CYTOTOXICITY OF NANOMATERIALS APPLICABLE IN RESTORATION AND CONSERVATION

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Abstract

Nanotechnologies represent new promising approach for conservation and preservation of cultural heritage. Nanomaterials (NMs) have been shown to improve efficacy, durability and quality of the restoration due to their unique properties that can be achieved and tuned by controlling particle physico-chemical characteristics. The same characteristics, however, affect the way how NMs interact with biological systems and may potentially underlie their enhanced or unexpected toxicity. In this pilot study, we compared the toxic potential of representatives of three of the most common oxide materials applicable in restoration: TiO_2 (standard and purified P25, a mixture of prevailing anatase with rutile crystalline modifications), SiO_2 (bare A200, and R805, R9200 as coated forms of A200), and ZnO. Using two *in vitro* cytotoxicity assays, WST-1 and LDH, evaluating metabolic activity and cell membrane integrity, respectively, we preliminary ranked the tested substances according to their cytotoxic potential, which may be used for their prioritization for further testing and applications. After 24h exposure, a dose-dependent decrease in cell viability was only detected in ZnO NPs and uncoated silica (A200). Hydrophobic coated silicas (R805 and R9200) and TiO_2 NPs (purified and unpurified P25) did not exhibit cytotoxic effects up to the highest tested concentration of 250 µg/mL. Toxicological data related to the physico-chemical characteristics will be applicable in developing both more efficient and safer nano-based products for restoration and conservation.

Keywords: Nanotechnology, cytotoxicity, restoration, work of art, safe-by-design

1. INTRODUCTION

Traditional techniques for preservation of cultural and heritage works of art (e.g. frescoes, oil-paints, sculptures) using organic solvents and polymers exhibit several limitations and drawbacks, such as promotion of chemical degradation or modification of permanent color. Moreover, exposure to organic solvents has been associated with various adverse health effects. Reducing toxicity might be a crucial factor for successful introduction of novel materials in restoration and conservation practices. NMs have been shown to improve efficacy, durability and quality of the restoration due to their unique properties that can be achieved and tuned by controlling particle chemistry, size, shape, crystal structure, surface, and other characteristics during their production [1]. The same characteristics, however, affect the way how NMs interact with biological systems and may potentially underlie their enhanced or unexpected toxicity. The ideal NM should exhibit low toxicity while maintaining desirable technical properties. By the controlled synthesis of NMs, individual characteristics can be designed in or out and affect both toxicity and performance. Controlling toxicity at an early stage of material or technology development is a core principle of so called safe-by-design approach. It is aimed at designing products or processes that bear an intrinsically low risk potential, instead of confining this potential by application of protective measures [2]. Designing out the hazard represents one of the main safe-by-design strategies, and in NMs can be achieved by e.g. NP doping, surface passivation, reduction of photo-catalytic

efficiency, formation of composites, or surface functionalization (reviewed by [2]). Simple *in vitro* assays able to examine basic toxicological endpoints offer a possibility to perform a preliminary screening evaluation of a wide range of NMs that may offer new or enhanced properties applicable in restoration practice.

In the present work, we used representatives of three of the most common oxide NMs: TiO_2 (standard and purified P25, a mixture of prevailing anatase with rutile crystalline modifications), SiO_2 (bare A200 and R805, R9200 as coated forms of A200), and ZnO (see **Table 1**). All tested samples are relevant for applications in restoration practice. P25 offers a solution to limit degradation of materials caused by pollutants (e.g. volatile organic compounds, NOx, SO_2) and pathogens due to its excellent photocatalytic properties in combination with high stability, availability and low-cost [3]. ZnO NPs exhibit antimicrobial and antifungal properties, resistance to moisture, UV, and leaching. Similarly to nano-TiO_2, nano-ZnO is relatively cheap and available [4]. Silica NPs may be especially beneficial in consolidants, where they reduce crack formation, prevent dimensional instability owing to swelling and shrinkage induced by changes in temperature and relative humidity [5]. Silica NPs have been reported to increase cohesion, compression and flexural resistances, and to reduce the porosity after setting, and overall to increase the durability [6].

In this pilot study, we compared the toxic potential of the NP samples using two *in vitro* cytotoxicity assays WST-1 and LDH, evaluating metabolic activity and cell membrane integrity, respectively, as diverse markers of cell viability.

2. METHODS

2.1. NMs

Five commercially available metal oxide NPs were tested. Their key characteristics of the tested NMs are reported in **Table 1**. All samples were provided in a powder form. NM dispersions were prepared by probe sonication according to the protocol published by Jensen et al. [7]. Before cell exposure, the NP dispersions were gradually diluted in the cell culture medium containing 1 % fetal bovine serum (FBS).

Table 1 Physico-chemical characterization of the tested NMs

Label	Manufacturer	Commercial name	Surface modification	Surface area BET* (m^2/g)	Diameter* (nm)
A200	Evonik Industries	Aerosil® SiO_2 A200	-	220	12
A805	Evonik Industries	Aerosil® SiO_2 R805	$-CH_3$ $(CH_2)_7$	125-175	12
A9200	Evonik Industries		$-CH_3$	150-190	12
P25	Evonik Industries	Aeroxide® TiO_2 P25	-	35-65	25
P25 purified	Evonik Industries	Aeroxide® TiO_2 P25	Purification	35-65	25
ZnO	Bochemie		-	90-110	12

2.2. Cytototoxicity assays

A549 cells (Human Lung Adenocarcinoma, ATCC No CCL-185™) were cultivated in MEM GlutaMAX™ Supplement medium (Gibco) with 10 % fetal bovine serum (FBS; Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5 % CO_2. For cytotoxicity assays, the cells were seeded in 96-well microplates at a concentration of 7,500 cells per well and incubated overnight to get attached. Before exposure, the cells were washed with phosphate saline buffer. Serial dilutions of NMs in the cell culture medium supplemented with 1 % FBS were added to the cells in the volume of 100 µL per well.

WST-1 assay: After the exposure period, the cell culture medium was removed and cells were rinsed twice with PBS. The Cell Proliferation Reagent WST-1 (Roche Diagnostics) was mixed with MEM with 1 % FBS in

a ratio of 1:10 and delivered to the test wells in a volume of 120 µl per well. The plate was incubated at 37 °C for 1h. To prevent interference of NPs adsorbed on the plate plastic with absorbance reading, 100 µL of supernatants from each well were transferred to a new plate. Absorbance was measured at 450 nm using a SpectraMax® M5 Plate Reader (Molecular Device). To determine the viability, background values of wells without cells were subtracted. Negative control (NC) cells were defined to be 100 % viable and the viability was expressed as a percentage of viability of NC.

LDH assay: At the end of the 24h incubation, 50 µL of the supernatant from each well was transferred into a new 96-well microtiter plate for the measurement of released LDH activity ($LDH_{supernatants}$). The rest of the medium was discarded. The survived cells were washed with PBS and lysed with 100 µL of 1 % Triton X-100 (Sigma-Aldrich) at 37°C for 30 min. Fifty µL of the supernatant from each well with lysed cells was transferred into a new plate for the measurement of LDH activity of survived cells ($LDH_{lysates}$). Fifty µL of the reaction mixture containing the detection dye and the catalyst from the Cytotoxicity Detection Kit (LDH) (Roche Diagnostics) was added to both the $LDH_{supernatants}$ and the $LDH_{lysates}$. Plates were incubated in the dark for 15 min. After adding 25 µL of 10 nM HCl to each well to terminate the reaction, the absorbance was measured at 490 nm using the SpectraMax® M5 Plate Reader. Background values of wells without cells were subtracted. The percentage of cell viability was calculated as ($LDH_{lysates}/LDH_{lysates}$ + $LDH_{supernatants}$) × 100. The NC represented 100 % viability and the results were expressed as a percentage of NC viability.

2.3. Statistical analysis

Three independent experiments were performed for each NP sample and both cytotoxicity assays. Data are presented as mean ± standard deviation (SD). To determine significance, an analysis of variance (ANOVA) followed by a Dunnett's test was performed (The Prism 5 program, GraphPad Software, San Diego, CA, USA). Statistical significance was established at p-level <0.05. IC50 values (concentrations that inhibited cell survival by 50 %) were calculated using four-parameter log-logistic models in the drc package [8] in the statistical software R (version 3.4.0.).

3. RESULTS AND DISCUSSION

NMs offer numerous benefits over the conventional chemicals when used in restoration and conservation practices, e.g. high compatibility with historic materials, very high chemical effectiveness due to their increased surface-to-volume ratio and a higher concentration of active substances, ability to penetrate into various and variable porous systems, deeper penetration, antimicrobial properties, self-cleaning, suitable optical properties (such as transparence), etc. [9]. The direct contact of the restorater with the applied subsances and the potential to their release after application emphasize the importance of their safety.

In the present study, we evaluated cytotoxicity as a basic toxicological endpoint of six NMs applicable in restoration and conservation practice that represent three most often used metal oxides. *In vitro* cytotoxicity assays represent a rapid screening tool applicable to preliminary ranking the tested substances and to their prioritization for further testing and applications. Since inhalation represents the main route of exposure to NPs during consolidation processes, A549 cell line was used as an *in vitro* model of human lung epithelial cells. We carried out two independent methods, WST-1 and LDH assays evaluating cell metabolic activity and cell membrane integrity, respectively. This combination allows better interpretation of the results and minimizes the risk that results would be affected by interference of NPs with the test system.

After 24h exposure, a dose-dependent decrease in cell viability was only detected in ZnO NPs and uncoated silica (A200). Hydrophobic coated silicas (R805 and R9200) and TiO_2 NPs (purified and unpurified P25) did not exhibit cytotoxic effects up to the highest tested concentration of 250 µg/mL (**Figure 1**).

Zinc is a vital component, essential for cellular metabolism and cell proliferation. However, high concentrations of Zn^{2+} can cause cell death by breakdown of the mitochondrial membrane potential [10]. It has been

postulated that internalized soluble metal oxide NPs dissolve in the acidic lysosomal fluid releasing ions [11]. To address the contribution of Zn^{2+} ions to the observed cytotoxicity of ZnO NPs, the cells were treated with $ZnCl_2$ as an ionic form of zinc and tested using the WST-1 assay that exhibited higher sensitivity in detection of ZnO cytotoxicity than the LDH assay. The results showed that $ZnCl_2$ had significantly higher toxic effects than ZnO NPs when the exposure concentrations were adjusted to provide the same dose of elemental Zn (**Figure 1E**, IC_{50} values in **Table 2**). The lower cytotoxic potential of ZnO NPs in comparison with $ZnCl_2$ may be explained by a gradual release of Zn^{2+} from the internalized NPs contrarily to complete dissolution of $ZnCl_2$ in the cell culture medium. Lower sensitivity of the LDH assay may be explained by a limited period of the released LDH present in the cell culture medium. At the selected time point (24h) the enzyme may be still kept inside the dying cells during programmed cell death. Contrarily, LDH can already be degraded after 24h if the cells die early after the exposure. The discrepancy between the results obtained using LDH and WST-1 assay shows the importance of employing cytotoxicity assays with different endpoints to avoid underestimation of the results.

A200, R805, and R9200 represent the same type of silica NPs differing only in their surface properties. According to an extensive literature review [12], silica NPs induce significant cytotoxic effects at or above the concentration of 25 µg/mL, which is in concordance with our results for uncoated SiO_2 NPs (**Figure 1C,D**). Based on the obtained results, organosilane coating rendering R805 (SiO_2-octyl), and R9200 (SiO_2-methyl) hydrophobic surface significantly reduced cytotoxic effects of the NPs towards the A549 cells (**Figure 1C,D**). The mechanism of reduction of cytotoxicity of silica NPs by the hydrophobic coating was not investigated in this study. However, it has been shown that reactive silanol groups (Si-OH) on the surface of bare silica are involved in ROS generation inside cells, as well as in cell membrane damage, predominantly mediated by hydrogen bonding [13]. Surface modifications reducing the abundance of surface silanol groups may thus decrease reactivity and related toxicity of silica NPs.

No cytotoxicity of P25 was detected in A549 cells in our study. However, in the available literature, results on cytotoxic effects of TiO_2 NPs (including P25) are very inconsistent. Experimental conditions, e.g. exposure to UV light may significantly affects the results [14]. The contaminants of P25 (Al_2O_3, SiO_2, Fe_2O_3, HCl; the content of all molecules other than TiO_2 based on ignited material is reported to be below 1 % (wt) did not decrease viability, thus the purification did not have any detectable effects on cytotoxicity of P25.

The tested NMs were ranked according to their cytotoxic potential expressed as IC50 (concentration causing 50 % decrease in cell viability) (**Table 2**). In general, soluble metal-oxide NPs, such as ZnO, are considered more toxic than those exhibiting limited solubility (e.g. TiO_2). Manipulation of NM surface chemistry in an easy and effective way to adjust both biological and performace (e.g. consolidating) properties.

Cell death is a basic toxicological endpoint, however, absence of cytotoxic effects alone does not guarantee safety of the tested NPs. Other toxic effects, such as genotoxicity (DNA damage), immunotoxicity, and others, should be taken into account. Nevertheless, *in vitro* cytotoxicity assays provide an effective preliminary tool to screen for low toxic NPs.

Table 2 IC_{50} values (the inhibition concentration that cause 50 % decrease in cell viability) of NPs and $ZnCl_2$ in A549 exposed for 24h. IC_{50} values for ZnO NPs and $ZnCl_2$ were recalculated to the amount of zinc ions (values in brackets). NA - not available (not tested).

Sample	WST-1 assay (µg/mL)	LDH assay (µg/mL)
ZnO	9.4 (7.6 Zn^{2+})	12.6
A200	87.4	88.0
$ZnCl_2$	9.8 (4.7 Zn^{2+})	NA

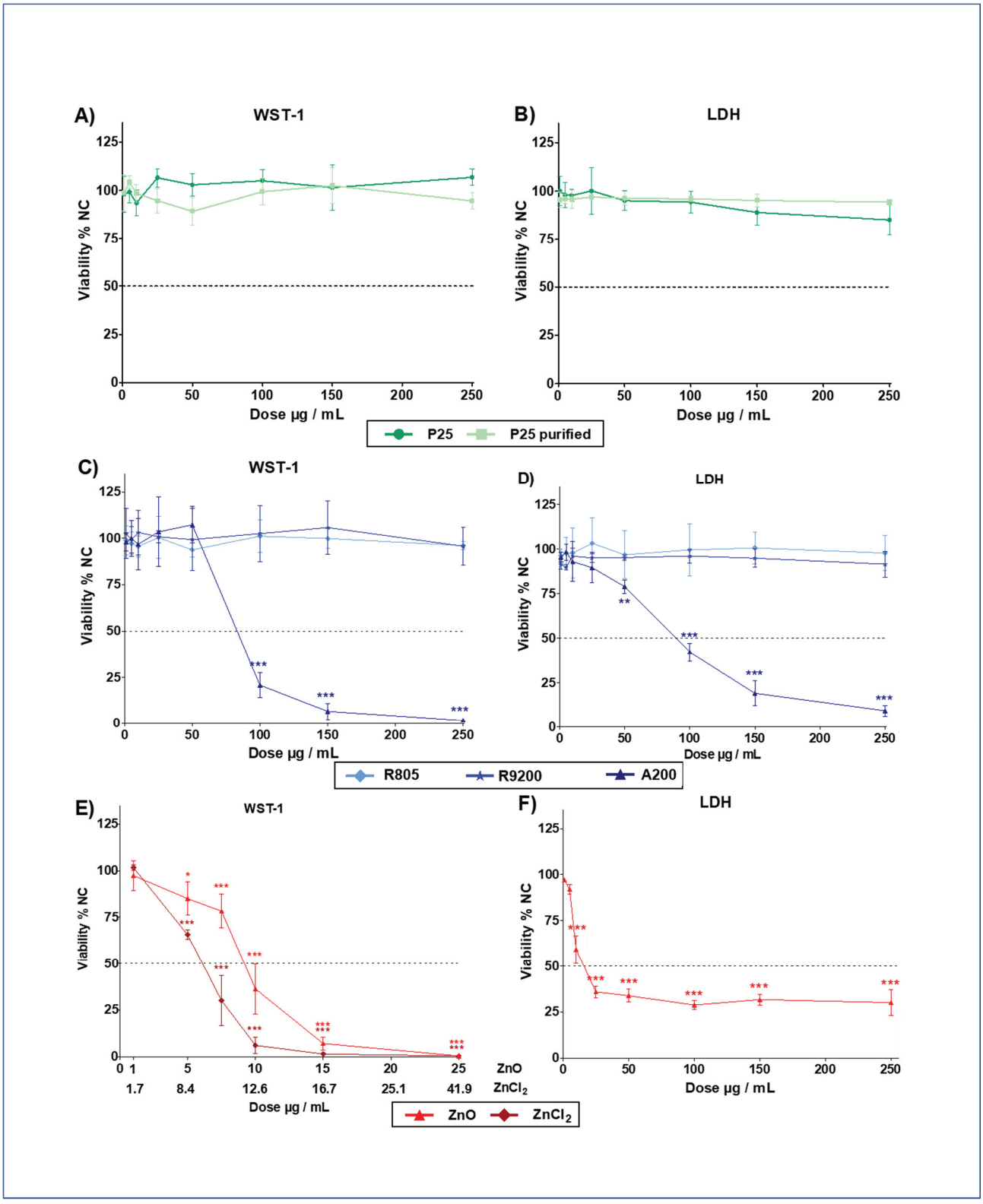

Figure 1 Cytotoxic effects of NPs and zinc salt ($ZnCl_2$) in A549 cells after 24h exposure measured by the WST-1 (A, C, E) and LDH assays (B, D, F). Results are expressed as % of cell viability (N=3). Concentrations of ZnO and $ZnCl_2$ are adjusted to provide the same concentration of molar Zn. Data were analyzed by ANOVA followed by Dunnett's Multiple Comparison. *p<0.05, **p<0.01 and ***p<0.001.

4. CONCLUSION

In this pilot study, we compared three of the most common oxide materials (TiO_2, ZnO a SiO_2) applicable in restauration practice in term of their cytotoxic effects *in vitro* towards the lung epithelial cell line A549 using WST-1 and LDH assays. The results showed that hydrophobic coating prevents cytotoxicity of silica NPs, which may be related to decreased abundance of surface silanol groups and reactivity. ZnO as soluble metal oxide NPs exhibited highest cytotoxicity, probably as a consequence of intracellular dissolution and release of Zn^{2+} ions. Toxicological data will be considered in combination with experimental data evaluating performance of the NPs in restauration processes with the aim to find develop more efficient and safer nano-based products for restoration and conservation.

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