Adverse outcome pathways-oriented toxicology in *in vitro* systems for implementing the safety-by-design of new nanomaterials: preliminary studies on Ag NPs

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Introduction: Silver nanoparticles (Ag NPs) are among the most used metal-based NPs owing to their antimicrobial activity. Concerns regarding the potential hazard due to the exposure to these NPs pose several open issues about their safe development and use. In this project, Ag NPs are used as coating agents for antibacterial textiles and as components to be embedded in creams. After developing a harmonized protocol for the preparation and characterization of the NP suspensions, their physical-chemical (p-chem) properties were evaluated by TEM and DLS. The cytotoxic effects on the human lung cell line, A549, of different Ag NPs – naked (Ag-NKD) and coated with polyvinylpyrrolidone (Ag-PVP) or hydroxyethyl cellulose (Ag-HEC) – were evaluated

Aim: hazard identification of new Ag-based NMs, designed according to a Safe-by-Design (SbD) approach, toward human health during their production and use. To date it has not yet been defined a unified standard protocol for characterization and exposure of biological models to NPs. The aim is also to address this issue and to study how different p-chem properties affect toxicological outcomes.

Methods

Ag-NPs used in this study were obtained within the ASINA project in form of suspension or powder (Ag-HECs and Ag-HECp, respectively). Comparison with reference materials (Ag-NKD and Ag-PVP) was done in order to assess the influence of different coatings on Ag NP toxicity.

First, it was necessary to establish a harmonized protocol for NPs preparation and characterization with the aim of reducing possible interference during stock suspensions preparation and cell exposure.

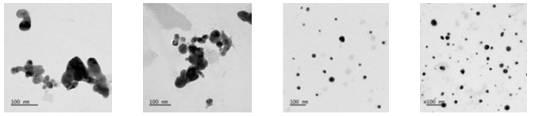
After NPs characterization using TEM and DLS, an adverse outcome pathways (AOPs)-oriented investigation strategy was developed. After identifying the main key events involved in Ag NPs toxicity, representative assays for cytotoxicity (MTT), oxidative stress (H_2DCFDA), inflammation (ELISA) and apoptosis/necrosis (Annexin V-PI), were selected to be performed on A549 cells, adenocarcinomic human alveolar basal epithelial cells; this cell line was chosen to represent the lung since inhalation is one of the main routes of human exposure to these nanoparticles.

Three key events involved in Ag NPs toxicity are:

- Oxidative stress
- Inflammation
- DNA damage

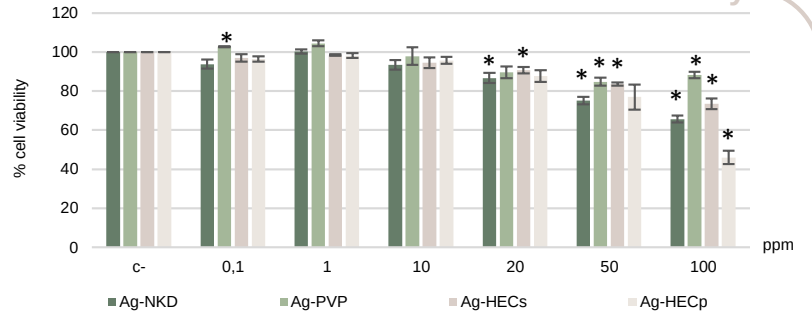
NPs characterization

		Ag-NKD	Ag-PVP	Ag-HECs	Ag-HECp
ζ-potential (mV) in mQ	t=0h	-27,57	-6,07	-4,71	9,92
Z-average in DMEM 1% FBS	t=0h	328,71 ± 76,9	361,4 ± 110,43	80,37 ± 2,4	158,08 ± 20,29
	t=24h	167,57 ± 7,55	185,11 ± 7,62	75,23 ± 4,91	85,79 ± 1,04
PdI in DMEM 1% FBS	t=0h	0,37 ± 0,13	0,43 ± 0,06	0,2 ± 0,02	0,32 ± 0,02
	t=24h	0,33 ± 0,1	0,2 ± 0,14	0,21 ± 0,05	0,51 ± 0,02



In the table, are reported the values of z-average (nm) ± SD and PDI ± SD. The ζ-potential of Ag-NPs was measured at 100 ppm in mQ water. It resulted negative for Ag-NKD, Ag-PVP and Ag-HECs, and positive for Ag-HECp. Z-average and PdI were measured through Dynamic Light Scattering (DLS) in cell culture medium (DMEM supplemented with 1% FBS) at 100 ppm. These two parameters were taken right after the preparation of the suspensions and after 24h of sedimentation. Ag coated with HEC is better dispersed and more stable. TEM images show the spherical shape and the dispersion of the NPs. Ag-HEC NPs are smaller and better dispersed, while both Ag-NKD and Ag-PVP agglomerate.

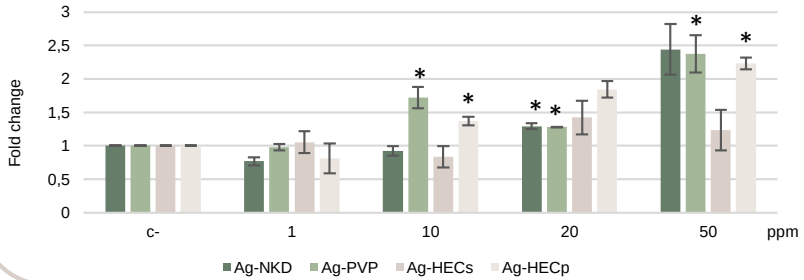
Cell viability



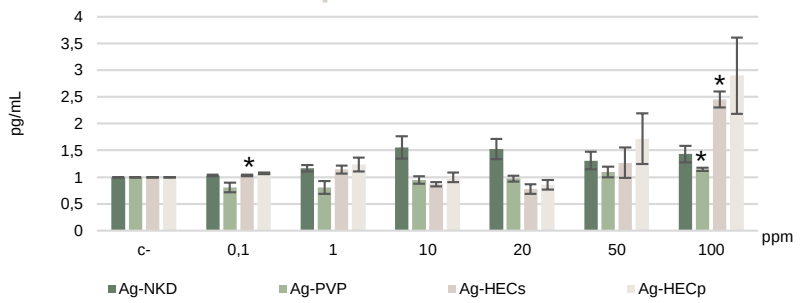
*The effects of Ag-NPs on A549 cell viability were evaluated through MTT assay. The viability decrease significantly after the exposure to higher concentrations of Ag-NKD, Ag-HECs (20, 50, 100 ppm), Ag-PVP (50, 100 ppm) and Ag-HECp (100 ppm). Data presented as mean (n=3) ± SE. Statistical analysis: ANOVA one way followed by t-test, *p < 0.05.*

ROS

*To investigate the capability of these NPs of inducing oxidative stress, ROS expression was assessed using H_2DCFDA as indicator. Ag-PVP increase significantly ROS expression at 10, 20 and 50 ppm. Ag-HECp increase ROS expression at 10, 20 and 50 ppm, but only 10 and 50 ppm are significant. Ag-NKD increase ROS expression at 20 and 50 ppm, but only the highest concentration is significant. Data presented as mean (n=3) ± SE. Statistical analysis: ANOVA one way followed by t-test, *p < 0.05.*

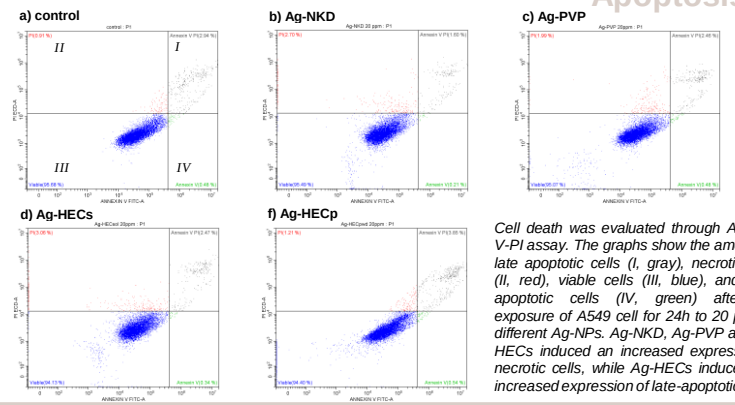


IL-8 production



*The IL-8 production was evaluated in order to investigate the inflammatory response. IL-8 levels increase significantly after the exposure to 100 ppm of Ag-PVP and Ag-HECs. There is an increase in the level of IL-8 after cell exposure with 100 ppm of Ag-HECp, but the result is statistically not significant. Data presented as mean (n=3) ± SE. Statistical analysis: ANOVA one way followed by t-test, *p < 0.05.*

Apoptosis



Cell death was evaluated through Annexin V-PI assay. The graphs show the amount of late apoptotic cells (I, gray), necrotic cells (II, red), viable cells (III, blue), and early apoptotic cells (IV, green) after the exposure of A549 cell for 24h to 20 ppm of different Ag-NPs. Ag-NKD, Ag-PVP and Ag-HECs induced an increased expression of necrotic cells, while Ag-HECs induced and increased expression of late-apoptotic cells.

Conclusions: The results demonstrate that the cellular responses to the Ag NP exposure strictly depend on their p-chem properties, and in particular on the properties of the coating polymer. Ag coated with HEC show a different toxicity profile after its reduction to a powder form followed by reconstitution with water. In fact, Ag-HECp has a stronger effect on cells viability. HECs is the only one able to increase the expression of late-apoptotic cells. Ag-PVP seems more effective in inducing oxidative stress and Ag-NKD appears less effective in promoting IL-8 expression, even though further investigations are required.

Future plans

- ✓ Gene expression analysis for markers of inflammation and oxidative stress;
- ✓ DNA damage investigation;
- ✓ To use the same approach for other NMs developed within the project;
- ✓ *In vitro* air-liquid interface (ALI) exposure method to simulate *in vivo* inhalation exposure;
- ✓ EpiDerm™ *in vitro* 3D tissue model to investigate the skin exposure route.

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