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The effect of agglomeration state of silver and titanium dioxide nanoparticles on cellular response of HepG2, A549 and THP-1 cells

Anna Lankoff[a,b,*], Wiggo J. Sandberg[c], Aneta Wegierek-Ciuk[b], Halina Lisowska[b], Magne Refsnes[c], Bożena Sartowska[a], Per E. Schwarze[c], Sylwia Meczynska-Wielgosz[a], Maria Wojewodzka[a], Marcin Kruszewski[a,d]

[a] Center for Radiobiology and Biological Dosimetry, Institute of Nuclear Chemistry and Technology, 16 Dorodna Str., 03-195 Warsaw, Poland
[b] Department of Radiobiology and Immunology, Institute of Biology, Jan Kochanowski University, 15 Swietokrzyska Str., 25-406 Kielce, Poland
[c] Norwegian Institute of Public Health, Lovisenberggate, 0456 Oslo, Norway
[d] Independent Laboratory of Molecular Biology, Institute of Rural Health, Jaczewskiego 2, 20-090 Lublin, Poland

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ABSTRACT

Nanoparticles (NPs) occurring in the environment rapidly agglomerate and form particles of larger diameters. The extent to which this abates the effects of NPs has not been clarified. The motivation of this study was to examine how the agglomeration/aggregation state of silver (20 nm and 200 nm) and titanium dioxide (21 nm) nanoparticles may affect the kinetics of cellular binding/uptake and ability to induce cytotoxic responses in THP1, HepG2 and A549 cells. Cellular binding/uptake, metabolic activation and cell death were assessed by the SSC flow cytometry measurements, the MTT-test and the propidium iodide assay. The three types of particles were efficiently taken up by the cells, decreasing metabolic activation and increasing cell death in all the cell lines. The magnitude of the studied endpoints depended on the agglomeration/aggregation state of particles, their size, time-point and cell type. Among the three cell lines tested, A549 cells were the most sensitive to these particles in relation to cellular binding/uptake. HepG2 cells showed a tendency to be more sensitive in relation to metabolic activation. THP-1 cells were the most resistant to all three types of particles in relation to all endpoints tested. Our findings suggest that particle features such as size and agglomeration status as well as the type of cells may contribute to nanoparticles biological impact.

1. Introduction

The rapid increase in development, production and utilization of nanoparticles (NPs) has led to increased efforts towards the elucidation of physico-chemical properties that may be advantageous from a technological point of view, but still minimize potential adverse health effects induced by the NPs. The potential hazardous properties are commonly ascribed to their extremely small size, with a large surface area per mass unit, but also to their potential ability to be translocated to compartments of the body and cells that are not accessible for larger particles (Oberdörster et al., 2005). A better understanding and a proper risk assessment of the NPs seems required also because of the use of NPs in different medical applications, giving the NPs access to tissues that may not be reached by inhalation, via dermis or the gastrointestinal (GI) tract (Quadros and Marr, 2010; Nohynek et al., 2010; Powell et al., 2010). Compared to larger-sized particles, NPs have a strong potential to agglomerate, both in ambient air and in liquid solutions such as the fluid compartments of the body. The agglomeration state of NPs might influence the deposition in the respiratory tract and the potential to be taken up via the skin and GI-tract. Furthermore, the extent of agglomeration might affect the ability of NPs to interact with cells in in vitro testing systems. The potential for interaction will depend on gravitation, diffusion and convection forces (Teeguarden et al., 2007; Lison et al., 2008). The electrolyte/salt content, the pH and the protein composition, either in culture medium or in body fluids, could affect the agglomeration process (Vippola et al., 2009). Several studies have shown that NPs may bind different proteins, depending on both the composition of the NPs and available proteins (Lundqvist et al., 2008; Ehrenberg et al., 2009; Kittler et al., 2009). The protein coronas have been shown to influence the NPs potential to agglomerate and affect active residues on the NPs, changing their reactivity against cellular targets (Lynch et al., 2009; Aggarwal et al., 2009; Caracciolo et al., 2010). Furthermore, uptake via different routes in the body might be altered. Several studies have examined how different dispersion procedures may affect the

* Corresponding author at: Center of Radiobiology and Biological Dosimetry, Institute of Nuclear Chemistry and Technology, 16 Dorodna Str., 03-195 Warsaw, Poland. Tel.: +48 22 504 12 81; fax: +48 22 504 13 41.
E-mail address: alankoff@gmail.com (A. Lankoff).

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agglomeration state of the NPs (Buford et al., 2007; Deguchi et al., 2007; Bihari et al., 2008; Murdock et al., 2008), whereas other studies have focused on how the exposure conditions influence the behaviour of the NPs and cellular responses (Herzog et al., 2009; Murdock et al., 2008; Geys et al., 2010). The motivation of this study was to examine how the agglomeration state of Ag particles from the nano- to submicron size and also TiO_2-NPs may affect the kinetics of cellular binding/uptake and ability to induce cytotoxic responses and to what extent this may depend on the cell type and the end-point examined. The three cell lines of diverse origin were chosen: A549 (human lung epithelial cell line), HepG2 (human liver cell line) and THP1 (human monocyte cell line), which represent different target tissues and routes of exposure to nanoparticles (Sung et al., 2009; Müller et al., 2010). The TiO_2-NPs and Ag-NPs were employed, as they are of high commercial relevance. These nanoparticles have been extensively studied unfortunately, no simple conclusions have emerged from the available studies due to the variability of parameters such as the physical and chemical properties of the particle, cell type, dosing parameters and the biochemical assays used (for reviews see Stone et al., 2007; Johnston et al., 2010).

2. Materials and methods

2.1. Chemicals

F12 Ham medium, RPMI 1640 medium, L-glutamine, albumin bovine serum, phosphate-buffered saline, Hepes buffer solution, ethanol, Dimethyl sufoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, glutaraldehyde, osmium tetroxide, lead (II) citrate tribasic trihydrate, uranyl acetate dihydrate and Epoxy embedding medium were purchased from Sigma–Aldrich Chemicals (Poznan, Poland). Williams medium and Fetal bovine serum were the products of Gibco (Invitrogen, Poland). Binding buffer and propidium iodide were obtained from Bekton Dickinson (San Diego, USA).

2.2. Cell cultures

Human epithelial cell line A549, human hepatic cell line HepG2 and human undifferentiated monocyte cell line THP-1 were purchased from the American Type Tissue Culture Collection (ATCC, Rockville, MD) and maintained according to ATCC protocols. Briefly, A549 were cultured in F12 Ham medium supplemented with 10% FCS and 2 mM L-glutamine, HepG2 were cultured in Williams medium supplemented with 10% FCS and 2 mM L-glutamine and THP-1 cells were cultured in RPMI medium supplemented with 5% FCS and 2 mM L-glutamine. The cells were incubated in a 5% CO_2 atmosphere at 37 °C.

2.3. Preparation of nanoparticles/particles

Spherical silver particles with nominal diameters of 20 ± 5 nm and 200 ± 50 nm were purchased from PlasmaChem (Berlin, Germany). Titanium dioxide nanoparticles (TiO_2-NPs) of nominal size 21 nm anatase/rutile were purchased from Degussa-Evonik (Essen, Germany) and kindly provided by the European Commission Joint Research Center depository. Two different protocols of particle dispersions were used. Protocol 1: particle stock solution was prepared by dispersion of 2 mg of particles in 800 μl of distilled water. Particle dispersion was then sonicated on ice using a probe sonicator (Bronson, USA) with 420 J/m^3 total ultrasound energy. 100 μl of 15% bovine serum albumin and 100 μl of a 10× concentrated phosphate-buffered saline were given immediately after sonication. Protocol 2: particle stock solution was prepared by dispersion of 2 mg of particles in 1000 μl of culture medium containing 15 mM Hepes buffer with 10% FCS. Particle dispersions were sonicated on ice using a probe sonicator (Bronson, USA) with 420 J/m^3 total ultrasound energy. Stock solutions were prepared before each experiment.

2.4. Dynamic light scattering measurements

Size distribution of particles was measured by dynamic light scattering measurements (DLS). The system measures multiple angles of light scatter and derives a bulk intensity plot of particle sizes. DLS was performed at 25 °C with a scattering angle of 90° on the Zeta-sizer Nano ZS (Malvern, Malvern Hills, UK). Stock solutions were diluted 1:4 in various full exposure media (RPMI 1640, Williams, F12K) and measured in triplicate with 20 sub-runs.

2.5. Zeta-potential measurements

Zeta-potential measurements for surface charge were performed at 25 °C in a folded capillary cell at 150 V and M3-PALS detection using non-invasive backscatter at 173° with an Avalanche photodiode, Q.E. > 50% at 633 nm (Malvern, Malvern Hills, UK). Stock solutions were diluted 1:8 in various full exposure media and measured in triplicate with 20 sub-runs zeta-potentials were calculated using the Smoluchowski limit for the Henry equation with a setting calculated for practical use (f(ka) = 1.5).

2.6. NP analysis by scanning (SEM) and transmission (TEM) electron microscopy

Observations were performed using a scanning electron microscope (SEM) type DSM 942 (Zeiss, Germany) in the secondary electron (SE) mode. SEM worked with parameters: high voltage EHT 2 kV, working distance WD = from 4 to 4.8 mm. Ag particles of nominal size 20 nm and 200 nm, as well as TiO_2-NPs of nominal size 21 nm were dispersed according to protocols 1 and 2. Stock solutions of the particles were diluted 1:10 in distilled water and deposited on the microscopic holders. After evaporation of the solvent (24 h), the samples were coated with a thin layer of Au (about 10 nm) using a vacuum evaporator (JEE-4X, JEOL, Japan) to protect the sample from heat destruction and to keep real parameters of the observed details. The images were collected at 10 kV and either 10,000× or 20,000× magnification. The morphology of NPs was also observed by transmission electron microscopy (TEM) (JEOL 1200 EXII, JEOL, Japan) operating at an acceleration voltage of 120 kV. The samples were prepared on the copper mesh covered with a carbon film as the carrier. Digital images were recorded by CD camera SIS Morada 11 megapixels and processed using AnalySIS. The cells were treated in 96-well plates with 50 μg/ml of Ag-particles and TiO_2-NPs for 2, 24 and 48 h. They were fixed with 2.5% glutaraldehyde, post-fixed with OsO_4 and dehydrated in graded concentrations of ethanol (Cai et al., 2007; Foley et al., 2002), then embedded in Epon. Ultra-thin sections (80 nm) were cut, counterstained with lead citrate and uranyl acetate and then observed with a TEM microscope.

2.7. Kinetics evaluation of cellular binding and uptake of silver and titanium dioxide particles into A549, HepG2 and THP-1 cells by flow cytometry

The kinetics of cellular binding and uptake of Ag particles and TiO_2-NPs into A549, HepG2 and THP-1 cells were examined by flow cytometry (Suzuki et al., 2007; Zucker et al., 2010). The approach is based on analysis of forward scatter (FSC) versus side scatter (SSC) of measured samples. Side scatter distribution ratio was chosen as a measure of cellular uptake and was calculated by dividing the SSC value in the particle-treated cells by the SSC value in control cells. Twenty-four hours after cell seeding, cells were incubated in 6-well plates, with 10, 50 and 100 μg/ml of particles for 2–72 h. After treatment with particles, cells were washed three times with PBS to remove loosely bound particles. After centrifugation, cells were re-suspended in 1 ml PBS for flow cytometry. Following gating, control and particle-exposed cells were run and plotted to examine the increase in side scatter (SSC). Data for 50,000 events were stored per point. Because the flow rate affects these measurements, they were always performed at low flow rates. The cytometer (Becton Dickinson LSR II flow cytometer equipped with 488 nm laser, FSC diode detector, and photomultiplier tube SSC detector) was set up to measure SSC logarithmically and FSC linearly.

2.8. Metabolic activation – MTT assay

The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was used to measure cellular metabolic potential of treated cells (Plumb, 2004). The effect of 20 nm, 200 nm Ag particles and 21 nm TiO_2-NPs (10, 50 and 100 μg/ml) on metabolic potential was assessed in treated cells 24, 48 and 72 h after of treatment. In brief, $3–4 \times 10^6$ cells/ml in 200 μl of culture medium were seeded into each of the 96 wells of multi-dish culture plates. Twenty-four hours after cell seeding, cells were incubated with varying concentrations of tested particles as indicated above. After treatment, cell cultures were rinsed three times with PBS, and 100 μl of 3 mg/ml MTT was added to each well. After 4 h incubation at 37 °C, the MTT solution was removed. Remaining insoluble formazan crystals were dissolved in 100 μl DMSO and absorbance was measured at 570 nm in plate reader spectrophotometer (Carry 50, Varian, Australia).

2.9. Cell death – propidium iodide (PI) staining

Twenty-four hours after cell seeding in 6-well plates, cells were incubated with 10, 50 and 100 μg/ml of NPs for 24, 48 and 72 h. Briefly, cells were washed two times with cold PBS and then resuspended in a 1× binding buffer at a concentration of 1×10^6 cells/ml. Cell suspension (100 μl) was incubated with 5 μl of PI (propidium iodide) at room temperature for 15 min in the dark. The cells were re-suspended in 400 μl of a 1× binding buffer (Lankoff et al., 2008). The fluorescence was determined using a LSR II flow cytometer (Becton Dickinson). A computer system BD FACS DiVa (version 6.0, Becton Dickinson) was used for data acquisition and analysis. Data for 20,000 events were stored.

2.10. Statistical evaluation

Statistical analysis of the obtained data was performed using Statistica 7.1 software (Stat Soft. Inc. Tulsa, USA). The data were expressed as mean ± standard deviation (SD) of at least three independent experiments. Data were evaluated by Kruskal–Wallis One Way Analysis of Variance on Ranks (ANOVA) followed by Dunnet's method. Sensitivity of cell lines was tested by two-ways analysis of variance