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Pan-European inter-laboratory studies on a panel of in vitro cytotoxicity and pro-inflammation assays for nanoparticles

Jean-Pascal Piret[1] · Olesja M. Bondarenko[2] · Matthew S. P. Boyles[3] · Martin Himly[3] · Ana R. Ribeiro[4] · Federico Benetti[5,14] · Caroline Smal[6] · Braulio Lima[7] · Annegret Potthoff[8] · Monica Simion[9] · Elise Dumortier[1] · Paulo Emilio C. Leite[4] · Luciene Bottentuit Balottin[4] · José Mauro Granjeiro[4] · Angela Ivask[2,15] · Anne Kahru[2] · Isabella Radauer-Preiml[3] · Ulrike Tischler[3] · Albert Duschl[3] · Christelle Saout[1] · Sergio Anguissola[10] · Andrea Haase[11] · An Jacobs[12] · Inge Nelissen[12] · Superb K. Misra[13] · Olivier Toussaint[1]

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Abstract The rapid development of nanotechnologies and increased production and use of nanomaterials raise concerns about their potential toxic effects for human health and environment. To evaluate the biological effects of nanomaterials, a set of reliable and reproducible methods and development of standard operating procedures (SOPs) is required. In the framework of the European FP7 NanoValid project, three different cell viability assays (MTS, ATP content, and caspase-3/7 activity) with different readouts (absorbance, luminescence and fluorescence) and two immune assays (ELISA of pro-inflammatory cytokines IL1-β and TNF-α) were evaluated by inter-laboratory comparison. The aim was to determine the suitability and reliability of these assays for nanosafety assessment. Studies on silver and copper oxide nanoparticles (NPs) were performed, and SOPs for particle handling, cell culture, and in vitro assays were established or adapted. These SOPs give precise descriptions of assay procedures, cell culture/seeding conditions, NPs/positive control preparation and dilutions, experimental well plate preparation, and evaluation of NPs interference. The following conclusions can be highlighted from the pan-European inter-laboratory studies: Testing of NPs interference with the toxicity assays should always be conducted. Interference tests should be designed as close as possible to the cell exposure conditions. ATP

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✉ Jean-Pascal Piret
jean-pascal.piret@unamur.be

✉ Olivier Toussaint
Olivier.toussaint@unamur.be

1 Research Unit in Cellular Biology (URBC), Namur Nanosafety Center (NNC), NAmur Research Institute for Life Sciences (NARILIS), University of Namur (UNamur), Namur, Belgium

2 Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics (NICPB), Tallinn, Estonia

3 Division of Allergy and Immunology, Department of Molecular Biology, University of Salzburg, Salzburg, Austria

4 Directory of Life Sciences Applied Metrology, National Institute of Metrology, Quality and Technology, Rio de Janeiro, Brazil

5 ECSIN-European Center for the Sustainable Impact of Nanotechnology, Veneto Nanotech S.C.p.A., Rovigo, Italy

6 Straticell Screening Technologies, S.A., Les Isnes, Belgium

7 Department of Biochemistry and Immunology, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

8 Fraunhofer-Institut für Keramische Technologien und Systeme (IKTS), Dresden, Germany

9 Laboratory of Nanobiotechnology, National Institute for Research and Development in Microtechnologies - IMT Bucharest, Bucharest, Romania

10 Centre for BioNano Interactions, University College Dublin, Belfield, Dublin 4, Ireland

11 Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany

12 Environmental Risk and Health Unit, Flemish Institute for Technological Research (VITO), Mol, Belgium

13 Materials Science & Engineering, Indian Institute of Technology Gandhinagar, Gandhinagar, India

14 Present Address: ECSIN-European Center for the Sustainable Impact of Nanotechnology, ECAMRICERT srl, Rovigo, Italy

15 Present Address: Future Industries Institute, University of South Australia, Mawson Lakes, Australia

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and MTS assays gave consistent toxicity results with low inter-laboratory variability using Ag and CuO NPs and different cell lines and therefore, could be recommended for further validation and standardization. High inter-laboratory variability was observed for Caspase 3/7 assay and ELISA for IL1-β and TNF-α measurements.

Keywords Inter-laboratory studies · Standard operating procedures · Interference · In vitro assays · Nanoparticles

Introduction

Nanomaterials (NMs) display many unique and enhanced properties (electrical, chemical, mechanical) compared to conventional materials (Hussain et al. 2015). Nowadays, an increasing number of products containing nanoparticles (NPs) are already available on the market. However, even if important efforts have been done to evaluate the safety of NMs, it is not yet well defined how NMs should be classified and labeled when following the globally harmonized system (GHS) (Schulte et al. 2014). An increasing number of studies assessed adverse effects of NMs on human health and the environment, thus contributing an essential part to the responsible development of nanotechnology (Arora et al. 2012; Kuempel et al. 2012; Oomen et al. 2014). In vitro toxicity testing is usually the initial phase to evaluate the potential health effects of chemicals (including manufactured NMs) and their mechanisms of toxicity (Ivask et al. 2014; Piret et al. 2012a). However, the major concern arises from the lack of international harmonized protocols to evaluate toxicity of NMs. To our best knowledge and agreeing with Nogueira et al. (2014), in vitro methods currently used for toxicological evaluation of chemicals and drugs have not been adapted and validated for NMs (Hartung 2008).

Substantial uncertainties, due to serious gaps in understanding the nano–bio interactions, biological uptake mechanisms, fate, distribution, bio-accumulation, are slowing down the risk assessment of NMs (Kuempel et al. 2012; Ribeiro et al. 2016). In the literature, conflicting and contradicting laboratory data on nanotoxicity started to emerge (Krug and Wick 2011). These discrepancies in observed results could result from different parameters such as different origin of NPs, various NPs production processes, presence or not of dispersant, different dispersion procedures, use of different test media, various assay procedures, and NPs interference with assays (Bondarenko et al. 2013; Kakinen et al. 2011; Vankoningsloo et al. 2010, 2012). For example, lactate dehydrogenase (LDH) assay used to evaluate the membrane integrity yielded erroneous results for Cu NPs and Cu–Zn NPs toxicity toward lung epithelial cells A549 in vitro (Karlsson et al. 2013), whereas carbon nanotubes were shown to interfere with MTT assay (Worle-Knirsch et al. 2006). Moreover, there is an absence or limited description of NMs physicochemical properties in nanotoxicological studies. These parameters largely determine NMs cellular uptake, transport, and fate (Zhu et al. 2013), but without adequate reporting of these properties, QSAR and QNAR approaches to model the toxicity of NMs are seriously hampered. In addition, an urgent need to adapt assays for NPs testing has been pointed out by the Organisation for Economic Co-operation and Development (OECD) in order to develop a set of methods suited for NPs toxicity evaluation (OECD 2009, 2013; Rasmussen et al. 2016).

Taking this into consideration, the main aim of EU FP7 project NanoValid was to develop and evaluate a set of reliable reference methods for physicochemical characterization and hazard identification of selected well-characterized NPs. In this study, NanoValid consortium partners mainly worked with silver (Ag) NPs (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2010)46&doclanguage=en) and copper oxide (CuO) NPs.

Ag NPs possess broad-spectrum bactericidal, virucidal, antifungal properties enabling their wide use in healthcare applications (Lansdown 2006; Sondi and Salopek-Sondi 2004). They have been introduced in the surface of medical devices such as surgical instruments, cardiovascular and contraceptive devices, contact lenses and bandages, preventing patients from infection diseases (Chen and Schluesener 2008; Cohen et al. 2007; Ge et al. 2014; Vigneshwaran et al. 2007). In parallel, Ag NPs have also been used in consumer goods like textiles (Lee et al. 2007; Vigneshwaran et al. 2007) as well as in washing machines and refrigerators. Therefore, the possibilities of exposure to the human body have increased.

CuO NPs are applied to different areas, including gas sensors catalysis, batteries, high temperature superconductors, solar energy conversion, and field emission emitters. Thus, they are good representatives of industrial NPs relevant to occupational risk assessment (Dutta et al. 2003). Besides that, CuO is an antimicrobial agent used in many consumer products (Gabbay et al. 2006).

In the framework of the FP7 NanoValid project, we addressed, by pan-European inter-laboratory studies (ILS), the suitability and the reliability of three different cell viability assays (MTS, ATP content, and caspase-3/7 activity) with different readouts (absorbance, luminescence, and fluorescence, respectively) and inflammation assays (TNF-α and IL-1β ELISAs) to evaluate Ag NPs and CuO NPs toxicity and pro-inflammatory effects. Common protocols for both cell cultures and in vitro assays were developed and shared between all consortium partners as well as same stock of cells and fetal bovine serum (FBS) batches. As NPs have the potential to interfere with assays at several stages, special attention was paid to assay interference and specific procedures to address this issue were included in the protocols of

Table 1 Summary of main physicochemical properties of Ag and CuO NPs obtained by transmission electron microscopy (TEM), Brunauer–Emmett–Teller (BET), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS)

NPs	Silver	Copper oxide
Supplier	Colorobbia	Intrinsiq materials
Form	PVP-stabilized colloidal suspension	Powder
Shape	Spherical	Spherical
TEM size	20.4 ± 6.8 nm (20.7 nm)	24.5 ± 8.1 nm (24.4 nm)
BET surface area	Not available	22.8 ± 3.7 m^2/g
XRD crystalline phase	Phase pure elemental silver	Tenorite
XPS elemental composition	Presence of C, H, and O indicating the presence of organic surfactant	Denoted the particles to be of CuO and not Cu_2O

Data obtained by TEM (median in brackets) and by BET were expressed as mean ± standard deviation (SD)

the in vitro tests. This study was one of few attempts to test the suitability of several in vitro assays by ILS at international level (Bonner et al. 2013; Xia et al. 2013).

Materials and methods

Nanoparticles characterization

Ag NPs were purchased from Colorobbia as 4% colloidal aqueous suspension stabilized by polyvinyl pyrrolidone (PVP). CuO NPs were supplied as powder by Intrinsiq Materials Ltd. Ag and CuO NPs were characterized using a range of analytical techniques and through inter-laboratory comparison studies. Hence, equipment details for characterization are not mentioned. The results obtained from the inter-laboratory characterization studies are summarized in Table 1. Shape and size of primary NPs were evaluated by TEM. Droplets from CuO NPs powder dispersed in ethanol or from 4% Ag NPs aqueous suspension were placed on TEM grids (carbon-coated copper grids) and dried under ambient conditions prior to TEM analysis. For BET analysis, CuO NPs powder was subjected to surface area measurements using nitrogen as the adsorbent gas molecules. Crystallography (XRD) and chemical composition (XPS) were conducted on CuO NPs powder or after drying of 4% Ag NPs aqueous suspension.

Preparation of nanoparticles stock suspension for in vitro studies

Ag NPs 4% stock solution was vortexed for 30 s on a benchtop vortex at full speed before dilution into cell culture medium. CuO NPs stock solution was prepared freshly at 1 mg/ml in distillated water by sonication before being diluted into cell culture medium for in vitro experiments. To minimize variation in sonication procedure between partners, ultra-sonication was performed at identical specific energy (E_{spec}). E_{spec} (KJ/m^3) is a function of the delivered acoustic power P (W), time t (s), and sample volume V (m^3) and therefore independent of used ultrasonic device. It was calculated using the following equation:

$$E_{spec} = \frac{P \times t}{V}$$

In this study, we used an E_{spec} of 6.318×10^5 kJ/m^3. After calorimetrically determination of P, each partner calculated the required sonication time to obtain the same E_{spec}.

Table 2 Mean diameter of Ag and CuO NPs in water or cell culture medium (CCM) suspensions at the beginning (0 h) and at the end of in vitro experiment (24 h) measured by DLS or CLS

Particles	Media	Technique	0 h (nm)	24 h (nm)
Ag NPs	Water stock solution	DLS	20, 116	
	A549 CCM		146	131
	THP-1 CCM		137	249
CuO NPs	Water stock solution	CLS	41 (*n*), 68 (*w*)	
	HepG2 CCM		33 (*n*), 60 (*w*)	21 (*n*), 77 (*w*)
	Caco-2 CCM		33 (*n*), 59 (*w*)	22 (*n*), 81 (*w*)

Size distribution of Ag and CuO NPs in water stock solution and cell culture medium

Size distribution of Ag NPs was performed using dynamic light scattering (DLS; Delsa Nano Beckman Coulter) in aqueous stock solution and after dilution at 12 µg/ml (highest concentration used during in vitro assays) in cell culture medium at the beginning (0 h) and at the end of in vitro experiment (24 h). A bimodal size distribution was observed, with primary particles at 20 nm and agglomerates around 116 nm in water stock solution (Table 2). In the different culture media, size distribution of Ag NPs was similar regardless of incubation time. A slight increase in the mean Ag NPs hydrodynamic diameter was only

observed after 24 h of incubation in THP-1 cell culture medium (Table 2).

Size distribution of CuO NPs in water stock solution and in HepG2 and Caco-2 cell culture media (100 µg/ml, corresponding to the highest concentration used in in vitro assays) was analyzed by centrifugal liquid sedimentation (CLS) using a DC24000 system Disc Centrifuge (CPS Instruments Inc.). Using weight (*w*) and number (*n*) distribution, mean size of agglomerates and primary particles was determined, respectively (Table 2). Primary particles and agglomerates of similar size were measured in all media (water and cell culture media) regardless of incubation time.

Cell culture and incubation

Different cell lines representing the main routes of exposure to NPs (inhalation, ingestion) and immune system were selected for this study. All cells were used at passages $X + 2$ to $X + 20$.

Respiratory tract

Human alveolar epithelial A549 cells were originally purchased from the American Type Culture Collection (ATCC). A549 cells were grown in modified Eagle Medium (MEM) with glutamax (Gibco-Invitrogen) + 10% fetal bovine serum (FBS, Gibco-Invitrogen) at 37 °C in a humidified 5% CO_2 atmosphere. For viability assays, A549 cells were seeded into 96-well plate at 10,000 cells/well.

Digestive tract

Human hepatoma HepG2 cells purchased from ATCC were grown in Dulbecco's Modified Eagle Medium (DMEM) with glutamax (Gibco-Invitrogen) + 10% FBS under standard cell culture conditions (37 °C, 5% CO_2, humidified). HepG2 were used at 10,000 cells/well in 96-well plate for MTS assay.

Human colon carcinoma Caco-2 cells obtained from the European Collection of Cell Cultures (ECACC) were cultured in Dulbecco's Modified Eagle Medium high glucose 4.5 g/l (DHG) with L-glutamine without pyruvate (Gibco-Invitrogen) supplemented with 1% nonessential amino acids and 10% FBS (37 °C, 5% CO_2, humidified). A density of 5000 cells/well (96-well plate) was used for MTS assay.

Immune system

Human monocytic THP-1 cells purchased from ECACC were grown in RPMI 1640 containing glutamax and Hepes (Gibco-Invitrogen) + 10% heat inactivated FBS (30 min at 56 °C) (37 °C, 5% CO_2, humidified). THP-1 cells were maintained at 2–8 × 10^5 cells/ml. Differentiation of THP-1 cells was induced by adding 0.5 µM phorbol 12-myristate 13-acetate (PMA, Sigma) for 24 h. Differentiated cells were washed with phosphate buffered saline (PBS, Sigma) before NPs exposure. For viability assays, differentiated THP-1 cells were treated at 50,000 cells/well in 96-well plates. For measurements of IL-1β and TNF-α secretion, THP-1 cells were seeded in 24-well plates at 300,000 cells/well to respect the same amount of cells per cm^2 between different sized well plates.

One percentage of antibiotics (penicillin–streptomycin) was added into all culture media only during incubation with NPs.

In order to reduce inter-laboratory variability due to cell culture conditions, all partners used same cell lines and FBS batches and followed the same culture protocols that were developed by the NanoValid consortium (THP-1, HepG2 and Caco-2 cell culture SOPs; available at www.nanovalid.eu) or adapted from the preexisting protocol from the FP7 European QualityNano (A549 cells). For THP-1 cell differentiation into macrophages, a common PMA batch was used by all partners. SOPs for the different assays were also set up within this consortium project or adapted from QualityNano preliminary protocols (MTS, ATP assays) and used by all partners.

Staurosporine (STS; Proteinkinase.de, PKI-STSP-001) and benzalkonium chloride (BC; Sigma, B6295) were used as positive control for MTS, ATP content, and caspase 3/7 activity assays. Lipopolysaccharide (LPS, L2880, Sigma-Aldrich) was used as positive control for ELISAs.

In vitro inter-laboratory studies

In vitro ILS described here were organized in the framework of the FP7 European project NanoValid including up to seven laboratories. The schematic representation of the tests performed is shown in supplementary Fig. 1 (Electronic Supplementary Material).

For the MTS, ATP content, and caspase 3/7 assays, a common 96-well plate layout (Rosslein et al. 2015) was used (supplementary Fig. 2; Electronic Supplementary Material).

The cytotoxic effect of increasing concentrations of NPs and STS-positive control was evaluated by technical triplicate on each plate. Experiments were repeated, in general, to obtain three biological replicates.

All wells received identical volumes of culture medium containing original NPs suspension solvent (water). Cell-free wells containing either cell culture medium alone (B7–G7) or positive control/NPs concentrations (columns 2 and 11, respectively), were included in each plate to serve as

blanks allowing the evaluation of assay background and serving as internal interference controls, respectively (supplementary Fig. 2; Electronic Supplementary Material).

Several acceptance criteria were defined, and only experiments passing these tests were considered:

- The absolute viability of the cells used in the assay has to be higher than 90% when cell seeding is performed.
- The cells in no-treatment wells (column 6) do not show obvious cytotoxicity at the end of the exposure period, as observed by microscopic observation.
- The coefficient of variation of the average luminescence/absorbance/fluorescence value of no-treated cells is lower than 30%.
- The difference between the average luminescence/absorbance/fluorescence value of the cells in no-treatment wells (column 6, 6B–6G) and the average luminescence/absorbance/fluorescence value of the cells in line B (B3–B5, 0 nM of STS and B8–B10, 0 µg/ml of NPs) should not be upper than 15% allowing estimation of cell seeding variability.
- The positive control treatment should induce a dose-dependent effect, and an effect of at least <70% cell survival (or significant higher caspase 3/7 activity) should be obtained, as compared to nontreated cells (for which the average cell survival is set to 100%).

To determine whether a test showed a low or a high inter-laboratory variability, the mean biological value of each laboratory, at each concentration, was compared to the overall mean (black curve in the different graphs).

High inter-laboratory variability was assumed when more than one laboratory with for at least three concentrations displayed biological values varying from the overall mean by more than one time the overall standard deviation (SD).

In addition, assays showing different biological profiles between laboratories were defined as high inter-laboratory variability assays.

MTS reduction assay

The conversion of MTS tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) into its reduced formazan form was assessed with the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, cells were incubated with MTS solution for 1–3 h at 37 °C before measuring 490-nm absorbance. Results were expressed as percentages of non-treated control cells. More details about complete procedure can be found in MTS SOPs available on NanoValid website (www.nanovalid.eu).

ATP content assay

After NPs incubation, A549 cells were washed with PBS (100 µl/well; 96-well plate) before being incubated with cell lysis solution (ATPLite, PerkinElmer) for 5 min. Cell lysates were transferred to a white flat-bottom 96-well plate. Substrate solution containing recombinant firefly luciferase and its substrate D-luciferin was added, and plate incubated for 5 min at room temperature on an orbital shaker at 700 rpm. Luminescence was measured in a microplate reader at 22 °C, and results expressed as percentages of controls. More details about complete procedure can be found in ATP SOP available on NanoValid website (www.nanovalid.eu).

Caspase 3/7 activity assay

Pro-apoptotic effect of Ag NPs was evaluated throughout detection of caspases 3 and 7 activation. A549 cells were seeded in a black 96-well plate with clear bottom (PerkinElmer) at a density of 10^4 cell/well. After 24 h of NPs incubation, 100 µl of Apo-ONE homogeneous caspase 3/7 Reagent (Promega) was added to wells, and plate shaken for 30 s before incubating 2 h at 25 °C in the dark. Caspases 3/7 activation was evaluated exciting rhodamine 110 at 485 ± 20 nm and recording fluorescence intensity at 528 ± 20 nm. Results were expressed as percentages of non-treated control cells. More details about complete procedure can be found in caspase 3/7 assay SOP available on NanoValid website (www.nanovalid.eu).

TNF-α and IL1-α ELISA detection

The pro-inflammatory potential of Ag NPs, at sub-toxic concentrations (determined by MTS assay), was evaluated by determining the tumor necrosis factor (TNF)-α and interleukin (IL)-1β release from the THP-1 cell line.

In order to have a similar Ag NPs dose per cm^2, and thus per cell, for the investigation of the pro-inflammatory response of NPs (in 24-well plates) and the evaluation of their toxicity (MTS assay; in 96-well plates), the volume of cell culture medium added per well was 600 µl instead of 500 µl commonly used with 24-well plates (supplementary Table 1; Electronic Supplementary Material). In addition, cell number per well was adapted to obtain similar cell densities (3×10^5 cells/well in 24-well plate; 5×10^4 cells/well in 96-well plates) independently of well plate used.

After incubation for 24 h with Ag NPs, cell culture medium was collected and centrifuged for 10 min at 14,000*g*. Quantification of TNF-α and IL-1β present in supernatants was performed using commercial ELISA kits (DY201-05, R&D Systems for human IL-1β; 900-K25,

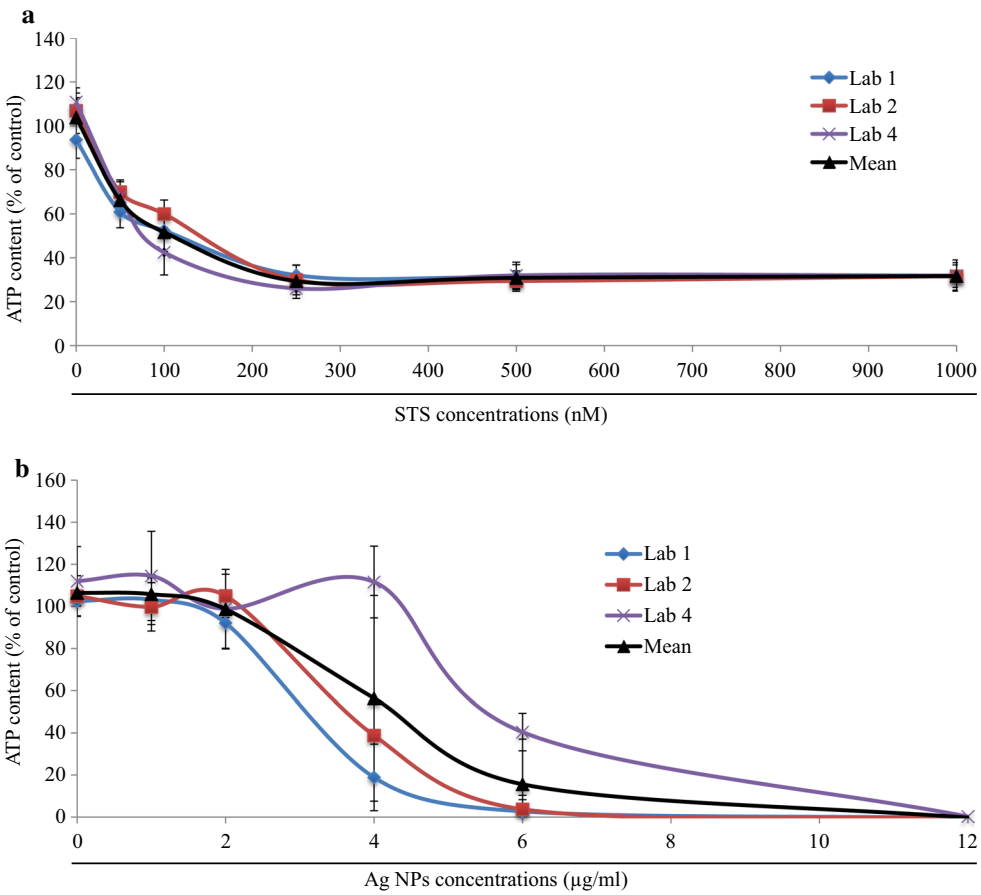

Fig. 1 Evaluation of the effect of STS (**a**) or Ag NPs (**b**) on the A549 cells viability by measuring the cellular ATP content after 24 h of incubation. Results are expressed as mean ± SD ($n = 3$) and presented as percentages of non-treated cells. *Black curve* represents the mean of all laboratories' results (color figure online)

Peprotech for human TNF-α). Further details can be viewed in the TNF-α and IL-1β ELISA SOP available on NanoValid website (www.nanovalid.eu).

Statistical analysis

Results are expressed as mean ± SD. Data were analyzed by Student's *t* tests.

Results and discussion

Effect of NPs on cell viability

In order to evaluate the potential cytotoxic effect of Ag NPs on human alveolar A549 cells, ATP and MTS assays were used. These two viability assays were assessed as they are based on different readout parameters (luminometry and colorimetry), and they are prolifically used in nanotoxicology studies, and due to their relative procedure simplicity.

In the MTS assay, tetrazolium salt is reduced by viable cells into a colored formazan product that is soluble in cell culture medium correlating with the number of living cells (Mosmann 1983). This conversion, accomplished by NAD(P)H produced by dehydrogenase, reflects cellular redox metabolism. NPs effects on viability can also be evaluated by determination of cellular ATP content using luciferase or other ATP-consuming reactions (Kangas et al. 1984). ATP is a marker of cell viability as it is present in all metabolically active cells and it is assumed its concentration decreases rapidly upon cell death. The ATP assay has been widely used in vitro toxicology for several decades (Crouch et al. 1993).

ATP assay

A549 cells were incubated with increasing concentrations of Ag NPs (1–12 µg/ml) or STS (50–1000 nM), a well-known protein kinase inhibitor that leads to cell death (Tang et al. 2000). As illustrated in Fig. 1a, increasing concentrations of STS rapidly decreased cell viability, measured by a drop in intracellular ATP content, reaching a maximal toxic effect at 250 µM. A very good intra- and inter-laboratory reproducibility was observed.

Ag NPs induced a dose-dependent decrease in A549 cell viability leading to an important drop of ATP content at a concentration of 6 µg/ml suggesting that ATP assay was suitable to evaluate the toxic effect of Ag NPs on A549

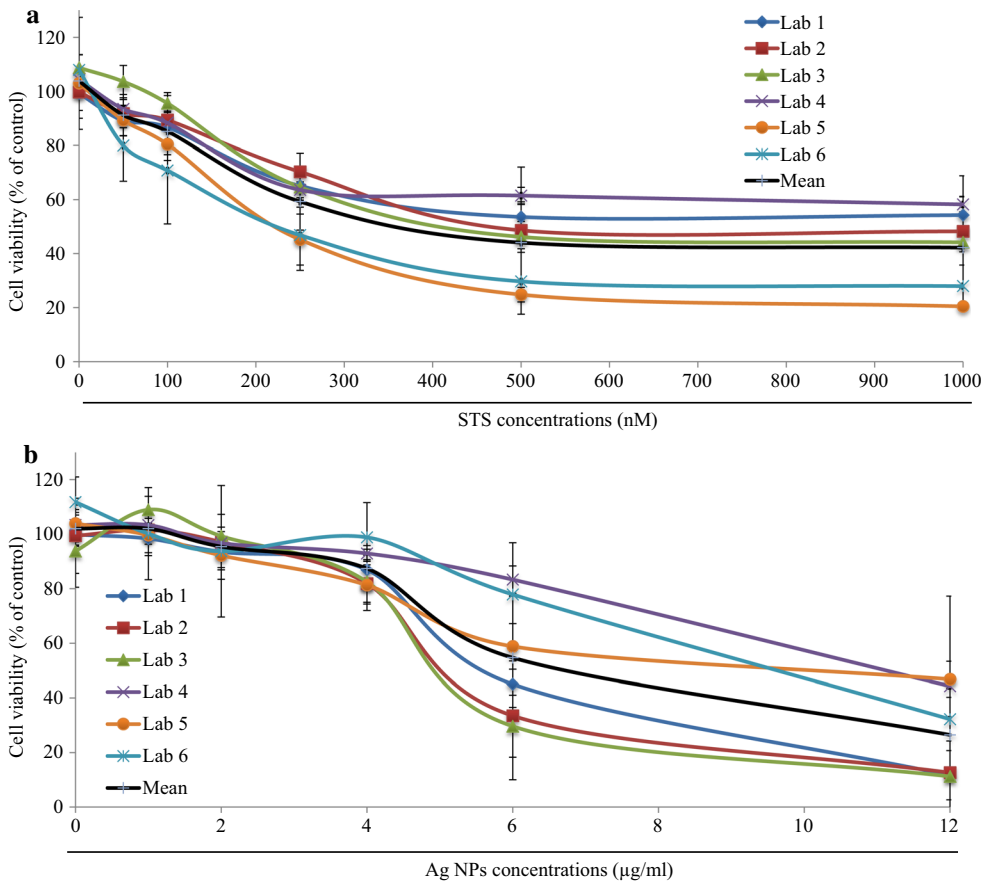

Fig. 2 MTS evaluation of the effect of STS (**a**) or Ag NPs (**b**) on A549 cell viability after 24 h of incubation. Results are expressed as mean ± SD ($n = 3$ or 2-Laboratory 5-) and presented as percentages of non-treated cells (control cells). *Black curve* represents the mean of all laboratories' (Lab) results (color figure online)

cells even if at mid-range Ag NPs concentrations the intra- and inter-laboratory variability was greater than when assessing the STS-positive control (Fig. 1b).

MTS assay

In addition to the ATP assay, Ag NPs cytotoxicity was evaluated by MTS assay. After 24 h exposure with Ag NPs or positive control STS, incubation medium was discharged and replaced by new medium containing MTS substrate. Formation of formazan salt was then measured. A dose-dependent toxic effect was observed after exposure of A549 cells to STS (Fig. 2a). All laboratories observed a dose-dependent decrease in A549 cell viability after incubation with Ag NPs (Fig. 2b) even if some variability in the toxic effect of Ag NPs was observed at the highest concentrations. Comparing the mean toxicological value of each laboratory, at each concentration, with the overall mean (black curve), a high inter-laboratory variability was assumed for this experiment. Toxicological values of two laboratories varied from the overall mean by more than one time the overall SD (see "Materials and methods" section) for at least three concentrations (Laboratory 3 at the concentrations of 0, 1, 2, and 6 µg/ml and Laboratory 6 at the concentration of 0, 4, and 6 µg/ml).

One parameter that could influence the inter-laboratory results reproducibility is the dispersion of NPs. Indeed, some NPs can be hydrophobic and/or form agglomerates that tend to sediment, reducing the opportunity of obtaining a well-dispersed NPs solution. Ag NPs were provided as a stable well-dispersed solution. After dilution in cell culture medium, Ag NPs size distribution revealed a monodispersed suspension (see Table 2). However, it was noticed by partners that Ag NPs tend to deposit on the inner walls of plastic disposables as recently described (Malysheva et al. 2016). This could lead to pipetting errors. In consequence, the MTS protocol followed by all partners was amended to ensure that careful pipetting of Ag NPs was used.

Considering this point, the suitability of MTS assay to evaluate the toxic effect of Ag NPs was investigated using another cell type, the human immune monocytic THP-1 cells. As shown in Fig. 3b, a better reproducibility and

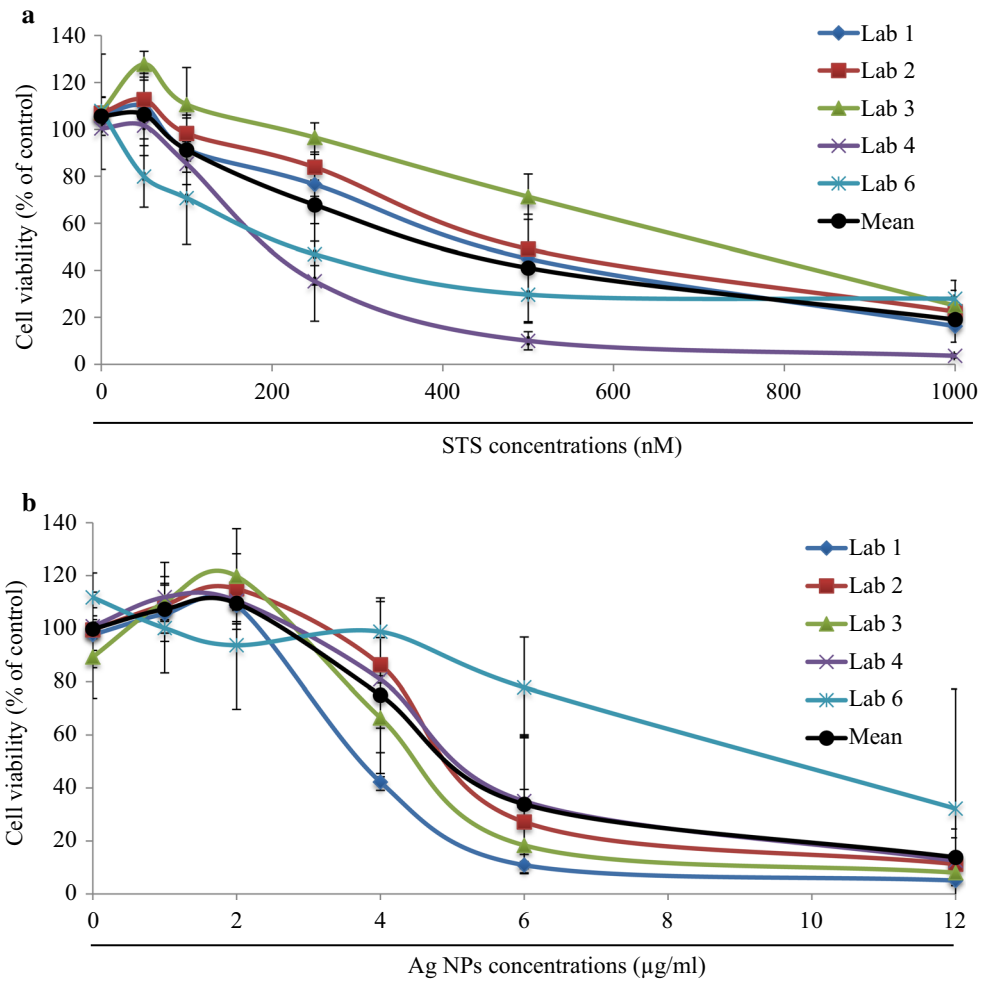

Fig. 3 MTS evaluation of the effect of STS (**a**) or Ag NPs (**b**) on THP-1 cell viability after 24 h of incubation. Results are expressed as mean ± SD ($n = 3$) and presented as percentages of non-treated cells. *Black curve* represents the mean of all laboratories' (Lab) results (color figure online)

higher consistency in the results were obtained by most of the laboratories upon THP-1 cells exposure to Ag NPs in comparison with Fig. 2. Only one laboratory displayed toxicological values that varied from the overall mean by more than one time the overall SD at all concentrations except at 1 µg/ml (Laboratory 6).

This underlines the importance of thoroughly homogenizing NPs suspensions. Altogether, these data suggested that MTS assay was suitable to evaluate the toxicity of Ag NPs on different cell lines.

Another MTS inter-laboratory study was conducted using CuO NPs. These NPs were chosen based on their antimicrobial properties and their use in several components including textiles and food packages (Delgado et al. 2011; Ren et al. 2009). CuO NPs toxicity was evaluated on both human hepatoma HepG2 cells and human intestinal Caco-2 cells. CuO NPs were sonicated using a common specific energy to obtain a well-dispersed CuO NPs stock suspension (see "Materials and methods" section).

As observed in Fig. 4a, c, the positive control BC induced a dose-dependent toxicity on both HepG2 and Caco-2 cells. Increasing concentrations of CuO NPs also led to a reproducible dose-dependent drop of HepG2 and Caco-2 cell viability (Fig. 4b, d). A limited inter-laboratory variability (as defined in "Materials and methods" section) was observed with only one laboratory displaying toxicological values that exceeded the overall mean by more than one time the overall SD (Fig. 4b, d). These data are in accordance with previous studies showing the high toxicity of CuO NPs on these cell lines (Cuillel et al. 2014; Piret et al. 2012a, b).

Altogether, these data suggested that MTS viability assay is suitable to evaluate the cytotoxicity of different NPs on several cell lines. This assay was consistent with good intra- and inter-laboratory reproducibility with both NPs, especially after amendment about Ag NPs handling and homogenization.

Pro-apoptotic effect of Ag NPs

Caspases 3/7 activation was evaluated by inter-laboratory comparison to test the pro-apoptotic effect of Ag NPs on A549 cells. Caspases 3 and 7 are two members of the caspase family known as apoptosis effectors that selectively cleave the DEVD sequence present in their substrates (Fan

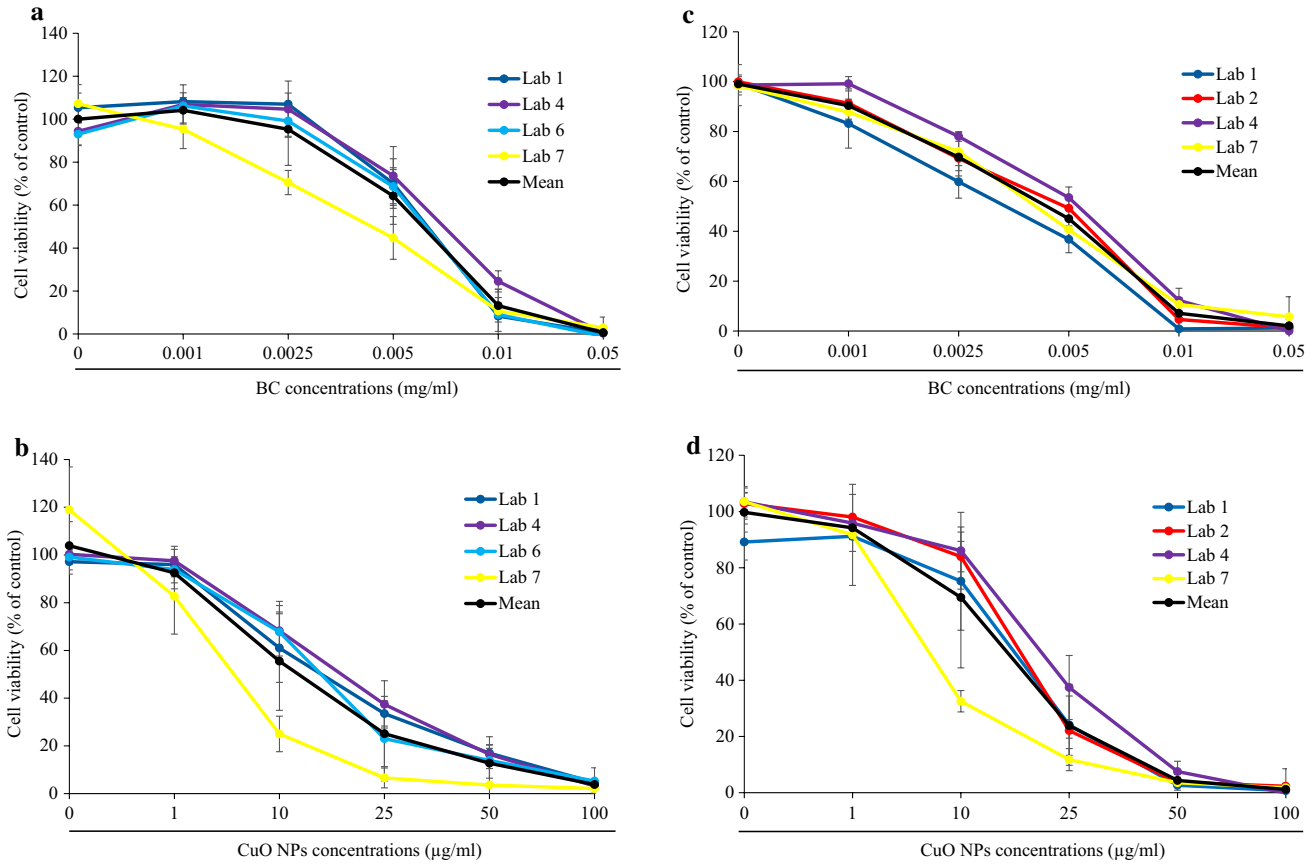

Fig. 4 MTS evaluation of the effect of benzalkonium chloride (BC) or CuO NPs on HepG2 or Caco-2 cell viability. HepG2 (**a**, **b**) and Caco-2 (**c**, **d**) cells were incubated with increasing concentrations of BC (**a**, **c**; mg/ml) or CuO NPs (**b**, **d**; µg/ml) for 24 h. Results are expressed as mean ± SD ($n = 3$) and presented as percentages of non-treated cells. *Black curve* represents the mean of all laboratories' (Lab) results (color figure online)

et al. 2005). The pro-apoptotic inducer STS was used as positive control (Chae et al. 2000). Despite variability in the caspases 3/7 activity levels at the highest STS doses, results obtained from different laboratories on A549 cells treated with increasing concentrations of STS revealed dose-dependent caspases 3/7 activation (Fig. 5a). In the presence of Ag NPs, partners obtained contrasting results on caspases 3/7 activity (Fig. 5b). Partners 2 and 7 did not find apoptosis induction at the tested concentrations, while the others found caspase activation. Different trends were observed, with partners 1 and 4 showing the highest caspases 3/7 activity at 2 and 4 µg/ml, respectively, and partner 6 reporting a dose–response curve. In comparison with the positive control, caspases activation induced by Ag NPs was much lower. A limited pro-apoptosis effect of Ag NPs could explain in part the observed variability.

Pro-inflammatory effects of Ag NPs

The pro-inflammatory effect of Ag NPs was tested, at sub-toxic concentrations (based on the MTS assay results), by measuring the amount of IL-1β and TNF-α secreted by human immune monocytic THP-1 cells.

A dose-dependent increase in the amount of IL-1β was observed after treatment of THP-1 cells by the positive control LPS confirming that THP-1 cells can positively respond when stimulated by a pro-inflammatory agent (Fig. 6a). In general, laboratories observed a similar secretion profile, characterized in all cases by a high fold induction of secreted IL-1β.

Inter-laboratory results were not consistent in the amount of IL-1β detected upon exposure of THP-1 cells to Ag NPs (Fig. 6b). Notably two partners observed a dose-dependent response at higher concentrations, while the remaining two partners only measured a limited induction of IL-1β at the highest concentration.

Comparing positive control values to those of Ag NPs exposures, it became evident that the amount of IL-1β released after Ag NPs treatment was much lower than those measured after LPS exposure suggesting a rather low pro-inflammatory effect of Ag NPs in THP-1 cells.

The positive control LPS also induced a clear and significant dose-dependent TNF-α secretion in THP-1 cells

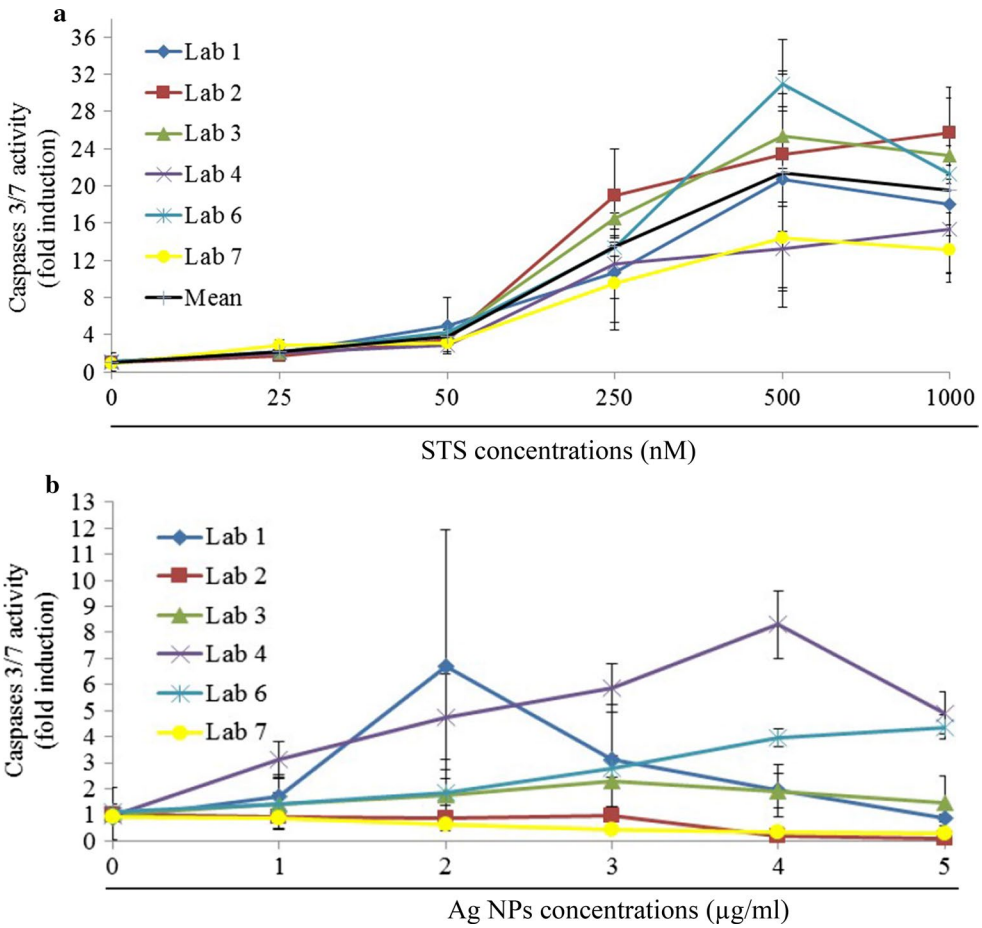

Fig. 5 Evaluation of the pro-apoptotic effect of STS (**a**) or Ag NPs (**b**) on A549 cells by measuring the caspases 3/7 activity after 24 h of incubation. Results are expressed as mean ± SD ($n = 2$ or 3) and presented as percentages of non-treated cells. *Black curve* represents the mean of all laboratories' (Lab) results (color figure online)

measured by all partners' laboratories. However, some variation in the fold inductions was found. A similar secretion profile of TNF-α after LPS exposure was observed by all partners with similar secretion levels except one partner where values far exceeded those obtained by the others (Fig. 6c). The amount of TNF-α secreted by THP-1 cells after Ag NPs exposure was low (Fig. 6d). Heterogeneous response profiles were found. The TNF-α response was not significant, and a clear dose response was not observed by any of the partners, corroborating the previously observed low pro-inflammatory effect of Ag NPs in THP-1 cells.

Evaluation of NPs interference with in vitro assays

When assaying the biological effects of NPs with in vitro assays, it is essential to ensure that NPs do not interfere with the reagents of tests that may generate erroneous results. Potential interference of Ag NPs with assays used in this study was considered by designing interference tests.

To evaluate the potential interference with ATP assay, Ag NPs were mixed with ATP solution (1 µM) before measuring the associated luminescence values. As shown in Table 3, Ag NPs decreased the luminescence values at all concentrations tested. This could be due to inhibition of luciferase enzyme activity as shown recently with citrate-coated silver NPs (Kakinen et al. 2013). Moreover, the level of inhibition was not consistent between laboratories at same NPs concentrations, which could be a source of the inter-laboratory variability.

According to these results, it might be concluded that ATP assay is a poor choice for the evaluation of toxicity of Ag NPs. However, in this interference test, NPs were present during the whole assay (incubation with ATP standard and measurement of the luminescence values). This is not realistic of the functional ATP assay, where medium containing Ag NPs was discarded and cells washed prior to cell lysis and further incubation with luciferase/luciferin solution. These steps would limit the risk of NPs interference, as many would be removed. Results described in Fig. 1b showed a dose-dependent toxic effect of Ag NPs. These data were supported by optical microscopy observations of A549 cell morphology, showing shrunken dead cells (data not shown).

Considering these results, two options would be recommended: If the tested NPs are shown to induce no interference during the interference test, the ATP cell viability assay is suitable to study the NPs effect on cell viability.

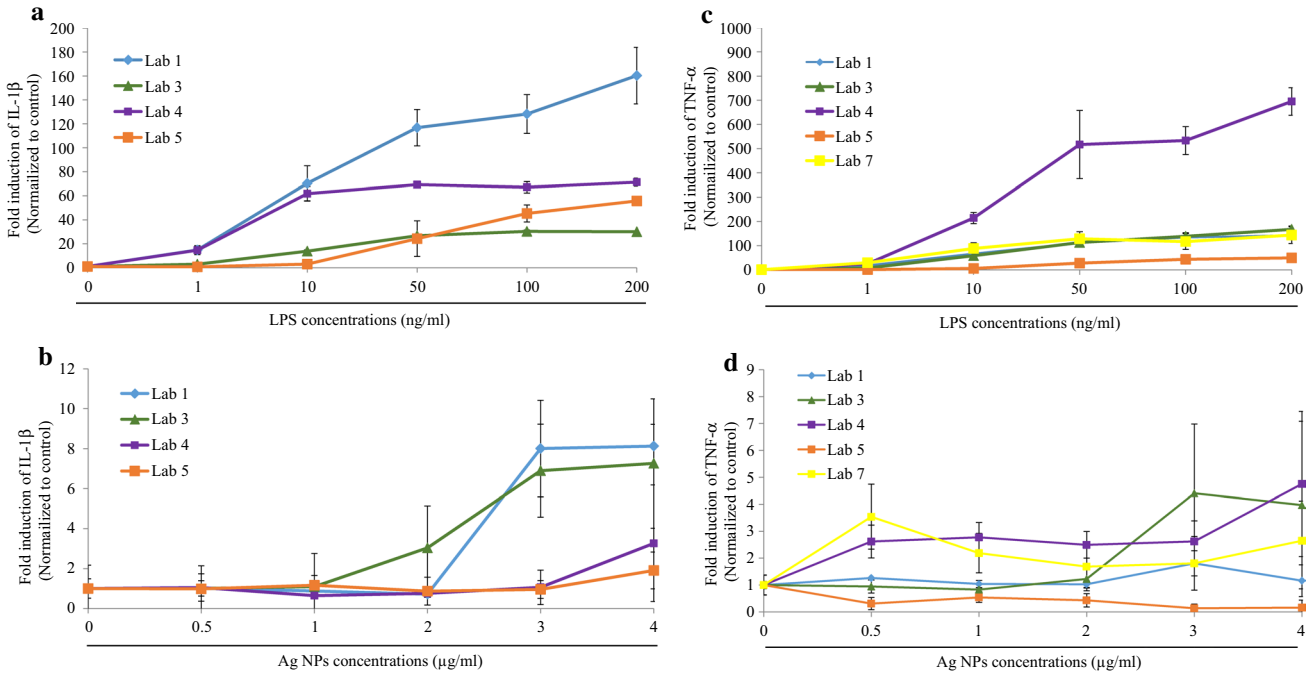

Fig. 6 Evaluation of the pro-inflammatory effect of LPS (**a**, **c**) or Ag NPs (**b**, **d**) on THP-1 cells by measuring the amount of secreted IL-1β (**a**, **b**) or TNF-α (**c**, **d**). THP-1 cells were incubated with increasing concentrations of LPS (ng/ml) or Ag NP (µg/ml) for 24 h and IL-1β and TNF-α were quantified in cell culture medium by ELISA. Results are expressed as mean ± SD ($n = 3$) and normalized to non-treated cells (color figure online)

Table 3 Evaluation of the Ag NPs interference with ATP assay

	Laboratory 1	Laboratory 2	Laboratory 4
ATP	100 ± 3.89	100 ± 1.22	100 ± 2.03
ATP + Ag NPs 1 µg/ml	21.08 ± 2.93	11.05 ± 1.13	/
ATP + Ag NPs 2 µg/ml	9.9 ± 1.96	0.42 ± 0.18	/
ATP + Ag NPs 4 µg/ml	4.69 ± 2.4	0.35 ± 0.18	/
ATP + Ag NPs 6 µg/ml	2.98 ± 0.19	0.29 ± 0.15	/
ATP + Ag NPs 12 µg/ml	0.3 ± 0.23	0.27 ± 0.07	0.08 ± 0.02

Comparison of results from several laboratories. ATP standard at 1 µM was mixed with increasing concentrations of Ag NPs and luminescence values were measured. Results are expressed as mean ± SD ($n = 3$) and presented as percentages of ATP standard without NPs (ATP)

If some interference is observed during the test, results obtained with the ATP cell viability assay should be considered with care and compared with those generated by other cell viability assays.

The potential for Ag NPs to interfere with the MTS assay was also considered. It has been reported that the presence of NPs can interfere with MTS optical density readings (Xia et al. 2013). Our data confirmed this observation since increasing concentrations of Ag NPs dose dependently distorted the optical density values measured by several laboratories (Table 4). The presence of NPs during the absorbance measurements in a WST-1 assay (WST-1 is a water-soluble tetrazolium salt similar to the hydrophilic MTS substrate) has

Table 4 Evaluation of the Ag NPs interference with MTS assay

	Laboratory 1	Laboratory 2	Laboratory 4	Laboratory 5
MTS substrate	100	100	100 ± 2.79	100 ± 1
MTS substrate + Ag NPs 1 µg/ml	104 ± 0.001	128	99 ± 5.88	103 ± 0.91
MTS substrate + Ag NPs 2 µg/ml	109 ± 0.002	116	103 ± 1.61	108 ± 0.84
MTS substrate + Ag NPs 4 µg/ml	119 ± 0.004	128	111 ± 3	119 ± 0.89
MTS substrate + Ag NPs 6 µg/ml	128 ± 0.004	146	121 ± 4.84	124 ± 2.32
MTS substrate + Ag NPs 12 µg/ml	153 ± 0.01	162	150 ± 1.27	138 ± 0.8

MTS substrate was incubated for 1h with increasing concentrations of Ag NPs and optical density values were measured. Results are expressed as means ± s.d. (n = 2 or 3), and presented as percentages of MTS substrate without NPs

Table 5 Interference test for caspases 3/7 assay

	Laboratory 1	Difference	Laboratory 2	Difference	Laboratory 3	Difference
STS	100 ± 4.24		100 ± 4.96		100 ± 1.93	
STS + Ag NPs 0.5 µg/ml	97.55 ± 1.15	2	105.15 ± 7.15	5	98.9 ± 9.1	1
STS + Ag NPs 1 µg/ml	98.38 ± 0.67	2	105.63 ± 14.74	6	98.9 ± 3.39	1
STS + Ag NPs 2 µg/ml	97.81 ± 2.42	2	101.11 ± 15.54	1	96.13 ± 3.86	4
STS + Ag NPs 4 µg/ml	93.17 ± 2.04	7	107.06 ± 3.55	7	94.1 ± 5.93	6
STS + Ag NPs 5 µg/ml	93.36	7	104.73 ± 3.04	5	88.56 ± 9.61	11
	Laboratory 4	Difference	Laboratory 6	Difference	Laboratory 7	Difference
STS	100 ± 35.31		100 ± 3.13		100 ± 42.82	
STS + Ag NPs 0.5 µg/ml	100.28 ± 22.4	0	84.36 ± 0.8	16	102.43 ± 34.95	2
STS + Ag NPs 1 µg/ml	94.28 ± 31.67	6	84.97 ± 0.86	15	95.7 ± 38.68	4
STS + Ag NPs 2 µg/ml	100 ± 27.26	0	85.13 ± 4.86	15	97.91 ± 43.43	2
STS ± Ag NPs 4 µg/ml	95.22 ± 12.06	5	85.4 ± 2.21	15	106.34 ± 48.77	6
STS + Ag NPs 5 µg/ml	98.31 ± 6.39	2	84.83 ± 1.18	15	123.83 ± 5.31	24

Comparison of results from several partners. Cells were treated with STS at 1 µM for 24 h to induce apoptosis and caspase activation. Cells were then incubated with Apo-ONE caspase-3/7 reagent and fluorescence readout analyzed in the absence or presence of increasing concentrations of Ag NPs. Results are expressed as mean ± SD ($n = 3$) and presented as percentages of STS at 1 µM without NPs

also been reported to lead to an underestimation of NPs toxicity (Guadagnini et al. 2015). To avoid this problem, incubation medium containing Ag NPs was discharged before addition of freshly prepared MTS-containing cell culture medium.

In an effort to minimize risk of NPs interference with MTS assay, several additional recommendations must be adopted:

1. After NPs exposure, addition of a washing step in order to eliminate as much as possible NPs before incubation with MTS substrate.
2. Following the incubation of cells with MTS substrate, transfer of colored solution to a new well plate to reduce risk of interference of potential NPs agglomerates attached to cell surface or internalized. In case of detachment of some of NPs agglomerates during this step, centrifugation of the colored solution could be considered before transfer into a new well plate.
3. As described in "Materials and methods" section, cell-free blank media containing the different NPs concentrations have been included in the 96-cell plate layout. Subtraction of their O.D. values to the absorbance values of corresponding cells incubated with the same NPs concentrations allows to take into account the background absorbance of potential residual NPs and interference with MTS measurements.

All these recommendations were considered to conduct the MTS inter-laboratory evaluation of toxic effect of CuO NPs, showing a good inter-laboratory reproducibility (Fig. 4).

Similarly to ATP and MTS assays, interference of Ag NPs with the components of caspases 3/7 assay was evaluated. A549 cells were incubated for 24 h with STS to induce apoptosis. Caspases activity was then measured in the presence or absence of increasing Ag NPs concentrations (Table 5). A difference not higher than 15% between STS condition and STS + NPs was stated as the limit to exclude NPs interference. Although laboratory 7 found values slightly higher than 15% in the presence of 5 µg/ml, it is possible to conclude that Ag NPs did not interfere with the assay.

In order to evaluate the potential interference of Ag NPs with IL-1β and TNF-α ELISAs, Ag NPs at 0, 0.5, 1, 2, 3, and 4 µg/ml were incubated with 250 pg/ml IL-1β or 2000 pg/ml TNF-α for 1 h at room temperature. After incubation, each sample was centrifuged to pellet particles and supernatant was collected. One portion of this supernatant was frozen at −70 °C, and the remaining was kept at 4 °C overnight. This assay provided a twofold interference assessment: the binding of proteins to NPs and the alteration of proteins by metal ions (released from Ag NPs; around 44–48%) (Böhme et al. 2015) during different storage conditions. As shown in Fig. 7a, a slight Ag NPs-dose-dependent decrease in IL-1β was observed for both storage conditions. No or a slight modification (at the highest Ag NPs concentration) of TNF-α was detected by ELISA when different storage conditions were used (Fig. 7b). Such interference test was also conducted after incubation of Ag NPs with known concentrations of each cytokine at 37 °C for 24 h. In these conditions, a drastic and significant decrease in both cytokines was measured (Fig. 7c, d).

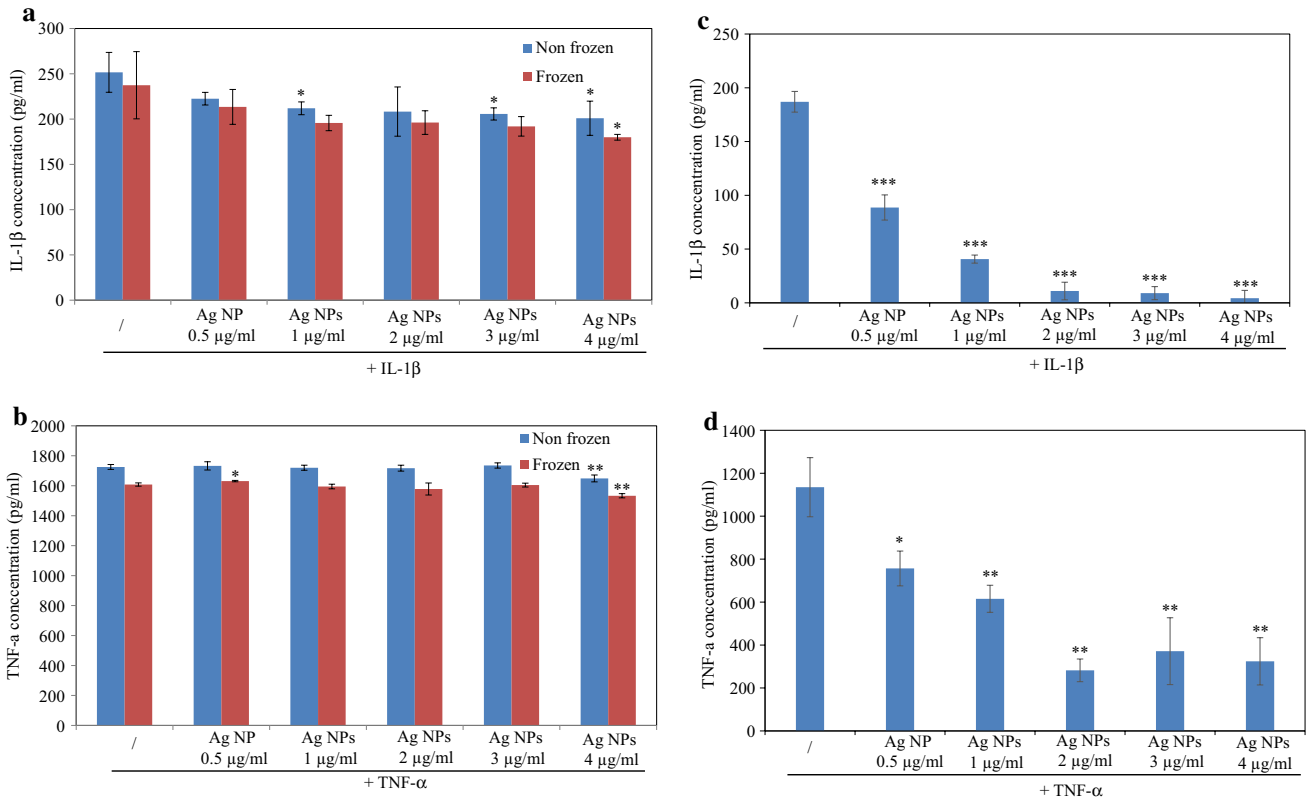

Fig. 7 Evaluation of the interference of Ag NPs with IL-1β (**a**, **c**) and TNF-α (**b**, **d**) ELISAs. IL-1β (250 pg/ml) or TNF-α (2000 pg/ml) were incubated 1 h at room temperature (**a**, **b**) or at 37 °C for 24 h (**c**, **d**) with increasing concentrations of Ag NPs before centrifugation. Supernatants were collected and stored overnight either at 4 °C (*blue columns*, non frozen) or at −70 °C (*red columns*, frozen) before analyzing the presence of IL-1β or TNF-α by ELISA (**a**, **b**) or directly analyzed after 24 h of incubation (**c**, **d**). Results are expressed as mean ± SD ($n = 3$) *$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$ versus TNF-α or IL-1β without NPs (color figure online)

Interference of NPs with cytokine detection by ELISA was reported by several authors (Guadagnini et al. 2015; Val et al. 2009). The importance of interference is related to the cytokine and NPs types. Checking potential interference of NPs before quantifying cytokines secretion by ELISA should be made standard to evaluate the importance of cytokines adsorption on NPs. In the case of ELISA interference, the inflammatory response induced by NPs could be, in most cases, analyzed at the transcriptional level by measuring the production of cytokines mRNAs by real-time PCR.

Variability observed during this ILS could be partly due to the NPs' interference with the in vitro assays. Indeed, Ag NPs were shown to interfere with MTS, ATP content, and inflammatory assays in the framework of interference tests. However, our data indicated an important interference only when NPs were not discarded before addition of MTS substrate or quantification of ATP or cytokines.

NPs could impair in vitro assays by different mechanisms such as (1) interaction with assay substrate as reported for carbon nanotubes with MTT assay (Worle-Knirsch et al. 2006). Here, Ag NPs were shown to dose dependently increase the MTS substrate's O.D. values suggesting that Ag NPs could absorb at selected MTS assay wavelength. In the ATP assay, the presence of Ag NPs drastically reduced luminescence values associated with amount of ATP. Such interference effect could be due to (2) quenching of emitted fluorescence by the NPs or due to (3) inhibition of luciferase enzyme activity (Kakinen et al. 2013). Ag NPs were also shown to interfere with the measurement of IL-1β and TNF-α possibly giving false negative interpretations and explaining in part the low pro-inflammatory effect of Ag NPs in THP-1 cells. Interestingly, interference was only observed after incubation with Ag NPs for 24 h at 37 °C suggesting that interference tests should be conducted under conditions comparable to the exposure conditions of cells. This interference could reflect the potential of Ag NPs to (4) adsorb cytokines leading to their sedimentation during centrifugation before conducting ELISA. Release of Ag ions from NPs (around 44–48%) described inside NanoValid project (Böhme et al. 2015) could also hamper cytokines detection by (5) altering and/

Table 6 Potential critical phases that could participate to variability in NPs toxicity evaluation

Protocol step	Critical phase
All steps	Verification of pipettes and instruments
Storage and preparation of NPs and positive control stock solution and dilutions in culture medium	Storage conditions Dispersion protocol Pipetting (same person) Pipetting technique (use of electronic multipipette) Low pipetting volume (10 µl minimum) Nature of NPs
Cells seeding	Cell counting method Edge effects
Exposure to test item	Removal of medium from cultures Equal volume of test items Incubation time of cell cultures with NPs
In vitro assay	Removal of NPs containing medium from cultures Washing step Interference of NPs with read out Air bubbles

or (6) degrading proteins. No interference of Ag NPs with caspase 3/7 assay was noticed suggesting that high inter-laboratory variability was not due to NPs impairing of the assay. Furthermore, (7) deposition of Ag NPs on the inner walls of plastic disposables (Malysheva et al. 2016) could lead to pipetting errors modifying the final concentration to which cells will be exposed and explain in part the low inter-laboratory reproducibility with this assay. Several critical points could also influence intra- and inter-laboratory variability (Rosslein et al. 2015). Table 6 summarizes critical phases that could participate to variability in NPs biological effects results. For instance, correctly calibrated instruments and pipettes are crucial points to consider before starting in vitro toxicity evaluation. Pipetting technique is also very important; human parameter representing one of the most important factors causing variability. Indeed, variability is often observed between two persons from same laboratory performing same in vitro assay all things being equal. To reduce the impact of this factor, the use of calibrated electronic pipettes can be recommended.

Altogether, these results suggest that interference tests should be designed as close as possible to the cell exposure conditions. In that context, cell-free blank media containing the different NPs concentrations should be included in the assay well plate to serve as internal interference controls and to evaluate background of potential residual NPs.

Efforts should also be made to eliminate the presence of NPs during the test procedure to reduce assay interference as much as possible.

Conclusion

Evaluating the biological effects of NPs is crucial for nanosafety. However, conflicting and contradicting laboratory data on relative toxic effects of individual nanomaterials have been described in the literature. Lack of well-defined harmonized protocols (SOPs) renders difficult to obtain reproducible and comparable toxicological data. Therefore, under the remit of European NanoValid project, inter-laboratory study involving seven laboratories was conducted to evaluate the suitability and reliability of classical in vitro assays commonly used in toxicology. Only few attempts to test the suitability of several in vitro assays by ILS at international level were conducted up to now (Bonner et al. 2013; Xia et al. 2013).

In that context, well-characterized NPs, common cell line clones, common FBS batches, and common NPs dispersion procedures were used following detailed cell culture and in vitro biological assays (SOPs) that were developed in the framework of NanoValid or adapted from preexisting protocols from QualityNano project. These SOPs give precise description of assay procedure, cell culture/seeding conditions, NPs/positive control preparation and dilutions, experimental well plate preparation, and interference tests (www.nanovalid.eu). In this study, we considered three in vitro viability assays (MTS, ATP content, and caspase 3/7 activity) with different readouts (absorbance, luminescence, and fluorescence, respectively) and one in vitro inflammation assay focusing on possible interference of NPs with each assay. High inter-laboratory variability was observed with the caspase 3/7 and ELISA assays in the evaluation of pro-apoptotic and pro-inflammatory effects of Ag NPs. In contrast, ATP and MTS assays gave consistent toxicity results with low inter-laboratory variability. Suitability and reliability of MTS to evaluate the toxic effect of NPs were confirmed using two NPs and several cell lines.

Using suitable assay interference tests, we were able to identify a number of reasons that could partly explain the inter-laboratory variability observed during ILS and in some cases, and assay modifications were made to reduce these interferences (MTS assay). One critical point is the presence of NPs during the assay. During MTS and ATP assays, incubation medium containing NPs is discarded and a washing step is included before starting the evaluation of the NPs' impact on cell viability. Conversely, release of pro-inflammatory cytokines is assessed in incubation medium in the presence of NPs that could hamper their detection via direct interaction (proteins adsorption) or resulting from proteins alterations mediated by released ions.

In conclusion, MTS and ATP assays are more adaptable to manage NPs interference and reduce this risk and thus

constitute good candidates to become reference assays for NPs toxicity testing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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