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Multi-omics approaches confirm metal ions mediate the main toxicological pathways of metal-bearing nanoparticles in lung epithelial A549 cells

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Abstract

The toxicity of silver (Ag) and zinc oxide (ZnO) nanoparticles (NPs) has been associated with their dissolution or ability to release metal ions while the toxicity of cerium dioxide (CeO_2) NPs has been related to their ability to induce or reduce oxidative stress dependent on their surface redox state. To examine the underlying biochemical mechanisms, multiple omics technologies were applied to characterise the responses at the molecular level in cells exposed to various metal-based particles and their corresponding metal ions. Human lung epithelial carcinoma cells (A549) were exposed to various Ag, ZnO, and CeO_2 NPs, Ag and ZnO micro-sized particles (MPs), Ag ions (Ag^+) and zinc ions (Zn^{2+}) over a 24h time course. Molecular responses at exposure levels that caused ~20% cytotoxicity were characterised by direct infusion mass spectrometry lipidomics and polar metabolomics and by RNAseq transcriptomics. All Ag, Zn and ZnO exposures resulted in significant metabolic and transcriptional responses and the great majority of these molecular changes were common to both ionic and NP exposures and characteristic of metal ion exposure. The low toxicity CeO_2 NPs elicited few molecular changes, showing slight evidence of oxidative stress for only one of the four CeO_2 NPs tested. The multiple omics analyses highlight the main pathways implicated in metal ions-mediated effects. These results can be used to establish adverse outcome pathways as well as strategies to group nanomaterials for risk assessment.

Keywords

nanoparticles, nanotoxicology, transcriptomics, metabolomics, lipidomics

1. Introduction

Although nanomaterials are increasingly used in many different applications, detailed knowledge on the underlying biochemical mechanisms by which they may induce harmful effects on humans and the environment is lacking. Several possible mechanisms of action have been proposed[1]. One of the proposed mechanisms of action is related to the release of metal ions. For both silver (Ag) and zinc oxide (ZnO) nanoparticles (NPs), toxicity is often related to their dissolution or ability to release metal ions[2, 3]. However, studies comparing the toxicity of these NPs with that of their ionic forms indicate that the toxicity of the NPs cannot always be fully explained by the release of metal ions[4, 5]. Another proposed mechanism of action is via the induction of oxidative stress through the generation of reactive oxygen species (ROS). Cerium dioxide (CeO_2) NPs may have oxidative as well as anti-oxidative properties, depending on their redox surface state. The ability to shift valence states from Ce^{3+} to Ce^{4+} or from Ce^{4+} to Ce^{3+} at the surface of the NPs is suggested to influence the ability of the CeO_2 NPs to either scavenge or generate reactive oxygen species (ROS), respectively, subsequently increasing or decreasing the ability to induce oxidative stress[6, 7]. However, previous studies comparing CeO_2 NPs with different valence states indicate that the mechanisms by which the redox surface status of NPs influences the toxicity are not yet fully understood[7].

Transcriptomics (gene transcriptional profiling), metabolomics (profiling of polar metabolites), proteomics (profiling of proteins) and lipidomics (profiling of lipids) are valuable non-hypothesis driven methods to gain insight into the mechanisms of actions or pathways leading to biological effects of NPs on living organisms, especially when these approaches are combined into a multi-

omics approach to explore a larger molecular landscape[8]. Computational modelling can be used to search for molecular signatures that can contribute to the discovery of molecular key (initiating) events within adverse outcome pathways (AOPs), i.e. mechanistically based molecular changes that are related to both an (upstream) molecular initiating event and (downstream) key events leading to higher levels of phenotypic change[9]. These signatures can be used in the screening, ranking and risk assessment of nanomaterials. When designing a multi-omic study, it is important to generate time-resolved data to be able to follow the different molecular responses within a pathway leading to a biological response[10]. Multi-omics approaches have not yet been widely used within the field of nanotoxicology[11, 12].

In this study, toxicological, analytical and computational methods are combined to 1) identify the molecular mechanisms by which Ag, ZnO and CeO_2 NPs induce toxicity and 2) investigate the influence of dissolution and redox surface state on the NP toxicity using transcriptomics, metabolomics and lipidomics. A549 lung epithelial cells were exposed to nano, micro and ionic forms of Ag, Zn or ZnO and various CeO_2 NPs over a 24 hour time course. A549 cells were selected as they are lung epithelial cells and inhalation is considered an important route of exposure in occupational settings and for consumers using spray products[13, 14]. To investigate the role of dissolution and ionic release on the pathways leading to adverse effects of metal (oxide) NPs, Ag and ZnO NPs and MPs as well as Ag^+ and Zn^{2+} were studied in parallel. In addition, CeO_2 NPs with different amounts of zirconium (Zr)-doping were studied as a means to investigate the effect of the redox surface state on the biological response. Zr-doping increases the Ce^{3+}:Ce^{4+}-ratio and is therefore expected to increase the antioxidant potential of the CeO_2

NPs[6, 7]. Concentrations of NPs and their equivalent ions that induced approximately 20% cytotoxicity after 24 hrs exposure were chosen for this study, aiming to achieve a similar level of cellular damage in all cases. Importantly, temporal responses were investigated by sampling after different exposure times (1, 6 and 24 hrs) to characterize the development of the toxicological responses over time.

2. Materials and Methods

2.1 Nanomaterials: selection, dispersion and characterisation

Ag, ZnO and CeO_2 NPs were selected because of their expected modes of action that involve either the release of ions or their ability to generate or scavenge ROS. An overview of the physicochemical characteristics of the selected materials is given in **Table 1**. When provided as powder the micro- and nano-sized particles were dispersed using the previously published protocol by Jensen et al.[15]. For a final stock concentration of 2.56 mg/mL the powder was pre-wetted with 0.5 vol% ethanol and dispersed in water with 0.05% w/v bovine serum albumin from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands), and sonicated for 16 minutes on ice using a 400 Watt Branson Sonifier S-450D set at 10% amplitude with a 3 mm probe (Branson Ultrasonics Corp., Danbury, CT, USA). When provided as dispersions, the NPs were vortexed for 15 s and sonicated for 5 mins in an ultrasonic bath (Branson CPX2800, 40 kHz, 110W) to re-disperse any possible agglomerates.

2.2 Cell culture and exposures

A549 cells were obtained from ATCC (VA, USA). The cells were cultured in tissue culture flasks in RPMI 1640 medium with Glutamax (Gibco, ThermoFisher Scientific Inc., Landsmeer, the Netherlands) supplemented with 10% Fetal Bovine Serum (FBS, Greiner BioOne BV, Alphen aan de Rijn, the Netherlands) and 1% penicillin/streptomycin (Gibco). Cells were cultured at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. The adherent cells were harvested by a short incubation with 0.5% EDTA trypsin in Ca/Mg free Dulbecco's Phosphate Buffered Saline (Gibco).

To determine the EC20 (effective concentration resulting in 20% cytotoxicity) dose-response studies were performed. A549 cells were harvested and counted 24 hrs before exposure. Twenty thousand ($2x10^4$) cells were seeded in wells of 96-well plates in 100 µL supplemented RPMI 1640 medium. After 24 hrs incubation a semi-confluent monolayer of cells was obtained and the cells were exposed to the various materials. Cell survival (i.e. cytotoxicity) was determined after 24 hrs of exposure by a colorimetric assay using cell proliferation reagent WST-1 (Roche, Sigma-Aldrich Chemie). All exposures were performed in triplicate. Dose-response modelling and derivations of the EC20 were performed using PROAST software [16] version 60.1. For the four CeO_2 NPs and Ag MP no EC20 was obtained, since the highest concentration tested (128 µg/mL) resulted in less than 20% cytotoxicity. An overview of the EC20 values and confidence intervals can be found in **Electronic Supplementary Information (ESI) 1.** Dissolution of nanoparticles and microparticles in cell culture medium was measured using ICP-OES **(ESI 2).**

131 **Table 1:** Physicochemical characteristics of the selected materials[a] and cell viability at doses applied in the omics study

Test material	Batch no	Short Description	Primary size (nm±SD)	Hydrodynamic size (nm±SD) measured with disc centrifuge	Hydrodynamic size (nm±SD) measured with DLS	Dose [b] (µg/mL)	Ion conc. [c] (µg/mL)	Cell viability (%)
Ag NP-NM300K	JRC-Ag<20nm-NM03002a000855b	Ag NPs dispersed in H_2O with 4% polyoxy-ethylene glycerol trioleate and 4% Tween 20.mean particle size 15 nm	< 20	n.m.	50 to 70	38.6	0.04	79
Ag MP	SIGMA- AgBulk - 2-3.5microns- 180215a	micro-sized Ag particles powder	>1000?	n.m.	n.m.	128	0.005	95
$AgNO_3$	-	ionic silver nitrate ($AgNO_3$) soluble powder	n.a.	n.a.	n.a.	**8**	5.08	83
ZnO NP-NM110	JRC-ZnOun-NM110-0801b	uncoated ZnO NPs powder mean particle size 150 nm, primary particle size 42 nm	151 ± 57	193 ± 3	275 ± 4	**15**	1.41	94
ZnO NP-NM111	JRC-ZnOTECS-NM111-2995b	ZnO NPs coated with triethoxy-caprylsilane powder mean particle size 140 nm, primary particle size 34 nm	141 ± 66	n.m.[d]	253 ± 1	**10**	0.989	89
ZnO MP	SIGMA – ZnO- 5 microns- 180215a	micro-sized ZnO particles powder	5000	n.m.	n.m.	**30**	1.46	82
$ZnCl_2$	-	ionic zinc chloride ($ZnCl_2$) soluble powder	n.a.[e]	n.a.	n.a.	24.6	11.80	67
CeO_2 NP-A	PROM-CeO2-20nm-batchCE026A-a	undoped CeO_2 NPs dispersed in H_2O	4.7 ± 1.4	39	172 ± 2	128	<LOD[f]	88
CeO_2 NP-C	PROM-ZrCeO2-batchCE026C-a	27% ZrO_2 -doped CeO_2 NPs dispersed in H_2O	4.6 ± 1.4	40	297 ± 4	128	<LOD	89
CeO_2 NP-E	PROM-ZrCeO2-batchCE025E-a	78% ZrO_2 doped CeO_2 NPs dispersed in H_2O	4.7 ± 1.4	41	358 ± 6	128	<LOD	89
CeO_2 NP-NM212	Umnicore-CeO2-NM212-RIVM-batch	uncoated CeO_2 NPs powder primary particles size 33 nm	28.4 ± 10.4	135 ± 4	213	128	<LOD	87

132 [a] CeO_2 NP-NM212, ZnO NPNM110, ZnO NP-NM111 and Ag NP-NM300K were characterized within the OECD sponsorship programme [17-19]. The other NPs were
133 characterized within the NanoMILE project (Lynch et al., in preparation); [b] Exposure doses for the A549 cells, adapted for incubation in the 6-well plates, are
134 shown in bold figures; [c] Ion concentration measured for the NPs and MPs in cell culture medium after 24 hrs (see ESI 2 for details) and estimated using the
135 molecular weight for $AgNO_3$ and $ZnCl_2$; [d] n.m. = not measured; [e] n.a. = not applicable; [f] <LOD = below limit of detection.

For the omics studies, $8x10^5$ cells per well were seeded in 6-well plates and cultured for 18 hrs, after which the cells were exposed to the determined EC20 concentrations or to 128 µg/mL for the particles where EC_{20} was not reached. Because the cytotoxicity of the A549 cells was higher in the 6-well plates compared to the 96-well plates, several concentrations were adapted and applied as presented in **Table 1**. Omics analyses were carried out on independent replicates from 14 exposure and control groups. Biological replication was, for polar metabolomics n=6, lipidomics n=6 and transcriptomics n=4. Different cell plates were exposed for t=1, t=6 and t=24 hrs to monitor changes in the molecular responses over time. At t=24 hrs, additional control wells were included to measure the actual cytotoxicity and possible interference of the materials with the viability assay. After exposure, the cells (approximately $2x10^6$ per well) were quickly washed with PBS (phosphate buffered saline) twice at room temperature after which the 6-well plates were deep frozen by quenching on liquid nitrogen (-196^oC) and stored at -80^oC until extraction for omics evaluation.

2.3 Omics Analyses

Brief descriptions of the methods used for omics analysis are shown. Full methodological details are provided in **ESI 3**.

2.3.1 Extraction of metabolites and lipids

Cells were harvested then vortexed in methanol:chloroform:water (v/v/v at 1:1:0.9) and the phases separated by centrifugation. The polar phase was dried in a speed vac concentrator

(Thermo Savant, Holbrook, NY) for 4 hr. The non-polar phase was dried under a stream of nitrogen for 5 mins. All dried samples were then frozen at -80°C until analysis.

2.3.2 Direct infusion mass spectrometry (DIMS)

The DIMS analysis method used was similar to previous studies[20, 21]. Dried extracts were re-suspended in 80:20 (v/v) methanol:water with 0.25% formic acid (for positive ion mode analysis of polar extracts) or 80 µL 2:1 methanol:chloroform with 5 mM ammonium acetate (for negative ion mode analysis of lipids). Samples were analysed (in quadruplicate) using direct infusion mass spectrometry (Q Exactive, Thermo Fisher Scientific, Germany) in positive ion mode (for polar metabolomics) or negative ion mode (for lipidomics), utilising a Triversa nanoelectrospray ion source (Advion Biosciences, Ithaca, NY, USA).

2.3.3 Metabolomics Data Processing

Mass spectra were recorded using the selected ion monitoring (SIM) stitching approach from m/z 50-620 (for polar metabolomics) or from m/z 50-1020 (for lipidomics) and then processed using custom-written Matlab scripts as previously reported[22, 23]. The resulting matrices of peak intensities (termed "DIMS dataset") were probabilistic-quotient normalised (PQN) and intensity-drift corrected using a Quality Control-Robust Spline Correction (QC-RSC) algorithm. Finally, the missing values were imputed using the k-nearest neighbours (KNN) algorithm. For multivariate analysis, generalized log (Glog) transformation of the DIMS dataset was performed.

2.4 RNA seq gene expression profiling

2.4.1 Sequencing

Total RNA was extracted from A549 cells using a micro RNeasy Kit (Qiagen, Crawley, UK). All RNA libraries were produced using the Biomek FxP (Beckman Coulter A31842) with Ultra Directional RNA Library Prep Kit (New England Biolabs E7420L) and NEBnext Multiplex Oligos for Illumina Dual Index Primers (New England Biolabs E7600S), using provided protocols and 500ng of total RNA. Multiplex library clustering and sequencing was performed upon the HiSeq2500 (Illumina) with HiSeq Rapid Cluster Kit v2 (Illumina GD-402-4002) at 12pM library concentration with 10% PhiX Control v3 spiked in (Illumina FC-110-3001). The sequencing run was carried out using HiSeq Rapid SBS Kit v2 (Illumina FC-402-4021).

2.4.2 RNA seq Data Processing

The binary base call (BCL) files were converted to FASTQ format (containing a biological sequence and its corresponding quality scores) using Illumina bcl2fastq conversion software (v1.8.4). Sequences were then trimmed using Trimmomatic (v0.36). Five low quality samples were identified and removed accordingly. The FASTQ files were aligned to the GENCODE human transcript sequences (release 25, GRCh38.p7) using Bowtie2 (v2.3.0). The resulting Sequence Alignment Map (SAM) data were converted into Browser Extensible Data (BED) format using SAMtools (v1.3.1) and bamToBed (v2.19.1). Finally, the RNA read counts were extracted from the BED files with a Python script. To provide gene-level analysis, the RNA reads were collapsed to the counts of their coding genes. The gene annotation information was retrieved from the Ensembl database (release 87).

2.5 Omics Data Analysis

Putative metabolite annotations were added using MI-Pack[24]. ANOVA, t-tests and principal components analyses (PCA) were performed in Genespring (v7.3.1. Agilent) using multiple testing corrections[25]. DESeq2[26] was used for differential gene expression analysis with a $q<0.05$ cut-off. Combined gene and metabolite pathway over-representation analyses were performed with IMPaLA[27], using gene identifiers and Human Metabolite Database (HMDB) identifiers [28] for each peak identified as significantly altered as input lists. Comparative pathway analyses were performed with Ingenuity Pathway Analysis (IPA; Qiagen) on combined sets of genes, lipids and polar metabolite identifiers. Raw transcriptomic data and experimental details are archived at ArrayExpress (accession number: E-MTAB-5734).

3. Results

In total, 259 polar metabolomic, 250 lipidomic and 156 transcriptomic samples passed the quality control metrics. Four RNAseq samples were removed prior to further analysis due to anomalously low counts. The full results of univariate analyses comparing each exposure group with its time-matched control group are shown in **ESI 4**. Comparisons were made with time-matched controls since gene expression and metabolite profiles varied significantly with time between the control groups sampled at 1h, 6h and 24h. **Figure 1** illustrates the overall numbers of significantly changing ($q<0.05$) genes and metabolites in comparison with time-matched controls. The numbers of molecular (transcript and metabolic) changes at 24h correlated significantly with cytotoxicity (Table 1) for all silver (Ag MP, Ag NP and Ag^+) exposures ($r^2=0.98$;

223 $p<0.01$) and all zinc (ZnO MP, ZnO NP and Zn^{2+}) exposures ($r^2=0.85$; $p<0.03$) but not for the CeO_2
224 NP exposures. All silver exposures resulted in significantly more changes than CeO_2 exposures,
225 and all zinc exposures led to the most numerous alterations. Silver exposures ranked from the
226 largest to the smallest effect in the order Ag^+, Ag NP and then Ag MP, whereas zinc exposures
227 ranked highest for Zn^{2+}, followed by ZnO MP, ZnO NP-NM110 and ZnO NP-NM111. These rank
228 orders matched the orders of ionic concentrations determined by dissolution analysis (Table 1).
229 CeO_2 NP exposures resulted in few gene expression or metabolic changes, and ionic dissolution
230 was below the limit of detection. Of these few changes, CeO_2 NP-A (undoped CeO_2 NPs) was the
231 only CeO_2 NP that increased metabolites putatively identified as cysteine at 1 hr (3.5 fold;
232 $q<0.032$) and γ-glutamylcysteine at 6h (1.5 fold; $q<0.02$).
233
234 PCA scores plots of transcriptomic and polar metabolomic data after 6h of exposure are shown
235 in **Figure 2**, with PCAs for all other timepoints, as well as for the lipidomics data, shown in **ESI 5**.
236 For silver there was grouping of replicate samples and separation from the controls was
237 apparent for all exposure groups. For zinc, replicate samples grouped tightly and clearly
238 diverged from the control group along PC1, with the degree of divergence corresponding with
239 the number of molecular changes outlined above (see also Figure 1). There was little or no
240 apparent grouping of the CeO_2 NP samples or divergence from the control group.
241 IMPaLA pathway over-representation analysis results are shown in full in **ESI 6**, while over-
242 representation of selected pathways is illustrated in **Figure 3**. All silver and zinc exposures at 6h
243 and 24h resulted in significant enrichment of the terms 'Response to metal ions' and
244 'Metallothioneins bind metals'. Terms relating to the heat shock response were enriched in the

same groups at 6h but not at 24h, except for the Ag^{+} and Zn^{2+} exposures where they persisted. Other enriched terms, including 'Translation', 'Nonsense-Mediated Decay', 'Apoptosis' and 'Immune System' were highlighted with all zinc exposures and either Ag^{+} or Ag NP exposure at 6h and sometimes also at 24 h. Zn^{2+}, ZnO MP and ZnO NP-NM110 repressed molecules related to DNA repair. CeO_2 exposures elicited few molecular changes and showed no enriched pathway annotations, apart from 'HIF-1 alpha transcription network' induced at 24h with CeO_2 NM212 and 'ID signalling pathway' repressed at 6 and 24h with CeO_2 NP-A,- C and E.

Ingenuity Pathway Analysis (IPA) Comparison Analyses were used to compare molecular pathway responses to the various silver and zinc exposures. **Figure 4** shows the top 20 canonical pathways and the top 20 'diseases and bio-functions' associated with the silver and zinc exposures, ordered by function or process from IPA's Pathway Activity Analysis function, representing predicted pathway activation or inhibition. Particularly prominent for silver was the Nrf2-mediated oxidative stress response pathway, predicted to be activated by Ag NP at 6h and by Ag^{+} at all timepoints. The Nrf2 pathway was also predicted to be activated by all Zn exposures at 6h, but repressed at 24h. The canonical pathway comparison of silver exposures was otherwise dominated by modulation of several molecular signalling pathways, particularly with Ag^{+} at 6h. All Zn exposures resulted in very similar profiles of predicted pathway activation, highlighting co-ordinated induction of signalling pathways at 6h, followed by repression at 24h, except for Zn^{2+} for which these pathways were predicted to still be activated at 24h. Data from CeO_2 NP exposures were not used due to the low numbers of responsive molecules.

To identify candidate nano-specific responses, t-tests were performed comparing, for silver, the Ag NP group versus respectively the control, Ag^+ and Ag MP groups. Molecules were only selected if statistically significantly (FDR<0.05) changed in all comparisons. A similar procedure was followed for ZnO NP-NM110 and ZnO NP-NM111. The results of these comparisons and IMPaLA pathway analyses using these data are shown in **Figure 5 and ESI 7**. For Ag NPs, 17.6% of transcriptional and 22% of metabolic changes were assessed as candidate nano-specific. The induced molecules associated with several pathways, particularly those concerned with amino acid metabolism, while the decreasing molecules associated with glycolysis and galactose metabolism and reduced transcription relating to phase II xenobiotic metabolism. ZnO NP-NM110 elicited only 12 (0.15%) candidate nano-specific changes and ZnO NP-NM111 elicited 22 (0.77%), mostly reduction in metabolites associated with galactose metabolism.

4. Discussion

In our study molecular changes were sought that were unique to the nanomaterial exposures, not appearing in response to the ionic or micro-sized particle exposures at any timepoint. These were termed 'candidate nano-specific responses' as only three timepoints were examined for each exposure, raising the possibility of these responses having occurred at an unexamined timepoint in the non-NP exposures. For silver there was evidence for candidate nano-specific changes supported by both the transcriptomics and metabolomics data (**Figure 5 and ESI 7**). These were related to increases in amino-acid transport, reduced glycolysis and galactose metabolism and reduced glucuronidation and xenobiotic metabolism. Potentially these changes

could result in lower capacity to detoxify organic xenobiotics and it could be instructive to compare modulation of organic xenobiotic toxicity in co-exposures with Ag NP or Ag^+. However, since no dispersant control (for Ag NP-NM300K) was included in the omics study, some of these nano-specific changes may be caused by the dispersant (water with 4% polyoxyethylene glycerol trioleate and 4% Tween 20), instead of the Ag NPs. Although previous studies with the same Ag NPs and its dispersant indicated that the dispersant was not cytotoxic to A549 cells up to 256 μg/mL, [29, 30] DNA damage was observed in the absence of cytotoxicity [31] in A549 cells. For zinc there was very little evidence of candidate nano-specific responses, limited to metabolomics changes related in pathway analyses to a reduction in galactose metabolism. Although candidate nano-specific responses were found for Ag NPs, by far the majority (>78%) of responses to the Ag NPs were also seen with Ag^+ and Ag MP, as was found by NMR metabolomics in HaCaT cells[32], implying that nano-specific toxicity is likely a minor component compared with that elicited by silver ions.

All silver and zinc exposures induced transcription of genes responsive to metal ions at 6h and 24h. Metallothionein induction was particularly notable, with *MT1A*, *MT1B*, *MT1F*, *MT1G*, *MT1X*, and *MT2A* highly and significantly induced with all Ag and Zn exposures but *MT1H* and *MT1E* induced only with Zn. Several of these *MT* transcript inductions exceeded 1000-fold, including *MT1B* with Ag NPs and Ag^+ at 6h and *MT1H* with ZnO NM-110, Zn^{2+} and Zn MP at 6h and 24h. Additionally the zinc transporter *SLC30A1* (ZnT-1), responsible for export of zinc ions, was induced by both Zn and Ag exposures. Ag ions have been shown to release Zn ions in fibroblasts[33].

Metallothioneins have long been considered biomarkers for metal ion exposure and oxidative stress [34] and metal-based NP studies frequently report their induction[35, 36]. Metallothionein induction may be viewed as an adaptive response enabling cells to bind and sequester metal ions and for Ag MP exposure this response appeared effective, resulting in only 5% cytotoxicity (**Table 1**) and few significant alterations in other biological pathways (**Figure 3; ESI 6**). However with the other Ag and Zn exposures resulting in higher cytotoxicity, it was apparent that this capacity was exceeded, leading to stress responses and cellular damage. The heat shock response was activated in all 6h and 24h Zn exposures and with Ag^{2+} and Ag NPs; transcripts encoding the molecular chaperones HSPA1A, HSPA1L, HSPA6, HSPA7, HSPB1, HSPH1, HSP90AA1, HSP90AB1, DNAJB1 and DNAJB6 were induced, implying a response to protein damage.

Oxidative stress is a well-established outcome following NP exposure, including Ag, ZnO and CeO_2, NPs[37, 38]. IPA highlighted the Nrf2 mediated oxidative stress response as activated by Ag^{+} and all 6h Zn exposures. Nrf-2 (*NFE2L2*) transcript was significantly but not highly induced (<1.6-fold) with Zn^{2+} and Zn MP and several key antioxidant enzyme transcripts were either mildly but significantly induced (*SOD1*, *TXN*, *GLRX*, *GCLM*, *GSS*) at 6h or mildly repressed (*CAT*, *GCLC*, *GSR*, *PRDX1*). Aldehyde oxidase *AOX1* was induced over 2-fold, as were heme oxygenase (*HMOX1*), several chaperones mentioned above and transcription factors *FOS*, *JUN* and *ATF4*. Transcription of *KEAP1*, a repressor of Nrf-2 signalling, was significantly repressed with ZnO NM-110, Zn^{2+} and Zn MP at 6h. Nrf2 pathway induction has previously been found for Ag NPs[39] and for ionic Ag and Zn[40]. Several polar metabolite peaks that were putatively annotated as

glutathione (GSH) followed a similar profile of a significant increase with Ag^{+} at 1h but a significant decrease with all zinc exposures at 6h and 24h. GSH is the major intracellular antioxidant and its depletion implies vulnerability to further oxidative damage. GSH has previously been found to decrease with ZnO NP treatment in mouse livers and kidneys[41, 42].

A metabolite peak putatively annotated as cysteine was particularly highly increased with all Ag exposures, and amino acid concentrations were significantly altered with most exposures. This may represent an adaptive reorganisation of amino acid synthesis and transport. Expression of transcripts involved in translation, including those encoding ribosomal subunits, was increased with Ag NP, ZnO MP and Zn^{2+} exposure. Interestingly nonsense mediated decay pathway transcripts were induced in the same exposures, implying an increase in mRNAs with premature stop codons. Potentially this could be due to an increased rate of DNA damage, as transcription of DNA repair genes was reduced, including those of the base excision repair, mismatch repair, nucleotide excision repair and double-strand break repair pathways, particularly with Zn^{2+}, Zn MP and ZnO NP-NM110. DNA damage has previously been described for Ag^{+}, Ag NPs[43, 44], Zn^{2+} and Zn NPs[45]. Transcription of the stress-inducible AP-1 transcription factor genes was increased by Zn^{2+} and ZnO NP exposure, and by Ag^{+}, including induction of *FOS*, *FOSB*, *FOSL1*, *JUN* and *JUNB*. Cell cycle gene transcription was significantly repressed with both Ag and Zn exposures. These effects have previously been seen with Ag NPs[43, 46, 47]. There was an induction of transcription associated with apoptosis and with immune signalling with Ag^{+} and all Zn exposures at 6h, persisting to 24h with Zn^{2+}. Both ionic and NP Ag and Zn can increase apoptosis in A549 cells[48, 49]. By 24h Zn^{2+} exposure, both transcripts and metabolites of the TCA

cycle were significantly reduced, indicating major disruption of cellular respiration pathways, consistent with the bioenergetic disruption reported for ZnO NP exposure of A549 cells[50].

These molecular alterations illustrate a progression from adaptive changes, such as metallothionein induction, to depletion of antioxidants, such as glutathione, repressed DNA repair and induction of apoptosis. Several of these molecular changes have been proposed as key events (KE) in the Adverse Outcome Pathway (AOP) paradigm[51]. Examples include increase in oxidative stress, activation of Nrf-2, depletion of glutathione, repression of DNA repair and increased apoptosis. It is however apparent that many additional pathways, genes and metabolites were altered during the exposures to Ag and Zn (**ESI 5 and 6**) and that using a cell line model one can only examine those events leading up to cell death.

For ZnO NPs and MPs, responses indicative of damage tended to peak at 6h, with a reduction by 24h, for example acute phase signalling (**Figure 5**), while with Zn^{2+} these changes persisted to 24h, consistent with the greater cytotoxicity caused by this treatment. The responses to Ag and Zn clearly demonstrated the importance of measuring molecular responses over time in order to robustly assess molecular toxicity. At 1h there were relatively few molecular pathway changes (**Figure 3**), at 6h molecular responses indicative of toxicity had developed, but at 24h for several exposures (Ag^{+}, Ag MP, ZnO NP-NM110, ZnO NP-NM111 and Zn MP) the responses had declined, or even reversed (**Figure 4**) whereas these persisted with the more cytotoxic $ZnCl_2$ exposure. This time-dependence of molecular response can be explained by adaptive changes, such as induction of metallothioneins that ameliorate cellular damage by sequestering

the metal ions, as illustrated by the Ag MP exposure. There may also be a time dependency in exposure to the metal ions, due to different uptake rates and dissolution kinetics of the different micro- and nano-sized particles resulting in different intracellular concentrations, or different intracellular compartmentalisation of the metal ions. In A549 cells and phagocytic murine macrophages, Ag NPs were associated with lysosomes[52], whereas ionic Ag^+ was bound to metallothioneins[53]. ZnO NPs showed intracellular dissolution in lysosomes of macrophages[54] and extracellular dissolution with only ions entering hepatocytes[55]. Additionally NP dissolution can also occur within the NP preparations[56] and in cell culture medium (ESI 2). The dynamic molecular responses detected could therefore reflect changing intracellular doses of metal ions. For future studies it is therefore recommended to obtain additional supporting data to estimate the intracellular doses, including time-resolved data on the bioavailable ion concentrations after exposure to the salts and cellular uptake rates of MPs, NPs and ions.

CeO_2 NPs led to relatively few significant alterations of transcription or metabolism compared with silver and zinc, reflecting both a decrease in molecular alterations with increasing EC20 concentrations (effective concentrations resulting in 20% cytotoxicity) and their low solubility (Table 1). A similar mild metabolomic and transcriptomic response to CeO_2 NPs was found by Taylor et al.[12] in algae. Among the few significant alterations induced by the CeO_2 NPs, only CeO_2 NP-A (undoped CeO_2 NP) exposure significantly increased metabolites putatively identified as cysteine and γ-glutamylcysteine, potentially representing an adaptive response to oxidative stress by increased uptake and synthesis of these glutathione precursors. Because these changes were not observed after exposure to CeO_2 NP-C (27% Zr-doped CeO_2 NPs) or

398 CeO_2 NP–E (78% Zr-doped CeO_2 NPs), this finding might indicate that modification of the
399 surface redox state by Zr-doping increases the ability to scavenge ROS, resulting in a decreased
400 induction of oxidative stress of the CeO_2 NPs. Since all CeO_2 NPs showed very low cytotoxicity and
401 exposure to only one of the four CeO_2 NPs showed any slight evidence of molecular response to
402 oxidative stress, the actual occurrence of oxidative stress, ROS or damage related to ROS was not
403 further investigated.

404

405 CeO_2 NP-A, -C and -E exposures all resulted in repression of ID family gene expression (**Figure**
406 **4**). Verstraelen et al. [57] similarly found repression of *ID2* transcription in A549 cells treated with
407 CeO_2 NPs. The ID, or Inhibitor of DNA binding, genes are binding partners of bHLH transcription
408 factors and are involved in regulation of a wide variety of biological processes, including
409 metastasis and vascularisation[58]. CeO_2 NP-NM212 elicited a different molecular response from
410 the other CeO_2 NPs, activating genes of the HIF1-alpha transcription factor network (**Figure 4**)
411 by 24h. This response was also shared by the 6h Ag and Zn exposures, with additional induction
412 of heme oxygenase (*HMOX1*). HIF1-alpha responsive genes are also commonly induced by
413 several metal ions and particles[59] and in cancer cells by ROS[60], leading to angiogenesis via VEGF.
414 This finding is consistent with CeO_2 and Ag NPs inducing angiogenesis[61, 62].

415

416

417 5. Conclusions

418 A time series experiment was used to determine the similarity of A549 cellular responses
419 following exposure to NPs and ions, as focussing on a single timepoint would have led to

420 erroneous conclusions in the absence of internal dose measurements. The majority of
421 molecular responses of A549 cells to the Ag and Zn NPs, such as metallothionein induction,
422 depletion of antioxidants, repressed DNA repair and induction of apoptosis, are similar to their
423 responses to Ag and Zn ions, respectively, confirming that the modes of action of these NPs are
424 largely mediated by dissolved metal ions rather than by the physical aspects of the NPs. Low
425 toxicity CeO_2 NPs elicited only minor molecular responses. Of the four CeO_2 NPs tested, only
426 CeO_2 NP-A elicited any molecular changes indicative of oxidative stress.

427

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Figure Legend

Figure 1: Number of genes significantly differentially expressed (black bars) and metabolite peaks significantly altered in concentration (clear bars) ($q<0.05$) in A549 cells after silver, zinc, or CeO_2 NPs, MPs and/or ionic exposures for 1, 6 or 24 h.

Figure 2: Principal components analysis scores plots of transcriptomics and metabolomics data from A549 cells exposed to silver, zinc, or CeO_2 for 6 hrs. For silver, control samples are shown in black, Ag NP treated in red, Ag MP in cyan, Ag^+ in blue. For zinc, control samples are shown in black, ZnO NP-NM110 in red, ZnO NP-NM111 in cyan, Zn^{2+} in blue and ZnO MP in pink. For CeO_2, control samples are shown in black, CeO_2 NP-A treated in red, CeO_2 NP-C in cyan, CeO_2 NP-E in blue and CeO_2 NP-NM212 in pink. PCAs for all timepoints are shown in **ESI 5**.

Figure 3: Heatmap illustrating selected pathway annotation terms significantly differentially represented ($q<0.05$) by IMPaLA among genes and metabolites induced (red) or repressed (green) in comparison with time matched control groups after exposure of A549 cells to silver, zinc, or CeO_2 (NPs, MPs or ions) for 1, 6 or 24h. Full data are shown in **ESI 6**.

Figure 4: The top 20 canonical pathways and the top 20 'diseases and bio-functions' associated with the silver and zinc exposures in A549 cells, ordered by function or process from Ingenuity Pathway Analysis (IPA) Pathway Activity Analysis function, representing predicted pathway activation (orange) or inhibition (blue) with maximum colour intensity set to z-score ≥2.

Figure 5: Heatmap illustrating selected pathway annotation terms significantly differentially represented ($q<0.05$) by IMPaLA among candidate nano-specific transcripts and metabolites induced (red) or repressed (green) in comparison with control, microparticle and ionic exposures over all timepoints. Full data are shown in **ESI 7**.

Supporting Information

ESI 1: Cytotoxicity of tested particles to A549 cells

ESI 2: Dissolution of NPs and MPs in cell culture medium using ICP-OES

ESI 3: Methods

ESI 4: Univariate analyses of omics data

ESI 5: PCA scores plots of omics data

ESI 6: IMPaLA pathway analysis of omics data

ESI 7: Candidate nano-specific molecular responses