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Cytotoxicity, cell uptake and microscopic analysis of titanium dioxide and silver nanoparticles *in vitro*

Katerina Tomankova [a,b,*], Jana Horakova [a,b], Monika Harvanova [b,c], Lukas Malina [a,b], Jana Soukupova [d], Sarka Hradilova [d], Kristina Kejlova [e], Jakub Malohlava [a,b], Libor Licman [a], Marketa Dvorakova [e], Dagmar Jirova [e], Hana Kolarova [a,b]

[a] Department of Medical Biophysics, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic
[b] Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic
[c] Department of Pharmacology, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic
[d] Department of Physical Chemistry, Faculty of Science, Regional Centre of Advanced Technologies and Materials, Slechtitelu 11, 783 71 Olomouc, Czech Republic
[e] Centre of Toxicology and Health Safety, National Institute of Public Health, Srobarova 48, 100 42 Prague, Czech Republic

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ABSTRACT

Commercially manufactured nanomaterials are used massively for modification of products of everyday use, including products intended for children. Therefore their potential risks have to be ultimately studied. Aside from toxicity of nanomaterials with known specific parameters, the end-consumer is potentially endangered by materials with unknown specification. Commercially available products are not usually accompanied by parameter/specification sheet providing the consumer with sufficient chemico-physical parameters allowing the evaluation of possible toxic effects.

The aim of this work was to evaluate the declared parameters of commercially available TiO_2 and Ag NPs employing chemico-physical methods and consequently *in vitro* cytotoxicity and genotoxicity tests performed on non-cancer cell lines. Based on the results of our complex study we can conclude that the data provided by the producers are not in good agreement with the performed measurements. Furthermore, all tested NPs penetrated into the SVK14 cells and all NPs had significant effect on the kinetics of ROS production in all cell lines (note: the ROS production has not been established as the major mechanism of cell damage elicited by Ag NPs). The study revealed greater cytotoxic potential of Ag NPs in comparison with TiO_2 NPs and all of the studied NPs caused significant DNA damage.

1. Introduction

In 2012 the released reports estimated that at least 1000 consumer products containing NPs were available on the market (Oosthuizen et al., 2012; Shukla et al., 2011; Singh and Ramarao, 2012). Titanium dioxide (TiO_2) represents one of the most frequently applied nanomaterials, which is primarily used as a pigment due to its brightness, high refractive index, and resistance to discolouration. Nearly 70% of all the TiO_2 NPs produced are used as pigments in paints, glazes, enamels, plastics, paper, fibres, foods, pharmaceuticals and cosmetics (e.g. toothpastes, UV sunscreens, shampoos, deodorants or shaving creams). Aside from this application TiO_2 can be also used as an antimicrobial agent, inorganic UV filter, and a catalyst for air and water purification, medical applications and energy storage (Botelho et al., 2014; Janer et al., 2014; López-Heras et al., 2014; Weir et al., 2012). In order to fulfil the required role, the particles have to be up to 100 nm in their diameter, which is, however, proved to be only in 36% of the produced NPs (Botelho et al., 2014; Holbrook et al., 2013; Weir et al., 2012). Another crucial parameter is the concentration, which varies in different products. In pharmaceutical products the lowest concentration of Ti was reported. The concentration was between 0.0001 and 0.014 μg Ti mg^{-1}. Similar concentrations of Ti, i.e. up to 0.01 μg Ti mg^{-1} can be found in cosmetic products. The highest amount of TiO_2 (up to 10%) can be, however, found in common sunscreens and even in products intended for children such as candies, sweets or chewing gums (Weir et al., 2012).

Silver nanoparticles (Ag NPs) are nowadays widely applied in products of everyday usage such as textiles, toys, toothpastes, cosmetics, household cleaning products, water disinfection, air cleaners,

Abbreviations: ROS, reactive oxygen species; MMP, mitochondrial membrane potential; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; IC50, 50% lethal concentration; NP, nanoparticle; DLS, dynamic light scattering; AAS, atomic absorption spectroscopy; AFM, atomic force microscopy; KMCA, *k*-mean cluster analysis; RT, room temperature.

* Corresponding author. Department of Medical Biophysics, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic. Tel.: +420 585 632 103; fax: +420 585 632 167.
E-mail address: katerina.tomankova@upol.cz (K. Tomankova).

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food packaging and coating for refrigerators. In general, we can find Ag NPs everywhere, where antibacterial effect is required (Cleveland et al., 2012). Therefore a logical application of Ag NP modified/based materials is in medical practice. Ag NPs are used for surface modification of medical devices such as catheters, dental materials, bandage materials for wound and burn treatment (Asharani et al., 2009; Chopra, 2007; Dobrzyńska et al., 2014; Greulich et al., 2011).

However, the use of composite materials involving NPs has certain limitations. The possible release of NPs represents one of the most frequently discussed toxicological threats. The danger to the environment was modelled by Cleveland et al. (2012) who studied commercially available socks modified by Ag NPs and tried to conclude the release risk and its impact on the environment. The socks were submerged into seawater, which enabled the release of approx. 95% of its total silver content within 2 hours. Similar experiment was performed also with wound dressings and a toy bear, all of them modified by Ag NPs. These products released approx. 99% and 82% of their total silver content after 12 h of being submerged in seawater, respectively (Cleveland et al., 2012). Therefore these nanomaterials can easily get into surface waters, where they are likely to interact with living organisms and/or it can lead to human exposure (Markus et al., 2013; Weir et al., 2012). Although sewage water treatment plants are capable of removing the majority of microscale objects from influent sewage, particles of diameter up to tens of nanometres cannot be fully eliminated. Consequently they can cause damage of the plants or elsewhere when released out from the plant (Weir et al., 2012).

More importantly, the NPs released into the environment may possibly penetrate through skin or membranes of the gastrointestinal tract and lungs (Fröhlich and Roblegg, 2012). After penetration into the organism, Ag NPs may circulate systematically and migrate to organs like the liver, spleen, lungs, kidneys or brain. Singh and Ramarao (2012) determined that the intracellular dissolution of Ag NPs occurs about 50 times faster than in water (Singh and Ramarao, 2012).

Mammalian cells proved to take up many NPs via mechanisms like pinocytosis, endocytosis dependent on caveolae and lipid raft composition, clathrin-dependent endocytosis and phagocytosis. The possible uptake, its kinetics, intracellular localisation and exocytosis, however, highly depends on fundamental characteristics such as NP size, surface characteristics, zeta potential and its resistance against aggregation. On the other hand, the uptake kinetics is also affected by the cell type (Asharani et al., 2009; Bartłomiejczyk et al., 2013). Therefore the *in vitro* studies of toxicity effects of the NPs are highly required because small particles often penetrate into the cells (Furukawa et al., 2011; Shukla et al., 2011; Valdiglesias et al., 2013). There they can initiate production of reactive oxygen species (ROS) resulting in induced cytotoxicity, which can even end up with DNA damage of the exposed tissue (Asare et al., 2012).

In the present study we focused on two most frequently applied materials, Ag and TiO_2 NPs, that were first characterised by physicochemical techniques and consequently evaluated from the biological point of view. The characterisation using SEM, AFM, and DLS, was necessary to obtain information on fundamental properties of the purchased NPs. Consequently the obtained data were compared with the data provided by the producers. Regarding the biological techniques we focused on the NP penetration into the SVK14 cells. Tests of *in vitro* cytotoxicity on SVK14, BJ and NIH3T3 cell lines included endpoints such as cell viability, production of ROS, mitochondrial membrane change, and DNA damage (comet assay).

2. Materials, methods and procedures

2.1. Materials and instruments

NIH3T3 cell line (mouse fibroblasts), SVK14 (human keratinocytes) and BJ (human fibroblasts from foreskin) were used as biological materials. The following chemicals were used: phosphate buffered saline (PBS, pH 7.4, in-house preparation), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Invitrogen), thiazolyl blue tetrazolium bromide (MTT, Sigma Aldrich), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide ($C_{25}H_{27}Cl_4IN_4$, JC-1, Sigma Aldrich), dimethyl sulphoxide (DMSO, Sigma Aldrich), HMP agarose (Serva), LMP agarose (Qbiogene), trypsin-EDTA (Sigma), ethanol (Sigma), foetal bovine serum (FBS, Sigma Aldrich), NaCl (Tamda), EDTA (ethylenediaminetetraacetic acid, Lachema), tris (tris(hydroxymethyl) aminomethane) (Sigma Aldrich), Triton X-100 (Serva), NaOH (Sigma Aldrich), SYBR Green (Invitrogen), Annexin V-Cy3 kit (APO-AC, Sigma Aldrich). In addition, we used 96, 6 and 24 well plates (TPP) for cell lines cultivation and 1 × 1 cm quartz slides (Meopta-optika) for Raman spectroscopy. The characterisation measurements were carried out with the following instruments: multi-detection microplate reader Synergy HT (BioTek), transmission microscope Olympus IX81 with DSU unit (Olympus), centrifuge (Biotech), electrophoretic tank (Bio-RAD), Atomic Force Microscope Bioscope Catalyst (Bruker) with SNL-A tip (Bruker). Concentration of silver was determined by means of ContrAA® 300 High-Resolution Continuum Source atomic absorption spectrometer (Analytik). Raman spectra were acquired using confocal Raman microscope, model 300 alpha R (WITec, GmbH). Excitation was performed with frequency doubled Nd: YAG laser (Spectra Physics Excelsior 532 nm). Zeiss EC Epiplan-Neofluar (50 × 0.8 NA, WD = 0.58 mm) dry objective was used. Results were processed using Phototox Version 2.0 software (ZEBET, Berlin, Germany), Comet Score (Tritek Corp, Sumerduck, VA, USA), Gwyddion version 2.28 (Czech Metrology Institute, Brno, Czech Republic), Nanoscope analysis (Bruker, Santa Barbara, CA, USA) and ASpect CS1.5.5.0 software (Analytic, Jena, Germany).

2.2. Used NPs

We studied 5 samples intended for commercial use (2 samples of titanium dioxide NPs and 3 samples of silver NPs). The dispersion of TiO_2 NPs (labelled as TiO_2), with the declared concentration equal to 8000 g L^{-1}, was purchased from Precheza, a.s. (product No. 180311/2) and the producer declared the diameter of the NPs to be 28 nm. Stabilisation was not carried out.

The second sample containing TiO_2 NPs (labelled as Nanorutil) was also purchased from Precheza, a. s. (product No. 150311/1) and its concentration was 16.4 mg L^{-1}. The producer declared the diameter of the NPs to be 128 nm. Stabilisation was performed using SiO_2/Al_2O_3.

The other three tested dispersions contained Ag NPs. First two samples were purchased from KC Rulc company (http://www.kcrulc.cz/en) with the Ag concentrations equal to 14 mg L^{-1} and 20 mg L^{-1}, respectively. The company declares that the dispersions consist of silver (99.99%) and ultrapure water. The first mentioned sample was manufactured on 12/10/2009 and the latter on 02/15/2013. Stabilisation was not carried out. None of the samples was accompanied by a product specification sheet. Therefore we did not have any information concerning particle diameter, size distribution or stability against aggregation.

Last of the tested samples (Vintr) was purchased from Lakshmi-Narayan company (http://www.lakshmi-narayan.eu); again not accompanied by a product specification sheet including stabilisation. However, the company declared the Ag concentration in the dispersion to be 40 mg L^{-1}.

2.3. Dynamic light scattering

The commercially available NPs were characterised with Zetasizer Nanoseries (Malvern Instruments, UK). This technique enables measurements of the diffusion of particles moving under Brownian motion, which is then converted into hydrodynamic diameter (d) and a size distribution (also called polydispersity; p) using the Stokes–Einstein relationship:

$$d = \frac{k_b T}{3\pi\eta(t)D},$$

where k_B is Boltzmann's constant, T is temperature in K, $\eta(t)$ is viscosity of the liquid in which the NPs are moving, and D is translational diffusion coefficient. Each of the measurements involved 3 independent measurements, each lasting 60 s. The particle diameter is then presented in "intensity" and "number" modes. Although the first mode is more objective, characterising the system as a whole, the values of hydrodynamic diameter can be artificially increased by a possible fraction of impurities in the sample (usually in micrometre range). On the contrary, the latter mode takes into account the quantity of the particles in the fractions and therefore the hydrodynamic diameter is not overestimated.

The information on the size distribution (polydispersity) was obtained from the same instrument, which uses the following equation:

$$p = \frac{\mu_2}{\Gamma_2},$$

for its calculation. In the equation μ_2 represents second moment of the size distribution and Γ^2 first squared moment of the size distribution. The values of polydispersity range from 0 for system entirely monodispersed (i.e. containing particles homogeneous in diameter and morphology) to 1 for system with really wide sized distribution.