indeed, in the gut, developmental pathways like Notch and Wnt, controlling essential processes such as stem cell renewal, cell proliferation, migration and differentiation, are regulated by NADPH oxidase, involved in the synthesis of ROS. A tight regulation is necessary, as too much or too little ROS may inhibit proliferation [98] or induce tissue destruction, exacerbating the pathogenesis of some IBDs [99]. In addition, innate immunity response also involves ROS produced by infiltrated immune cells such as macrophages in the case of acute gut inflammation [100]. As a major regulator of cell antioxidant response [87, 88], Nrf2 plays a role

in these processes in the gut and needs to be tightly regulated. Consequently, defects or aberrant activation of Nrf2 have been correlated with some human cancers or exacerbation of hepatic steatosis [99].

2.5 Inflammatory properties of AgNPs

The use AgNPs in the treatment of burn and wound healing has been recognised for decades, explaining their development in nanomedical applications such as sutures, wound dressings and medical instruments. As discussed in *Chapter 1*, this could be attributed to their antimicrobial properties. However, their capacity to improve healing and decrease inflammation is also discussed in literature.

The majority of *in vivo* studies seem to underline anti-inflammatory properties of AgNPs on pre-inflamed models such as allergic rhinitis [101], burn wound models [102] or allergic dermatitis [103, 104]. In humans, beneficial effects of AgNPs were observed on *Psoriasis* patients. Regarding the gut, Bhol *et al.* have observed a decrease of the secretion of inflammatory cytokines in rats subject to chemically-induced colitis, which had been already observed after ingestion of a dosis of 0.4mg AgNPs/kg/day during 5 days [105].

However, *in vitro* studies seem less unanimous concerning the inflammatory properties. Some studies have suggested anti-inflammatory properties of AgNPs, based on the inhibition of cytokine secretion upon activation by external stressors in Hacat cells [106], peripheral blood mononuclear cells [107, 108], primary nasal epithelial cells [109] or macrophages [110,111]. On the opposite, other authors have observed an upregulation of inflammatory cytokines secretion in macrophages [112, 113], umbilical vein endothelial cells [114] or neural cells [115]. In addition, Shi *et al.* have shown a phosphorylation of IKK and IκBα, and a nuclear localization of p65, suggesting an activation of NF-κB pathway [114]. Similar observations were made in murine macrophages by Nishanth *et al.* and Paul *et al.* [116, 117] and in Jurkat T cells by Eom and Choi [118]. However, Bastos *et al.* and Skladanowski *et al.*

did not observe any NF-κB activation [119, 120]. Concerning IECs, different studies have been performed either in Lovo cells (a colonic cell line) [121] or models based on Caco-2 cells. These studies have observed an increased expression or secretion of the pro-inflammatory chemokine IL-8 in both Lovo cells [121] and Caco-2 cells [122–124].

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Part 2

Objectives

Thesis aims & General strategy

Antimicrobial properties of silver as well as the advances of nanotechnology have led to the development of silver nanoparticles (AgNPs) that can be found, among many others, in more and more food-related products and applications. Nevertheless, the migration of silver from coatings and packaging has been reported in literature. In addition, AgNPs are also present in some unauthorised food supplements, claimed to treat infections or boost immune defences. All these applications should result in the ingestion of AgNPs by most consumers. Even if exposure assessments are complicated by analytical limitations, the increasing use of AgNPs will undoubtedly raise the exposure of humans to them. However, numerous studies have observed a toxicity of AgNPs in different *in vitro* cellular models. Furthermore, regarding inflammation, studies are not unambiguous. The majority of *in vivo* studies suggested anti-inflammatory properties of silver, presenting beneficial properties for wound and burn treatments. Nevertheless, *in vitro* studies are less unanimous, as described in *Chapter 2*.

Among existing literature, only few studies have focused on the gut, despite the fact that this organ presents a major role in immunity and is exposed to the highest concentrations of ingested components, compared to internal tissues. Due to their specialisation for nutrients absorption, they could also reach higher concentrations in their cytosol than other cells.

In this context, we postulated that AgNPs could affect inflammatory response

of enterocytes. For this purpose, differentiated Caco-2 cells, widely accepted as an *in vitro* model of the intestinal barrier, were used for their similarity with small intestine enterocytes.

As a preliminary work, the cytotoxicity of AgNPs had to be estimated, in order to define the concentration range to investigate inflammatory processes. However, such as many nanoparticles, AgNPs are prone to interact with assay components or analytes, perturbing the cytotoxicity assessment. Cytotoxicity assays should thus be carefully selected to avoid or minimise the risk of bias due to interferences with nanoparticles.

As the general purpose of this thesis was to investigate the effect of AgNPs on the intestinal barrier, we addressed this question by specific objectives throughout the thesis.

- The first objective aimed at evaluating the effect of AgNPs on the NF-κB pathway, as it is commonly involved in the induction of inflammatory cellular responses against various stresses. The ability of AgNPs to affect this signalling cascade was estimated in absence or presence of NF-κB-inducing stimuli, composed of a cocktail of cytokines and lipopolysaccharide previously developed in our laboratory [1]. The activation of NF-κB was evaluated at different levels.

- The second objective focused on the ability of AgNPs to induce interleukin-8 (IL-8) secretion, as this chemokine is the major pro-inflammatory cytokine secreted by enterocytes. In addition, the possibility of Nrf2 involvement in this secretion was estimated, as this pathway is responsible for cellular responses against oxidative stress inducers.

- The third objective concerned the involvement of ionic silver in the observed effects of AgNPs. These ions are present in suspensions of AgNPs, subsequently to oxidation of metallic silver constituting the nanoparticles. This objective was treated transversally through the three results chapters to eval-

uate if the reported results with AgNPs could be, at least partly, mediated by the nanoparticles *per se*.

The first and second objectives are addressed in *Chapters 4* and *5*, respectively, while the preliminary work about cytotoxicity is presented in *Chapter 3*. The third objective concerning the involvement of ionic silver will be addressed transversally in the three results chapters.

Part 3

Results

III

Challenges in cytotoxicity assessment of silver nanoparticles in Caco-2 cells

Foreword

The assessment of nanomaterials cytotoxicity is a prerequisite to nanotoxicology studies to define concentration ranges to use during *in vitro* assays. In addition, it could provide keys about the mode of action behind the toxicity of AgNPs. However, such as other metallic particles, silver nanoparticles (AgNPs) present two main challenges in the assessment of their cytotoxicity: (i) a possible interference between nanoparticles and cytotoxicity assay components, biasing the assessment of their toxicity and (ii) the involvement of silver ions, present at various amounts in AgNPs suspensions due to oxidation of metallic silver. This chapter addresses both questions by assessing AgNPs toxicity in our cell cultures. In addition, this cytotoxicity assessment was used to define a subcytotoxic concentration range to be used for the experiments of *Chapter 4* and *Chapter 5*.

Challenges in cytotoxicity assessment of silver nanoparticles in Caco-2 cells

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Highlights

- Silver nanoparticles interfere with all tested cytotoxicity assays, to a various extent.
- Neutral red assay, resazurin assay and ATP content measurement seem more appropriate to assess the toxicity of silver nanoparticles, as they minimise the risk of bias.
- The dissolved fraction of silver nanoparticles suspensions presents a lower toxicity than pristine suspensions, suggesting a nanoparticle specific toxicity.
- The assay of the cellular ATP content is more affected at lower concentrations than metabolic activity and lysosomal integrity in differentiated Caco-2 cells.

Abstract

Owing their antimicrobial properties, silver nanoparticles are increasingly found in the agri-food sector, in applications such as coatings, sprays or even unauthorised food supplements. Their presence in the gastro-intestinal tract following their ingestion can lead to a health impact, but nevertheless, some gaps remain concerning their toxicity. In this context, *in vitro* cellular models could help to address these questions by assessing different toxicity endpoints such as ATP production, metabolic activity, lysosomal integrity or necrosis.

However, because of their high reactivity, silver nanoparticles present two main challenges in their toxicity assessment: (i) the risk of interference with cytotoxicity assays, which could lead to bias and (ii) the presence of silver ions, formed by their oxidation, whose amount depends on many parameters such as nanoparticles properties, environing medium or storage conditions.

In this study, three cytotoxicity assays *e.g.* the neutral red, resazurin assay and ATP measurement were chosen as they minimise the risk of bias due to interference. The toxicity of silver nanoparticles suspensions on differentiated Caco-2 cells was compared with the soluble silver fraction of these suspensions, separated by ultrafiltration on an appropriate membrane. The concentration of AgNPs leading to a decrease of 50% of the ATP content (the "effective concentration 50'"), compared to control cells, was estimated at 38.3μg/mL, while the metabolic activity and the lysosomal integrity achieve 50% of control cells at higher concentrations, respectively 96.4 and 98.5 μg/mL. Silver nanoparticles suspensions present a higher toxicity than the soluble silver it contained, suggesting the involvement of particles *per se*.

This study confirms the need of appropriate controls for cytotoxicity assessment, on one hand, to choose assays with minimal interference and, on the other hand, to investigate the involvement of dissolved silver in the silver nanoparticles toxicity.

List of abbreviations

Ag^+ silver ions

AgNPs silver nanoparticles

DMEM Dulbecco modified Eagle's medium

EC50 effective concentration 50

HBSS Hank's balanced salt solution

LDH lactate dehydrogenase

NBT nitroblue tetrazolium

NMs nanomaterials

NR neutral red

ROS reactive oxygen species

TX Triton-X100

3.1 Introduction

Silver nanoparticles (AgNPs) are increasingly incorporated in consumer goods, mainly for antimicrobial purposes [1]. In particular, their use in the agri-food sector has gained popularity throughout all food production phases, making silver the most frequently nanomaterial found in biocidal and food packaging material [2]. Although they are not intentionally incorporated as additive in human food [3,4], the presence of AgNPs was evidenced as a subpopulation in silver pearls used for pastry decorations and containing the food colouring agent E174 [5]. Moreover, migration from packages to food could occur and the presence of silver has indeed been observed in boneless chicken breasts [6] or orange juice [7] stored in an AgNPs-embedded packaging material. This migration process seems more efficient at acidic pH [8–11]. A majority of the silver is found in

the form of oxidised silver. However, the migration of pristine AgNPs has also been evidenced [8, 10, 12–14], suggesting that the oxidation of silver is not the only reason of its migration from packages. von Goetz *et al.* (2013) [10] have measured a migration of 4.2 μg/mL of silver for a new box, in the worst case scenario. Even if this amount is relatively low, their widespread applications could increase the consumer exposure to AgNPs. In addition, AgNPs are found as aqueous dispersions of colloidal silver, claimed for having benefits on the immune system [3, 15–17]. Consumption of these compounds could lead to up to 0.02 mg/kg body weight x day of exposure [18]. Finally, incorporation of AgNPs in dental materials, such as implants, endodontic materials, resins or orthodontic adhesives has been reported in literature and could lead to their unintentional ingestion [19, 20].

Although numerous studies have been published about AgNPs toxicity, challenges remain in their toxicity assessment. Such as some other metallic nanoparticles, because of their high surface area, AgNPs have the ability to generate silver ions (Ag^+) as a subproduct of their oxidation by dissolved oxygen [21, 22]. Indeed, some AgNPs suspensions could contain these ions in proportions up to 90% of total silver content, depending on different nanoparticles parameters, such as their size, nature of stabiliser, concentration, synthesis method or storage conditions [23–27]. These Ag^+ form the "soluble silver fraction" present in aqueous AgNPs suspensions [28]. Due to the low solubility of silver salts, these Ag^+ could present other forms in medium culture, rather chloride precipitates than phosphate in physiological conditions [22]. Even if the cytotoxicity of AgNPs has been largely studied in literature, the involvement of soluble silver in this toxicity is rarely investigated and should be taken into account [23, 29]. Different strategies have been developed to address this problem of silver dissolution. Generally, studies compared the effect of AgNPs with a soluble silver salt, *e.g.* silver nitrate or silver acetate as a source of Ag^+ [30–34]. Zhao *et al.* used cysteine, which is able to complex soluble silver, therefore inactivating its action [35]. Only some studies have separated AgNPs from soluble silver fraction, although

Beer *et al.* recommended it when studying metallic nanoparticles like copper or silver [23]. Since the dissolution of nanomaterials (NMs) could affect both toxicodynamic and toxicokinetic parameters, this item should be addressed in nanotoxicology studies [36]. The separation of soluble silver from the rest can be achieved by ultracentrifugation [23, 37], ion exchange resin [25], extraction with the detergent Triton X-114 [38, 39] or filtration through a membrane with the appropriate porosity [35, 40]. The latter option was chosen for this study.

In vitro assays have been developed as an attracting alternative to *in vivo* models, more suitable for mechanistic studies, being less expensive and easier to perform. Furthermore, they are in agreement with the ethical need to reduce animal testing and to protect the animal welfare [41–43]. The *in vitro* assessment of NMs adverse effects involves the application of cytotoxicity assays. Cytotoxicity can be defined as "*the adverse effects deriving from reactions with structures and/or processes crucial for cell maintenance such as proliferation, survival, and normal biochemical/physiology*" [44]. The number of developed cytotoxicity assays is important. Most of the time, they are based on the measurement of an endpoint associated with the viability or functionality of cells. Available commercial kits are based on subsequent biochemical reactions, leading to absorbance, fluorescence or luminescence changes, measuring different endpoints [44, 45].

The measurement of necrosis largely focuses on the assessment of membrane integrity. Assessment of lactate dehydrogenase (LDH) activity in the extracellular medium is widely used in membrane leakage assays [29]. LDH is a cytosolic enzyme, released in extracellular medium when cellular membrane integrity is altered by direct necrosis or post-apoptotic necrosis [29, 46]. Reduced viability leads to a leakage of LDH in extracellular medium. The amount of LDH is generally expressed in comparison to the total releasable LDH activity upon treatment of cells with a detergent like *e.g.* Triton-X100 (TX).

Among cytotoxicity tests, the neutral red (NR) assay is one of the most used [47]. It is based on the NR dye that enters in cells by dif-

fusion and concentrates in acidic vesicles and, in particular, lysosomes. In these compartments, it gets protonated and be concentrated inside these vesicles since the diffusion of the charged form of the molecule through cellular membranes is considerably slowed down. Cell death or reduction of lysosomal pH gradient decrease the amount of dye in cells. After washing, the dye is extracted with an acidic ethanol solution. As a result, the absorbance of the obtained solution reflects the cell viability but also lysosomal integrity [47, 48].

The other major common measured endpoint is the assessment of metabolic activity [29]. This endpoint is representative of both the number of cells and the functional viability of these cells [42]. For this purpose, tetrazolium salts are considered as the golden standards [46]. These dyes are reduced in colourful formazan salts by viable and metabolically active cells. MTT is the most known, even if other tetrazolium salts have been developed *e.g.* MTS, XTT, WST-1 [49]. It is generally assumed that the tetrazolium reduction occurs due to numerous enzymes, mainly cytoplasmic, *e.g.* oxidoreductases, dehydrogenases, oxidases and peroxidases [49]. More recently, alternative assays have been applied to nanotoxicology, such as the resazurin. Similarly to tetrazolium salts, this dye is reduced into the fluorescent resorufin, measuring the cellular redox potential. However, biological mechanisms behind this reduction remain unclear [46]. In addition to these techniques, the measurement of ATP amount in cells seems a relatively sensitive way to estimate cell viability [42].

Even if a lot of cytotoxicity assays are already available on the market, nanotoxicological studies present challenges in cytotoxicity assessment, due to the high reactivity of NMs, which leads to possible interferences with classical assays [41, 42, 45, 50–53]. Nanoparticle specific properties like absorbance or fluorescence, adsorption of proteins or catalytic activity could interfere with assay components and affect the response.

First, interference could occur due to intrinsic properties of NMs *e.g.* absorbance, fluorescence or formation of trouble precipitates [45]. In addition, because of their high specific surface area, they could adsorb assay

components or analytes or react with assay components, which affect the response [45, 50]. Therefore, different controls have to be performed to choose the appropriate cytotoxicity assays and avoid bias in nanotoxicology studies: (i) the absorbance and fluorescence of NMs suspensions, (ii) the interference between NMs and assay components, which is called "direct" interference and (iii) the "indirect" interference between NMs and the analyte [45]. Although these recommendations are well recognised in literature, Ong *et al.* have shown that 95% of nanotoxicology studies published in 2010 did not report any kind of interference control, which could explain partially the contradictory results observed in literature [41, 45]. To avoid any bias, nanotoxicology authors recommend to use at least two different toxicity assays [42, 50, 53].

characterisation

In this context, this study aims at evaluating the cytotoxicity of AgNPs in an *in vitro* model of the intestinal barrier, composed of differentiated Caco-2 cells. After choosing cytotoxicity assays to avoid any bias due to interferences, the cytotoxicity of AgNPs suspensions was evaluated, assessing the involvement of the soluble silver fraction in these AgNPs suspensions. For this purpose, AgNPs were separated from soluble silver by centrifugal ultrafiltration and ICP-MS measurements and cytotoxicity assays were performed with filtrates, containing only soluble silver.

3.2 Material and methods

3.2.1 Cell culture and exposure

Caco-2 cells from a human colon adenocarcinoma (clone 1 from Dr. M. Rescigno, University of Milano, IT) were cultivated between p+10 and p+30 at 37 °C under a water-saturated atmosphere with 10% (v/v) CO_2. Caco-2 cells were grown in tissue culture flasks (Corning incorporated, Corning, NY) in Dulbecco modified Eagle's medium (DMEM) with 4.5 g/L glucose (Lonza, Basel, CH), supplemented with 10% (v/v) foetal

bovine serum (Biowest, Nuaillé, FR), 1% (v/v) non-essential amino acids 100X (Lonza), 1% (v/v) L-glutamine 200 mM in a 0.85% NaCl solution (Lonza). Caco-2 cells were seeded at a cell density of ca. 63 000 cells/cm^2 on 48-well plates (Corning) precoated with type I collagen (Sigma-Aldrich, St. Louis, MO). Cells were cultivated for 21 days to achieve cell differentiation and incubated during 3h with AgNPs suspensions or soluble Ag diluted five times in Hank's balanced salt solution (HBSS) to obtain the concentration annotated in charts.

3.2.2 Reagents

AgNPs $<$ 20nm (NM-300K, JRC repository, Ispra, IT) were purchased from Fraunhofer institute (Schmallenberg, DE) as a dispersion in water containing 10.16% (w/w) of silver, 4% of polyoxyethylene glycerol trioleate and 4% of polyoxyethylene (20) sorbitan mono-laurate (Tween 20). The characterisation was performed by Klein *et al.* [54]. Stock solution was kept at room temperature and stored under nitrogen atmosphere to avoid oxidation and modify the ionic silver content. AgNPs suspensions were prepared freshly in milliQ water (Millipore, Burlington, MA) and diluted in HBSS (Lonza) directly on cells to obtain the target range of concentrations. Nitric acid (70%, highest purity), used for ICP-MS measurements, was purchased from VWR (Leuven, BE).

3.2.3 Separation of soluble silver from silver nanoparticles suspensions

Soluble silver was separated from AgNPs suspensions at different concentrations in milliQ water as reported in figure 3.1. Suspensions were centrifuged on ultrafiltration devices (Vivaspin turbo 4 10kDa, Sartorius, Goettingen, DE) at 3220 g during 10 min. The absorbance between 350 and 750nm of filtrates was measured by a UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA). No absorbance was observed for filtrates. Initial suspensions of AgNPs are further mentioned as "AgNPs suspensions" while the filtrates are named "soluble Ag".

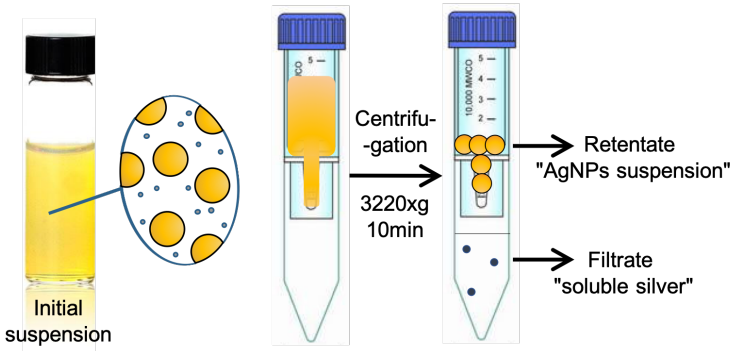


Figure 3.1: Principle of the separation between AgNPs and soluble silver.

3.2.4 ICP-MS

Following the ultrafiltration, filtrates (containing soluble silver) and initial solutions (containing total silver) were acidified with 4% (v/v) nitric acid (VWR). Silver content was then quantified using an ICP-MS (7900 ICP-MS, Agilent, Santa Clara, CA). The calibration curve was performed from 0.010 to 50 μg Ag/L with several dilutions of a multi element standard solution (Chem-lab, Zedelgem, BE, certified ISO/IEC 17025). The analytical method validity was verified every 10 measurements with 3 quality controls at 0.5, 5 and 25 μg Ag/L (Merck, Readington, NJ). The limit of quantification was 0.010 μg Ag/L. Samples were diluted so that silver concentrations were in the calibration range.

3.2.5 Interference and cytotoxicity endpoints assays

Five cytotoxicity assays were pre-selected to assess the cytotoxicity of AgNPs (Figure 3.2).

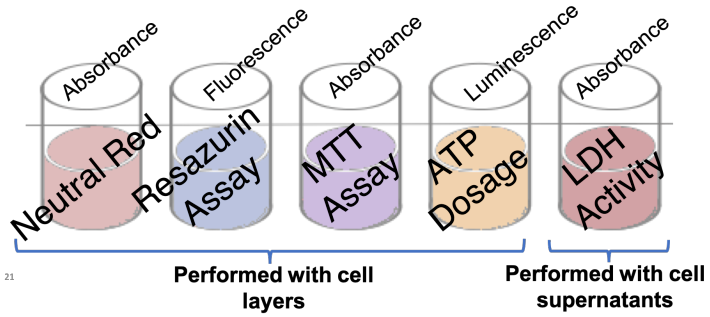


Figure 3.2: Pre-selected cytotoxicity assays for interference estimation with AgNPs, performed either on cell layers or on cell supernatants after incubation with AgNPs, based on absorbance, fluorescence or luminescence measurements.

The LDH leakage assay (Cytotoxicity Detection Kit (LDH), Sigma-Aldrich) and the MTT assay (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich) were not performed to evaluate the cytotoxicity because of a risk of bias due to interference, as it will be described in results. Lysosomal activity was estimated with a NR assay. NR (Sigma-Aldrich) was dissolved in HBSS to reach a concentration of 33 μg/mL and filtered through a filter 0.22 μm (Millex-GP filter unit, 0.22 μm PES, Merck-VWR). Cells were then exposed to 750 μL of NR solution at 37 °C for 3h. After washings with PBS, dye was extracted from cells with 300 μL of extraction solution (50% ethanol, Sigma- Aldrich; 1% acetic acid, Sigma-Aldrich; 49% milli-Q water). The absorbance of 100μL was measured at 540nm.

ATP content in cells was estimated using a luciferase assay (CellTiter-Glo®, Promega, Madison, WI). After equilibration of 15min with 150μL HBSS at room temperature, 150μL of reagent was added to the wells. The luminescence was then recorded with a luminometer (10s of shake, 0.5s integration time, Fluoroskan Ascent FL, Thermo Fisher Scientific). Metabolic activity was estimated by means of a resazurin assay (CellTiter-Blue®, Promega). Cells were incubated with 357μL of reagent during 30min at 37 °C followed by a fluorescence measurement (530nm excita-

tion wavelength and 584nm emission wavelength) in a fluorimeter (Fluoroskan Askent FL, Thermo Fisher Scientific).

Different controls were performed to evaluate a possible interference between AgNPs and cytotoxicity assays: (i) the measurment of the intrinsic absorbance, luminescence and fluorescence of AgNPs suspensions, (ii) a direct interference between AgNPs and assay components and (iii) an indirect interference between AgNPs and the analyte *e.g.* the LDH enzyme or the ATP molecule.

The intrinsic absorbance of AgNPs suspensions was measured at a concentration of 15μg/mL suspension prepared in milli-Q water. The absorbance was measured between 350 and 750nm with a spectrophotometer (Thermo Fisher). The luminescence and fluorescence of AgNPs suspensions, using 530nm excitation and 584nm emission wavelengths, were measured with a fluorimeter-luminometer (Fluoroskan Askent FL).

For interaction between AgNPs and assay components, 100μL of the assay reagent were mixed with AgNPs diluted 40 times and assay reagent diluted 2 times in HBSS. Interference between AgNPs and assay components was estimated by mixing in a 96 well plates (Nunc, Roskilde, DK) 100μL of the assay reagent with 100μL of AgNPs diluted 40 times to obtain the target concentration reported in results. Assay was then performed following manufacturer's instructions. For the assays normally performed on cell layers (NR, MTT, resazurin and ATP measurement), in the absence of cells the extraction of the dye was not possible but the different extraction or lysis solutions were added to the mixture of the assay reagent and AgNPs.

The interaction between AgNPs and the analyte was assessed for the LDH leakage assay and ATP measurement. For LDH assay, a fraction containing fresh LDH was obtained by incubating differentiated Caco-2 cells with TX during 30min. For the ATP measurement, a disodium salt of ATP was used (Promega).

Finally, a supplementary control was performed to evaluate the interaction between ROS and the two metabolic activity assays *e.g.* the MTT

and the resazurin assays. Superoxide was generated by illumination of riboflavin under an intense light at concentrations ranging from 25 to 300μM during 1h in the presence of either MTT reagent at 2mM or the resazurin reagent. For the resazurin assay, the fluorescence of 200μL of this mixture was measured using 530nm excitation and 584nm emission wavelengths.

3.2.6 Statistical analysis

Statistical analyses for cytotoxicity curves were performed using R software (version 3.5.0, [55]). The function *drm* of the package *drc* was used to estimate the four parameters of a log-logistic model for the dose-response curves. Parameters "e" (representing the effective concentration 50) and "b" (representing the sharpness of the curve) were compared with the function *compParm* with type 1 error (α) set at 0.05. Other statistical analyses were performed with JMP Pro14 (SAS Institute, USA). First, normality of the data distribution and homoscedasticity were verified with respectively Shapiro-Wilk test and Levene test to determine which comparison tests should be used. When data had a normal distribution and equal variances, ANOVA-1 was applied followed by a Dunnet's post-hoc analysis. In any other cases, Student's t-test were performed with type I error (α) set at 0.05 and a Bonferroni-Šidák correction of the p-values for multiple comparisons [56]. All data are expressed as mean $\pm$ standard error of the means.

3.3 Results

3.3.1 Interference between cytotoxicity assays and AgNPs

Neither luminescence, nor fluorescence at wavelengths used for the assays were detected for AgNPs suspensions (data not shown). Absorbance spectrum of AgNPs displays an absorption peak at 410nm (Figure 3.3), a wavelength that is not used for the chosen cytotoxicity assays. However, they absorb a low amount of light at 490nm, a wavelength used for LDH and MTT assays, which could impact the response to these tests at high concentrations of AgNPs.

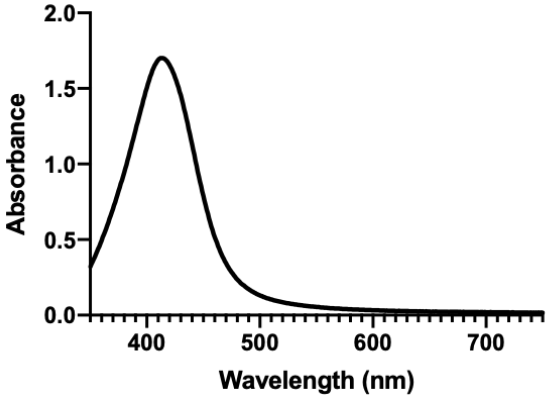


Figure 3.3: Absorbance spectrum of an aqueous AgNPs suspension at a concentration of 15μg/mL. The mean from 3 replicates is represented.

The interaction between AgNPs and assay components was then evaluated for NR, ATP, resazurin, and MTT assays, by mixing AgNPs with the reagents used for the different assays. An important interference was observed for the MTT assay in the presence of AgNPs (Figure 3.4E) and a small but significant interference for the NR assay (Figure 3.4A), while no interference was evidenced for the luminescent ATP measurements (Figure 3.4B) or the fluorescent resazurin assay (Figure 3.4C), except at the highest concentration of AgNPs. This interference with NR and

MTT would lead to an overestimation of cell viability, minimising the observed toxicity. However, for cytotoxicity measurements, both assays were performed on cell layers, after removal of AgNPs suspensions and subsequent washings. As a result, the concentration of AgNPs in contact with the NR dye or with the resazurin reagent is thought to be low, leading to a negligible interference. An increase of absorbance was observed for the LDH assay, proportional to the concentration of AgNPs (Figure 3.4D).

For the two assays where the endpoint is a precise analyte (LDH assay and ATP measurement), the possibility of an interference between AgNPs and the analyte, *i.e.* the LDH enzyme or the ATP molecule, was then assessed. The LDH indirect interference is reported in figure 3.5A. Fresh LDH was obtained by exposure of differentiated Caco-2 cells to TX, which leads to a release of cytosolic LDH in the supernatant, due to cell lysis. This supernatant was then used as a source of LDH for subsequent interference analysis. The same analysis was performed for ATP content measurement, using an ATP salt as the analyte (Figure 3.5B). A similar interference was observed for both assays, being significant from a concentration of 11.25μg/mL of AgNPs.

An additional control was performed for the MTT assay, based on the structure similarity with nitroblue tetrazolium (NBT) reagent [49]. This molecule also contains a tetrazolium ring such as other cytotoxicity assays *i.e.* XTT, MTS or WST-1 but is rather used to assess the intracellular generation of reactive oxygen species (ROS) [49]. We investigated if the MTT tetrazolium reagent was able to react with ROS to generate the formazan dye absorbing at 490nm. For this purpose, MTT reagent was incubated during 1h with different concentrations of riboflavin. Riboflavin is known to undergo photooxidation and to generate thereby superoxide anion. After this incubation, the absorbance at 490nm was measured and is reported in figure 3.6B as "mix." values. As both MTT tetrazolium salt and riboflavin alone absorb at 490nm, the sum of their respective absorbance is also reported in the figure 3.6B as "ind." values. For all tested concentrations of riboflavin, the absorbance of the mixture

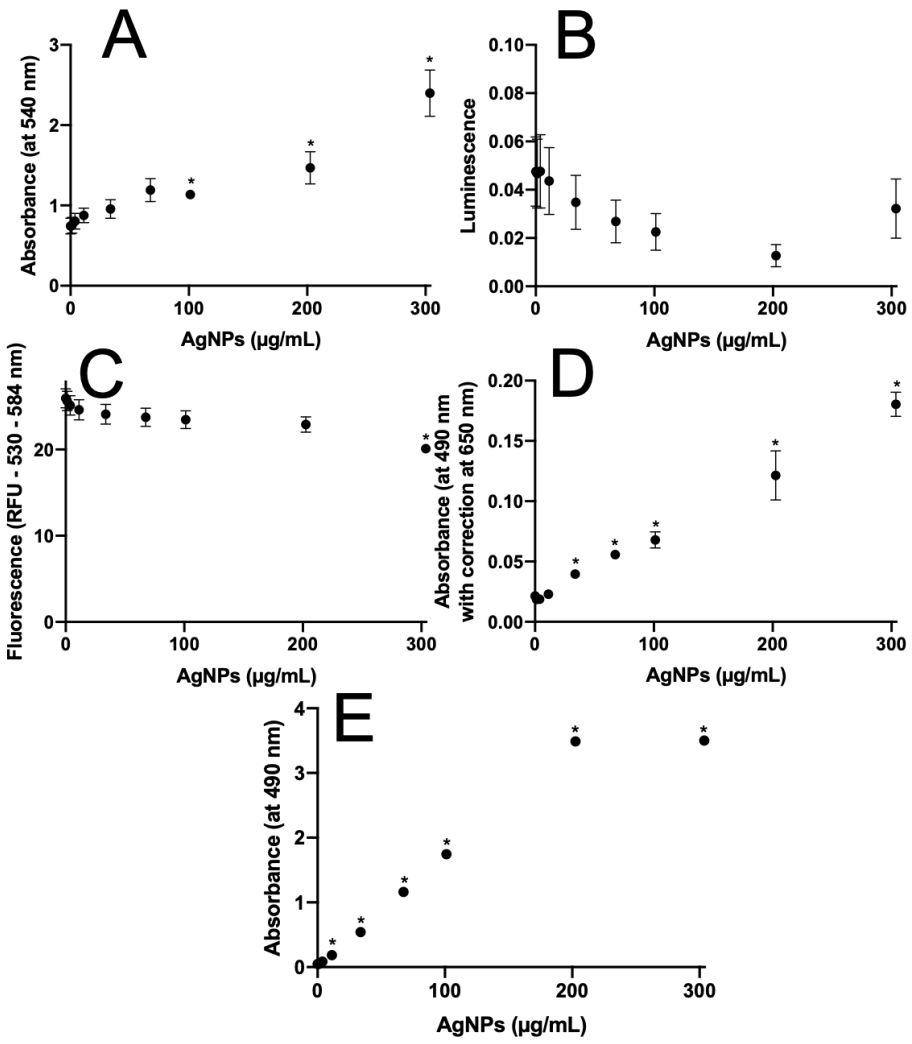


Figure 3.4: Direct interference between AgNPs and (A) NR assay (absorbance at 540nm), (B) ATP content measurement (luminescence), (C) resazurin assay (fluorescence), (D) LDH leakage assay (absorbance at 490nm with a correction at 650nm) and (E) MTT assay (absorbance at 490nm), measured by mixing different concentrations of AgNPs with the assay reagents, and the extraction solution for (A), (B) and (E). The mean ± SEM from 2 repetitions with 3 replicates is represented. A star means a result significantly different from control treatment ($P<0.05$; Student's t test with Bonferroni-Šidák correction).

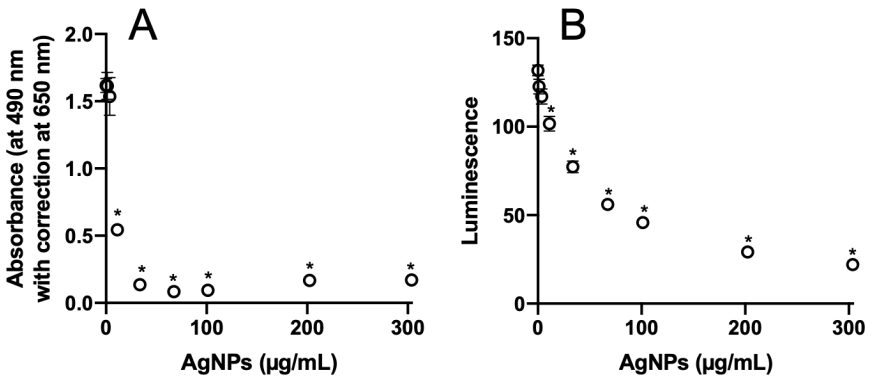


Figure 3.5: Indirect interference between AgNPs and (A) LDH assay (absorbance) and (B) ATP content measurement (luminescence), obtained by mixing different concentrations of AgNPs with the assay reagents and the analyte (extract of cells treated 30min with TX for (A) and ATP disodium salt 100μM for (B). The mean ± SEM from 2 repetitions with 3 replicates is represented. A star means a result significantly different from control treatment ($P<0.05$; Student's t test with Bonferroni-Šidák correction.)

with MTT tetrazolium salt was significantly higher than individual values, suggesting that MTT tetrazolium could react with the superoxide radical generated by riboflavin under light. As a result, cytotoxicity measurement through MTT assay should be avoided if oxidative stress is involved in the toxicity mechanism of the studied toxicants. Indeed, it could lead to an underestimation of the cytotoxicity as the MTT reagent would be reduced by ROS, generating formazan salt. The same control was performed for the resazurin assay, but no interaction was observed in the presence of riboflavin (data not shown).

3.3.2 Soluble silver content in AgNPs suspensions with ICP-MS measurements

After separation of the soluble silver fraction from AgNPs suspensions by centrifugal ultrafiltration, the amount of silver was measured by ICP-MS measurements in the soluble silver fraction and in initial AgNPs suspen-

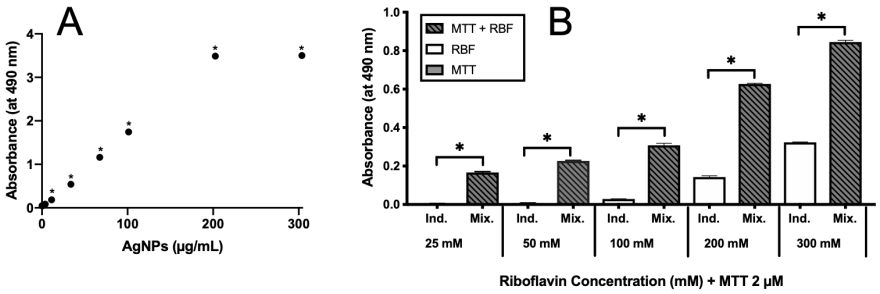


Figure 3.6: (A) Absorbance of MTT after incubation with different concentrations of AgNPs and (B) MTT conversion into formazan by ROS-producing riboflavin. Ordinate axis shows absorbance at the formazan emission peak wavelength (490 nm) of the individual compounds (MTT and riboflavin RBF) summed together (ind) and of the mixture of both compounds (mix). Data are expressed as mean ± SEM and come from 2 repetitions with 4 replicates (for A) and 1 repetition with 3 replicates (for B). A star represents a result significantly different from control treatment ($P<0.05$; Student's t test with Bonferroni-Šidák correction.)

sions. Results are expressed as a percentage between silver contained in soluble silver fractions, after ultrafiltration, and total silver content found in initial AgNPs suspensions, before filtration. The proportion of silver in the soluble silver fraction seems constant for all the different concentrations (Figure 3.7). The arithmetic mean of all experimental data points is 3.9%, suggesting that only a minor fraction of silver is oxidised in the AgNPs suspension used for this study. As a control of the separation process, it was checked that filtrates did not absorb light at any wavelength between 350 and 750nm, which suggests that AgNPs were not able to pass through filters (Figure S1). As an additional control of the separation process, a solution of Ag^{+} at a concentration of 15μg/mL was ultrafiltered. Silver content in filtrates was analysed with ICP-MS and 89% of the expected silver content was retrieved.

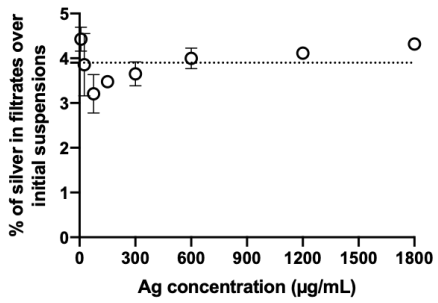


Figure 3.7: Total silver measured in filtrates obtained after centrifugal ultrafiltrations of AgNPs suspensions at different concentrations. Data are expressed as a percentage of silver compared to initial suspensions, before the separation process. For each concentration, mean ± SEM comes from 3 replicates. The dotted line is set at 3.9%, the arithmetic mean of all experimental measures.

3.3.3 Compared cytotoxicity of AgNPs and soluble silver fraction

The cytotoxicity of AgNPs was then evaluated with the cytotoxicity assays that did not present any risk of bias due to interferences. In addition, the involvement of the soluble silver fraction present in AgNPs suspensions was also assayed in order to evaluate its contribution to the global cytotoxicity.

After separation with ultrafiltration devices, it was not possible to retrieve the nanoparticles because they were retained in the filter. Filtrates, containing soluble Ag, as well as initial AgNPs suspensions were diluted 5x in HBSS, directly on differentiated Caco-2 cells to avoid the loss of silver due to the formation of silver chloride precipitate. Three cytotoxicity assays *i.e.* ATP content, resazurin assay and NR assays were performed after 3h incubation with silver solutions. Results are expressed as a percentage of control cells, treated neither with AgNPs nor soluble silver.

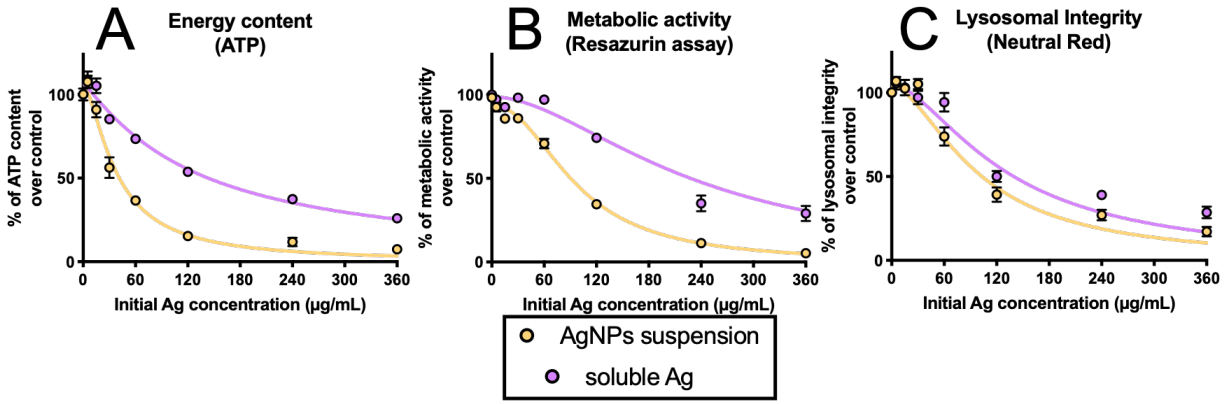
To statistically compare the toxicity of soluble silver fraction over AgNPs suspension, experimental curves were fitted on experimental data

points with log-logistic at 4 parameters models (equation in figure 3.8), recommended for dose-response curves [57]. The parameter "d", that represents the lowest possible response, was fixed at 0% of viability while the other parameters ("c" for the highest response, "b" for the steepness of the curve and "e" for the effective concentration 50 (EC50)) were estimated by the model (Figure 3.9D). For each cytotoxicity assay and each condition (AgNPs suspension or soluble Ag), the parameters "e" and "b" were compared to decide if curves were different.

$$f(x,(b,c,d,e)) = c + \frac{d-c}{(1+\exp(b(\log(x)-\log(e))))}$$

Figure 3.8: Equation of the log-logistic at 4 parameters model used to fit experimental data points. The parameter "b" is representative of the curve steepness, the parameters "c" and "d" are respectively the higher and the lower limit of the model and the parameter "e" estimates the EC50 of the curves [57].

Starting from 15μg/mL AgNPs, ATP content (Figure 3.9A) decreased steeply with AgNPs concentration, reaching 15% of ATP content of the negative control for a concentration of 120μg/mL. The EC50, estimated at 38.3μg/mL, is significantly lower for AgNPs suspension than for soluble Ag, suggesting that the effect is not due to only the soluble fraction but also to nanoparticles *per se*. Indeed, the ATP content of cells treated with the soluble fraction only did not decrease so steeply. In addition, the steepness and EC50 of both curves estimated by the log-logistic model were also statistically different.



D

	Energy content (ATP)			Metabolic activity (Resazurin assay)			Lysosomal activity (Neutral Red)		
	AgNPs suspension	soluble Ag		AgNPs suspension	soluble Ag		AgNPs suspension	soluble Ag	
e - EC-50	38.3 ± 2.7	128.2 ± 10.8	*	96.4 ± 3.6	231.0 ± 8.2	*	98.5 ± 7.2	131.3 ± 9.4	*
b - steepness	1.5 ± 0.1	1.1 ± 0.1	*	2.2 ± 0.2	1.8 ± 0.1		1.7 ± 0.2	1.6 ± 0.2	

Figure 3.9: Comparative cytotoxicity of AgNPs observed in Caco-2 cells incubated during 3h with AgNPs suspensions not filtered (AgNPs suspensions) or with soluble silver (soluble Ag) present in filtrates of AgNPs suspension after centrifugal ultrafiltration devices to separate AgNPs from soluble silver. The cytotoxicity was estimated by (A) ATP content, representative of energy content, (B) Resazurin assay, representative of metabolic activity of cells and (C) NR assay, representative of lysosomal activity. Log-logistic model estimated parameters are reported in (D). A star represents a significant difference between the estimated parameter for AgNPs suspensions and soluble Ag curves. Data are presented as means ± SEM, coming from 3 repetitions with 3 replicates.

The resazurin assay, evaluating the metabolic activity of cells was also performed for cells treated with either AgNPs suspensions or only the soluble fraction of these suspensions (Figure 3.9B). Similarly to ATP content, both curves presented a different decrease of metabolic activity. AgNPs suspensions decreased steeply the metabolic activity, although to a lower extent than for the ATP content. Cells treated with 120μg/mL AgNPs suspensions were 34.5% as active as control cells while cells treated only with the soluble fraction of this suspensions reached 73% of the control metabolic activity. Even if the steepness of curves was not statistically different, the EC50 were significantly different *i.e.* 96.4μg/mL for AgNPs suspensions and 231μg/mL for the soluble fraction.

The third cytotoxicity assay estimating the lysosomal integrity, the NR assay is presented in figure 3.9C. In viable cells, the NR dye is concentrated in acidic vesicles *e.g.* lysosomes, where it gets protonated and retained. If the integrity of these organites is altered, NR assay would lead to a lower response, as observed when increasing the concentration of AgNPs. Even if the difference between AgNPs suspensions and soluble Ag is less important than for the two other assays, EC50 was significantly different, reaching 98.5 μg/mL for AgNPs suspensions and 131.3μg/mL for soluble silver. Curves steepness was not statistically different.

3.4 Discussion

Unlike more conventional chemicals, nanoparticles and in particular AgNPs present two main challenges in their cytotoxicity assessment. On one hand, the possibility of interferences between AgNPs and the selected cytotoxicity assay is not negligible, which could impair conclusions about their effect on cell viability and physiology. On the other hand, such as other metallic particles, silver from AgNPs is known to partially oxidise in Ag^+. As the dissolution may vary with the physicochemical parameters of AgNPs, the environment, and the presence of organic compounds [23–27, 36], the Ag^+ proportion varies a lot between

AgNPs suspensions, adding some complexity to the risk assessment of AgNPs.

During this study, we have observed an interference between AgNPs and all cytotoxicity assays classically used for *in vitro* toxicity assessment. Even if AgNPs used in this study did not harbour any luminescence or fluorescence that could interfere with the cytotoxicity assays measurements, other interferences have been observed. Indeed, AgNPs seem to inhibit the LDH activity, as an extract of cells treated with TX presents a lower activity in the presence of AgNPs, which would lead to an underestimation of the necrosis and increase false negative results. Han *et al* also observed this inhibition of LDH by AgNPs of 35nm [58]. They suggested an adsorption of the LDH protein on the surface of AgNPs leading to its inactivation. Oh *et al.* suggested a combination of this effect and the inactivation of the LDH by ROS subsequently generated by AgNPs [59]. In addition, Ag^{+}, present in AgNPs suspensions, following their oxidation, could also inhibit the LDH activity [60].

The same interference was observed for ATP content measurement by luminescence, which tends to underestimate the amount of cellular ATP. This could be due to an interaction between AgNPs and luciferase, the enzyme used in the ATP measurement. As shown by Kakinen *et al.*, luciferases could form a corona around AgNPs, modifying the enzyme secondary structure and decreasing its activity [61]. However, while LDH assay is performed on cell supernatant in the presence of AgNPs, the ATP content is measured on cell layers washed to remove as much as possible AgNPs suspensions. As a result, the amount of remaining AgNPs is thought to be low, minimising the risk of bias.

At high concentrations of AgNPs, an increase of absorbance was observed for neutral red and a decrease of fluorescence for the resazurin assay, which could be due to the turbidity of highly concentrated AgNPs suspensions [62]. Similarly, Breznan *et al.* observed a decrease of resazurin fluorescence in the presence of carbon nanotubes [63]. However, such as performed for the ATP content measurement, cell layers were washed before applying cytotoxicity reagents and AgNPs concentrations

remaining present are thought to be marginal.

Furthermore, both MTT and resazurin assays showed an interference with AgNPs, with an increased response due to AgNPs, suggesting a reduction of the assay reagent by AgNPs. Interference with tetrazolium salts has already been observed in literature, with a reduction of MTT by silicon nanoparticles [64] or a MTS reduction by AgNPs [65,66]. This reduction led to an overestimation of cell viability, minimising the toxicity of AgNPs. However, this interference is again thought to have a negligible impact on cytotoxicity assessment, because this assay was performed with rinsed cell layers, after removing AgNPs incubation solutions.

A similar interaction was observed between MTT assay and ROS, produced by illuminated riboflavin. Nevertheless, this interference could not be avoided by washings, as these species are generated inside cells, which explains why this assay was excluded for the rest of this study. Indeed, the MTT structure presents similarities with NBT molecule, which is classically used to estimate superoxide levels in cells (Figure 3.10) [49]. Wang *et al.* have performed a similar experiment in which they observed that both MTT and XTT were reduced by superoxide radical, generated by a xanthine-xanthine oxidase system [67]. According to Berridge *et al.*, MTT is reduced by activated neutrophils, immune cells generating reactive oxygen species and could be used to assess superoxide dismutase activity [49]. In contrast, this interaction was not observed for the resazurin assay.

As a result, tetrazolium salt assays should be carefully applied if superoxide levels are modulated by the experimental conditions [67]. Hence, the use of resazurin assay that measures also the metabolic activity should be considered.

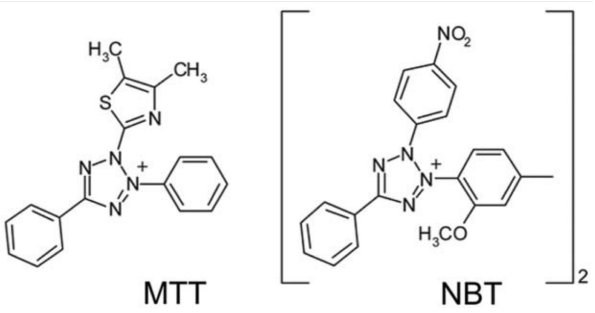


Figure 3.10: Chemical structures of (A) MTT, used for metabolic activity estimation and (B) NBT, used as a dye for superoxide detection. Adapted from Berridge *et al.* [49].

These observations confirm the specific recommendations for nanotoxicology studies to use at least two different toxicity assays with minimal risk of bias due to interference [42, 50, 53].

Based on all of these interferences, we selected the resazurin assay, the neutral red assay and the measurement of ATP for the rest of this study because it was possible, by careful washings of cell layers, to remove the majority of AgNPs and minimise possible bias in cytotoxicity assessment. However, as these cytotoxicity assays do not measure the same endpoints, they generate different results *i.e.* EC50 [42]. A dose-response effect to AgNPs was observed for the three chosen cytotoxicity assays, affecting negatively the different tested endpoints. EC50 were estimated by fitting a 4 parameters log-logistic model, classically used to modelise dose-response curves [57]. For the ATP measurement, the estimated EC50 was around 38.3μg/mL, while the two other assays had a similar EC50, around 96.4 and 98.5μg/mL for respectively metabolic and lysosomal activities. It suggests that ATP content is affected at a lower concentration of AgNPs, at which the metabolic activity and lysosomal integrity are relatively preserved. This premature loss of cytoplasmic ATP upon AgNPs exposure could be related to the intracellular ROS production, the major mechanism for AgNPs cytotoxicity reported in literature [34, 65, 68–70]. Indeed, AgNPs are known to impair mitochon-

drial respiratory chain [71, 72] leading to an ATP depletion, which has been observed by several authors [65,73]. Both ATP depletion and intracellular ROS production have consequences for the cellular metabolism, which could be observed with the decrease of lysosomal integrity and metabolic activity. As ATP is related to chemical energy in cells, its deficiency disturbs transport processes, biological syntheses and metabolic activities of cells [65, 74]. In addition, ATP is required for the maintenance of an acidic pH in lysosomes [75, 76], which retains the neutral red dye in its protonated form [47]. The ATP drop in AgNPs-treated cells could explain the decrease of neutral red storage in cells [47]. Both lysosomal integrity and metabolic activity are impacted starting from a concentration of 60μg/mL. At this concentration of AgNPs, the ATP content represents only 36.66% of control cells and could not be able to maintain these cellular processes.

Even if numerous studies have been published about AgNPs *in vitro* toxicity, they are difficult to compare, because different parameters could impact the response of cells *e.g.* duration of exposure, chosen endpoint, cell type, physico-chemical properties of AgNPs, which could also affect the cellular uptake or doses [77–81]. For the latter, as they depend on the medium in which they are dissolved, it gives rise to an additional variety between studies. For the majority of published studies, AgNPs are dissolved in culture medium, often containing serum and various proteins. However, proteins could impact the physico-chemical properties of AgNPs, affecting therefore their uptake and toxicity [82, 83].

Furthermore, the possible dissolution of AgNPs could also affect the cytotoxicity assessment of AgNPs. Such as other metallic nanoparticles, AgNPs are known to oxidise, to a different extent, and form Ag^+. This phenomenon requires oxygen and is favored by the acidity and presence of oxygen [84]. The involvement of these Ag^+ in the cytotoxicity of raw AgNPs suspensions should be taken into account, to estimate the actual toxicity of AgNPs.

We first estimated the proportion of soluble silver in aqueous AgNPs suspensions. In water, these ions form the soluble fraction of AgNPs

suspensions. In other media containing ions such as chloride or phosphate, they could form precipitates such as AgCl or Ag_3PO_4 or complexes constituted of silver and chloride. In this study, the separation was thus performed in water to avoid the formation of solid compounds that could not go pass through filters. Different techniques have been used in literature to separate dissolved silver from silver nanoparticles, varying from dialysis [27], membrane filtration [26, 37], ultracentrifugation [23, 31, 37, 85] or cloud point extraction [38, 39]. Speciation of silver in suspensions used for cytotoxicity assays is often performed in studies as a way to characterise AgNPs. However, only a few studies have used the result of the separation to perform cytotoxicity assays. In this study, it was not possible to retrieve the AgNPs fraction after the filtration, because particles were trapped by the filter. As a result, we only used the soluble fractions that have passed through the filter and compared their cytotoxicity with that of initial AgNPs suspensions that were not filtered and contained simultaneously pristine AgNPs and soluble silver.

Results indicated that, even if Ag^+ ions are not free in HBSS, they are considerably toxic. However, they are not the only responsible for the toxicity of AgNPs suspension, as a difference between these two curves was observed for the three selected assays. According to Beer *et al.*, who have tested the soluble fraction of AgNPs suspensions, after separation by ultracentrifugation, the involvement of silver ions in the toxicity depends on their amount. If AgNPs suspensions contain less than 5.5% Ag^+ there could be an additional effect of AgNPs *per se*, while if they contain more Ag^+, the toxicity come only from these ions. The soluble silver content was estimated in this study at *ca.* 3.9% of total silver content, for AgNPs aqueous suspensions from JRC (NM-300K), which could explain that we observed an additional toxicity coming from AgNPs. This amount of soluble silver was similar to Van der Zande *et al.*, who have shown, also by ultrafiltration, that suspensions of AgNPs from the same supplier contained in average 7% of silver in soluble form [26]. More recently, Köser *et al.* reported a dissolved silver fraction in water of 4% with ultrafiltration for NM-300K AgNPs [37], which is similar to

our value of 3.9% measured in the same conditions. Different parameters could impact the proportion of Ag^+, such as the synthesis method [23], AgNPs concentration [25] or size [24], or the coating used to stabilise AgNPs suspensions [26]. Indeed, Kittler *et al.* showed with dialysis that AgNPs suspensions could release up to 90% of their weight in the soluble fraction [27]. Kittler *et al.* have also reported an increase in toxicity of AgNPs during storage, probably arising from their dissolution in Ag^+ [27]. However, for NM-300K, Köser *et al.* reported that the amount of dissolved silver was relatively stable over time, increasing up to 5.5% seven days after the preparation. Indeed, in the case of NM-300K, the nanoparticles used in this study, molecules of the coating (Tween 20 and polyoxyethylene glycerol trioleate) seem to constitute a reservoir of dissolved Ag^+, but also to protect AgNPs from additional dissolution [13, 37].

Finally, the culture medium could also affect the effect of the soluble silver fraction on cells. In pure water, Ag^+ are free and soluble. However, silver is known to precipitate with the majority of anions, such as chloride and phosphate present in culture medium. According to Loza [22] phosphate concentration in culture mediums are not sufficient to precipitate silver phosphate. Depending on the relative proportion of silver and chloride and the presence of other ions, different forms of silver will be present, either soluble such as chloride complexes ($AgCl_3^{2-}$, $AgCl_2^-$) or insoluble (AgCl precipitates) [37]. The amount of chloride in HBSS is similar to chloride concentrations present in the chyme leaving the stomach [86, 87]. Based on this concentration of chloride in HBSS, the insoluble form AgCl seems predominant for the silver concentration range used in this study, probably decreasing the bioavailability and subsequent toxicity of Ag^+ [88, 89], which could explain that AgNPs exhibit an additional toxicity, coming from particles themselves and not soluble silver.

3.5 Conclusion

From nanotoxicology studies, two main challenges arise from AgNPs toxicity estimation: on one hand, the possibility of interference with classical toxicity assays and on the other hand, the presence of various amounts of dissolved silver ions in AgNPs suspensions subsequent to their oxidation. In this study, AgNPs interfered with almost all the cytotoxicity assays tested, some of them leading to an underestimation of AgNPs toxicity while other tend to overestimate it. Even if choosing tests without any interference appears almost impossible, due to a large spectrum of interferences with AgNPs, the use of appropriate controls and washings could minimise the risk of bias in the cytotoxicity assessment of AgNPs. In addition, the cytotoxicity should be assessed by different tests, even if, as they do not measure the same endpoint, they could lead to different concentration range for the observed effects. The soluble fraction could always be used as an additional control for metallic particles, in order to discriminate the effects due to particles *per se*. The amount of ions, depending on many parameters, should be routinely reported in nanotoxicology studies.

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Supplementary data

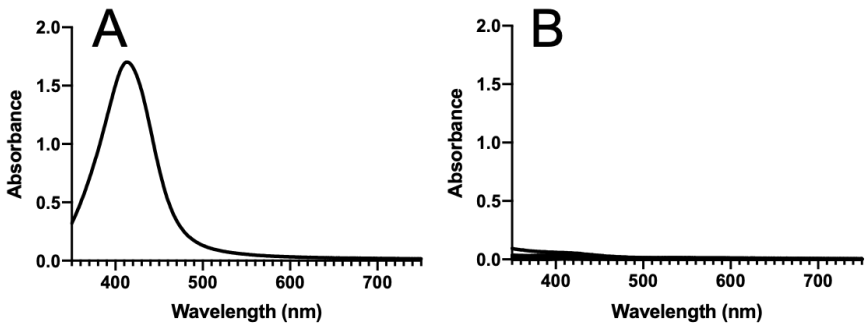

Figure S.1: Absorbance of filtrates before (A) and after (B) centrifugal ultrafiltration, suggesting that no nanoparticles were present in the soluble silver fraction. The mean of three replicates is represented.

IV

Silver nanoparticles effects on the NF-κB cascade activation

Foreword

This chapter addresses the first and third objectives of this thesis *i.e.* the ability of silver nanoparticles (AgNPs) to affect the inflammation-related NF-κB pathway and the involvement of silver ions in this process, respectively. This pathway was chosen for its involvement in inflammatory response of cells to external stresses. The effect of AgNPs was evaluated on two different levels; on one hand, on resting Caco-2 cells in order to evaluate if they could activate this pathway and, on the other hand, on Caco-2 treated with a NF-κB-inducing-cocktail in order to estimate if AgNPs could inhibit this activation. The previous chapter helped to set a subcytotoxic concentration range for this study.

Silver nanoparticles effects on the NF-κB cascade activation

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Abstract

Antimicrobial properties of silver and the advances of nanotechnology have led to the development of silver nanoparticles that can be found increasingly in food related applications. Upon migration in food, they can be ingested and interact with the gastrointestinal tract. Although their cytotoxicity has been largely studied, their effect on inflammation remains controversial. In this context, the present study aims at assessing the effect of silver nanoparticles on the inflammatory signalling cascade NF-κB in human intestinal cells. For this purpose, Caco-2 cells were exposed during 3h with non-cytotoxic concentrations of silver nanoparticles (up to 15 µg/mL), either in the absence or presence of inflammatory stimuli. Then, an assessment of NF-κB cascade activation was performed at different levels *i.e.* nuclear translocation of p65, production of a NF-κB responsive luciferase and expression of NF-κB target genes. Results first show that non cytotoxic concentrations of silver nanoparticles induce neither the translocation of p65 nor the expression of NF-κB target genes. It also indicates that, in the presence of inflammatory stimuli, silver nanoparticles inhibit p65 nuclear translocation and the expression

of a luciferase under the control of NF-κB response elements, suggesting an inhibition of the NF-κB cascade by silver nanoparticles. This inhibition is mainly mediated by nanoparticles but not silver ions release in silver nanoparticles suspensions, which amounts to *ca.* 5% of the total silver retrieved in the nanoparticles suspensions.

Keywords

Silver nanoparticles - *in vitro* - NF-κB pathway - Caco-2 cells

Highlights

- Non cytotoxic concentrations of silver nanoparticles do not induce p65 translocation, avoiding NF-κB canonical pathway activation.
- NF-κB target genes are not induced upon exposure to non cytotoxic concentrations of silver nanoparticles.
- Silver nanoparticles inhibit the positive control-induced nuclear translocation of p65.
- Silver nanoparticles decrease the positive control-induced production of NF-κB luciferase.
- These results suggest that silver nanoparticles inhibit NF-κB canonical pathway.

List of abbreviations

Ag^+ silver ions

AgNPs silver nanoparticles

BSA bovine serum albumin

DAPI 4',6-diamidino-2-phenylindole

DLS dynamic light scattering

DMEM Dulbecco modified Eagle's medium

HBSS Hank's balanced salt solution

IECs intestinal epithelial cells

IL-6 interleukin-6

IL-8 interleukin-8

LPS lipopolysaccharide

NMs nanomaterials

PBS phosphate buffer saline

TEM transmission electron microscopy

TNF-α tumor necrosis factor-α

TX Triton-X100

4.1 Introduction

Engineered nanomaterials (NMs) have been developed with promises of higher reactivity and efficiency owing their smaller size although this parameter could also raise their toxicity and interactions with living organisms [1]. In particular, an increased interest has emerged in using silver nanoparticles (AgNPs) in food products [2,3] because of their antimicrobial properties making them, among all NMs, the most commonly found in consumer products, with 50% of the consumer products containing silver [4]. Even if not incorporated on purpose in food, their presence varies in applications from farm to fork [2]. Nevertheless, different studies have underlined their migration in food from packaging materials containing AgNPs [5–10] resulting in their ingestion. Although amounts remain relatively low, the increasing use of AgNPs could dramatically raise the consumers exposure. Additionally, high contamination by AgNPs could also occur after ingestion of some unauthorised food supplements, claimed for having benefits on the immune system, whose consumption could lead to up to 0.02 mg/kg BW/day exposure [2, 11].

This widespread presence of AgNPs in food-related products will lead to contact of AgNPs with intestinal epithelial cells (IECs). IECs can be mimicked *in vitro* by Caco-2 cells [12]. Although originally isolated from a colonic tumor, they differentiate spontaneously in cells presenting many characteristics of small intestine enterocytes [13]. While immune cells are a major actor in inflammatory processes, IECs also play a role as they form the first barrier encountered by luminal antigens and interact with immune cells through various ways. Furthermore, IECs respond to inflammatory mediators secreted by immune cells with changes in the epithelial permeability. In turn, they modify the exposure of immune cells to luminal antigens. In addition, IECs secrete by themselves inflammatory mediators to communicate with their environment [14]. NF-κB is a major transcription factor family governing these processes. The activation of the pathway leads to the phosphorylation of IκB, which is the signal for its linkage to ubiquitin, followed by its degradation by the protea-

some. This event releases the NF-κB dimer containing a nuclear addressing sequence, which then translocates to the nucleus where it regulates the expression of over 200 genes involved in processes such as apoptosis, cell adhesion, proliferation, cellular stress but also inflammation [15, 16]. NF-κB activation could be achieved *in vitro* in Caco-2 cells by using an inflammatory cocktail that consists in a mixture of three inflammatory cytokines and lipopolysaccharide (LPS) to allow a maximal secretion of inflammatory mediators such as interleukin-8 (IL-8), interleukin-6 (IL-6), PGE-2 or NO [17]. The expression of NF-κB target genes such as NFKB1, NFKBIA but also receptors IFN-γR1, TLR3 and NOD2 involved in NF-κB activation was observed in response to the inflammatory cocktail [18]. The canonical NF-κB pathway, in which the p50-p65 dimer is central, is involved in inflammatory processes. Dimers without p65 are preferentially involved in developmental processes through the alternative NF-κB pathway.

AgNPs are recognised for their antimicrobial properties in wound and burn healing [19], but also for their capacity to improve healing in applications such as dressings and pomades. Anti-inflammatory properties are also attributed to AgNPs, underlined by *in vivo* studies on different pre-inflamed models such as allergic dermatitis [20, 21], burn wound models [22] or allergic rhinitis [23]. Ingestion of AgNPs also lead to anti-inflammatory effect, as observed by Bhol *et al.* [24] on a rat ulcerative colitis model. A beneficial effect of AgNPs was also shown on human *Psoriasis* patients [25]. However, *in vitro* studies are less unanimous concerning the inflammatory properties of AgNPs. Although some studies showed pro-inflammatory properties of AgNPs [26–29], many observed that AgNPs seem to inhibit the induction of inflammation by external stressors in Hacat cells [25], peripheral blood mononuclear cells [30, 31], primary nasal polyepithelial cells [32] or macrophages [33, 34]. While some studies evaluated the inflammatory properties of AgNPs, only a few of them have focused on the subjacent mechanism behind these inflammatory properties [35] and, in particular, the involvement of the NF-κB pathway.

The aim of the experiments presented here was to analyse the effect of AgNPs on the NF-κB signalling pathway either on its initiation in uninflamed Caco-2 cells or on the activation of this pathway in cells stimulated with an inflammatory cocktail in order to mimic *in vitro* inflammation.

4.2 Material and methods

4.2.1 Cell culture and exposure

Caco-2 cells from a human colon adenocarcinoma (clone 1 from Dr. M. Rescigno, University of Milano, IT) were cultivated between p+10 and p+30 at 37°C under a water-saturated atmosphere with 10% (v/v) CO_2. Caco-2 cells were grown in tissue culture flasks (Corning Incorporated, Corning, NY) in Dulbecco modified Eagle's medium (DMEM) with 4.5 g/L glucose (Lonza, Basel, CH), supplemented with 10% (v/v) fetal bovine serum (Biowest, Nuaillé, FR), 1% (v/v) non-essential amino acids 100X (Lonza), 1% (v/v) L-glutamine 200 mM in 0.85% NaCl solution (Lonza) and 1% (v/v) penicillin-streptomycin (Lonza). Caco-2 cells were seeded at a cell density of *ca.* 63 000 cells/cm^2 either in inserts (polycarbonate membrane with 0.4 μm pore diameter, Costar Transwell Permeable Supports, Corning incorporated) for p65 translocation experiments, or in culture well plates (Corning) precoated with type I collagen (Sigma-Aldrich, St. Louis, MO) for the other experiments. Cells were cultivated during 21 days upon differentiation. The day of the experiment, differentiated Caco-2 cells were exposed in Hank's balanced salt solution (HBSS) for 3h to the different treatments, whose volume was adapted between inserts and wells taking the surface area into account.

4.2.2 Reagents

AgNPs $<$ 20nm (NM-300K, JRC repository, Ispra, IT) were purchased from Fraunhofer institute (Schmallenberg, DE) as a dispersion in water containing 10.16% (w/w) of silver, 4% (w/w) of polyoxyethylene glycerol

trioleate and 4% (w/w) of polyoxyethylene (20) sorbitan mono-laurate (Tween 20). Stock solutions of AgNPs were prepared in milliQ water (Millipore, Burlington, MA) and diluted in HBSS (Lonza) directly on cells to obtain a range of concentrations between 1.5 and 15 μg/mL, which was chosen to avoid any cytotoxicity (Figure S2).

Solutions of silver ions (Ag^+) were prepared from silver nitrate salt (Sigma-Aldrich) in milliQ water at concentrations between 1.88 and 30 µg/mL. Similarly to AgNPs solutions, they were diluted in HBSS directly on cells to obtain a range of concentrations between 93.75 ng/mL and 1.5 µg/mL.

The inflammatory cocktail was constituted of tumor necrosis factor-α (TNF-α) (50 ng/mL), interleukin-1β (25 ng/mL), interferon-γ (50 ng/mL) and lipopolysaccharide (1 μg/mL) (all coming from Sigma-Aldrich) and was developed to obtain the highest secretion by Caco-2 cells of IL-8, IL-6, NO and PGE-2, targets of NF-κB pathway, as established by Van de Walle *et al.* [17].

Formaldehyde, Triton-X100 (TX), bovine serum albumin (BSA), 4',6-diamidino-2-phenylindole (DAPI) were purchased by Sigma-Aldrich. Nitric acid (70%, highest purity), used for ICP-MS measurements, was purchased from VWR (Leuven, BE).

4.2.3 p65 immunostaining and confocal observation

After 3h incubation, Caco-2 cells were washed with phosphate buffer saline (PBS), as it was performed several times between each of the following steps. Cells were fixed with 4% (v/v) formaldehyde (Sigma-Aldrich) during 15 min and permeabilised for 3 times during 5 min in 0.3% (v/v) TX. Blockage was performed during 1h in 0.3% TX and 2% (w/w) BSA and cells were incubated overnight with an anti-p65 rabbit antibody (Cell signaling technology, Danvers, MA) diluted 1:400 in a 0.3% (v/v) TX and 1% (w/w) BSA solution. Cells were then incubated for 1h with a secondary goat anti-rabbit antibody, coupled with FITC dye (FITC-Goat Anti-Rabbit IgG; Thermo Fisher Scientific, Waltham,

MA). 30 min before the end, DAPI was added at a final concentration of 1.5 μg/mL. Finally, cells were mounted with Ultracruz Mounting Medium (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were observed and 3 images per slide were taken with a Zeiss LSM 710 confocal laser microscope (Carl Zeiss Microimaging GmbH, Jena, DE). Images were processed with ImageJ software (v1.8.0) [36] with FigureJ plugin [37]. A post-hoc semi-quantification of p65 localisation was performed by the software, based on the confocal images. Two regions were defined on images. The DAPI staining allowed to define the "nuclear" region while the "cytosolic" region was selected by inverting the previous selection. The integrated density of FITC was measured in both "nuclear" and the "cytosolic" regions. The ratio of both integrated densities was used as a quantification of the nuclear presence of p65.

4.2.4 Plasmid preparation, transient transfection and luciferase assay

The determination of NF-κB activity was measured with plasmids. Proliferating Caco-2 cells were transiently co-transfected with a reporter plasmid containing the gene *luc2P* encoding a firefly luciferase whose expression is under the control of a promoter containing NF-κB response elements (pGL4.32, Promega, Fitchburg, WI, represented in figure 4.1A), and a transfection control plasmid, containing the gene *hRluc* coding for a *Renilla* luciferase with a consitutively induced HSV-TK promoter (pGL4.74, Promega, represented in figure 4.1B). Both were kindly provided by Prof. R. Rezsohazy (LIBST, UCLouvain, Louvain-la-Neuve, BE). In combination with the plasmids, the cationic polymer jetPEI (Polyplus transfection, Illkirch, FR) was added on cells for transfections. For each well, a solution containing jetPEI (7.5mM), 250 ng of pGL4.32 and 50 ng of pGL4.74 in 100 μL of NaCl 0,15M was added on cells for 24h. The assay was performed on 7-days post-transfection cells allowing both the combination of cell robustness and a great luciferase expression, as represented in figure S3. 7 days post-transfection, after a 3h treatment,

luciferase activities were assessed with Dual-Luciferase Reporter Assay (Promega) using a Glomax luminometer (Promega). Firefly luciferase activities were normalised with *Renilla* luciferase to avoid experimental variation due to transfection efficiency.

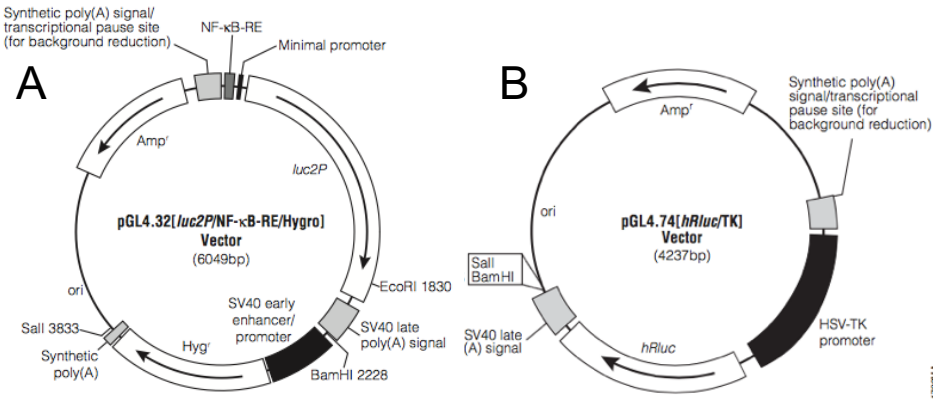

Figure 4.1: Plasmids used for transfection (A) pGL4.32 containing response elements to NF-κB and the gene *luc2P* encoding a firefly luciferase and (B) pGL4.74 containing a HSV-TK promoter, constitutively induced in Caco-2 cells and *hRluc* encoding for a *Renilla* luciferase.

Each experiment was repeated three times with individual treatments being assayed in triplicate.

4.2.5 Cytotoxicity assay

The cytotoxicity of AgNPs was estimated on Caco-2 cells cultivated for 8 days, representative of cells used for reporter assay studies (Figure S4) and on differentiated cells, cultivated for 21 days (Figure S2). After 3h of exposure to different concentrations of AgNPs varying between 2.5 and 60 µg/mL, cells were washed and ATP content was estimated by using a luciferase assay (CellTiterGlo, Promega) following manufacturer's instructions. The luminescence was recorded with a luminometer (10s of shake, 0.5s integration time, Fluoroskan Ascent FL, Thermo Fisher Scientific).

4.2.6 Separation of soluble silver from silver nanoparticles suspensions

Ag^+ was separated from AgNPs suspensions freshly prepared at different concentrations in milliQ water. Suspensions were centrifuged on ultrafiltration devices (Vivaspin turbo 4 10kDa, Sartorius, Goettingen, DE) at 3220xg during 10 min. The absorbance between 350 and 750nm of filtrates was measured by a UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA). As AgNPs absorb at *ca.* 410nm, which is not the case for Ag^+, it allowed a control of the filtration process. No absorbance was observed for filtrates (Figure S1B), which suggests that, as expected, AgNPs were retained at the surface of filters.

4.2.7 ICP-MS

Following the ultrafiltration, filtrates (containing Ag^+) and initial suspensions (containing total silver) were acidified with 4% (v/v) nitric acid (70%, highest purity). Silver content was then quantified using an ICP-MS (7900 ICP-MS, Agilent, Santa Clara, CA). The calibration curve was performed from 0.010 to 50 μg Ag/L with several dilutions of a multi element standard solution (Chem-lab, Zedelgem, BE, certified ISO/IEC 17025). The analytical method validity was verified every 10 measurements with 3 quality controls at 0.5, 5 and 25 μg Ag/L (Merck, Readington, NJ). The limit of quantification was 0.010 μg Ag/L. Samples were diluted so that silver concentrations were in the calibration range.

4.2.8 Gene expression study

After 3h incubation with treatments, total RNA was extracted from differentiated Caco-2 cells cultivated during 21 days (each sample containing *ca.*1.10^6 cells) by a treatment with Qiazol (Qiagen, Germantown, MD) and a purification with Aurum Total RNA Mini Kit (Bio-rad Laboratories, Hercules, CA) according to manufacturer's protocol. RNA quality and quantity were estimated with a Nanodrop instrument (Thermo

Fisher Scientific) measuring the absorbance at 230, 260 and 280nm. Total RNA integrity was assessed by gel electrophoresis on 1.5% (w/v) agarose displaying neither contamination with genomic DNA nor degradation of RNA. A RT-PCR was performed using 1 µg of total RNA using the iScript cDNA synthesis kit (Bio-rad Laboratories) following manufacturer's instructions. After retrotranscription, cDNA coming from three different wells similarly treated were pooled to have enough material for the qPCR analyses and diluted to a concentration of 2ng/μL. qPCR were performed using Takyon SYBR Green low ROX (Eurogentec, Liège, BE) on a Viia7 384-well real-time PCR instrument (Thermo Fisher Scientific) according to manufacturer's instructions. Primer information are reported in table 4.1. A BLAST search over the human genome sequence was carried out to assess the specificity of primers. The absence of primer dimers and secondary structures was verified *in silico* with the software Amplify4 (Bill Engels, University of Wisconsin, WI). Five reference genes were used and three of them (β-actin, HPRT1 and SDHA) were selected as the most suitable for normalisation of data using the Biogazelle algorithm in qBase+ 3.2 software (Biogazelle, Ghent, BE). Relative expressions were calculated with the $\Delta\Delta$Ct method taking into account multiple reference gene normalisation and specific primer PCR efficiencies [38]. Control cells were exposed to neither silver nor inflammatory cocktail and their expression level was set at 1. Efficiency and linearity for each couple of primers was estimated with a preliminary qPCR performed on a pool of all the samples for experimental validation and efficiencies estimation, which are reported in figure S5. Primer efficiencies were all between 1.8 and 2.0.

4.2.9 Statistical analysis

All statistical analyses were performed with JMP Pro14 (SAS Institute, Cary, NC). First, normality of the data distribution and homoscedasticity were verified with respectively Shapiro-Wilk test and Levene test to determine which comparison tests should be used. When data had a

normal distribution and equal variances, ANOVA-1 was applied followed by a Dunnet's post-hoc analysis. In any other cases, Student's t-tests were performed with type I error (α) set at 0.05 and a Bonferroni-Šidák correction of the P-values for multiple comparisons [39]. For gene expression analysis, the same t-test was applied on log-transformed data, as the reference condition (control) had no variance.

Table 4.1: **Primers used in the analysis of mRNA in the current study**.

Targeted gene	Accession number	Amplicon size (bp)	PCR efficiency	Primer	Sequence 5'-3'	Reference
Nuclear factor-κB subunit 1	NM_003998	137	1.966	NFKB1 Forward NFKB1 Reverse	GAAGCACGAATGACAGAGGC GCTTGGCGGATTAGCTCTTTT	[40]
Nuclear factor-κB inhibitor α	NM_020529	113	1.913	NFKBIA Forward NFKBIA Reverse	ACCTGGTGTCACTCCTGTTGA CTGCTGCTGTATCCGGGTG	[40]
Beta actin	NM_001101.4	119	1.908	β-actin Forward β-actin Reverse	TCCAAATATGAGATGCGTTGTT AATGCTATCACCTCCCCTG	Designed
Hypoxanthine phosphoribosyltransferase 1	NM_000194.3	94	1.887	HPRT1 Forward HPRT1 Reverse	TGACACTGGCAAAACAATGCA GGTCCTTTTCACCAGCAAGCT	[41]
Succinate dehydrogenase complex flavoprotein subunit A	NM_001294332.1	86	1.891	SDHA Forward SDHA Reverse	TGGGAACAAGAGGGCATCTG CCACCACTGCATCAAATTCATG	[41]
Tyrosine 3-monooxygenase	NM_003406	94	1.922	YWHAZ Forward YWHAZ Reverse	ACTTTTGGTACATTGTGGCTTCAA CCGCCAGGACAAACCAGTAT	[42]
Glyceraldehyde-3-P dehydrogenase	NM_001256799.2	111	1.905	GADPH Forward GADPH Reverse	ACCCACTCCTCCACCTTTGAC GTCCACCACCCTGTTGCTGTA	[43]

4.3 Results

4.3.1 Characterisation of the nanomaterial

The characterisation of dry particles from this batch was performed by Klein *et al.* (2011) [44]. We also performed an additional characterisation of AgNPs in HBSS (67.5μg/mL) by transmission electron microscopy (TEM) (Figure 4.2), UV-visible spectrophotometry (Figure S1) and dynamic light scattering (DLS). The hydrodynamic diameter measured by DLS was 57.75nm with a polydispersity index of 0.226.

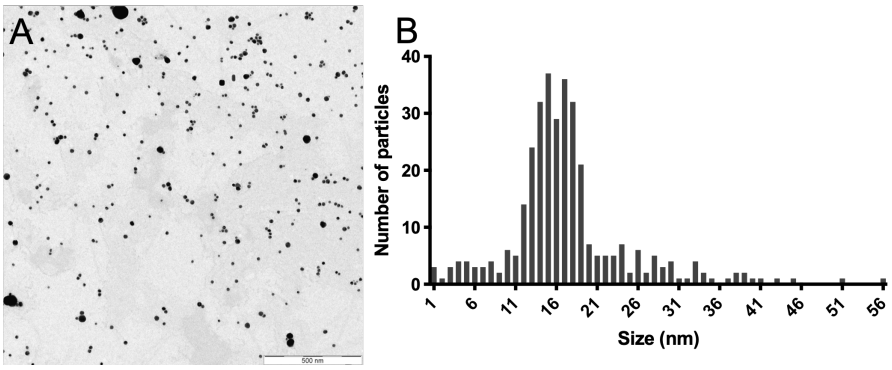

Figure 4.2: TEM image (10 000x) (A) and size distribution (B) of a AgNPs suspension (NM-300K) prepared at 67.5μg/mL and dropped on a TEM grid. The scale bar represents 500nm.

4.3.2 AgNPs do not activate NF-κB canonical pathway

The effect of 3h incubation of Caco-2 cells with AgNPs to NF-κB cascade was evaluated through three different techniques that are presented below.

First, the localisation of p65 was estimated by immunostainings, whose images are reported in figure 4.3A. When cells were incubated with the inflammatory cocktail, the localisation of p65 changed as images display a clear nuclear localisation, indicating that p65 was present within nuclei. The subcellular p65 localisation has been quantified. The "nuclear" region was defined through DAPI staining, and the integrated

density of FITC in this region and in the opposite region, corresponding to the "cytosolic" zone, were measured. Values reported in figure 4.3B are expressed as a ratio between the nuclear and cytosolic integrated densities of FITC reflecting the nuclear presence of p65. No significant difference between any concentration of AgNPs and the control can be observed from this graph, as observed in figure 4.3A. On the contrary, the inflammatory cocktail led to a 4-times increase compared to control cells confirming the nuclear localisation of p65 observed in figure 4.3A.

The ability of AgNPs to activate the NF-κB signalling pathway was also evaluated by a reporter assay system, constituted of a firefly luciferase responsive to NF-κB response elements and a *Renilla* luciferase constitutively expressed. Preliminary experiments indicated that the expression of luciferases was still important seven days after the transfection and then dramatically decreased (as shown in figure S4). As a result, transfected Caco-2 cells were exposed during 3h to AgNPs or the inflammatory cocktail 7 days after their transfection (Figure 4.4). Control cells indicate the basal level of NF-κB expression. Cells treated with the inflammatory cocktail present a huge firefly luciferase activity, set at 100%. No significant difference of relative luciferase activity was observed between the three tested concentrations of AgNPs and the control, suggesting that AgNPs treatment was unable to activate the NF-κB response elements contained in the promoter of the firefly luciferase. The cytotoxicity of AgNPs to cells cultivated in same conditions was controlled and concentrations up to 15 μg/mL led to only a small decrease of ATP content (Figure S4).

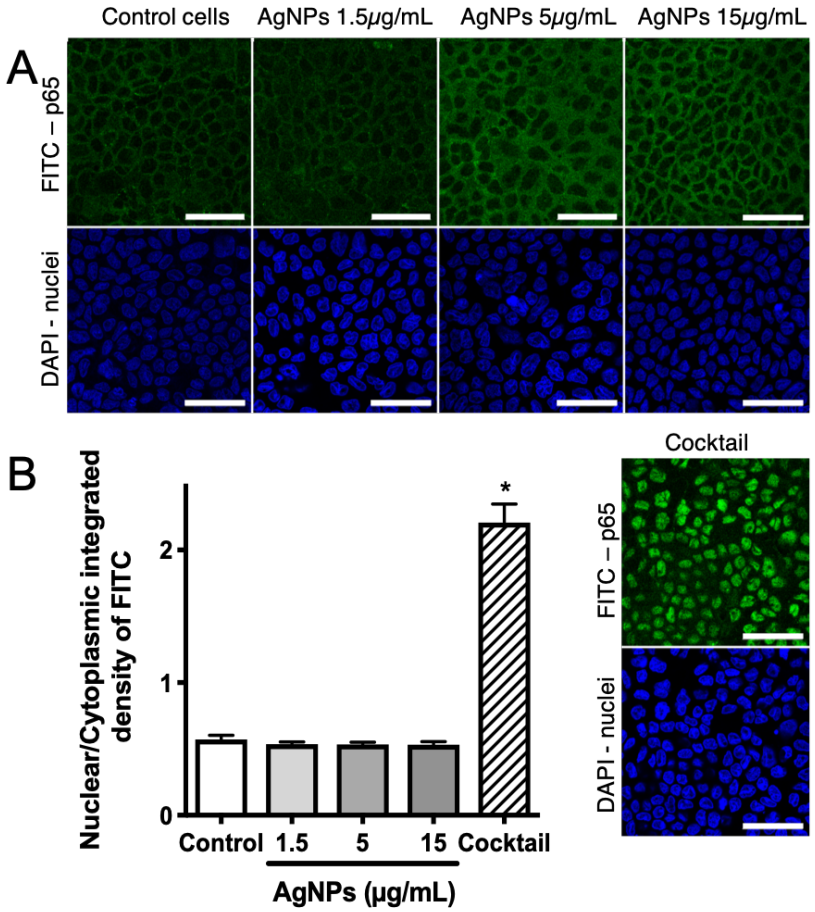

Figure 4.3: Laser scanning confocal microscopy images (A) and quantification of the nuclear presence of p65 (B) in cells treated or not with different concentrations of AgNPs. Green regions in (A) reflect the presence of p65 immunostained by a FITC dye. DAPI staining is reported in blue. Images were obtained by confocal laser scanning microscope and chosen to be representative of all the images. The white line represents the scale bar set at 50 μm. Control cells were treated during 3h with HBSS while cocktail-treated cells were incubated during 3h with inflammatory cytokines and LPS. The ratio of nuclear integrated density of FITC probe over its cytoplasmic integrated density reflecting the nuclear presence of p65, was quantified with the software ImageJ. Images and quantification come from 3 repetitions with 3 replicates. For each replicate, 3 images were taken. A star indicates a significant difference with the control consisted in untreated cells ($P<0.05$, Student's t-test with Bonferroni-Šidák correction).

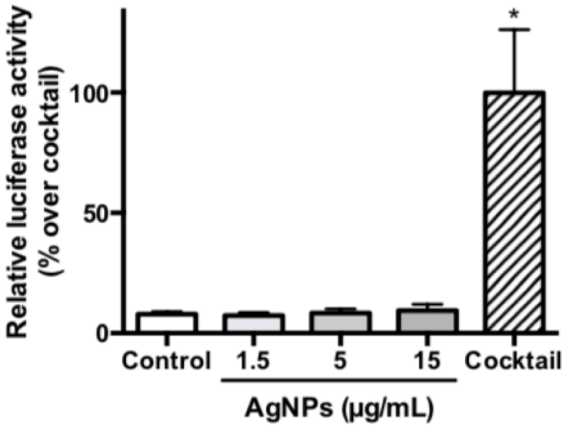

Figure 4.4: Relative luciferase activity of lysates from Caco-2 cells at 7 days post-transfection with a plasmid NF-κB responsive and a reference plasmid. Firefly luciferase activities were normalised with *Renilla* luciferase activities to give the relative luciferase activities and are expressed as percentages of cells treated with the inflammatory cocktail. Means $\pm$ SEM are presented in the graph and come from 3 repetitions with 3 replicates. A star indicates a significant difference with the control consisted in untreated cells ($P<0.05$; Dunnett's ANOVA).

The activation of NF-κB was finally assessed by qPCR analysis of two target genes of the canonical pathway, NFKB1 and NFKBIA [45] while NFKB1 encodes for p105, a 105kDa precursor of p50, involved in the NF-κB canonical pathway. NFKBIA encodes for inhibitor-κBα, which sequesters dimers in the cytosol and whose degradation leads to the activation of NF-κB classical pathway by nuclear translocation of dimers. Both genes are target genes induced by NF-κB canonical pathway [46, 47]. Differentiated Caco-2 cells were treated during 3h with AgNPs or inflammatory cocktail. Control cells consisted of cells treated with HBSS during 3h. As expected, the inflammatory cocktail induced significantly the expression of NFKB1 and NFKBIA (Figure 4.5). AgNPs had no significant impact on the expression levels of both genes, suggesting that AgNPs do not affect the activation of the NF-κB cascade.

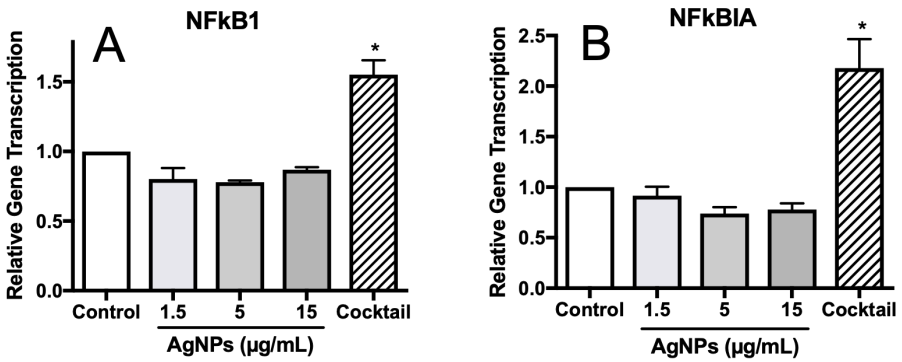

Figure 4.5: Relative gene transcription of (A) nuclear factor-κB subunit 1 (NFKB1) and (B) nuclear factor-κB inhibitor α (NFKBIA) after 3h exposure to AgNPs or inflammatory cocktail. Values are expressed as mean ± SEM (n=4). A star indicates a significant difference with the control composed of untreated cells ($P<0.05$, Student's t test).

4.3.3 AgNPs inhibit the inflammatory cocktail activation of NF-κB

The effect of AgNPs was then assessed on cells submitted to an inflammatory stress able to activate the NF-κB pathway. A well-defined nuclear localisation of p65 was observed for cells incubated with the inflammatory cocktail alone (Figure 4.6A).

Upon the addition of 15 μg/mL AgNPs, this intense fluorescence was faded and some nuclei were not stained at all (indicated by the white arrows on the figure). The ratio of nuclear over cytoplasmic integrated density of the FITC probe, reflecting the nuclear presence of p65, was quantified with the software ImageJ and expressed as percentages of cells treated with the inflammatory cocktail. This quantification of p65 in figure 4.6B shows a progressive decrease of nuclear p65, up to 30% of nuclear staining compared to the inflammatory cocktail alone. These results suggest that AgNPs inhibit or delay the nuclear translocation of p65 induced by the inflammatory cocktail.

AgNPs suspensions are subject to oxidation of Ag to Ag^+ to variable extents depending on nanoparticle size, chemical nature of the coating,

method of synthesis or concentration [48–52]. ICP-MS measurements showed that the AgNPs suspensions used in this study contained between 3 and 5% of total silver in the form of Ag^+ (Figure S6). To evaluate whether the observed effects were due either to AgNPs or to ionic silver found in AgNPs suspensions, we performed an additional control in which cells were treated with Ag^+ at a concentration of 0.75 μg/mL, corresponding to 5% of the higher tested concentration of AgNPs, in the presence of the inflammatory cocktail. Ag^+ affected the nuclear localisation of p65 only to a minor extent, compared to the inflammatory cocktail alone, which suggests that the effect on the NF-κB pathway is due to the AgNPs.

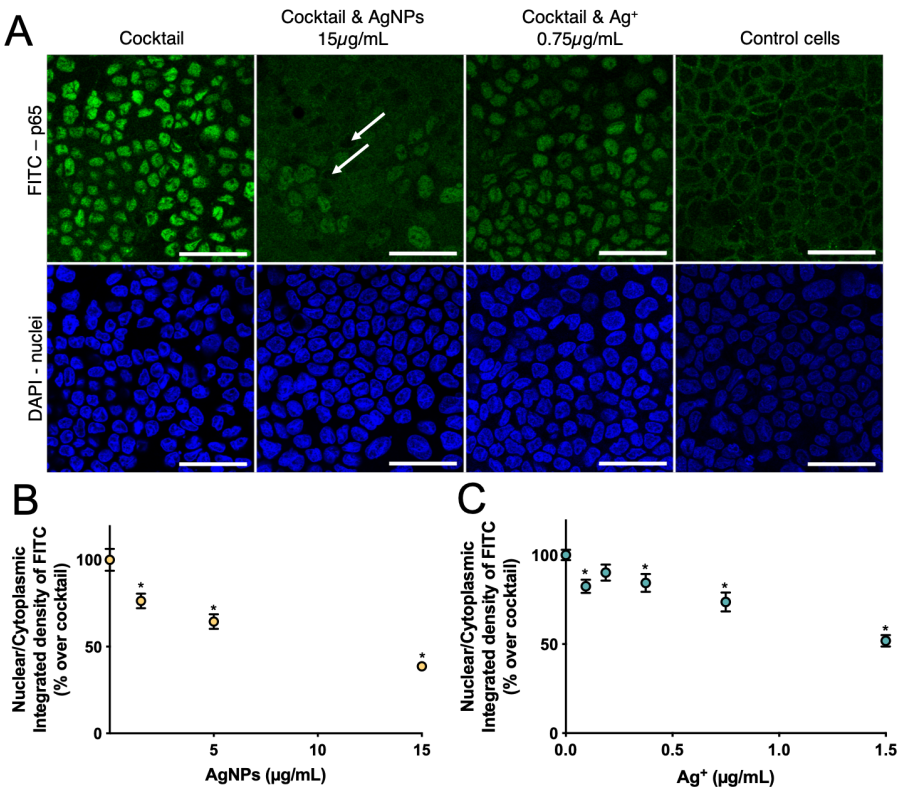

Figure 4.6: Images of cells treated with the inflammatory cocktail in the presence or absence of AgNPs or Ag^{+} (A) and quantification of nuclear presence of p65 treated with different concentrations of AgNPs (B) or Ag^{+} (C), co-incubated with the inflammatory cocktail. Representative images for each condition are reported in (A) and were obtained by confocal laser scanning microscope. The white line represents the scale bar, set at 50 μm. Arrows are pointed towards cells without green staining. Means $\pm$ SEM are presented in the graph and come from 3 repetitions, with 3 replicates with 3 images for each replicate. A star indicates a significant difference with cells treated with the inflammatory cocktail ($P<0.05$; Student's t-test).

These observations are confirmed by the quantification of p65 nuclear presence for AgNPs (Figure 4.6B) and Ag^+ (Figure 4.6C). In order to compare results between these two treatments, Ag^+ concentrations were calculated for AgNPs as 5% of AgNPs concentration and, for silver nitrate, assuming its complete solubility. As reported on figure 4.6B, AgNPs suspensions decreased to a higher extent the cocktail-induced activation of NF-κB pathway. Silver nitrate solutions, containing the same amount of Ag^+, were less efficient. This observation suggests that a part of the NF-κB inhibition was due to AgNPs specific effects, as discussed for figure 4.6A.

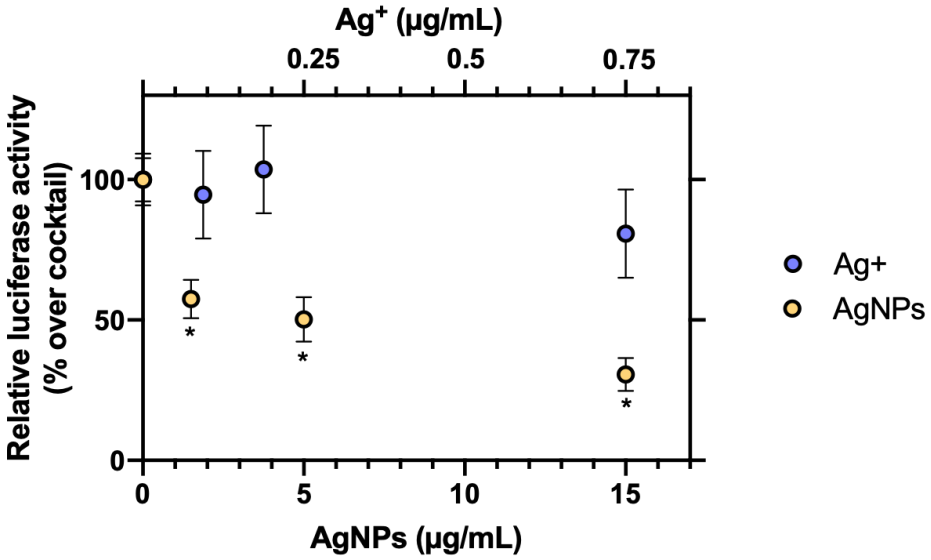

Figure 4.7: Relative luciferase activities of lysed cells after a coincubation with the inflammatory cocktail and AgNPs (in yellow) or Ag^+ (in purple). Firefly luciferase activities were normalised with *Renilla* luciferase values to give the relative luciferase activities and are expressed as percentages of cells treated with the inflammatory cocktail. Concentrations are expressed as total Ag concentrations in AgNPs suspensions (in the lower X-axis) and as concentration of Ag^+ contained in silver nitrate solutions (in the upper X-axis), assuming its complete solubility in water. Both conditions were compared, assuming that AgNPs suspensions contain, in the worst case scenario, 5% of silver as Ag^+. Means ± SEM are presented in the graph and come from 3 repetitions with 3 replicates. A star indicates a significant difference with the control consisted in untreated cells ($P<0.05$; Dunnett's ANOVA).

The presence of AgNPs inhibited significantly the production of NF-κB-dependent luciferase, as compared to the inflammatory cocktail alone (Figure 4.7). The involvement of Ag^+ in the inhibition of NF-κB pathway was evaluated by incubation of cells with different concentrations of silver nitrate, a soluble salt. Assuming that AgNPs suspensions contain *e.g.* 5% of silver in the ionic form, there seems to be a nano-specific inhibition of NF-κB activation by the inflammatory cocktail. Indeed, the

concentration of 15 μg/mL of AgNPs decreased the luciferase activity to 30%. On the opposite, 0.75 μg/mL of Ag^+, containing the same amount of ionic silver but not the nanoparticles, decreased the luciferase only to 80% of relative luciferase activity, not significantly different from cells treated with the inflammatory cocktail alone.

4.4 Discussion

In this study, the ability of AgNPs to modulate the NF-κB signalling pathway was assessed in Caco-2 cells, as an *in vitro* model of the human intestinal mucosa. In a first part, experiments performed on uninflamed cells indicated that AgNPs were unable to evidence the nuclear translocation of the p65-containing NF-κB dimer. After 3h incubation with AgNPs, p65 was not detected in nuclei. In turn, the activity of a luciferase, whose expression is under the control of NF-κB binding sequence in its promoter, was unaffected by the presence of AgNPs. Similarly, NF-κB target genes *e.g.* NFKB1 and NFKBIA mRNA expression was not increased upon AgNPs incubation, which strongly suggests that the NF-κB signalling pathway has not been activated in these experimental conditions. Possible bias through the interference with AgNPs were minimised by a washing of cell layer before lysis to recover luciferases. Moreover, results are expressed with a normalisation over a constitutively expressed luciferase, to avoid interference but also a bias due to a possible cytotoxicity. If the treatment was cytotoxic, the activity of this luciferase should also decrease. Furthermore, cytotoxicity assays performed in the same conditions as the luciferase assays did not evidence any important effect on ATP content (Figure S4). A change of relative activity of NF-κB-induced luciferase should then reflect a variation in NF-κB activation.

Contrary to this, the presence of the inflammatory cocktail induced an important nuclear translocation of p65, consistent with a huge increase of NF-κB-responsive luciferase and a dramatical rise of NF-κB target genes, suggesting that our *in vitro* model reacts adequately to NF-κB stimulation upon 3h exposure to inflammatory stimuli.

A cytoplasmic localisation of p65 has also been observed in HaCaT skin cells treated with 10 nm AgNPs, suggesting an absence of NF-κB activation and even an inhibition of its activation at high concentrations of AgNPs (40 μg/mL [53]. Moreover, the expression of NF-κB signalling pathway target genes was not affected by AgNPs, as observed by gene expression arrays [54]. On the opposite, other studies have indicated a nuclear localisation of p65 [55] or an increased expression of p65 and p50 [56] both in RAW264.7 murine macrophages but also in Jurkat T cells [57].

Skladanowski *et al.* did not observe an activation of NF-κB pathway in mouse fibroblasts treated up to 10 μg/mL AgNPs [58]. However, in our study, low concentrations of AgNPs were used to avoid cytotoxic doses that lead to cell detachment and could impair the NF-κB measurements. Even if these concentrations are low, they were able to induce effects on cytokines production [59], but we can not exclude that higher concentrations would not activate NF-κB signalling cascade.

Caco-2 cells were used in this study to investigate the impact of ingested AgNPs on IECs. Such as IECs, Caco-2 cells are known for their lower sensitivity to LPS than other cell lines [60]. In contrast, the inflammatory cocktail is composed of LPS but also inflammatory cytokines and in particular IL-1β, which was the major cytokine for inflammatory responses induced by the cocktail in Caco-2 cells [17] and its receptor is constitutively expressed in IECs [61]. This absence of response to AgNPs could be due to a specificity of IECs. Indeed, the toxicity of AgNPs was at least partially mediated by an interaction with the membrane receptor TLR2 [62], a receptor that interacts with bacterial lipopeptide to induce cellular responses. Indeed, TLR2 siRNA or TLR2 blocking antibodies partially reversed AgNPs-induced apoptosis. It has been suggested that AgNPs could interact with some Toll-like receptors (TLR) [63] that interact with bacterial components and activate immune responses through NF-κB signalisation. However, TLR such as TLR2 or TLR4 are less expressed in IECs, probably to maintain an immune balance towards the commensal microbiota [64–66].

The effect of AgNPs on NF-κB pathway highly depends on the cell line used, which could explain a part of this discrepancy in literature [67]. NF-κB pathway was induced by AgNPs in HepG2 liver cells while this was not the case in A549 lung cells [67]. The effect on NF-κB may depend on the basal activation of this pathway. However, in our study, the difference between inflammatory cocktail-treated cells and control cells was important, unlike to what was observed in A549 cells [67], suggesting that other factors could be involved. Furthermore, different characteristics of NMs, such as size, shape, or coating can affect their properties and their ability to interact with the cells and there might be different AgNPs with different immunomodulatory properties [52, 68, 69]. Although the activation of NF-κB is not always described in literature, the secretion of pro-inflammatory cytokines following AgNPs has however been observed [27, 70].

The second part aimed at studying the effect of AgNPs on the activation of NF-κB pathway by known inducers, *e.g.* inflammatory cytokines and bacterial LPS, regrouped in an "inflammatory cocktail" previously developed [17]. For this purpose, immunostainings of p65 and NF-kB dependent luciferase production were performed on cells incubated with the inflammatory cocktail in the presence or absence of AgNPs. Both experiments underline a lower cocktail-induced activation of NF-κB pathway, suggesting an inhibition of this cascade by AgNPs. Some *in vitro* studies have also reported a decrease of inflammatory cytokines production in inflamed models like infected macrophages [34], peripheral blood monocytes cells or Jurkat T cells stimulated with phytohaemagglutinin [30, 31, 71] or RAW264.7 murine macrophages incubated with LPS [33]. These results are consistent with various *in vivo* studies performed in inflamed conditions such as burns [22], allergic dermatitis [20, 21, 25] or allergic rhinitis [23], which showed an inhibition of inflammatory responses by AgNPs. A benefical effect of AgNPs was also shown on human *Psoriasis* patients [25]. Concerning the gut, AgNPs decreased colonic inflammation by suppressing the expression of pro-inflammatory cytokines in rats with ulcerative colitis [24].

Our results suggest that the inhibition of cocktail-induced NF-κB activation occurs at the nuclear translocation of NF-κB dimers containing p65 or upstream in the cascade. An assumption could be that AgNPs chemically interact with cocktail components and inactivate their action. However, while AgNPs and cocktail components were present together for the luciferase assays, this was not the case for the cells used for immunostainings of p65. Indeed, as these cells were grown on inserts, AgNPs were incubated at the apical side together with LPS while the other components of the cocktail were placed in the basolateral compartment. This was performed in order to mimic the *in vivo* environment in which cytokines come from immune cells localised in the subjacent tissues while LPS comes from the commensal microbiota [17]. As explained earlier, LPS has only a minor effect on Caco-2 cells, as they harbor only a few specific receptors. The major determinant of inflammatory response induced by the inflammatory cocktail seems to be interleukin-1β and this cytokine was not present in the same compartment as AgNPs during the immunostaining experiments. Therefore, the inhibition of the cocktail-induced NF-κB pathway activation cannot be explained by a direct interaction of AgNPs with LPS.

Another interaction could occur between AgNPs and receptors involved in NF-κB stimulation. As mentioned earlier, AgNPs could interact with TLR2 and trigger NF-κB responses [62]. However, in some cases, this interaction was shown to inactivate the receptor in lung cells [72, 73]. It was hypothesised that the binding of AgNPs to TNF receptors could disrupt the normal binding cycle of these receptors, causing a lower presence at the cell membrane and a decreased effect of TNF-α. There is no evidence for a similar disturbance of cytokine receptors by AgNPs and it would only partially explain the inhibition of cocktail induced NF-κB activation. In the experiments using immunostainings, AgNPs were applied at the apical side of Caco-2 cells in the absence of the inflammatory cytokines. However, this could explain the absence of inhibition by Ag^+. In addition to a direct interaction with cytokine receptors, the AgNPs inhibition of NF-κB activation could also come

from a decreased expression of receptors like NOD2, TLR2 and TLR4 initiated by AgNPs [34,74]. Finally, the effect of AgNPs could also occur at phosphorylation steps by kinases, which appear to play a key role in NF-κB activation [75].

4.5 Conclusion

In summary, non-cytotoxic concentrations of AgNPs do not activate the NF-κB canonical pathway as observed by an absence of nuclear translocation of p65, a major constituent of NF-κB dimers. Furthermore, AgNPs were unable to induce the expression of NF-κB target genes in Caco-2 cells either constitutive or artificially added by transient transfections. Moreover, AgNPs seem to inhibit the stimulation of NF-κB by an inflammatory cocktail constituted of LPS and pro-inflammatory cytokines. These observations suggest that AgNPs could harbor anti-inflammatory properties even if this has to be further explored with the analysis of secreted cytokines following AgNPs exposure.

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Supplementary data

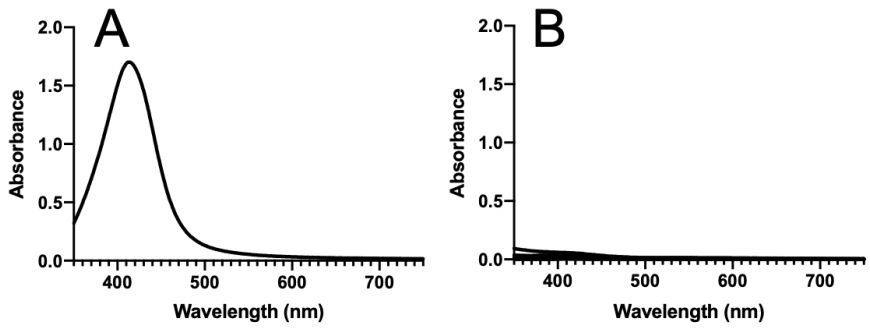

Figure S.1: Absorbance between 350 and 750nm of (A) AgNPs suspension at 15μg/mL in water and (B) the same suspensions after ultrafiltration on 10kDa centrifugal devices.

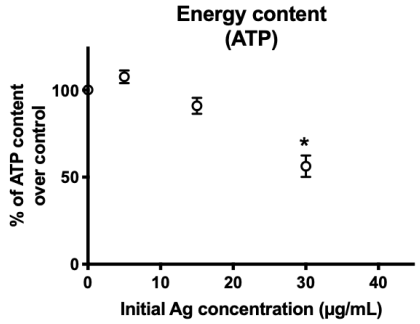

Figure S.2: ATP content in Caco-2 cells after treatment with different concentrations of AgNPs, expressed in percentage of control cells. Caco-2 cells were cultivated during 21 days to have similar conditions than cells used for p65-immunostaining analysis and gene expression experiments. Values (mean ± SEM) come from 3 repetitions with 3 replicates per condition. As far as the AgNPs concentration increased, the cell viability decreased almost proportionally ($P<0.05$, Student's t-test with Bonferroni-Šidák correction).

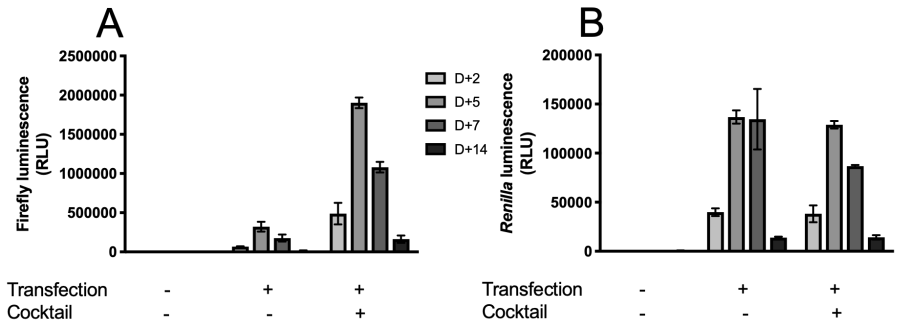

Figure S.3: Firefly (A) and *Renilla* luminescence (B) after lysis of cells treated with or without the inflammatory cocktail 2, 5, 7 or 14 days after cell transfection with both plasmids. Values (mean ± SEM) come from one repetition with two replicates per condition. For both luciferases, the luminescence level at 14 day post-transfection drastically decreased. For this reason, the assay was performed on 7 days post-transfection cells allowing the combination of cell robustness and a great luciferase expression.

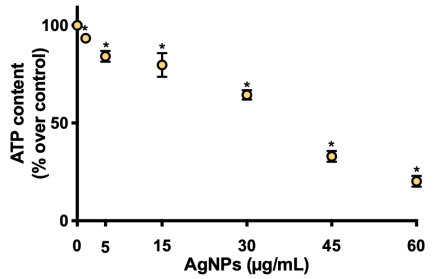

Figure S.4: ATP content in Caco-2 cells after treatment with different concentrations of AgNPs, expressed in percentage of control cells. Caco-2 cells were cultivated during 8 days to have similar conditions than cells used for luciferase assays. Values (mean ± SEM) come from 3 repetitions with 4 replicates per condition. As far as the AgNPs concentration increased, the cell viability decreased almost proportionally. The concentration of 15 μg/mL AgNPs decreases ATP content up to 80% of control cells. A star indicates a significant difference with the control consisted in untreated cells ($P<0.05$, Student's t-test with Bonferroni-Šidák correction).

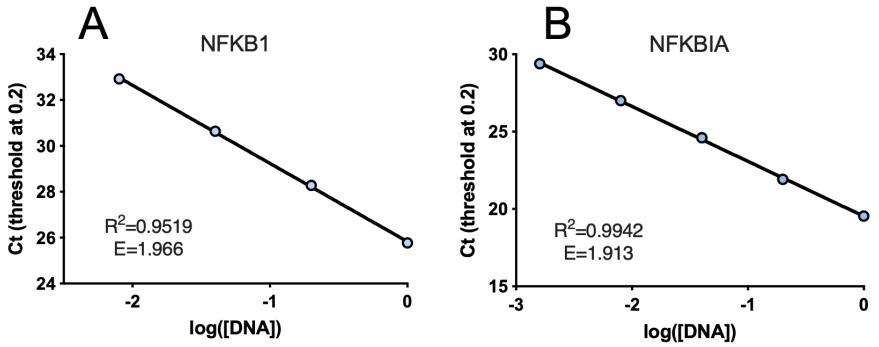

Figure S.5: qPCR efficiency estimation through calibration curves for (A) NFKB1 and (B) NFKBIA genes. qPCR were performed with different concentrations of sample cDNA, represented as logarithms of cDNA concentration in ng/μL. Ct (mean $\pm$ SEM) were measured with a fluorescence threshold set at 0.2 and come from 3 technical replicates of cDNA pooled from all samples.

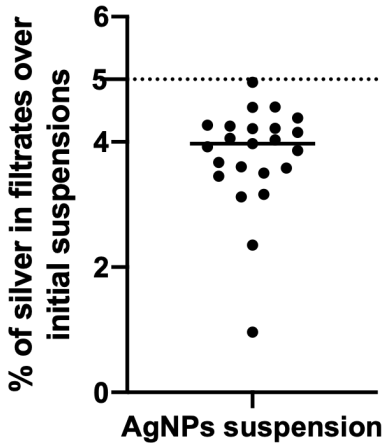

Figure S.6: Total silver measured in filtrates obtained after centrifugal ultrafiltrations of AgNPs suspensions at different concentrations, between 7.5 and 1800 μg/mL. Data are expressed as a percentage of silver compared to initial suspensions, before centrifugal ultrafiltrations. Each point represents a replicate while the plain line represents the mean of all values. The dotted line is set at 5%, the value used as a control in figures 4.6 and 4.7.

V

Soluble silver ions from AgNPs induce a polarised secretion of interleukin-8 in differentiated Caco-2 cells

Foreword

In the previous chapter, we investigated the effect of AgNPs on NF-κB signalling cascade, generally involved in inflammatory processes. It suggested that AgNPs were unable to induce the NF-κB cascade, and could even inhibit its activation induced by the inflammatory cocktail. This effect seemed at least partly result from AgNPs themselves. We decided to further investigate the inflammatory properties of AgNPs by examining the secretion of a pro-inflammatory chemokine, interleukin-8, by Caco-2 cells. The involvement of silver ions, as well as the possible role of Nrf2 pathway in this inflammatory chemokine secretion were evaluated. In this context, this chapter responds to the second and third objectives of the thesis.

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Soluble silver ions from silver nanoparticles induce a polarised secretion of interleukin-8 in differentiated Caco-2 cells

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Keywords

Silver nanoparticles; *in vitro*; intestinal inflammation; interleukin-8; Caco-2 cells

Highlights

- Silver nanoparticles induce the polarised secretion of interleukin-8 into the apical compartment of Caco-2 cells.
- Interleukin-8 production is Nrf2 dependent.
- This increased secretion of interleukin-8 is likely exerted by soluble Ag ions and not by nanoparticles *per se*

Abstract

Because of their antimicrobial properties, silver nanoparticles are increasingly incorporated in food-related and hygiene products, which thereby could lead to their ingestion. Although their cytotoxicity mediated by oxidative stress has been largely studied, their effects on inflammation remain controversial. Moreover, the involvement of silver ions (originating from Ag^0 oxidation) in their mode of action is still unclear. In this context, the present study aims at assessing the impact of silver nanoparticles on the secretion of the pro-inflammatory chemokine interleukin-8 by Caco-2 cells forming an *in vitro* model of the intestinal mucosal barrier. Silver nanoparticles induced a vectorised secretion of interleukin-8 towards the apical compartment, which is found in the medium 21 hours after the incubation. This secretion seems mediated by Nrf2 signalling pathway that orchestrates cellular defence against oxidative stress. The soluble silver fraction of silver nanoparticles suspensions led to a similar amount of secreted interleukin-8 than silver nanoparticles, suggesting an involvement of silver ions in this interleukin-8 secretion.

List of abbreviations

Ag^+	silver ions
AgNPs	silver nanoparticles
ARE	antioxidant response element
DLS	dynamic light Scattering
DMEM	Dulbecco modified Eagle's medium
GALT	gut-associated lymphoid tissue
HBSS	Hank's balanced salt solution
HO-1	heme oxygenase-1
IECs	intestinal epithelial cells
IFN-γ	interferon-γ
IL-10	interleukin-10
IL-6	interleukin-6
IL-1β	interleukin-1β
IL-8	interleukin-8
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
NBT	nitro blue tetrazolium
Nrf2	nuclear factor-erythroid 2-related factor
ROS	reactive oxygen species
SEM	standard error of the mean
TEM	transmission electron microscopy
TNF-α	tumor necrosis factor-α

5.1 Introduction

With the advances of nanotechnology, manufactured nanoparticles came on the market with promises of improved optical, catalytical, electronical or antimicrobial properties [1–4]. However, their smaller size and higher reactivity could also raise their toxicity. Among them, silver nanoparticles (AgNPs) are increasingly used in consumer products because of their antimicrobial properties [5]. According to the Project of Emerging Nanotechnologies [6], AgNPs are found in the highest number of consumer products containing nanoparticles, with 50% of the consumer products containing silver [6]. In particular, "health and fitness" and "food and beverages" applications are the most represented categories for AgNPs, potentially resulting in their ingestion [7]. The presence of AgNPs has also been proven in the food additive E174 added in pastry decorations such as chocolates or silver pearls [8]. Aside this example, AgNPs are not directly incorporated in food but can migrate into it from packaging materials, cooking instruments, cleaning sprays or storage boxes [5, 9–14]. Even if the potential migrated amount remains low, the increasing use of AgNPs in consumer products could dramatically raise the consumer exposure to AgNPs. Moreover, AgNPs constitute some unauthorised food supplements whose consumption can lead to up to 0.02 mg/kg BW/day exposure [15]. After ingestion, these AgNPs will come in contact with the gut. Although the cytotoxicity of AgNPs has been largely studied, their effect on inflammation remains controversial.

Because of its major role in inflammation, we decided to focus on the production of interleukin-8 (IL-8) by intestinal epithelial cells (IECs), a chemokine involved in inflammatory processes. For this purpose, Caco-2 cells were used as an *in vitro* model of the gut mucosal barrier. Although isolated from a colonic adenocarcinoma [16], they differentiate spontaneously in cells presenting characteristics similar to small intestine enterocytes such as the presence of active tight junctions and efflux pumps [17–19]. These cells are thus commonly used as a simple *in vitro* model of the small intestine in pharmaco-toxicology studies. In par-

ticular, they are the most widely used for nanomaterial translocation assessment [20, 21].

Although toxicity of AgNPs has been extensively reported in the literature, the involvement of silver ions (Ag^+) in their mode of action still remains unclear. These ions, as a subproduct of Ag^0 oxidation [22], are commonly found in AgNPs suspensions in variable proportions that can go even up to 69% of total silver content [23], depending on the synthesis method [23], the AgNPs size [24] and concentration [25] or the chemical nature of their coating [26]. Together with different chloride complexes [27], Ag^+ forms the "soluble Ag fraction" in AgNPs suspensions. Although silver salts are known to be toxic [28], only a few studies have addressed this issue. Most of the published reports concerning AgNPs have not distinguished the effect of soluble Ag from the global observed effects [29]. Papers addressing this issue have generally compared the effect of a silver salt such as acetate or nitrate as a source of Ag^+ [30–34]. Only a few tests have been performed with the soluble fraction separated from AgNPs suspensions although this was recommended by Beer *et al.* (2012) for all studies concerning metallic nanoparticles such as silver or copper [23]. In addition, AgNPs toxicity was suppressed by cysteine, which inactivates soluble Ag by complexing the ions [29]. Another way to assess the involvement of this soluble Ag is to separate it from the suspension, either by ultracentrifugation [23] or by filtration through a membrane with an appropriate cut-off [29]. Silver ions could also be separated from the rest of the suspension by ion exchange resin [25] although this is less used in literature.

The gut is a major immune organ, with about 60% of the total immunoglobulin content of the human body [35]. Within this organ the gut-associated lymphoid tissue (GALT) containing the largest pool of immune cells is found [36]. However, due to the presence of overwhelming potentially immune-stimulatory bacterial and food antigens, this tissue should respond adequately to stimulations. A complex regulation takes place in the gut to allow pathogens recognition while avoiding any unwanted response to the "normal" gut microflora. IECs form the first

barrier encountered by luminal antigens and should respond appropriately to have a role in the regulation of immune response [37]. For this purpose, they can secrete chemokines, cytokines and eicosanoids [38] to communicate with immune cells and to direct them selectively towards antigens. In particular, IL-8 is an important mediator for these cells, being the major secreted product of infected epithelial cells [39], *e.g.* Caco-2 cells [40, 41]. This chemokine produced among others by IECs, has the ability to attract neutrophils to guide them to the site of inflammation and it is thus commonly classified as a pro-inflammatory cytokine [42, 43].

As the involvement of oxidative stress in the toxicity of AgNPs has been extensively proven [21, 44–47], we have decided to evaluate whether a transcription factor *i.e.* Nuclear factor-erythroid 2-related factor (Nrf2) could be involved in this crosstalk as it orchestrates the defence against oxidative stress. Under normal conditions, Nrf2 is sequestrated by the cytosolic protein Keap 1, which leads to its proteasomal degradation. Keap1 contains a series of reactive cysteine residues, acting as a sensor through reaction with electrophiles or oxidants. This modification releases Nrf2 that then translocates in the nucleus and regulates the expression of its target genes, containing an antioxidant response element (ARE) in their promoters [48]. They modulate the cellular response to stress such as phase 2 detoxifying enzymes, thiol molecule generating system, reactive oxygen species (ROS) removing enzymes or stress response proteins [48–50]. Among these target genes, heme oxygenase-1 (HO-1) is one of the most used to assess the activation of Nrf2 as it has been probably the best characterised ARE [51]. This enzyme, also named "heat shock protein 32", catalyzes the heme degradation, releasing free iron, bilirubin and carbon monoxide [52]. Moderate levels of these three molecules could exert anti-inflammatory and anti-oxidant properties [53]. Indeed, HO-1 seems to have a major role in the protection against acute and chronic inflammation of the gut [54], its induction being associated with a protective response that contributes to the preservation of the gastro-intestinal tract [54]. Activation of Nrf2 [30, 55–59] and/or induc-

tion of HO-1 [24, 28, 58–64] have been largely observed as a response to AgNPs.

In this study, we investigated if this Nrf2 cascade could play a role in the secretion of IL-8 mediated by AgNPs as IL-8 displays an ARE in its promoter [65]. This study aims at evaluating if AgNPs could modulate the secretion of IL-8 in Caco-2 cells. Moreover, we investigated the involvement of soluble Ag and Nrf2 signalling pathway in this secretion.

5.2 Material and methods

5.2.1 Cell culture and exposure

Caco-2 cells from a human colon adenocarcinoma (clone 1 from Dr. M. Rescigno, University of Milano, IT) were cultivated between p+10 and p+30 at 37°C under a water-saturated atmosphere with 10% (v/v) CO_2. Caco-2 cells were grown in tissue culture flasks (Corning incorporated, Corning, NY) in Dulbecco modified Eagle's medium (DMEM) with 4.5 g/L glucose (Lonza, Basel, CH), supplemented with 10% (v/v) fetal bovine serum (Biowest, Nuaillé, FR), 1% (v/v) non-essential amino acids 100X (Lonza), 1% (v/v) L-glutamine 200 mM in a 0.85% NaCl solution (Lonza) and 1% (v/v) penicillin-streptomycin (Lonza). Caco-2 cells were seeded at a cell density of ca. 63 000 cells/cm^2 either in inserts (polycarbonate membrane with 0.4μm pore diameter, Costar Transwell Permeable Supports, Corning incorporated) for IL-8 quantification or in culture well plates (Corning incorporated) precoated with type I collagen (Sigma-Aldrich, St. Louis, MO) for the other experiments. Cells were cultivated during 21 days upon differentiation. The day of the experiment, differentiated Caco-2 cells were exposed in Hank's balanced salt solution (HBSS) during 3h to the different treatments, whose volume was adapted between inserts and wells taking the surface area into account.

5.2.2 Reagents

AgNPs < 20nm (NM-300K, JRC repository, Ispra, IT) were purchased from Fraunhofer institute (Schmallenberg, DE) as a dispersion in water containing 10.16% (w/w) of silver, 4% (w/w) of polyoxyethylene glycerol trioleate and 4% (w/w) of polyoxyethylene (20) sorbitan mono-laurate (Tween 20). Stock solutions of AgNPs were prepared in milliQ water (Millipore, Burlington, MA) and diluted in HBSS (Lonza) directly on cells to obtain a range of concentrations between 1.5 and 15μg/mL, which was chosen to avoid any cytotoxicity (data not shown).

The inflammatory cocktail was constituted of tumor necrosis factor-α (TNF-α) (50ng/mL), interleukin-1β (IL-1β) (25ng/mL), interferon-γ (IFN-γ) (50ng/mL) and lipopolysaccharide (LPS) (1μg/mL) (all coming from Sigma-Aldrich), and was developed to obtain the highest secretion by Caco-2 cells of targets of NF-κB pathway *e.g.* IL-8, IL-6, NO and PGE-2 as established by Van de Walle *et al.* (2010) [40].

Nitric acid (70%, highest purity), used for ICP-MS measurements, was purchased from VWR (Leuven, BE).

5.2.3 Cytokines measurement

Concentrations of cytokines IL-8, interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and interleukin-10 (IL-10) were measured with specific ELISA kits (Becton Dickinson Biosciences, Franklin lakes, NJ). Nrf2 signalling cascade was inhibited by a pre-incubation of 24h with ML-385 at 10μM (Cayman Chemicals, Ann Arbor, MI). Cells were incubated with the different treatments during 3h after which media were harvested. Cells were then washed once and culture medium was added for a duration called "the recovery period", varying between 0 and 21h. Media were collected again and used to quantify different cytokines by ELISA that contained capture and detection antibodies, recombinant standard and streptavidin-horseradish peroxidase and were performed according to the manufacturer's instructions.

5.2.4 Separation of soluble Ag from AgNPs suspensions

Soluble silver was separated from AgNPs suspensions prepared freshly at different concentrations between 7.5 and 75μg/mL in milliQ water using Vivaspin turbo 4 centrifugal ultrafiltration devices containing polyethersulfone membranes with a molecular weight cut off at 10kDa (Sartorius, Göttingen, DE). Suspensions were centrifuged on ultrafiltration devices at 3220g during 10 minutes. The absorbance of filtrates was measured between 350 and 750nm by a UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA). As AgNPs absorb at ca. 410nm, which is not the case for Ag^{+}, it allowed a control of the filtration process. No absorbance was observed for filtrates (Figure S5.3). Solutions were diluted 5 times in HBSS directly on cells to avoid a loss of silver due to precipitates with chlorides or phosphates. The process is illustrated in figure 5.1.

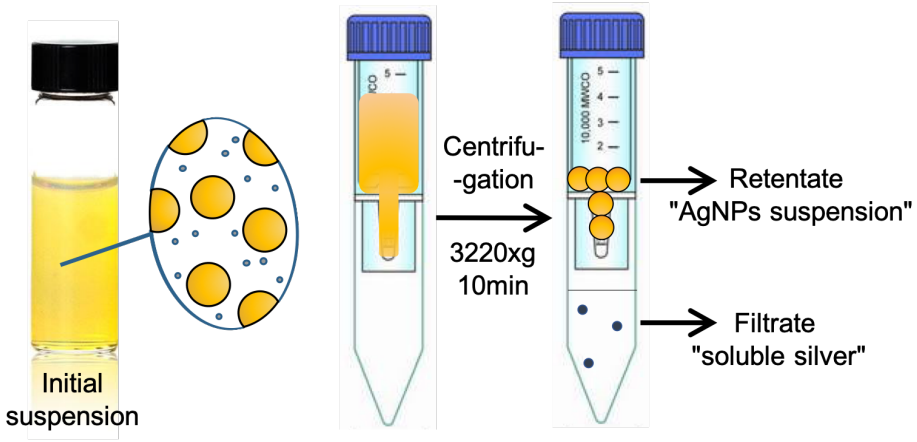

Figure 5.1: Principle of the separation between AgNPs and soluble Ag.

5.2.5 ICP-MS

Following the ultrafiltration, filtrates (containing soluble Ag) and initial suspensions (containing total silver) were acidified with 4% (v/v) nitric acid. Silver content was then quantified using an ICP-MS (7900 ICP-

MS, Agilent, Santa Clara, CA). The calibration curve was performed from 0.010 to 50 μg Ag/L with several dilutions of a multi element standard solution (Chem-lab, Zedelgem, BE, certified ISO/IEC 17025). The analytical method validity was verified every 10 measurements with 3 quality controls at 0.5, 5 and 25 μg Ag/L (Merck, Readington, NJ). The limit of quantification was 0.010 μg Ag/L. Samples were diluted so that silver concentrations were in the calibration range.

5.2.6 Involvement of oxidative stress

Oxidative stress was assayed with a soluble tetrazolium salt reacting with ROS, nitro blue tetrazolium (NBT) (Sigma-Aldrich). After 3h treatment with AgNPs, cells were washed once and incubated during 2h with NBT diluted at 0.4mg/mL in HBSS. Cells were rinsed and formazan salt was extracted with 1M NaOH aqueous solution containing 50% (v/v) DMSO (Sigma-Aldrich). Absorbance was measured at 680nm and expressed as a percentage of untreated cells.

5.2.7 Gene expression study

Cells were seeded in 6 well plates and 21 days later exposed for 3h to different treatments. Total RNA was then extracted from each well (containing *ca.* 1.10^6 cells) by a treatment with Qiazol (Qiagen, Germantown, MD) and a purification with Aurum Total RNA Mini Kit (Bio-rad Laboratories, Hercules, CA) according to manufacturer's protocol. RNA quality and quantity were estimated with a Nanodrop instrument (Thermo Fisher Scientific) measuring the absorbance at 230, 260 and 280nm. Primer efficiencies were all between 1.8 and 2.0.

Total RNA integrity was assessed by gel electrophoresis on 1.5% (w/v) agarose displaying neither contamination with genomic DNA nor degradation of RNA. 1μg of total RNA was used for retrotranscription with the iScript cDNA Synthesis Kit (Bio-rad Laboratories) following manufacturer's instructions. cDNA coming from 3 different wells similarly treated were pooled to have enough material for the qPCR analyses

and diluted to a concentration of 2ng/μL. qPCR were performed using Takyon SYBR Green low ROX (Eurogentec, Liège, BE) on a Viia7 384-well real-time PCR instrument (Thermo Fisher Scientific) according to manufacturer's instructions. A BLAST search over the human genome sequence was carried out to assess the specificity of primers. The absence of primer dimers and secondary structures was verified *in silico* with the software Amplify4 (Bill Engels, University of Wisconsin, WI). Five reference genes were used and three of them (β-actin, HPRT1 and SDHA) were selected as the most suitable for normalisation of data using the Biogazelle algorithm in qBase+ 3.2 software (Biogazelle, Ghent, BE). Primer information are reported in table 5.1 Relative expression were calculated with the $\Delta\Delta$Ct method taking into account multiple reference gene normalisation and specific primer PCR efficiencies [66]. Control cells were exposed to neither silver nor inflammatory cocktail and their expression level was set at 1. Efficiency and linearity for each couple of primers was estimated with a preliminary qPCR performed on a pool of all the samples for experimental validation and efficiencies estimation, which are reported in figure S1.

Table 5.1: Primers used in the analysis of mRNA in the current study.

Targeted gene	Accession number	Amplicon size (bp)	PCR efficiency	Primer	Sequence (5'-3')	Reference
Chemokine (CXC motif) ligand 8	NM_000584.3	125	1.916	IL-8 Forward IL-8 Reverse	CTGGCCGTGGCTCTCTTG GTAAGGACCCATCGGAGAAGC	[67]
Heme oxigenase-1	NM_002133	151	1.973	HMOX1 Forward HMOX1 Reverse	ATGACACCAAGGACCAGAGCC GGGTGGAAAGGTTTGGAGTATG	[68]
Beta actin	NM_001101.4	119	1.908	β-actin Forward β-actin Reverse	TCCAAATATGAGATGCGTTGTT AATGCTATCACCTCCCCTG	Designed
Hypoxanthine phospho ribosyltransferase 1	NM_000194.3	94	1.887	HPRT1 Forward HPRT1 Reverse	TGACACTGGCAAAACAATGCA GGTCCTTTTCACCAGCAAGCT	[69]
Succinate dehydrogenase complex flavoprotein subunit A	NM_001294332.1	86	1.891	SDHA Forward SDHA Reverse	TGGGAACAAGAGGGCATCTG CCACCACTGCATCAAATTCATG	[69]
Tyrosine 3-monooxygenase	NM_003406	94	1.922	YWHAZ Forward YWHAZ Reverse	ACTTTTGGTACATTGTGGCTTCAA CCGCCAGGACAAACCAGTAT	[70]
Glyceraldehyde-3-P dehydrogenase	NM_001256799.2	111	1.905	GADPH Forward GADPH Reverse	ACCCACTCCTCCACCTTTGAC GTCCACCACCCTGTTGCTGTA	[67]

5.2.8 Cytotoxicity assay

The cytotoxicity of ML-385, a specific inhibitor of Nrf2 pathway [71], was estimated with a LDH assay (Cytotoxicity detection kit, Sigma-Aldrich). Caco-2 cells were differentiated during 21 days in 48 well-plates. Cells were incubated during 3h in the absence or presence of ML-385 at 10μM and medium was used for LDH activity assessment according to manufacturer's instructions. Cells treated during 0.5h with Triton-X100 1% (v/v) HBSS (Sigma-Aldrich) were used as a positive control of lysis, set at 100% necrosis.

5.2.9 Statistical analysis

All statistical analyses were performed with JMP Pro14 (SAS Institute, Cary, NC). First, normality of the data distribution and homoscedasticity were verified with respectively Shapiro-Wilk test and Levene test to determine which comparison tests should be used. When data had a normal distribution and equal variances, ANOVA-1 was applied followed by a Dunnet's post-hoc analysis. In any other cases, Student's t-test were performed with type I error (α) set at 0.05 and a Bonferroni-Šidák correction of the p-values for multiple comparisons [72]. For gene expression analysis, the same t-test was applied on log-transformed data, as the reference condition (control) had no variance.

5.3 Results

5.3.1 Characterisation of the nanomaterial

The characterisation of dry particles from this batch was performed by Klein *et al.* (2011) [73]. We also performed an additional characterisation of AgNPs in HBSS (67.5μg/mL) by transmission electron microscopy (TEM) (Figure 5.2), UV-visible spectrophotometry (Figure 5.3A) and dynamic light Scattering (DLS). The hydrodynamic diameter measured by DLS was 57.75nm with a polydispersity index of 0.226. In addition,

the absorbance of soluble silver fraction was measured between 350 and 750nm as a control of the ultrafiltration process. The characteristic absorbance peak at 410nm, found in AgNPs suspensions (figure 5.3A) is not retrieved in filtrates (figure 5.3B).

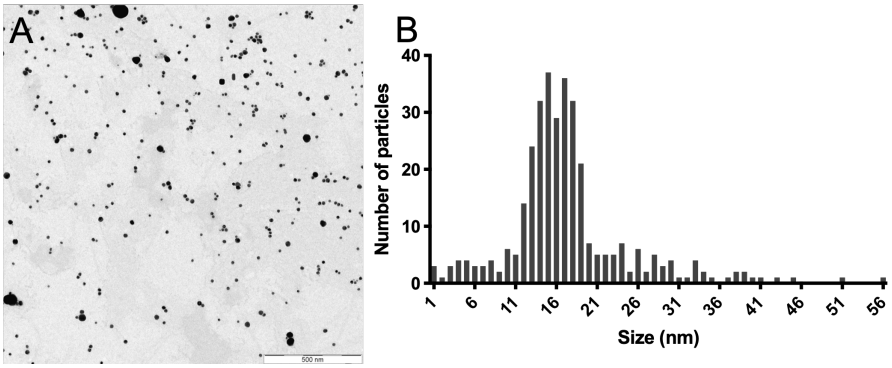

Figure 5.2: TEM image (10 000x) (A) and size distribution (B) of a AgNPs suspension (NM-300K) prepared at 67.5μg/mL and dropped on a TEM grid. The scale bar represents 500nm.

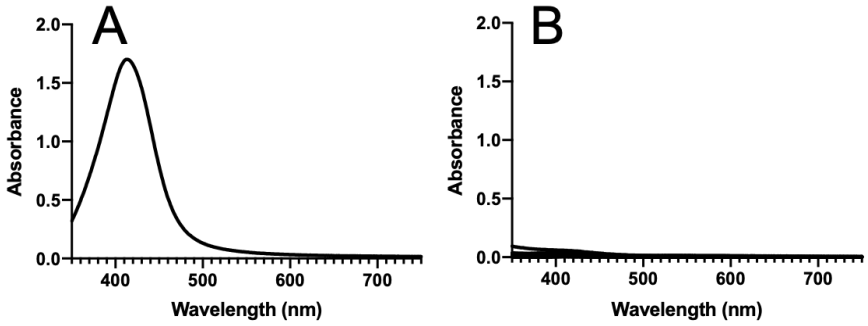

Figure 5.3: Absorbance between 350 and 750nm of (A) AgNPs suspension at 15μg/mL in water and (B) the same suspensions after ultrafiltration on 10kDa centrifugal devices.

5.3.2 Experimental setup for IL-8 measurement

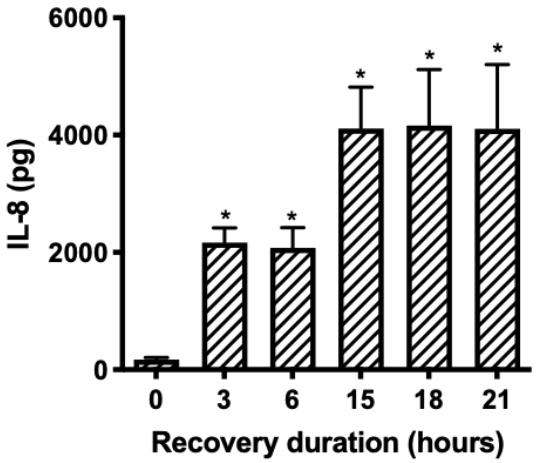

Figure 5.4: Kinetics of IL-8 secretion by differentiated Caco-2 cells after 3h exposure to the inflammatory cocktail (TNF-α, IL-1β, IFN-γ and LPS) and then allowed to recover for different durations in fresh culture medium. Samples were collected after 0, 3, 6, 15, 18 and 21h in culture medium and IL-8 was measured by ELISA. Data are expressed as mean $\pm$ standard error of the mean (SEM) of IL-8 amount secreted per well and come from 2 independent repetitions with 4 replicates. A star indicates a significant difference with the untreated cells ("Control') ($P<0.05$, Student's t-test with Bonferroni-Šidazk correction).

Differentiated Caco-2 cells were exposed to AgNPs during 3h, to mimic an acute ingestion. A preliminary experiment was performed to verify if this duration was sufficient to allow the production of the inflammatory chemokine IL-8 by testing the amount of IL-8 produced after a recovery period varying between 0 and 21h after the 3h incubation with an inflammatory cocktail composed of LPS and three cytokines, *i.e.* TNF-α, IL-1β and IFN-γ (Figure 5.4). No recovery period (0h in figure 5.4) led to a production of 175pg IL-8 per insert. The level of IL-8 protein increased with the duration of recovery, reaching a plateau after 15h recovery at 4100pg of IL-8 per insert, which suggests that IL-8 requires time to be produced and its secretion following the inflammatory stimulus is thus

delayed after the incubation.
Therefore, for the other experiments presented in this study, when IL-8 protein was measured, the 3h incubation with the different treatments was followed by a 21h recovery period to collect the total amount of IL-8 produced due to the treatments.

5.3.3 Interleukin-8 secretion in response to AgNPs exposure

The ability of AgNPs to induce IL-8 secretion was then investigated on Caco-2 cells cultivated in bicameral inserts. AgNPs increased the amount of IL-8 secreted by Caco-2 cells (Figure 5.5A). This effect was significant even at the concentration of 1.5μg/mL with 780pg and went up with the dose as 15μg/mL led to the secretion of 1135pg IL-8. However, this effect was not proportional: 2/3 of the effect was already observed with 1.5μg/mL AgNPs, suggesting that the secretion of IL-8 tends to level off with increasing concentration of AgNPs.

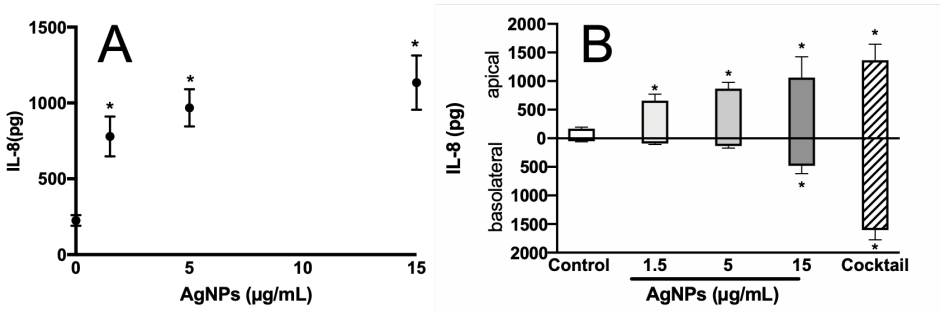

Figure 5.5: IL-8 secretion by Caco-2 cells incubated with AgNPs expressed (A) as the total (sum of apical and basolateral) secretion of IL-8 after 3h incubation with AgNPs or the inflammatory cocktail, followed by 21h in fresh culture medium, measured by ELISA and (B) by compartment (apical in the upper part, basolateral in the lower part) in the same conditions. Data are expressed as mean ± SEM of IL-8 amount secreted per insert and come from 3 independent repetitions with 3 replicates. A star indicates a significant difference with the untreated cells ("Control") ($P<0.05$, Student's t-test with Bonferroni-Šidazk correction).

IL-8 was differentially induced in either the apical or basolateral compartments (Figure 5.5B). Indeed, all tested concentrations of AgNPs led to a significant rise of IL-8 in the apical compartment while the basolateral amount of IL-8 was affected only with 15 μg/mL AgNPs. This was not the trend observed with the inflammatory cocktail, with a higher secretion in the basolateral compartment. This situation was more representative of the physiological situation, as the basolateral compartment corresponds *in vivo* to the blood.

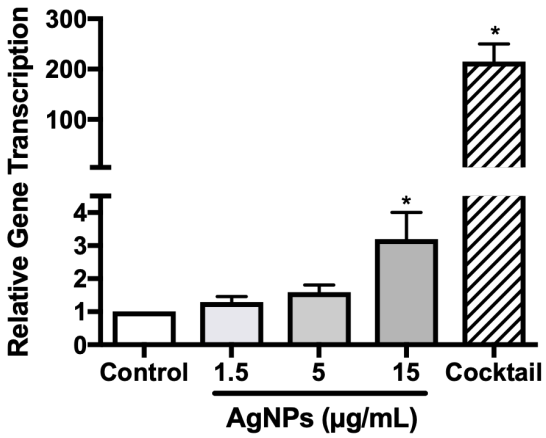

Figure 5.6: Relative gene transcription of IL-8 after 3h exposure with different concentrations of AgNPs or the inflammatory cocktail. Data are expressed as mean ± SEM and come from 4 independent repetitions with 3 technical replicates for each measure. A star indicates a significant difference with the control consisted in untreated cells ("Control") ($P<0.05$, Student's t-test with Bonferroni-Šidazk correction).

This effect of AgNPs on the expression of IL-8 after 3h incubation is confirmed at the transcriptional level, as IL-8 mRNA rose with increasing concentrations of AgNPs, up to three times for the concentration of 15μg/mL AgNPs (Figure 5.6). As expected, the inflammatory cocktail dramatically increased the amount of IL-8 mRNA.

The expression levels of IL-6, MCP-1 and IL-10 were also measured in these conditions but were below sensitivity of the ELISA for all the

experimental conditions (except IL-6 for cells incubated with the inflammatory cocktail treatment) (data not shown).

5.3.4 Effect of soluble Ag ions on IL-8 secretion

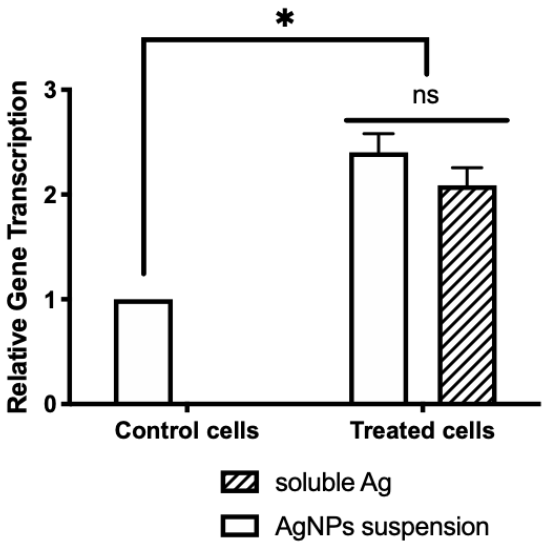

Figure 5.7: Expression of IL-8 measured as relative gene transcription of IL-8 after 3h incubation. Data are expressed as mean ± SEM and come from 4 independent biological replicates with 3 technical replicates. No significant difference was observed between soluble Ag and AgNPs although they were both different from untreated cells ("Control") ($P<0.05$, Student's t-test with Bonferroni-Šidazk correction).

AgNPs suspensions contained AgNPs but also soluble Ag deriving from the metal oxidation. A centrifugal ultrafiltration of AgNPs suspension was performed before the incubation with cells to evaluate the impact of this soluble Ag on AgNPs induction of IL-8. Initial suspensions are termed "AgNPs suspension" while the filtrates after centrifugal ultrafiltration are named "soluble Ag". No particles were able to pass through the filters (Figure S5.3). ICP-MS measurements showed that AgNPs suspensions contained up to 5% of soluble Ag, able to pass through ultrafiltration devices (Figure S2). No significant difference could be ob-

served between AgNPs and soluble Ag contained in AgNPs suspensions on IL-8 gene transcription (Figure 5.7), suggesting that the rise of IL-8 mRNA is mediated by the soluble fraction of AgNPs suspensions.

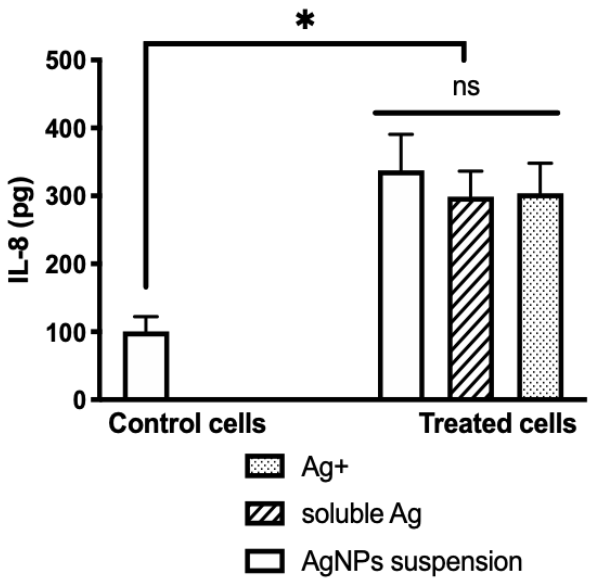

Figure 5.8: Total amount of IL-8 produced after 3h exposure to treatments followed by 21h recovery period in fresh culture medium. Treatments consists in HBSS ("Control"), AgNPs at a concentration of 15μg/mL filtrated (soluble Ag) or not (AgNPs suspension) with ultrafiltration devices or silver nitrate containing 0.75μg/mL Ag^{+}. Data are expressed as mean ± SEM and come from 3 independent repetitions with 3 replicates. A star indicates a significant difference caused by treatment, while "ns" means non-significant difference ($P<0.05$, ANOVA with Tukey-Kramer post-hoc test).

The involvement of soluble Ag was also investigated at the level of IL-8 protein, through ELISA measurements (Figure 5.8): as above, differentiated Caco-2 cells were incubated with AgNPs suspensions or soluble Ag during 3h followed by 21h recovery period and total IL-8 was measured. In addition to these conditions, a supplementary control was performed to investigate the influence of soluble Ag on this increase of

IL-8. As the majority of this soluble Ag should be in the form of ionic silver, the supplementary control consisted in a solution of silver nitrate (a soluble silver salt) containing 0.75μg/mL Ag^+ to compare it with AgNPs suspension at 15μg/mL.

AgNPs suspensions induce a significant secretion of IL-8 compared to control cells. Nevertheless, no significant difference was observed between AgNPs, soluble Ag and Ag^+, suggesting that the secretion of pro-inflammatory chemokine IL-8 is mediated by the soluble fraction of AgNPs suspensions, probably Ag ions as observed by a similar increase for a solution of Ag nitrate containing the same amount of Ag.

5.3.5 Involvement of oxidative stress and Nrf2 signalling pathway in production of IL-8

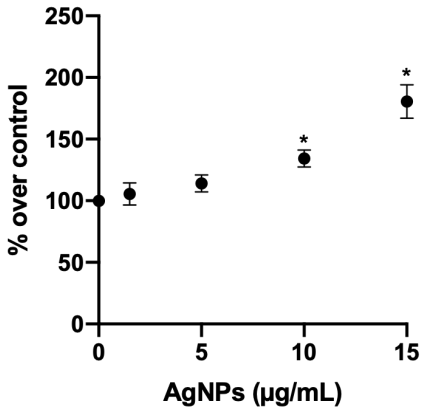

Figure 5.9: Generation of intracellular ROS measured by NBT assay, in differentiated Caco-2 cells incubated with different concentrations of AgNPs. Results are expressed as a percentage of the absorbance measured after incubation of untreated cells with NBT. Data are expressed as mean ± SEM and come from 4 independent repetitions with 3 replicates. A star indicates a significant difference with the untreated cells ($P<0.05$, Student's t-test with Bonferroni-Šidazk correction).

The generation of intracellular ROS was then evaluated with NBT reduction assay (Figure 5.9). For this experiment, cells were incubated

with AgNPs during 3h, washed, and incubated with NBT reagent during 1h. As NBT reacts with ROS to form blue formazan precipitates, it is correlated with the presence of intracellular ROS. AgNPs, from a concentration of 10μg/mL, led to a significant production of blue formazan precipitate, suggesting a generation of intracellular ROS consecutive to AgNPs presence. A positive control, *i.e.* tert-butyl hydroperoxide led to the production of 200% blue formazan precipitate compared to the untreated control (data not shown).

As the presence of superoxide anion radical is a marker of oxidative stress in cells, the involvement of Nrf2 signalling pathway, sensor of oxidative stress, was investigated. The expression of HO-1, a target enzyme of Nrf2 cascade was analysed by qPCR assay (Figure 5.10). AgNPs induced the transcription of HO-1 proportionally to their concentration. Levels of HO-1 mRNA were significantly different from untreated cells from a concentration of 5μg/mL AgNPs.

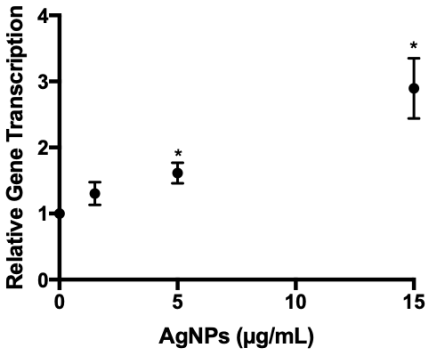

Figure 5.10: Relative gene transcription of HO-1 upon 3h exposure of differentiated Caco-2 cells with different concentrations of AgNPs. Data are expressed as mean ± SEM and come from four independent repetitions of three technical replicates. A star indicates a significant difference with the untreated cells ($P<0.05$, Student's t-test with Bonferroni-Šidazk correction).

The activation of the Nrf2 transduction cascade was further analysed using ML-385, a specific inhibitor of Nrf2 [71]. The HO-1 expression due

to AgNPs was measured in cells previously pre-incubated with ML-385 to investigate if Nrf2 pathway is involved. According to results of an LDH assay, this incubation with ML-385 was not toxic (Figure S3). The induction of HO-1 by AgNPs was counteracted when cells were pre-incubated with ML-385 in the presence of AgNPs, with a 4-fold decrease of HO-1 RNA levels. This tends to confirm that a concentration of 15μg/mL AgNPs activates Nrf2 signalling pathway and that this effect was inhibited by ML-385. The pre-incubation with ML-385 also significantly decreased the production of IL-8 mRNA by AgNPs, suggesting that Nrf2 could be a key to explain the mechanism of AgNPs induction of IL-8.

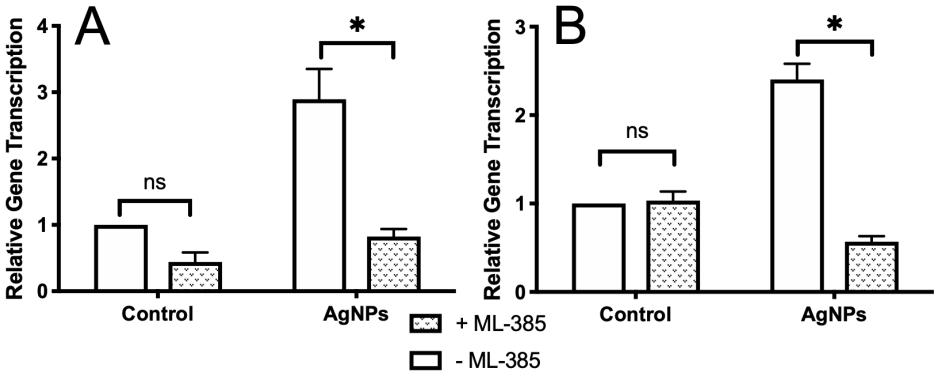

Figure 5.11: Relative gene transcription of (A) HO-1 and (B) IL-8 upon 3h exposure of Caco-2 cells to 15μg/mL AgNPs in cells pre-incubated or not during 24h with ML-385 at 10μM. Data are expressed as mean ± SEM and come from 4 independent repetitions of 3 technical replicates. A star indicates a significant difference caused by ML-385 while "ns" means non-significant difference (P<0.05, Student's t-test with Bonferroni-Šidazk correction.

5.4 Discussion

With their increasing use in food-related consumer products, it is very likely that a certain proportion of these AgNPs are ingested and that they come in contact with IECs from the gut. Although the important

role of these cells in immune regulation against luminal potential antigens has been well described, the effect of AgNPs on inflammation in IECs still remains largely unknown. This study analyses the implication of one of the key players of these mechanisms, *i.e.* IL-8 upon exposure of human intestinal Caco-2 cells to AgNPs and underlines the involvement of soluble Ag and Nrf2 signalling cascade in the secretion of IL-8. A preliminary characterisation of AgNPs was performed by TEM, DLS and spectrophotometry in the medium used for cell incubations and showed that nanoparticles were present in the material.

After a 3h incubation of differentiated Caco-2 cells with AgNPs followed by a recovery period, IL-8 levels increased time-dependently during this period, reaching a plateau after 21h. IL-8 needs time to be synthesized within the cells and to be secreted. This also suggests that the protein of IL-8 is stable and not degraded during this duration. mRNA was extracted immediately after the incubation with AgNPs to avoid its degradation, because less time is required to produce mRNA than the translated protein. Indeed, IL-8 mRNA was already produced as soon as 45 minutes after the stimulus [74], attaining a peak 2h after the treatment [75]. Moreover, it was also shown that IL-8 mRNA was not stable and decreased after these 2h [76]. The recovery period for mRNA quantification was thus not relevant.

Our results indicated that AgNPs induced the IL-8 expression in Caco-2 cells as the quantity of the corresponding mRNA and related protein increased upon exposure to AgNPs. IL-8 levels were shown to decrease in the presence of AgNPs after 3h incubation [32]. However, when cells have time to produce IL-8, this tendency is inverted and IL-8 is secreted in response to AgNPs stimulus as observed after the 21h recovery period. Similar studies have also demonstrated the production of inflammatory cytokines in presence of AgNPs and, in particular, IL-8 both at the mRNA and at the protein level, on macrophages [45, 76–79], mesenchymal stem cells [80–83], peripheral blood mononuclear cells [83, 84], liver cells [60] or keratinocytes [85]. This phenomenon occurs at subtoxic concentrations. Higher concentrations of AgNPs led to a cell

mortality, decreasing the amount of secreted cytokines [80]. In our study, the highest tested concentration of 15μg/mL was not cytotoxic (data not shown).

With regard to IECs lines, this induction of IL-8 has also been observed on Lovo cells (colonic cell line) with a AgNPs-size-dependent increase of IL-8 after 24h incubation [86]. Similarly, an increase of IL-8 due to AgNPs was shown in Caco-2 cells [21, 87, 88], which is in accordance with our observations on Caco-2 cells.

This IL-8 production by Caco-2 cells seems to be polarised towards the apical compartment, as basolateral level of IL-8 was only increased at the highest concentration of 15μg/mL AgNPs. Apical secretion of IL-8 could appear surprising, as this chemokine is involved in the recruitment of subepithelial immune cells. However, this is consistent with other studies where the stimulus was applied apically [89–91]. Moreover, the presence of IL-8 has been observed *in vivo* in the colon of healthy volunteers suggesting the apical secretion of IL-8 by enterocytes [92]. It was hypothesised that the polarised response of IECs could depend of the compartment stimulated [91]. When TNF-α was added apically, IL-8 was secreted only in the apical compartment, contrary to a basolateral stimulation with TNF-α leading to a bilateral release of IL-8. This would also explain the basolateral secretion of IL-8 following incubation with the inflammatory cocktail that consists in cytokines applied basolaterally and LPS in the apical compartment. Apically applied LPS has only a minor role in the secretion of IL-8 by Caco-2 [40, 91, 93], contrarily to basolaterally applied cytokines [40], which could polarize the secretion of IL-8 towards the basolateral compartment. In addition, it was discussed that the presence of IL-8 in the intestinal lumen could improve the chemotactic gradient of IL-8 and facilitate the transepithelial migration of neutrophils towards the inflammatory stimulus [91]. Luminal IL-8 could also have a role in autocrine signalisation between IECs based on the presence of CXCR1, a receptor for IL-8, in the apical pole of Caco-2 cells, as well as in duodenal and colonic enterocytes [43]. In addition, the apical incubation of Caco-2 with IL-8 led to changes in

the expression of genes involved in the regulation of cell differentiation and lipid metabolism, which suggests that apical IL-8 produced following AgNPs exposure could have a role in the communication between IECs [43]. glsIL-6, MCP-1 and IL-10 cytokine levels were also measured in our study but their levels remained below the detection limit. Similar results about an increase of IL-8 but not of other tested cytokines (IL-1β, IL-6 and TNF-α) have been described after 2h incubation of U937 human macrophages with 2.5μg/mL AgNPs [76].

As AgNPs are known to oxidise and to form Ag^+, the involvement of these ions in the observed effects of AgNPs should be taken into account. In this study, we separated the soluble Ag fraction from AgNPs by centrifugal ultrafiltration, which allows to discriminate particles that stay on the filter from soluble Ag ions able to pass through. As filtrates do not present an absorbance peak around 410nm such as AgNPs suspensions, it suggests that no particle was able to pass through the filter. This filtration was performed in water to avoid the formation of any precipitates. Indeed, because of the low solubility of silver salts, these Ag^+ could be present under other forms in the culture medium, with chloride salts precipitating at a higher rate than phosphate salts [94]. Ultrafiltration followed by ICP-MS measurements is commonly used to quantify the amount of soluble Ag ions present in suspensions. After separation, soluble Ag passing through the filter can also be incubated with cells and compared with total AgNPs suspensions, containing AgNPs but also soluble Ag ions. Any additional effect when cells were treated with AgNPs suspensions would therefore originate from AgNPs. The release of IL-8 after incubation of cells with AgNPs suspensions was mainly mediated by the soluble fraction of silver in this suspension. Furthermore, a silver nitrate solution containing the same amount of Ag led to a similar secretion of IL-8. This is consistent with reports that the toxicity of AgNPs suspension on A549 cells, originating from a lung adenocarcinoma, was mainly mediated by the soluble Ag fraction [23]. Some studies have also found that Ag^+ ions, in the form of silver nitrate, were able to induce the secretion of IL-8 by intestinal cells [21]. The involvement of these

ions could be a key to explain discrepancies between the different studies published about inflammatory properties of AgNPs. The importance of soluble Ag fraction relies on different parameters such as the synthesis method [23], the silver concentration [25], the size of AgNPs [24], their shape [95] or their coating [26].

NF-κB pathway is the main signalling cascade that regulates IL-8 secretion through the presence of element responses to NF-κB in IL-8 promoter [96,97]. Indeed, the inflammatory cocktail, comprised of cytokines and LPS, well-known inducers of NF-κB, induced a large expression of IL-8. However, we have shown in a previous work that AgNPs were not able to activate the NF-κB signalling cascade, and even decreased its activation by the inflammatory cocktail (unpublished results). Other signalling cascades involved in the induction of IL-8 such as Nrf2 could also modulate the expression of this chemokine because the promoter of IL-8 also contains an ARE [65].

As Nrf2 is induced as a response to oxidative stress in order to orchestrate cellular defences against oxidative stress, a NBT assay was used to show that AgNPs induced an oxidative stress, which is in agreement with other comparable studies performed on intestinal cells [30,32,98,99]. Moreover, different studies have observed a protective effect of a pretreatment of cells with N-acetylcystein, a precursor of glutathione [100] and a strong ROS scavenger, against AgNPs effects on cell viability [101], DNA damages [102], cell cycle [62,101], mitochondrial membrane potential [101] or even glucose consumption [103]. N-acetylcystein is also a silver ion chelator and this property could potentiate its protective effect on AgNPs toxicity. Similarly to N-acetylcystein, Nrf2 activation seems to alleviate deleterious effects of AgNPs. Indeed, Kang *et al.* (2012) have shown that the knockdown of Nrf2 cascade in cells increased dramatically their sensitivity to AgNPs [62, 102].

To investigate the importance of this transduction cascade in the IL-8 secretion, a specific inhibitor of Nrf2, ML-385, which attaches to the DNA binding domain of Nrf2, avoiding its interaction with the promoter of target genes and therefore reducing its transcriptional activity [71]

was used. The inhibition of Nrf2 with ML-385 led to a complete suppression of the HO-1 mRNA induction by AgNPs, as expected but also of IL-8, suggesting an involvement of Nrf2 in the AgNPs-induced IL-8 upregulation. It has been reported that an increased Nrf2 expression led to an increased expression of IL-8 at mRNA and protein level [65]. This is consistent with our observation that when Nrf2 is repressed (by the inhibitor ML-385), IL-8 transcription is blocked.

Different transcriptomic studies have indicated that Nrf2 was activated by AgNPs [30, 55–59]. In contrast, a few studies have observed a lower activation of Nrf2 in the presence of AgNPs [103, 104]. This inconsistency could be due to different physico-chemical properties of AgNPs but also a difference between cell lines [51]. For example, upon incubation with different metals, A549 cells were unable to induce Nrf2 to a high level in contrast with what was observed for HepG2 hepatocarcinoma cells. This could be explained by their high level of intracellular glutathione conferring an important antioxidative capacity. Moreover, they harbor a mutation in Keap 1, a major regulator of Nrf2 cascade [30, 51].

AgNPs activate the production of HO-1 as observed by the induction of its mRNA. HO-1 is commonly used as a target gene to assess the activation of Nrf2, because it has probably the best characterised ARE of any Nrf2 target gene [51]. HO-1 is also induced in liver cells [60], HeLa cells [28], in macrophages [76], in an alveolar barrier model [64] and in Caco-2 cells [30], suggesting an activation of the Nrf2 cascade, as its expression is known to be dependent on the cellular redox status and Nrf2 signalling pathway [105, 106]. However, it can also be induced by other pathways such as AP-1 or NF-κB [107, 108]. In our case, we have observed a complete inhibition of HO-1 production by the specific inhibitor of Nrf2, ML-385 suggesting that, in our experimental conditions, HO-1 is induced exclusively by Nrf2. Moreover, it seems that AgNPs induce HO-1 by a Nrf2 dependent pathway, as HO-1 induction by AgNPs does not occur in Nrf2 knockdown cells [102]. HO-1 protects cells from AgNPs toxicity, as the presence of a specific inhibitor decreases dramatically viability of cells while an activator of HO-1 protects them [102].

This Nrf2 pathway is activated to coordinate cellular responses to stress and seems to regulate the IL-8 secretion induced by AgNPs. Besides its pro-inflammatory properties, IL-8 could also have a protective role in enterocytes leading to the expression of genes involved in the regulation of cell differentiation and lipid metabolism, which could help to initiate responses against the potential loss of integrity due to chemicals [43].

5.5 Conclusion

In summary, Caco-2 cells produce IL-8 in response to AgNPs exposure. This secretion is polarised towards the luminal compartment and is activated, at least partially, by a Nrf2 dependent pathway suggesting the involvement of oxidative stress in this secretion. This IL-8 secretion seems to be mediated by the soluble fraction composed of Ag^{+} ions present in AgNPs suspensions, suggesting that it is not specific to nanoparticles.

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Supplementary data

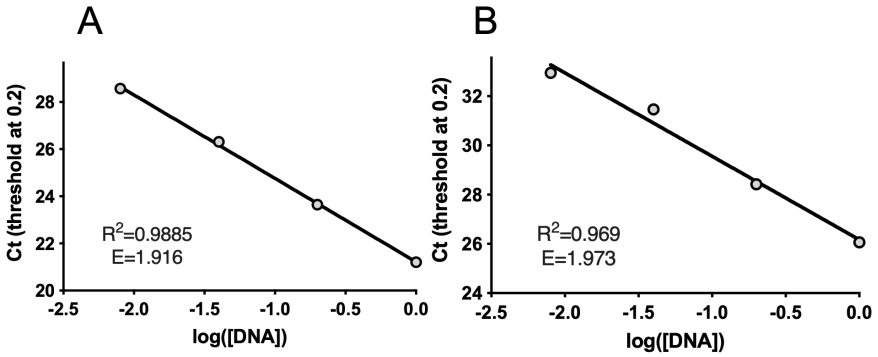

Figure S.1: qPCR efficiency estimation through calibration curves for (A) IL-8 and (B) HMOX1 genes. qPCR were performed with different concentrations of sample cDNA, represented as logarithms of cDNA concentration in ng/μL. Ct (mean$\pm$SEM) were measured with a fluorescence threshold set at 0.2 and come from three technical replicates of cDNA pooled from all samples.

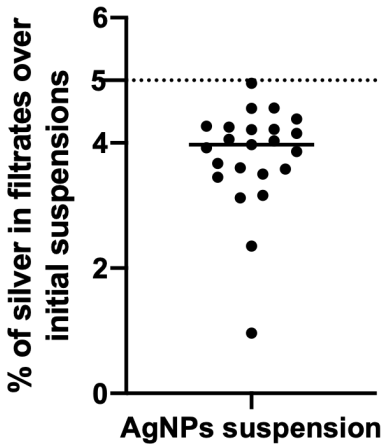

Figure S.2: Total silver measured in filtrates obtained after centrifugal ultrafiltrations of AgNPs suspensions at different concentrations, between 7.5 and 1800μg/mL. Data are expressed as a percentage of silver compared to initial suspensions, before centrifugal ultrafiltrations. Each point represents a replicate while the plain line represents the mean of all values. The dotted line is set at 5%, the value used as a control in Figure 5.8.

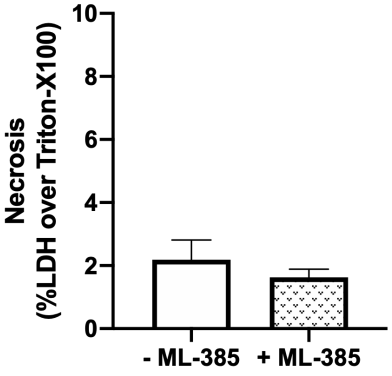

Figure S.3: Cytotoxicity of ML-385 estimated by a LDH necrosis assay in differentiated Caco-2 cells incubated 24h with ML-385. Data are expressed as a percentage of LDH activity measured in Triton-X100 treated cells, reported as mean±SEM from 3 repetitions of 2 replicates.

Part 4

Discussion

VI

General discussion & Perspectives

List of abbreviations

Ag^+ silver ions

AgNPs silver nanoparticles

EC50 effective concentration 50

HBSS Hank's balanced salt solution

HO-1 heme oxygenase-1

IL-8 interleukin-8

NMs nanomaterials

ROS reactive oxygen species

VI.1 General discussion

With the advances of nanotechnology, manufactured nanoparticles arrived on the market with promises of higher efficiency compared to bulk material. However, their smaller size and higher reactivity could also raise their toxicity [2]. Among them, silver nanoparticles (AgNPs) were introduced, largely, for their antimicrobial properties. Although ingestion seems to be a preferential route for AgNPs exposure [3] with applications varying from farm to fork [4], this exposure pathway is also less studied [5, 6]. Nevertheless, the intestinal epithelium forms a major interface in contact with nanomaterials (NMs) because of its huge exposed surface and its role of barrier to limit the contact of luminal compounds with subjacent tissues [5]. Indeed, van der Zande *et al.* showed that the highest amount of AgNPs was retrieved in the gastric wall, just followed by the small intestine epithelium in rats that had ingested AgNPs [7].

In this context, we postulated that AgNPs could affect inflammatory response of enterocytes. For this purpose, we used an *in vitro* model of the gut composed of Caco-2 cells.

As a preliminary work, we had to validate cytotoxicity assays to estimate AgNPs toxicity while minimising the risk of bias due to interferences with the nanoparticles.

Compared to conventional chemicals, the estimation of NMs cytotoxicity is complicated by their higher reactivity and small size, which could lead to various interactions with cytotoxicity assays components and lead to bias. Although this risk of interference has been largely reported in literature [8–16], only a few published studies reported the appropriate controls. Indeed, Ong *et al.* have shown that 95% of studies published in this domain in 2010 did not report any kind of interference controls [11].

In *Chapter 3*, we have observed that AgNPs interfered with all the tested cytotoxicity assays. However, based on the assay protocol and with the help of appropriate washings, it was possible to minimise over- or underestimation for three cytotoxicity assays *i.e.* the neutral red and resazurin assays and ATP content mea-

surement. In addition, we highlighted a major interference between reactive oxygen species (ROS) and MTT, a tetrazolium salt classically used in cytotoxicity assays. As a lot of toxic compounds are known to involve ROS production in their toxicity, this test should be avoided in the majority of toxicity studies and could be replaced by resazurin, which also assesses the cellular metabolic activity.

In this context of interferences, nanotoxicology experts advise to report interference assays and to use at least two cytotoxicity assays [9, 13, 16]. Nevertheless, as cytotoxicity assays generally do not measure the same viability endpoint *e.g.* lysosomal integrity, metabolic activity, energy content,..., they do not give the same toxicity results but rather a concentration range for toxic effects. Moreover, they could be compared together, to provide evidences for a general mechanism of toxicity.

In this study, we have noticed a dose-response effect to AgNPs for the three tested cytotoxicity assays. Inhibition of ATP content occurred at a lower concentration (with an effective concentration 50 (EC50) estimated around 38.3μg/mL, suggesting that it was first affected. This effect could be explained by evidences in literature of the involvement of oxidative stress in AgNPs-mediated toxicity [17–20], among others. Furthermore, *Chapter 5* indicated an induction of heme-oxygenase 1 in cells treated with AgNPs, an enzyme involved in cellular stress defences and a target gene of Nrf-2 pathway, involved in oxidative stress cellular response (as presented in *Chapter 2*). The inhibition of metabolic activity and lysosomal integrities occurred at higher concentrations of AgNPs, with an EC50 estimated around 96.4 and 98.5μg/mL, respectively. At these concentrations, ATP content is low (reaching 36.66% of the control level), which could at least partly contributes to the decrease of metabolic activity and lysosomal acidity, both requiring ATP.

Even if cytotoxicity of AgNPs has been largely studied, the comparison with these studies is almost impossible as *in vitro* NMs toxicity depends on many parameters such as their physico-chemical properties but also the cell line, the duration of exposure, the medium used to prepare NMs solutions, the cytotoxicity assays,... A part of the variation between cell lines could be attributed to different levels of antioxidants between cells and should be taken into account [21]. As a result, a complete cytotoxicity assessment, preceded by appropriate controls to avoid interfer-

ences, should be performed on different cell lines, representative of various tissues but also containing different levels of antioxidants, and should be performed for the introduction of each new NMs, even if only one property such as the size, the coating molecule, the shape,... is different from a previous one.

In addition, the *Chapter 3* should be seen as a preamble to estimate a subcytotoxic concentration range for subsequent *Chapter 4* and *Chapter 5*. Concentrations up to 15μg/mL AgNPs were not cytotoxic and could be used to estimate inflammatory properties of AgNPs without bias due to the loss of cells.

The first and second objectives of this thesis were to evaluate inflammation-related effects of AgNPs on NF-κB signalling pathway and IL-8 secretion, respectively.

In *Chapter 4*, we observed that AgNPs were unable to activate the NF-κB pathway, while they inhibited the activation of this pathway by an inflammatory cocktail composed of lipopolysaccharide and pro-inflammatory cytokines (*e.g.* TNF-α, IL-1β and IFN-γ). Even if this inhibition is generally sought for the treatment of inflammatory chronic diseases, such as Crohn or ulcerative colitis diseases, it could have deleterious impacts in the gut, as it governs also the immune response towards potential aggressors (as presented in *Chapter 2*).

In contrast, we have shown in *Chapter 5* a polarised secretion of IL-8 towards the apical compartment, confirmed by an increased expression of IL-8 mRNA. This result is intriguing, as the normal expression of IL-8 is controlled by the activation of NF-κB signalling cascade. The normal role of IL-8 is the attraction of neutrophils, which are found in the *mucosa*, at the basolateral side of enterocytes. However, the apical secretion of IL-8 could be seen as communication between enterocytes rather than an inflammatory response of cells. Indeed, Rossi *et al.* observed a presence of CXCR1, a receptor to IL-8, in the apical membrane of enterocytes, related to the regulation cell differentiation and lipid metabolism [22]. This communication between enterocytes could explain the absence of NF-κB pathway involvement and the slow release of IL-8, requiring a recovery period to be produced, while in the case of the inflammatory cocktail, this release was already seen as soon as after a 3h-

incubation.

As a pathway implicated in the response of cells to oxidative stress, Nrf2 could be involved in this AgNPs-induced IL-8 release. We have indeed observed that an inhibition of this pathway, by the good inhibitor ML385, led to a decrease of IL-8 release following AgNPs incubation. Future experiments could help to investigate this Nrf2 involvement.

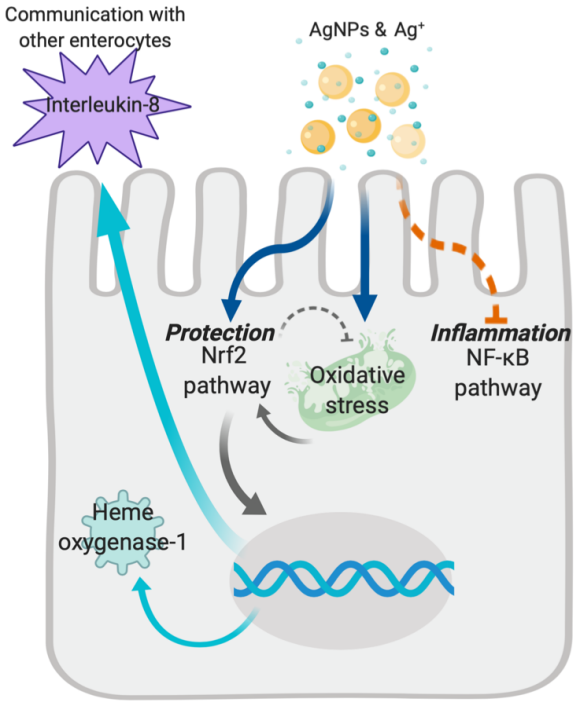

Figure VI.1 – A schematic explanation for IL-8 secretion in Caco-2 cells exposed to AgNPs during 3h. AgNPs and/or silver ions (Ag^+) seem to induce Nrf2 pathway, either directly or indirectly through ROS production while inhibiting NF-κB pathway. It could be related to heme oxygenase-1 (HO-1) and IL-8 apical secretion.

Based on these results, a possible explanation for AgNPs-induced IL-8 secretion in Caco-2 cells is reported in figure VI.1. Even if NF-κB pathway do not seem to be activated by AgNPs, they could induce Nrf2 signalling pathway either directly or

through ROS formation. This Nrf2 activation, generally related to a cellular defence against oxidative stress, seems to lead to HO-1 expression and IL-8 apical secretion, which could have a role of communication between enterocytes. It seems mainly mediated by the soluble silver fraction, composed of Ag^+, and not by nanoparticles themselves.

The third objective concerned the involvement of ionic silver in the processes investigated during this study.

Despite many studies have been published on this subject, there is still debate whether the toxicity of AgNPs is mediated by particle *per se* [23–28], by soluble silver present in AgNPs suspensions [29–31], or by a combination of both [5, 28, 32].

In AgNPs, silver is present at the +0 oxidation stage and can be oxidised in suspensions by oxygen or hydrogen peroxide, specially under acidic conditions. Indeed, all inorganic complexes and precipitates with silver are at the +1 oxidation state (Ag^+), requiring an oxidation to be formed. This oxidation process poses an additional challenge in the toxicity assessment of metallic NMs as amount of oxidised compounds are found at various extents in NMs suspensions. For instance, AgNPs suspensions could contain up to 90% of the total silver in the form of Ag^+ [33]. In this case, the observed toxicity should probably not come from the 10% of metallic silver forming nanoparticles. On the opposite, in this study, the AgNPs suspensions used contained only between 3 and 5% of silver in the form of Ag^+. As a result, due to this low amount of ionic silver, AgNPs particles *per se* should be responsible, at least partially, of the observed toxicity, as suggested by Beer *et al.* [32]. The amount of ionic silver in AgNPs suspensions depends on many parameters (Figure VI.2) *e.g.* the synthesis method [32], the AgNPs concentration [34] or size [35, 36], the coating used to stabilise AgNPs suspensions [7], or storage conditions [33]. As a result, an appropriate separation of soluble silver from AgNPs should always be performed.

In pure water, Ag^+ is soluble but in culture medium, Ag^+ moves from soluble to insoluble fraction over time, forming various kinds of inorganic complexes, either soluble such as chloride complexes *e.g.* $AgCl_3^{2-}$ and $AgCl_2^-$, or insoluble *e.g.* AgCl or Ag_3PO_4. Due to the really low value of K_s for silver sulfide, the presence

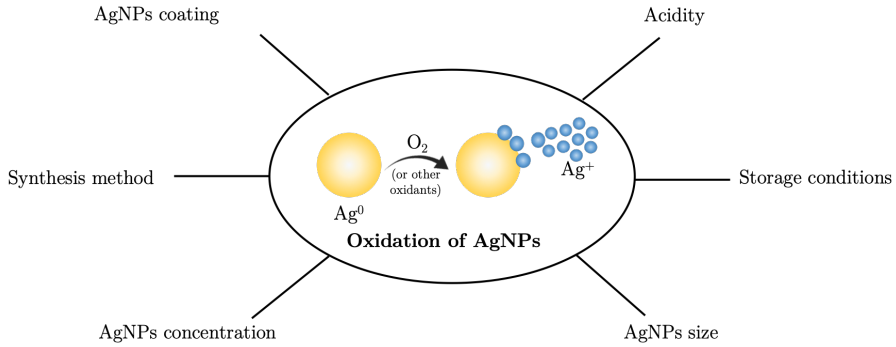

Figure VI.2 – Parameters affecting AgNPs oxidation, resulting in the formation of Ag^+ species.

of this anion could be seen as an antidote to the toxicity of AgNPs, according to Levard *et al.*, avoiding subsequent release of Ag^+ and decreasing their toxicity [37]. Silver salts are generally insoluble, as reported in figure VI.3. Even if silver phosphate is largely insoluble, its formation is less probable to occur in classical culture media than silver chloride, due to the relative concentrations of chloride and phosphate [38]. The formation of silver chloride seems to affect the oxidative dissolution of AgNPs by forming an impermeable layer of AgCl precipitated at the surface of AgNPs which limits the contact between oxygen and metallic silver in the core of the nanoparticle [30]. Several authors have indeed observed a lower toxicity in the presence of chloride [28, 39, 40], probably because the silver chloride, as a solid, is less bioavailable than soluble complexes [41, 42].

A simulation with the Medusa/Hydra chemical equilibrium software was performed to estimate the speciation of Ag^{+1} oxidation state in Hank's balanced salt solution (HBSS) (figure VI.4). The concentration of AgNPs suspension (NM-300K; the suspensions used in this study) containing the same amount of soluble silver is represented on the upper X-axis and was estimated with the ICP-MS results obtained in this thesis, around 5% of total silver. Silver chloride is insoluble while the other species, *e.g.* free Ag^+, and chloride complexes are soluble. As mentioned by Loza *et al.*, the phosphate concentration is not sufficient to form silver phosphate [38]. As these precipitation and complexation processes depends on the ratio

Compound	Formula	Ks (25°C)
Silver sulfate	Ag_2SO_4	1.20×10^{-5}
Silver chloride	AgCl	1.77×10^{-10}
Silver oxide	Ag_2O	4.00×10^{-11}
Silver carbonate	Ag_2CO_3	8.46×10^{-12}
Silver phosphate	Ag_3PO_4	2.82×10^{-18}
Silver sulfide	Ag_2S	5.92×10^{-51}

Figure VI.3 – Table of solubility constant for silver salts, adapted from Levard *et al.* [39]

between chloride and silver, the speciation of Ag^+ relies on chloride content but also on the total concentration of Ag^+. This amount is expected to remain constant over time scale used for this study, as Köser *et al.* reported that the amount of dissolved silver for NM-300K was relatively stable over time, increasing up 5.5% seven days after the preparation [43]. For the concentration range used to evaluate the effect of AgNPs on inflammation, in *Chapter 4* and *Chapter 5* (represented by the vertical red line), the predominant species of Ag^+ in this culture medium should be soluble. At higher concentrations, chosen for the cytotoxicity assessment of AgNPs in *Chapter 3*, the majority of oxidised silver should be precipitated as AgCl. In addition to this phenomenon, Li *et al.* suggested that chloride content could affect the aggregation of AgNPs, also modifying its bioavailablity [44]. An additional part of the variability between cell lines and species could thus be attributed to the various amount of chloride in their media and should be taken into account when performing studies to compare sensitivities to NMs [40]. The amount of chloride in HBSS is 140mM, in a similar concentration range than in most physiological fluids, and in particular with the chyme leaving the stomach after digestion [45, 46]. As a result, a similar speciation of Ag^+ compounds could be expected.

In this study, we obtained divergent responses concerning the involvement of silver ions in AgNPs cytotoxicity. In the first part, a cytotoxicity comparison

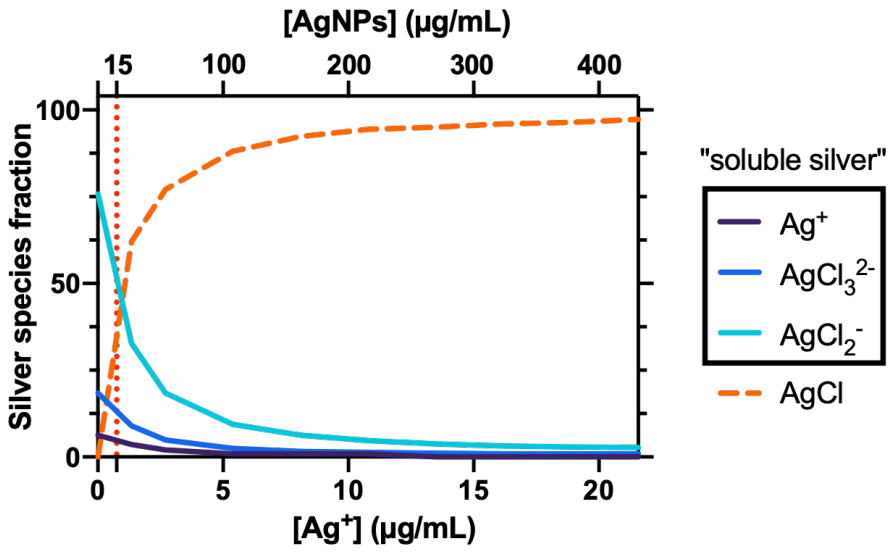

Figure VI.4 – Speciation of Ag^{+1} oxidation state compounds in HBSS, based on Ks tables at 25°C, using the software Medusa/Hydra [47]. The Y-axis represents the proportion of each silver compound, while the lower X-axis represents the total concentration of Ag^{+}. The upper X-axis represents the concentration of AgNPs containing this amount of Ag^{+}. The vertical red line represents the highest concentration used for *Chapter 4* and *Chapter 5*.

between AgNPs and soluble silver species showed that AgNPs suspensions were more toxic than the amount of soluble silver that it contained, suggesting a partial nanoparticle-specific toxicity (*Chapter 3*). The same observation was made for the inhibition of cocktail induced NF-κB pathway activation, as the concentration of ionic silver contained in AgNPs suspensions was not as efficient as AgNPs suspensions themselves (*Chapter 4*). On the opposite, the pro-inflammatory secretion of IL-8, which was polarised towards the apical pole of cells, was similar for AgNPs and the soluble silver content of AgNPs suspensions (*Chapter 5*). A part of this discrepancy could be attributed to the variation of Cl/Ag ratio. Indeed, the concentration range used for cytotoxicity assessment was higher, and for almost all tested concentrations, the predominant species of Ag^{+} was AgCl, an insoluble salt. As a result, cells were in contact with AgNPs and AgCl, but not with any soluble species. In this context, AgNPs could have an impact in the observed toxicity. In contrast, the

concentration range used for inflammatory assessment of AgNPs covers concentrations for which the main species of Ag^+ are soluble. As they are more bioavailable than insoluble forms of silver, they could explain the majority of the observed effects in the case of IL-8 secretion. However, this could not explain the nanoparticle specific inhibition of cocktail-induced NF-κB pathway. Nevertheless, it should be noted that for these experiments, the separation of AgNPs and soluble silver performed in the two other chapters was not used.

Even if the question of silver ions present in AgNPs suspensions could be addressed by a preliminary separation by ultrafiltration or ultracentrifugation, the oxidation of AgNPs could also occur inside cells. According to many authors, AgNPs are endocytosed, reach lysosomes where, due to an acidic pH, the oxidation of AgNPs is favoured, which releases silver ions. These ions could escape either by lysosomal rupture or, may be, via transporters for metals, and interact with biological molecules such as nucleic acids, metabolic enzymes (through their sulfhydryl groups) or other sulfur-containing molecules like antioxidants [28].

For toxicological risk assessment, the concentration of AgNPs to which cells are exposed should also be addressed. For this purpose, experimental concentrations obtained in *in vitro* studies should be compared with real-life scenarios. Concerning AgNPs, our dietary exposure is difficult to estimate, because, most of the time *i.e.* with the exception of some "food supplements", they are not directly incorporated in the ingested food, but are rather present after migration from surfaces in contact with food. However, due to their ubiquitous presence in the highest number of consumer products containing NMs [4, 48–51], the human exposure to AgNPs could be attributed from various sources. Even if low amounts of AgNPs migrate from these sources, their addition could lead to an important exposure to AgNPs over almost all the life. In addition, AgNPs are emerging as one of the fastest growing product from the industry of nanotechnology [52], and the human exposure is supposed to increase. An exception is the use of colloidal silver, as an unauthorised but frequent food supplement. As they are ingested, some estimations of the exposure to silver in this particular case are easier to perform. For people ingesting food supplements based on colloidal silver, the Danish Environmental Protec-

$$C_{(\mu g/mL)} = \frac{\text{Total daily intake}_{(\mu g)}}{V \,.\, n \,.\, 1000}$$

Figure VI.5 – Equation for the conversion of total daily exposure into realistic intestinal concentrations, adapted from Ribonnet *et al.* [54]. The Total daily intake is expressed in μg; V is the volume of the gastro-intestinal fluid, expressed in L per meal; n is the number of daily meals.

tion Agency has estimated an intake of 0.02 mg/kg body weight/day [10, 53], which would reach 1.5mg for a 75kg-human and could be converted to realistic intestinal concentrations by equation presented in figure VI.5. Ribonnet *et al.* stated a "dilution hypothesis" to convert daily exposure to realistic intestinal concentrations [54]. They assumed that, in the worst case, the dilution of the ingested compound occurs in 1L of gastro-intestinal fluids per meal (V=1L) with one meal per day (n=1). With these assumptions, the amount of AgNPs ingested through food supplements (1.5mg) would lead to a worst case intestinal concentration of 1.5μg/mL, used in this thesis for *Chapter 4* and *Chapter 5*. This concentration was already sufficient to increase IL-8 secretion in response to AgNPs, as well as inhibit of NF-κB activation.

To conclude, we have highlighted during this work different challenges posed by AgNPs in the estimation of their effects on cells that could also occur for NMs in general.

The toxicity of NMs depends on so many parameters *e.g.* their size, shape, the presence of coating molecules appearing during the production process or upon exposure to food or biological fluids, their composition but also NMs-independent characteristics such as cell line, chosen cytotoxicity endpoint, medium, duration of exposure ... This highlights that, unlike for conventional chemicals, an appropriate evaluation of their toxicity should be performed for each NMs with a different property. In this context, *in vitro* cellular models should help to address these questions as an attracting alternative to *in vivo* models, in particular for what concerns mechanistic items. Indeed, they are less expensive, easier to perform and without ethical restrictions, which render them more suitable for systematic and compre-

hensive toxicity assessment [8–10]. However, because of difference in sensitivity between cell lines [21], representative cell lines should be chosen as models for target body tissues such as Caco-2 for small intestinal cells. The problem of a possible dissolution or, on contrary of aggregation / agglomeration, in particular for metallic NMs should also be addressed, because it is an additional source of variation between studies. For this purpose, a separation between particles and dissolved ions could be performed freshly before each cytotoxicity assessment.

The debate about the use of nanotechnology is still ongoing, as well as regulations about its use, which are constantly changing. Even the definition of nanoparticles and nanomaterials is still debated. The recent introduction of nanomaterials specificities in the European REACH regulation, whose new obligations entered in application in 2020, could help to clarify the nanomaterials presence in products distributed over Europe. In addition, the recent interdiction of titanium dioxide nanoparticles in food in France has again focused the media attention on possible misuses of nanotechnologies. However, nanomaterials give promising opportunities, in particular in medical applications. For instance, the use of metallic particles has recently been shown to potentiate treatments against cancer, as reviewed by Kwatra *et al.* [55]. In 2016, Li *et al.* have shown an improvement by gold nanoparticles of proton therapy toxicity in A431 epidermoid carcinoma cells [56]. More recently, in 2019, Bonvalot *et al.* reported the enhancement of radiotherapy by hafnium oxide nanoparticles in a clinical trial with patients presenting advanced soft-tissue sarcoma [57]. Concerning AgNPs, they could emerge as an alternative to antibiotics to help against bacterial resistance before the development of new antimicrobial products. Nevertheless, such as other antimicrobial products, their use should be more strictly regulated and confined to medical applications such as burn treatments, but should be counterbalanced with their toxic effects, as well as the risk of bacterial resistance emergence.

VI.2 Insights for future research

- As ingested NMs underwent the mechanical and chemical digestion before

arriving in the small intestine, an appropriate evaluation of how this process could impact these NMs should be performed. Even if metallic particles are not subject to the action of digestive enzymes such as trypsin or pepsin, the presence of these enzymes, as well as the pH modifications following digestion could impact the physico-chemical properties of NMs and in turn, their toxicity. A part of this work is performed by Laurie Laloux, a PhD student working currently on this topic with AgNPs.

- In this thesis, the separation between AgNPs and silver ions was performed in water, to avoid the formation of precipitates that could decrease the amount of silver ions. If performed in HBSS, this separation would lead to two other fractions: the first one containing AgNPs and insoluble species, retained at the surface of the filters, while the second fraction would contain only soluble species *i.e.* chloride complexes and free ions. They could be used to compare the toxicity of insoluble silver with soluble silver species. As a result, for the concentration range used in *Chapter 3*, the soluble fraction should have no effect, as it should not contain any silver, while, at a lower silver concentration range, such as the one used for IL-8 secretion experiments, the soluble fraction would contain chloride complexes that could act on this secretion.

- The *in vitro* model of the intestinal barrier could be improved by the use of HT-29MTX cells to obtain a mucus layer on enterocytes [58] or by the addition of Raji cells to convert some Caco-2 cells in a M-cell phenotype [59]. Both models could be combined in a triculture to assess the effect of the mucus in combination with the presence of M-cells [60]. However, these improved models should be applied carefully, since increasing the complexity of the cell culture systems could complicate the analysis of results.

- As the difference between cell lines could be attributed at least partially to a difference in antioxidant levels [21], the involvement of Nrf2 pathway, responsible of cellular responses to oxidative stress, should be deepened. The expression of this pathway between the different cell lines should also be questioned, as some classical cell lines are from cancerous origin, which could impact their expression of Nrf2.

- In addition, for metallic particles forming insoluble salts or soluble complexes with various anions, the impact of the concentration of these anions in culture medium should be questioned, and compared to biological mediums in which NMs could be found.

- A localisation of NMs could help to investigate their mechanism of action. For this purpose, characteristics of NMs such as the presence of a plasmonic surface phenomenon, high electronic density or intrinsic fluorescence could help to visualise inside cells. A preferential presence of NMs in lysosomes, mitochondria or nuclei could be a key to assess their impacts on cells. Furthermore, it could provide evidences for a NMs-specific toxicity.

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Part 5

Appendix

A

Appendix A: List of scientific communications

A.1 Papers as first author

- M. Polet, L. Laloux, Y.-J. Schneider, "Cytotoxicity of silver nanoparticles in an *in vitro* model of the intestinal barrier", *Toxicology letters*, vol 205, supplement 1, p S270, 2018.
- M. Polet, L. Laloux, S. Cambier, J. Ziebel, A. Gutleb, Y.-J. Schneider, "Soluble silver ions from silver nanoparticles induce a polarised secretion of interleukin-8 in differentiated Caco-2 cells", *Toxicology letters*, available online, https://doi.org/10.1016/j.toxlet.2020.02.004, 2020.

A.2 Papers in collaboration

- A. Martirosyan, M. Polet, A. Bazes, T. Sergent, and Y.-J. Schneider, "Food nanoparticles and intestinal inflammation: a real risk?", in *Inflammatory bowel disease*. IntechOpen, 2012.
- A. Martirosyan, M. Polet, and Y.-J. Schneider, "*In vitro* safety assessment of nanosilver with improved cell culture systems", *BMC Proceedings*, vol 7, p10, 2013.
- A. Martirosyan, K. Grintzalis, M. Polet, L. Laloux, and Y.-J. Schneider, "Tuning the inflammatory response to silver nanoparticles via quercetin in Caco-2 (co-) cultures as model of the human intestinal mucosa", *Toxicology letters*, vol 253, pp. 36-45, 2016.
- L. Laloux, M. Polet, and Y.-J. Schneider, "Interaction between ingested engineered nanomaterials and the gastrointestinal tract: *In vitro* toxicology aspects", in *Nanotechnology in Agriculture and food science*. Wiley, 2017.
- Z.B. Barka, K. Grintzalis, M. Polet , C. Heude, U. Sommer, H.B. Miled, K.B. Rhouma, S. Mohsen, O. Tebourbi, and Y.-J. Schneider, "A combination of NMR and liquid chromatography to characterize the protective effects of Rhus tripartita extracts on ethanol-induced toxicity and inflammation on intestinal cells", *Journal of pharmaceuticals and biomedical analysis*, vol 150, pp. 347-354, 2018.

A.3 Oral presentations

- Polet M., Schneider Y.-J. (2017). Inflammatory properties of silver nanoparticles in an *in vitro* intestinal barrier model. Interuniversity PhD Student Day 2017. Liège (Belgium).
- Polet M., Laloux L., Van Craynest C. (2017). Interaction between silver nanoparticles and the gastro-intestinal tract: an *in vitro* study. Colloquium in honour of Professor Yves-Jacques Schneider. Louvain-la-Neuve (Belgium).

A.4 Posters

- Laloux L., Polet M., Claes S., Martirosyan A., Schneider Y.-J. (2013). Challenges in the risk assessment of ingested silver nanoparticles: modifications in the gastro-intestinal tract and interactions with food components. Beltox 2013 Meeting - Louvain-la-Neuve (Belgium).
- Polet M., Grintzalis K., Schneider Y.-J. (2014). Inflammatory properties of silver nanoparticles on an in vitro model of the intestinal barrier. Nanotox 2014 Meeting - Antalya (Turkey).
- Polet M., De Vos M., Schneider Y.-J. (2014). Cytotoxicity assessment of ingested silver nanoparticles on an intestinal coculture model. Beltox 2014 meeting - Geel (Belgium).
- Polet M., Laloux L., Schneider Y.-J. (2015). Silver nanoparticles interference with classical cytotoxicity assays. Nano in Belgium 2015 meeting - Bruxelles (Belgium).
- Polet M., Vandenkerckhove J., Schneider Y.-J. (2015). Silver nanoparticles effects on intestinal inflammation *in vitro*. PhD student day 2015 - Louvain-la-Neuve (Belgium).
- Polet M., Massart I., Schneider Y.-J. (2015). Silver nanoparticles effects on the NF-κB cascade activation. Beltox 2015 meeting - Antwerpen (Belgium).
- Polet M., Schneider Y.-J. (2017). Propriétés inflammatoires des nanoparticules d'argent dans un modèle in vitro de la barrière intestinale. Journées Francophones de nutrition 2017 meeting - Nantes (France).
- Polet M., Leurquin A., Schneider Y.-J. (2018). Silver nanoparticles inflammation on an *in vitro* intestinal barrier. Nanotox 2018 meeting - Neuss (Germany).
- Polet M., Cytotoxicity of silver nanoparticles in an *in vitro* model of the intestinal barrier. Eurotox 2018 meeting - Bruxelles (Belgium).

B

Appendix B: Quality controls for qPCR experiments

According to "Minimal information for publication of quantitative real-time PCR experiments" (MIQE) guidelines [61], different quality controls should be included to validate qPCR results. Some of them, such as PCR efficiency estimations, are reported in the material and methods and supplementary materials the corresponding article. Quality controls for RNA are reported in this Appendix C.

The quality and quantity of RNA were evaluated by the ratio A_{260}/A_{280} and A_{230}/A_{260}, measured in ultrapure water by a Nanodrop instrument (Thermo Fisher Scientific). Samples used for qPCR analyses had a ratio A_{260}/A_{280} above 1.96 and a ratio A_{230}/A_{260} above 1.6, suggesting an absence of genomic DNA contamination and other contaminants. However, as these ratios only provides indication of RNA purity, a gel electrophoresis was also performed.

The absence of genomic DNA contamination as well as the quality of RNA was evaluated by running RNA samples on a gel electrophoresis on 1.5% (w/v) agarose, stained with GelRed nucleic acid stain (Biotium, CA, USA). Pictures obtained are

Treatment	Sample	Cells	Concentration (ng/µL)	A260/280	A260/230
HBSS	1a	A	118,3	2,03	2,07
		B	95,8	1,99	2,11
		C	477,8	2,05	2,15
		D	201,5	2,08	2,09
	1b	A	109,7	2,1	1,95
		B	143,6	2,08	2,16
		C	228,3	2,1	2,02
		D	114,2	2,16	1,98
	1c	A	93	2,07	2,03
		B	147,1	2,1	1,97
		C	328,4	2,08	2,11
		D	136,3	2,12	2,14
AgNPs 1.5µg/mL	2a	B	95,0	1,99	2,01
		C	389,4	2,06	2,16
		D	91,1	2,06	1,74
	2b	A	114,1	2,11	2,05
		B	230,6	2,11	2,12
		D	223,5	2,11	2,24
	2c	A	126	2,11	2,17
		B	118,0	2,06	2,12
		C	327,4	2,12	2,2
		D	277,7	2,1	2,17
AgNPs 5µg/mL	3a	A	250,6	2,05	1,74
		B	109,2	2,03	2,22
		C	147,9	2,06	1,87
		D	200,8	2,08	2
	3b	A	79,8	2,09	1,94
		B	150,8	2,03	2,15
		C	245,2	2,09	2,22
		D	165,8	2,1	2,14
	3c	A	109,9	2,11	1,62
		B	108,2	2,1	2,06
		C	337,1	2,1	2,09
AgNPs 15µg/mL	4a	A	237,1	2,07	2,15
		B	138,8	2,12	2,23
		C	136,8	2,05	1,91
		D	174,9	2,08	1,66
	4b	A	131,1	2,05	1,76
		B	94,8	2,08	2,08
		C	245,7	2,06	1,93
		D	159,4	2,17	2,45
	4c	A	144,9	2,15	1,94
		C	275,8	2,11	2,17
		D	156,2	2,17	1,97

Treatment	Sample	Cells	Concentration (ng/µL)	A260/280	A260/230
Agsol 1.5µg/mL	5a	A	173,2	2,05	1,79
		C	177,8	2,06	1,86
		D	397,1	2,08	2,18
	5b	A	212	2,1	2,18
		B	74,0	1,96	1,81
		C	364,4	2,08	2,18
		D	158,6	2,13	1,84
	5c	A	161	2,14	2,12
		B	190,1	2,04	2,01
		C	389,2	2,08	2,23
		D	115,9	2,12	1,62
Agsol 5µg/mL	6a	A	268,9	2,06	2,15
		C	336,7	2,07	2,12
	6b	A	113,2	2,15	1,88
		B	174,2	2,01	1,91
		C	248,7	2,07	2,14
		D	129,1	2,13	2,34
	6c	A	135,1	2,12	1,72
		C	216,7	2,09	2,13
Agsol 15µg/mL	7a	A	141	2,04	2,03
		B	110,5	2,02	1,72
		C	117,6	2,06	2,09
		D	137,8	2,09	1,77
	7b	A	113,9	2,11	1,7
		C	357,8	2,09	2,21
		D	117,9	2,19	2,47
	7c	A	139,6	2,08	1,97
		B	123,6	2,14	2,2
		C	263,4	2,11	2,12
		D	136,4	2,12	1,97
Inflammatory cocktail	8a	A	233,9	2,03	2,07
		B	80,2	2,08	1,61
		C	137	2,06	2,13
		D	193,9	2,06	2,12
	8b	B	149,5	2,12	2,35
		C	222,4	2,08	2,04
		D	81,9	2,19	2,42
	8c	A	152,8	2,13	2,06
		B	64,7	2,31	2,48
		C	288	2,08	2,14
		D	110,2	2,1	2,37

Figure B.1 – Absorbance measurements and estimation of RNA concentration in samples and ratios by A_{230}, A_{260}, A_{280}.

presented in figure B.2. Two bands were retrieved in each sample, corresponding to ribosomal RNA and suggesting an absence of RNA degradation. No high molecular weight band was observed, suggesting an absence of genomic DNA contamination.

Before performing the reverse-transcription, cDNA coming from between 2 and 4 independent repetitions similarly treated (reported as A, B, C and D in figure B.1) were pooled to have enough material for the qPCR analyses and diluted to a concentration of 2ng/μL, based on RNA concentration estimation by the Nanodrop instrument.

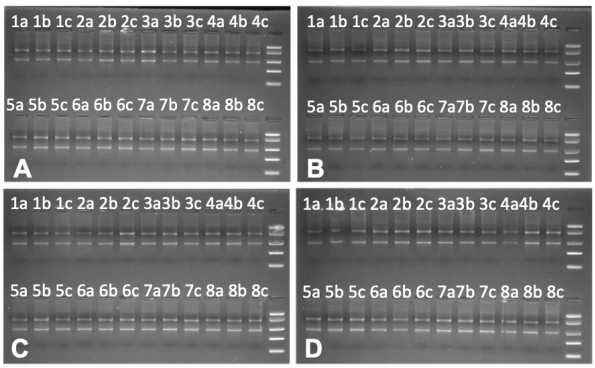

Figure B.2 – Gel electrophoresis of RNA samples after migration of a 1.5% agarosis gel.