APPENDIX

Annex A. Interference assays

Nanoparticles display a lot of unique properties such as high surface area, optical and magnetic properties and increased catalytic properties. These characteristics increase the interest on AgNPs by the industry but create also problems in toxicological studies. They can for example adsorb substances on their surface, interfere with visible and fluorescent light in absorption detection systems, and disrupt methods based on redox reactions. Interferences of AgNPs with traditional *in vitro* toxicity assays are well reported in the literature (Kroll *et al.*, 2009; Dhawan & Sharma, 2010; Monteiro-Riviere *et al.* 2009) so verifying interactions of AgNPs with assays is mandatory in any toxicological study.

In order to draw reliable conclusions from the cytotoxicity assays of this thesis, results obtained from them must be approved and validated. The interference of AgNPs with the assay reagents was thus beforehand verified for the three different cytotoxicity assays performed during this thesis: CellTiter-Glo, CellTiter-Blue and Crystal violet.

1. Methods

After preparing AgNPs solutions two times concentrated (compared to those used throughout the study: 0, 11.25, 33.75 and 101.25 µg/ml), these are mixed with assay reagents solutions followed by measurement of the absorbance or emitted fluorescence at the wavelengths used for the assays. Each interference test had two repetitions and three replicates per repetition (N=2, n=3). Interference test method and results for each cytotoxicity assay are described in detail in the next sections.

1.1 AgNPs solution preparation

First, the 4 following AgNPs solutions 2X concentrated were prepared at each AgNPs concentrations: 0, 22.5, 67.5 and 202.5 µg/ml; HBSS is used as a diluent. Only 1 ml of each solution were needed for the interference tests.

- Undigested AgNPs without food matrix.
- Digested AgNPs without food matrix.
- Undigested AgNPs with food matrix.
- Digested AgNPs with food matrix.

1.2 AgNPs interference with CellTiter-Blue®

As a reminder, CellTiter-Blue® assay is a general cell viability assay based on the reducing properties of viable cells. After incubation with the different AgNPs treatment, the remaining viable cells reduce the blue dye contained in CellTiter Blue assay, resazurin into resorufin a fluorescent purple compound. Measured fluorescence is directly proportional to the number of alive cells, or generally speaking, the cell viability.

To avoid under- or overestimations of the cell viability after cells being in contact with AgNPs, it is necessary to verify that AgNPs remaining on cells after washing will not disrupt the assay.

a) Method

First 6 ml of CTB solution was prepared, mixing HBSS (5ml) and CellTiter Blue reagent (1ml).

In a black 96 well plate, 50 µl of each AgNPs solutions 2X concentrated, 50 µl of HBSS and 100 µl of CTB solution were added per well. Triplicates have been carried out for each different treatment. Fluorescence (Ex.: 530 nm; Em.: 584 nm) is read after 30 min incubation at 37 ° C.

1.3 AgNPs interference with CellTiter-Glo®

The CellTiter-Glo assay is used to measure the content of ATP intracellularly, one of the most sensitive markers of cell viability. The reagent contains among others the luciferase, an enzyme that converts luciferin into oxyluciferin. This reaction that occurs only in the presence of ATP, emits light that can be detected with a luminometer. The quantity of light released is thus directly proportional to the amount of ATP contained in viable cells.

The disruption of proteins by AgNPs is no longer to be proven. So, any interaction with the luciferase could inactivate the enzyme and might distort results. AgNPs interference with CellTiter-Glo is measured through 2 tests: the “direct” and the “indirect” interference assays. In the first one, AgNPs interaction with CellTiter Glo reagent is directly assessed. In the indirect method, an ATP solution is added in order to induce a positive response.

a) Method

For the measure of the direct interference, each well of a white 96 well plate was filled with 50 µl of each AgNPs solution 2X, 50 µl of HBSS and 100 µl CellTiter Glo solution defrosted previously. After 2 min agitation, luminescence is read immediately during 20 min.

For the measure of the indirect interference, a stock solution of ATP was prepared at 5.8 mg/ml mixing ATP powder in HBSS. This stock solution was then diluted 50 times in HBSS, leading to a final concentration of 210.52 µM. In each well of a white 96 well plate, 50 µl of each AgNPs solutions 2X concentrated, 50 µl of ATP solution at 210.52 µM and 100 µl of CellTiter-Glo were added. Luminescence was then read during 20 minutes after a quick agitation of 2 minutes.

1.4 AgNPs interference with crystal violet dye

Crystal violet dying is an indirect method that evaluate the number of cells still adherent to the bottom of the wells. Crystal violet attaches to the DNA and proteins of the cells. So, the amount of CV fixed is correlated to the number of adherent cells. To measure the amount of fixed CV, the cell lines were first washed to eliminate dead cells and CV excess that did not get intracellularly. Then, the portion of intracellular CV was extracted and dissolved in an extractive solution. Absorbance of the solution is then correlated with the number of adherent cells.

a) Method

Two stock solutions were first prepared: a coloring solution at 0.125 % (w/v) crystal violet in 5 % (v/v) EtOH with 2% (w/v) PFA and an extractive solution containing 30 % (v/v) acetic acid in miliQ water.

Then, each well of a transparent 96 well plate was filled with 50 µl AgNPs solutions 2X concentrated, 50 µl HBSS and 100 µl of the coloring solution. The plate was incubated for 10 min at RT and then 50 µl of the extractive solution were added. The resulting solution was then diluted in the extractive solution in a new plate until an absorbance lower than 1 is reached. Absorbance is read at 595 nm.

2. Results

First, the interference with CellTiter-Blue will be examined. Regarding Figure 29, there was only a slight fluorescence, between 15 and 20 that remained constant with increasing AgNPs concentration and observed for the 4 treatments. The constancy of the curves reveal that AgNPs would not actually interfere with the assay but either would the solutions of HBSS and food matrix. We can indeed highlight the fact that the curves did not start at the origin, pointing out the intrinsic fluorescence of HBSS, digestive enzymes and the food matrix. However, since this intrinsic fluorescence was the same for all conditions, it should not interfere with the results. Besides, this fluorescence was negligible compared to the usual response of the test being about 120.

For the interference with the crystal violet, same conclusion as CTB interference can be drawn. In all treatments, the absorbance measured was nearly zero with increasing concentrations of AgNPs proving that AgNPs did not interact with crystal violet but that constituents of the digesting solutions did, in a nonsignificant way however. The intrinsic fluorescence of HBBS, digestive enzymes and food matrix was also exhibited but since it was practically the same for all treatments, it should not interfere with the results.

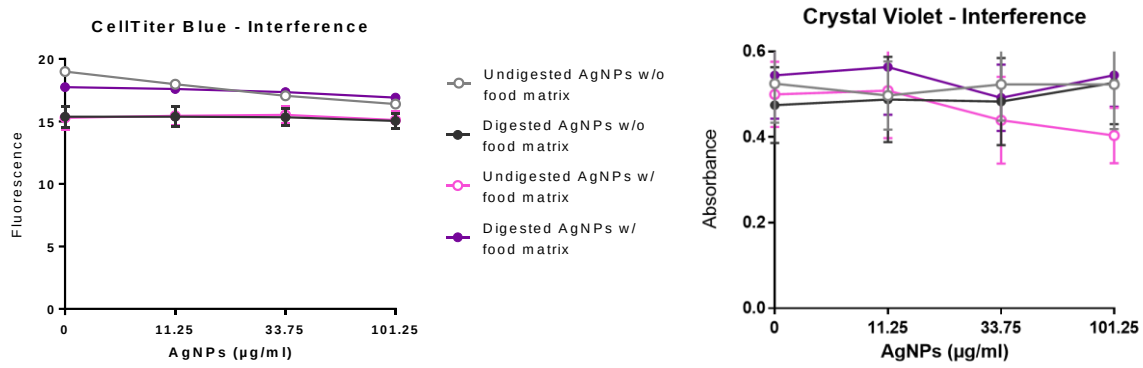

Figure 29. Interference between AgNPs at different concentrations (0, 11.25, 33.75, 101.25 µg/ml) included in HBSS or in a food matrix, digested or not, and CellTiter-Blue ® assay (left) or crystal violet assay (right). (N=2, n=3).

Looking at the interference with CellTiter-Glo assay on Figure 30, we observed that digested treatments showed a slightly higher luminescence than the undigested ones that is reduced as the AgNPs concentration rose. Luminescence is at 0.35 upon addition of digestive enzymes while no luminescence could be measured when pristine AgNPs were added with or without the food matrix. This observation highlights first, the intrinsic luminescence of digestive enzymes and secondly, the disruption of the luciferase by the digested AgNPs. However, since the values of luminescence were very close to zero (<0.5) for every treatment and negligible compared to the response obtained with cells (>100), we can assume that there is no interference with the direct method.

When ATP was added, we see that all treatments reacted in the same way, showing that AgNPs interacted indeed strongly with the assay. Luminescence was decreased by half at the highest concentrations of AgNPs revealing that if AgNPs are in contact with CTG assay, they will interfere probably with the luciferase and results should be underestimated. As the AgNPs concentration increased, viable cells will look non-viable. It must not be forgotten, however, that AgNPs treatments are removed and the cell lines washed with a buffer solution before the cytotoxicity assays. The concentrations of AgNPs remained on cells should thus very low and interference should be negligible in this case.

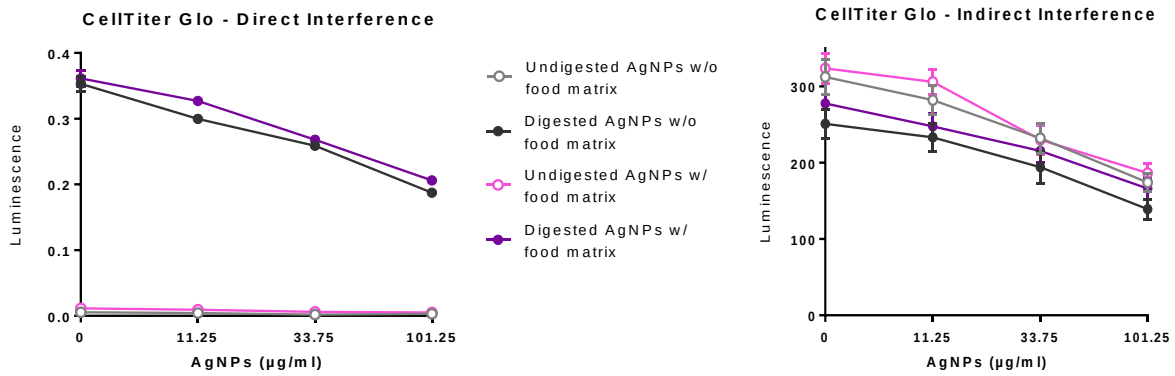

Figure 30. Direct (left) and indirect (right) interference between CellTiter-Glo® reagent and digested or undigested AgNPs solution at different concentrations (0, 11.25, 33.75, 101.25 µg/ml) included in HBSS or in a food matrix (N=2, n=3).

As a general conclusion of all interference assays, AgNPs are assumed to not interfere in a significant way with the reagents of CellTiter-Blue, CellTiter-Glo and crystal violet assays. Indeed, AgNPs treatments were removed from the cell lines before testing the toxicity so interference is unlikely to happen in the same way as the previous tests have demonstrated. The interference shown here, is for the matter, negligible whereas AgNPs concentrations tested were those used in the experiments and that would probably not be in contact with the assays since AgNPs are removed from cells after the treatment and cells are rinsed.