Endothelial cell activation, oxidative stress and inflammation induced by a panel of metal-based nanomaterials

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Abstract

The importance of composition, size, crystal structure, charge and coating of metal-based nanomaterials (NMs) were evaluated in human umbilical vein endothelial cells (HUVECs) and/or THP-1 monocytic cells. Biomarkers of oxidative stress and inflammation were assessed because they are important in the development of cardiovascular diseases. The NMs used were five TiO$_2$ NMs with different charge, size and crystal structure, coated and uncoated ZnO NMs and Ag which were tested in a wide concentration range. There were major differences between the types of NMs; exposure to ZnO and Ag resulted in cytotoxicity and increased gene expression levels of HMOX1 and IL8. The intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression were highest in TiO$_2$ NM-exposed cells. There was increased adhesion of THP-1 monocytic cells onto HUVECs with Ag exposure. None of the NMs increased the intracellular ROS production. There were no major effects of the coating of ZnO NMs. The TiO$_2$ NMs data on ICAM-1 and VCAM-1 expression suggested that the anatase form was more potent than the rutile form. In addition, the larger TiO$_2$ NM was more potent than the smaller for gene expression and ICAM-1 and VCAM-1 expression. The toxicological profile of cardiovascular disease-relevant biomarkers depended on composition, size and crystal structure of TiO$_2$ NMs, whereas the charge on TiO$_2$ NMs and the coating of ZnO NMs were not associated with differences in toxicological profile.

Introduction

The research on toxicological properties of nanomaterials (NMs) is increasingly important due to the rapid expansion of occupational, scientific and commercial use (Nel, 2013). The cardiovascular system is considered to be affected by NM exposure because of the analogy with ambient air particles that are associated with an increased risk of acute morbidity and mortality of cardiovascular diseases (Brook & Rajagopalan, 2010; Delfino et al., 2005; Mills et al., 2009). The principal cause of cardiovascular mortality and hospitalizations is atherothrombosis, which can lead to acute myocardial infarction in patients with coronary heart disease (Mills et al., 2007). A recent review showed that pulmonary exposure to ambient air particles as well as engineered NMs was associated with the development of vasomotor dysfunction and atherosclerosis in animal experimental models and humans (Møller et al., 2011). It has been suggested that the size, crystal structure and charge of TiO$_2$ particles are important variables for oxidative stress and inflammation (Johnston et al., 2009), but the effects of these variables have not been thoroughly investigated in regard to vascular endpoints. Oxidative stress and inflammation are implicated in the initiation and development of endothelial dysfunction, which plays an important role in the pathogenesis of a wide range of cardiovascular diseases (Elahi & Matata, 2006; Elahi et al., 2009). Atherosclerosis is also an inflammatory disease where leukocytes generate both reactive oxygen species (ROS) and cytokines (Hansson & Libby, 2006). Endothelial cells express intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the membrane during inflammation, which is believed to be a critical step in the initiation of atherosclerosis (Libby, 2002). VCAM-1 mediates the arrest and adhesion of inflammatory cells to the vascular endothelium (Carlos et al., 1990), whereas ICAM-1 is involved in the stable adhesion step and migration of monocytes across the endothelial barrier (Javahid et al., 2003). Thus, adverse effects of NM exposure on endothelial cells and monocytes could be relevant endpoints in hazard characterization with respect to atherothrombosis.

We hypothesized that adverse effects of NMs depend not only on the chemical composition and particle size, but also on the surface charge, crystal structure and coating. To this end, we investigated the effect of exposure to a panel of metal-based NMs on: (i) cytotoxicity; (ii) expression of adhesion molecules ICAM-1 and VCAM-1; (iii) ROS production; (iv) pro-inflammatory response (IL8, TNF, CCL2); (v) oxidative stress response (HMOX1); and (vi) cell adhesion of monocytes onto endothelial cells. These end points were measured in human umbilical vein endothelial cells (HUVECs) and/or THP-1 monocytic cells with a panel of eight engineered NMs, including Ag, five different TiO$_2$ with different sizes or crystal structure and/or surface charge, and coated and uncoated ZnO in a wide concentration range. Monocyte adhesion to HUVECs was only performed for four NMs, selected to address the importance of size for TiO$_2$ and effects related to the most cytotoxic NMs in terms of ZnO and Ag. The panel of NMs was part of the European research program,
Engineered NanoParticle Risk Assessment (ENPRA). NMs were provided by the program of NMs health and safety research of the Organisation for Economic Co-operation and Development (OECD). TiO₂ NMs were used for the assessment of effects of size, structure and charge with negatively and positively charged NMs made specifically for the ENPRA study. TiO₂ is used as a food-coloring agent, white pigment in toothpaste and UV-light-blocking agent in sunscreens. TiO₂ is also commonly used in paints, where it typically contains particle surface modifications in order to provide special properties of the paints (Christensen et al. 2011). The ZnO NMs were selected for the assessment of the impact of coating because they are used as surface-coated preparations, for instance in sunscreens (Schluesener, 2013).

Methods

Handling and preparation of NMs

The NMs with the prefix ‘‘NM’’ were obtained from the European Commission Joint Research Centre (Ispra, Italy): NM 101 (Hombikat UV100; TiO₂, anatase, 7 nm), NM 110 (BASF Z-Cote; zinkite, uncoated, 100 nm), NM 111 (BASF Z-Cote; zinkite coated with triethoxyacrylylsilane, 130 nm) and NM 300 (RAS GmbH; Ag capped with polyoxylaurat Tween-20). NRCWE 004 (TiO₂, rutile, 94 nm) was purchased from NaBond Technologies Co., Hong Kong, China. NRCWE 001 (TiO₂, rutile, 10 nm) was purchased from NanoAmor (Houston, TX) and used for the production of NRCWE 002 (amino-TiO₂ with positive charge) and NRCWE 003 (carboxy-TiO₂ with negative charge) as described previously (Kermanizadeh et al., 2012a). The handling and the preparation, including dispersion of the NMs was standardized among the laboratories participating in ENPRA (Jacobsen et al., 2010). The preparation of suspensions is described in detail in section S1 in the supplementary material. In brief, the NMs (2.56 mg/ml) were dispersed in 0.22 µm filtered Milli-Q water or in RPMI cell culture medium to the final concentrations. The NMs were then centrifuged and washed twice before they were transferred into black 96-well plates. The continuous measurement for 3 h was chosen to respond to the absorbance measured in non-exposed cells. The endotoxin content was measured by the Limulus Ameobocyte Lysate (LAL) pyrogent assay (Lonza, Walkersville, MD). None of the NMs (256 µg/ml) displayed a positive reaction of endotoxin in the LAL assay, which had a sensitivity of 0.06 EU/ml.

The main physical and chemical properties of the NMs have previously been published (Kermanizadeh et al., 2012a). TEM size and TEM characteristics are reproduced in Table 1. The size of the NMs in suspensions with or without heat-inactivated fetal bovine serum (FBS) was evaluated by the Nanoparticle Tracking Analysis. The NMs were suspended in either 0.22 µm filtered Milli-Q water or in RPMI cell culture medium with 10% FBS. The results are shown in Table 1. The particles were sonicated as described above and diluted to a final concentration of approximately 1 µg/ml. The samples were analyzed on a Nanosight LM20 (Nanosight Ltd, Malvern Instruments, Malvern, UK) with a blue (405 nm) laser.

Cell cultures and NM exposure

THP-1 cells (American Type Culture Collection, Manassas, VA) were used as surrogate cells for human circulating monocytes in the cardiovascular system and kept in an undifferentiated state (Qin, 2012). The cells were cultured in RPMI cell medium with 10% FBS (EU origin) from Gibco®, Life Technologies Europe BV (Naerum, Denmark), as previously described (Danielsen et al., 2009). THP-1 cells are cultured in suspension. Thus, the exposure concentrations are given per volume of medium rather than per culture area.

The HUVECs and growth medium were purchased from Cell Applications (San Diego, CA). The cells were cultured in Endothelial Cell Growth Medium, with 2% serum as previously described (Mikkelsen et al., 2011). The cells were used at passages 2 to 5 to maintain morphologic and phenotypic characteristics of endothelial cells.

The single cultures were exposed to the same 2-fold concentration range between 2 and 256 µg/ml (corresponding to 0.64–80 µg/cm²) in most experiments. For gene expression measurements the concentrations of 2, 8 and 32 µg/ml were excluded. For the cell adhesion assay the highest concentrations of 128 and 256 µg/ml were excluded and NMs included in the assay were the small and large TiO₂ (NRCWE 001 and NRCWE 004, respectively), uncoated ZnO (NM 110) and Ag (NM 300). Cellular uptake of the NMs, assessed by flow cytometry, is described in section S2 in the supplementary material. All measurements were repeated on three different days of analysis and they were analyzed in triplicate on each day of analysis.

Cytotoxicity

The cytotoxicity was measured with the colorimetric WST-1 assay from Roche Diagnostics GmbH (Mannheim, Germany). The amount of formazan dye formed in the assay directly correlates with the number of metabolically active cells. The assay was carried out as described in the manufacturer’s instruction. In brief, cells (5 × 10⁴ THP-1 or 2 × 10⁴ HUVECs) were seeded in 96-well plates and exposed to NMs for 24 h. The cells were subsequently washed and incubated in fresh media with 10% WST-1 reagent for 2 h. The absorbance was measured at 450 nm, with 630 nm as reference, using an ELISA reader (Multiskan Ascent, Thermo Labsystems, Thermo Fisher Scientific Inc, Waltham, MA). The results are shown as percent cytotoxicity, where 100% corresponds to the absorbance measured in non-exposed cells. The concentration, which is cytotoxic to 50% of the cells (LC50), was extrapolated from the concentration-response curve. The assay included two tests for interference between WST-1 and the NMs (2.56 and 256 µg/ml): (1) Cell medium + NMs and (2) Cell medium + WST-1 + NMs. The tests did not show any interference between NM exposure and WST-1 absorbance (results are shown in Table S1 in the supplementary material).

ROS production

The intracellular ROS production was measured in THP-1 cells and HUVECs as well as in a cell-free experiment by the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay as previously described (Danielsen et al., 2011). In brief, 2 × 10⁴ HUVECs were seeded in black 96-well plates and incubated in cell culture medium for 24 h before the incubation with DCFH-DA (Cayman Chemical, Ann Arbor, MI). The HUVECs were incubated with 2 µM DCFH-DA for 15 min and washed twice with Hank’s balanced saline solution. The THP-1 cells (5 × 10⁴ cells) were incubated with 2 µM DCFH-DA for 15 min and spun down and washed twice before they were transferred into black 96-well plates. The fluorescence in the cells was measured continuously on a fluorescence spectrophotometer at 485 and 538 nm (Fluoreskan Ascent FL, Thermo Labsystems) at 37°C for 3 h. The continuous measurement for 3 h was chosen because (1) the probe might be toxic to the cells during a longer exposure, (2) ROS production reaches a plateau after 3 h incubation that maybe due to the toxicity of the probe or that
Table 1. Main physical and chemical characteristics.

| NM code | Type (supplier information) | Average size (TEM) | Primary characteristic (TEM) | Size (nm) in water (NTA) | Size (nm) in RPMI (NTA) |
|---------|-----------------------------|--------------------|-----------------------------|--------------------------|-------------------------|
| NRCWE 001 | TiO₂ (Rutile) | 10 nm (XRD) | Irregular euhedral particles. | Mean (SD): 129 (39) | Mode: 112 |
| NRCWE 002 | TiO₂ (Rutile) | 10 nm (XRD) | Irregular euhedral particles. | Mean (SD): 126 (38) | Mode: 111 |
| NRCWE 003 | TiO₂ (Rutile) | 10 nm (XRD) | Irregular euhedral particles. | Mean (SD): 123 (35) | Mode: 113 |
| NRCWE 004 | TiO₂ (Rutile) | 94 nm (XRD) | Five particle types were identified: (1) irregular spheres, 1–4 nm (av.); (2) irregular euhedral particles, 10–100 nm; (3) fractal-like structures in long chains, 100–200 nm; (4) irregular polyhedral particles, 1–2 µm; (5) irregular particles with jagged boundaries, 1–2 µm. | Mean (SD): 202 (104) | Mode: 132 |
| NM 101 | TiO₂ (Anatase) | 7 nm | Two structures found; type 1 show agglomerates in the 50-1500 nm range. | Mean (SD): 171 (65) | Mode: 145 |
| NM 110 | ZnO (uncoated) | 100 nm | Mainly 2 euhedral morphologies: (1) aspect ratio close to 1 (20–250 nm range and few particles of approx. 400 nm); (2) ratio 2 to 7.5 (50–350 nm). Minor amounts of particles with irregular morphologies. | Mean (SD): 162 (54) | Mode: 138 |
| NM 111 | ZnO (coated) | 130 nm | As NM110, but with different size distributions. (1) particles with aspect ratio close to 1 (~90% in the 20–200 nm range); (2) particles with aspect ratio 2 to 8.5 (~90% in the 10–450 nm range). | Mean (SD): 202 (63) | Mode: 191 |
| NM 300 | Ag | <20 nm | Mainly euhedral morphology; minor fractions have either elongated (aspect ratio up to ~5) or sub-spherical morphology. | Mean (SD): 48 (19) | Mode: 42 |

Average size, TEM size and TEM characteristics are reproduced from (Kermanizadeh et al., 2012b).

XRD: X-ray diffractogram; NTA: nanoparticle tracking analysis.

the probe is utilized, (3) a longer time outside the incubator can affect the viability of the cells. Carbon black particles (Printex 90, 10 µg/ml) were used as positive control because it has been shown that this concentration increases the ROS production by 4-fold in both THP-1 cells and HUVECs (Danielsen et al., 2011; Vesterdal et al., 2012). All experiments were repeated three times and the accumulated ROS production over time was calculated as the area under the curve. The results are reported as fold-increase compared with the control. The concentrations with statistically significant cytotoxicity, indicated by the WST-1 assay, are excluded from the figures and the statistics.

ICAM-1 and VCAM-1 surface expression

The surface expression of ICAM-1 and VCAM-1 was measured with a modified ELISA procedure (Frikke-Schmidt et al., 2011; Hemmingsen et al., 2011). HUVEC cells were seeded in transparent 96-well plates (2 × 10⁶ cells/well) and allowed to attach for 24 h. The NMs were added to the cells in triplicate and incubated for 24 h. The cell culture medium was discarded and the cells were incubated with primary ICAM-1 (cat. no. BBA17, R&D Systems, Abingdon, UK) or VCAM-1 (cat. no. BBA19, R&D Systems, Abingdon, UK) antibody. The cells were washed three times with warm cell culture medium containing 1% bovine serum albumin, followed by incubation with the secondary rabbit anti-goat IgG antibody (cat. no. A5420, Sigma, St. Louis, MO) that was diluted 1:1000 in phosphate buffered saline (PBS) with 0.1% Tween. The plates were then washed five times with ice-cold PBS/0.1% Tween. To measure the membrane-bound antibodies, 100 µl phosphatecitrate/o-phenylenediamine solution with 20 µl of 30% H₂O₂ was added to each well, and the plate was incubated for 30 min in the dark. The absorbance was measured in an ELISA reader (Multiscan Ascent, Thermo Labsystems) at 450 nm. The proinflammatory cytokine TNF (100 ng/ml) was used as a positive control. The concentrations with statistically significant cytotoxicity, indicated by the WST-1 assay, are excluded from the figures and the statistics.

Gene expression

The mRNA levels of the inflammatory genes (TNF, IL8 and CCL2) and the oxidative stress response gene (HMOX1) were measured in THP-1 cells. A total of 10⁶ cells/well were seeded in 6-well plates. The cells were treated with 4 ml of NMs in final concentration of 0, 4, 16, 64,128 and 256 µg/ml and incubated for 3 h. The RNA was extracted using TRIzol reagent (Invitrogen™, Life Technologies Europe BV, Naerum, Denmark) according to the manufacturer’s protocol, followed by DNase treatment (Promega Biotech AB, Madison, WI). Approximately, 500 ng of RNA was used for cDNA synthesis in a reaction volume of 20 µl
using High Capacity cDNA Reverse Transcription Kit, and the synthesis were carried out on the GeneAmpPCR system 2700 (both from Applied Biosystems®, Life Technologies Europe BV, Naerum, Denmark). Quantitative real-time PCR reactions were carried out in ABI PRISM 7900HT (Applied Biosystems®, Life Technologies Europe BV, Naerum, Denmark), using TaqManGene Expression Assays from Applied Biosystems®, Life Technologies Europe BV, Naerum, Denmark. The assay IDs of the genes were as follows: \( \text{TNF} \), \( \text{HS00174128_m1} \); \( \text{IL8} \), \( \text{HS00174103_m1} \); \( \text{CCL2} \), \( \text{HS00234140_m1} \); \( \text{HMOX1} \), \( \text{HS00157965_m1} \). We used 18S rRNA as reference gene (Eukaryotic 18S rRNA Endogenous Control, 4352930E, Applied Biosystems®, Life Technologies Europe BV, Naerum, Denmark). The gene expression level is reported as the ratio between the mRNA level of the target gene and the 18S rRNA reference gene using the comparative \( 2^{-\Delta Ct} \) method. The concentrations with statistically significant cytotoxicity, indicated by the WST-1 assay, are excluded from the figures and the statistics.

**THP-1 cell adhesion assay**

The adhesion was assessed by measurement of 5-bromo-2-deoxyuridine (BrdU)-labeled THP-1 cells onto HUVECs as previously described (Forchhammer et al., 2012). Briefly, \( 2 \times 10^4 \) HUVECs were seeded in 96-well plates and incubated overnight. The cell medium was removed and \( 5 \times 10^3 \) BrdU-labeled THP-1 cells were added to each well. The co-cultures were then exposed to NMs or the positive control TNF (100 ng/ml) for another 24 h. After exposure, the cell medium was removed and the cells were washed twice with PBS. The supernatants (cell medium and PBS) were transferred to another 96-well plate. After centrifugation, the BrdU content both in the co-culture and supernatant was determined according to the TiO2 exposed cells, which have been verified by non-parametric tests. The validity of the nested ANOVA analysis was accepted on the basis of normal distribution of the residuals. For concentrations with statistically significant cytotoxicity, assessed as decreased WST-1 activity, we excluded results for other endpoints from the figures and the statistical analysis because they could be secondary effects. We kept concentrations of NM 101 (32 and 64 \( \mu \)g/ml) in the analysis of ICAM-1 expression and NM 300 (16 \( \mu \)g/ml) in the analysis of gene expression despite cytotoxicity in order to keep a balanced design in the statistical analysis. Statistical significant effects were accepted at 5% level in the overall nested ANOVA and in the post-hoc least significant difference (LSD) tests. The statistical analysis was performed in Statistica 5.5 (StatSoft, Inc., Tulsa, OK).

**Results**

**Cytotoxicity**

We initially screened all NMs in the concentration range of 2–256 \( \mu \)g/ml for cytotoxicity by means of the WST-1 assay (concentration-response curves are shown in the supplementary material, Figures S1 and S2 for HUVECs and THP-1 cells, respectively). All the TiO2 NMs, except NRCWE 003, generated statistically significant cytotoxicity at the highest concentrations (128 and 256 \( \mu \)g/ml, \( p < 0.05 \)) in HUVEC cells, whereas NRCWE 002 and NM 101 also showed increased cytotoxicity at lower concentrations (64 and 32 \( \mu \)g/ml, respectively, \( p < 0.05 \)). The ZnO NMs (NM 110 and NM 111) were both cytotoxic from the concentration of 32 \( \mu \)g/ml (\( p < 0.05 \)) and Ag (NM 300) from the concentration of 64 \( \mu \)g/ml (\( p < 0.05 \)). LC50 was reached following exposure to ZnO at 29 \( \mu \)g/ml (NM 110) and 28 \( \mu \)g/ml (NM 111), and Ag at 57 \( \mu \)g/ml (NM 300). The cytotoxicity pattern was slightly different in THP-1 cells. All the TiO2 NMs were non-cytotoxic for all concentrations. Similar to the HUVECs, both of the ZnO were cytotoxic at the concentration of 32 \( \mu \)g/ml (\( p < 0.05 \)). However, the Ag (NM 300) was cytotoxic at a lower concentration (16 \( \mu \)g/ml, \( p < 0.05 \)) in the THP-1 cells as compared to HUVECs. LC50 was reached following exposure to ZnO at 43 \( \mu \)g/ml (NM 110) and 46 \( \mu \)g/ml (NM 111) and Ag at 24 \( \mu \)g/ml (NM 300). The increased WST-1 formation at low concentration for some of the NMs could be due to an increased metabolic activity because the exposure triggered cellular stress. Thereby, the conversion of tetrazolium salts to formazan is increased until the concentration is so high that the cells are overwhelmed and consequently the metabolic activity drops.

**Assessment of effect of size, crystal structure and charge (TiO2 NMs)**

The TiO2 NMs had in general a low ability to generate ROS in cell-free dispersions and within cells during a 3 h exposure period (concentration-response curves for each of the NMs are shown in the supplementary material, Figure S3). The exposure to NRCWE 002 was associated with increased ROS production at 256 \( \mu \)g/ml in cell-free conditions (1.22-fold, \( p < 0.05 \)) and within THP-1 cells (1.29-fold, \( p < 0.05 \)). The NRCWE 004 was also associated with a statistically significant increase in the ROS production at the highest concentration in cell-free conditions (2.30-fold, \( p < 0.05 \)). The surface expression of ICAM-1 and VCAM-1 on HUVECs after exposure to TiO2 NMs is shown in Figure 1. The expression level of ICAM-1 on the membrane of the HUVECs was increased in cells exposed to TiO2 (NRCWE 004 and NM 101) at the highest concentrations (32 and 64 \( \mu \)g/ml, \( p < 0.05 \)). These exposure-concentrations showed some cytotoxicity for NM 101 in the WST-1 assay (29 and 47% reduced viability, respectively). NRCWE 004, NM 101 and NRCWE 002 increased the levels of VCAM-1 at the highest concentrations (32 or 64 \( \mu \)g/ml, \( p < 0.05 \)).
The mRNA expression levels of CCL2, IL8, TNF and HMOX1 in THP-1 cells after exposure to TiO₂ particles for 3 h are shown in Figure 2. The assessments of gene expression were carried out in the full concentration range up to 256 µg/ml because the exposure to TiO₂ was shown to be non-cytotoxic for THP-1 cells. The mRNA levels of CCL2 and IL8 were significantly increased compared to the control at the highest concentrations (64–256 µg/ml depending on the type of TiO₂). The level of TNF was only significantly increased in NRCWE 004 (128 µg/ml, \( p = 0.01 \)) exposed cells, but the highest concentration of 256 µg/ml showed borderline statistical significance (\( p = 0.07 \)). The mRNA level of HMOX1 was significantly increased in cells exposed to NRCWE 003 (256 µg/ml) and NRCWE 004 (from 64 µg/ml).

NRCWE 001 and NRCWE 004 were selected for the assessment of adhesion of THP-1 cells to HUVECs because they had shown the widest difference in potency for ICAM-1 and VCAM-1 expression on HUVECs and expression of inflammation genes in THP-1 cells (CCL2, IL8 and TNF). Nevertheless, neither NRCWE 001 nor NRCWE 004 increased the attachment of THP-1 cells onto HUVECs in co-cultures that were exposed for 24 h, whereas there was increased adhesion of THP-1 cells after exposure to the positive control TNF (\( p < 0.05 \), Figure 3).

The overall summary of comparison of the effect of size, crystal structure and surface charge of TiO₂ NMs is outlined in Table 2. In general, there was a somewhat stronger response for the anatase (NM 101) as compared to the rutile (NRCWE 001) TiO₂ NMs, assessed mainly by the expression of ICAM-1 and VCAM-1 on HUVECs. In addition, the size of rutile TiO₂ particles was an important variable in as much as the larger particle (NRCWE 004) was more potent than the smaller rutile NM (NRCWE 001) in generating increased expression of cell adhesion molecules on HUVECs and gene expression in THP-1 cells. The positively charged amino-TiO₂ (NRCWE 002) and negatively charged carboxy-TiO₂ (NRCWE 003) were generally associated with low or statistically non-significant effects and it therefore indicates that the charge had limited importance for the endpoints in this investigation.

Assessment of the effect of surface coating on ZnO NMs

The ZnO particles did not induce ROS production in the cell-free assay or within cells (Figure S4). HUVECs had increased levels of both ICAM-1 and VCAM-1 at the highest and non-cytotoxic concentrations of ZnO NMs (8 or 16 µg/ml, \( p < 0.05 \), Figure 4). The mRNA levels of CCL2 were unaltered in THP-1 cells exposed to either ZnO NMs, whereas the expression of TNF was increased at 4 and 16 µg/ml for the coated ZnO (NM 111) (\( p < 0.05 \), Figure 5). The expressions of IL8 and HMOX1 were increased at 16 µg/ml for both ZnO NMs (\( p < 0.05 \), Figure 5).
Accordingly, surface coating of ZnO NMs had limited importance for these endpoints. After the exposure to co-cultures of THP-1 cells and HUVECs to the uncoated ZnO (NM 110), the adhesion of THP-1 cells was not statistically increased, although the average adhesion at 8 and 16 µg/ml was nominally higher than that achieved by TNF exposure (Figure 3).

Assessment of effects of Ag NM

The Ag NM did not induce ROS production in the cell-free assay or cultured cells (Figure S5). The expression of ICAM-1 and VCAM-1 on HUVECs was increased at 16 and 32 µg/ml (p < 0.05, Figure S6), which were non-cytotoxic concentrations assessed by the WST-1 assay. The mRNA expression in THP-1 cells was unaltered for CCL2 and TNF; increased at 4 and 16 µg/ml for IL8 (p < 0.05) and at 16 µg/ml for HMOX1 (p < 0.05, Figure 5). There was also an increased adhesion of THP-1 cells onto HUVECs at 32 µg/ml (p < 0.05, Figure 3), although it should be noted that this concentration was associated with cytotoxicity in THP-1 cells (only 35% cell viability as assessed by the WST-1 assay). Figure S7 in the supplementary material shows a representative image of the co-culture exposed to Ag NM, examined by combined Differential Interference Contrast (DIC) and fluorescence microscopy in a Leica AF6000 inverted widefield microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Discussion

This study focused on the impacts of a panel of metal-based NMs with different size, crystal structure and coating on oxidative stress, inflammation and cardiovascular-related biomarkers in human cell cultures of monocytes and endothelial cells.

Our data show that the acute cytotoxic effects of the NMs differ in the HUVECs and monocyctic THP-1 cells. Such differences in cytotoxicity could be due to the different cell type, type of medium or whether the cells are adherent or growing in suspension. For both cell types Ag and coated and uncoated ZnO were highly cytotoxic, assessed by the WST-1 assay. The ZnO NMs were the most toxic NMs for HUVECs, whereas Ag was the most toxic NM for the THP-1 cells. Parallel studies conducted as part of the ENPRA project with the same panel of NMs showed similar results in hepatocytic and renal cell lines, where Ag was more toxic than the ZnO NMs (Kermanizadeh et al., 2012b, 2013). In contrast, the TiO2 NMs showed no cytotoxicity in THP-1 cells and only moderate cytotoxicity in HUVECs, which did not reach LC50 values. The FBS content in the cell culture medium may also play a major role for NM-induced cell cytotoxicity, which has been suggested to be due to better bioavailability (Kermanizadeh et al., 2013). The HUVEC and THP-1 cell culture medium contains 2 and 10% FBS, respectively. This difference in serum content could have an impact on the NM size and affect cellular uptake due to differences in protein coronas (Walkey & Chan, 2012). Nevertheless, the NM sizes in HUVEC and THP-1 cell culture medium revealed no clear pattern due to the FBS content (Table 1). THP-1 cells and HUVECs appeared to have similar uptake of NMs with NRCWE 004 showing particularly high uptake as described in Table S1. The uptake was evaluated by the Flow Cytometric Light Scatter analysis where cell granularity is
used as a marker of particle uptake (Suzuki et al., 2007). It has been argued that this method does not distinguish between particles attached to the cell membrane and internalized particles (Vranic et al., 2013). However, we have documented excellent agreement between flow cytometry and confocal microscopy for uptake of gold nanoparticles in HUVECs (Klingberg et al., 2014). The uptake of TiO$_2$ and silver nanoparticles has in several studies been evaluated using flow cytometry in combination with confocal or dark field microscopy to verify a dose-dependent internalization (Suzuki et al., 2007; Vranic et al., 2013; Zucker et al., 2010, 2013).

In relation to oxidative stress, TiO$_2$ NMs did not generate intracellular ROS during a continuous measurement for 3 h in any of the cell types, which is comparable with a previous study, where only a large TiO$_2$ (220 nm) induced ROS production in HUVECs and two other TiO$_2$ with smaller sizes (95 and 17 nm) did not (Mikkelsen et al., 2013). Similarly, neither ZnO NMs nor Ag increased the intracellular ROS production in our experiments. Interestingly, only the TiO$_2$ NMs induced ROS production in hepatocytes, whereas the two ZnO NMs and Ag induced ROS production in a renal cell line (Kermanizadeh et al., 2012a, 2013). The ROS production was measured by means of DCFH once after 6 or 24 h exposure to the study on hepatocytes (Kermanizadeh et al., 2012a), whereas we used continuous measurements for 3 h and expressed the results as a fold-change in “area under the curve”. The study on renal cells used dihydroethidium and flow cytometry for the measurement of ROS production (Kermanizadeh et al., 2013). Therefore, the discrepancy in ROS production between the studies using the same NMs and dispersion procedure could be due to differences in cell type and cell culture medium or due to other differences in the experimental method, such as the fluorescent probe and exposure time. Two types of TiO$_2$ NMs (NRCWE 004 and NM 101), both ZnO NMs and the Ag were able to induce ICAM-1 and VCAM-1 expression in a concentration-dependent manner. Overall, our results indicate that the upregulation of ICAM-1 and VCAM-1 does not depend on cellular oxidative stress in terms of intracellular ROS production for the presently tested NMs. However, it should be emphasized that the DCFH assay detects a variety of hydroxyl-, superoxide-, NO radicals, peroxynitrite and other ROS (Wardman, 2007).

It has been suggested that the proinflammatory cytokine TNF plays a key role in particle-elicited inflammation by functioning as a mediator for expression and secretion of chemokines (Driscoll et al., 1997). Other proinflammatory cytokines include IL8 and monocyte chemoattractant protein 1 (CCL2 alias MCP-1). IL8 exhibits mostly neutrophil chemotactic activity (Roebuck, 1999), whereas CCL2 is a chemoattractant for...
Table 2. Summary of findings and interpretation of size, crystal structure and charge for effects of TiO₂ NMs and effects of ZnO coating.

| Material | TiO₂ | TiO₂ | TiO₂ | TiO₂ | TiO₂ | ZnO | ZnO | Ag |
|----------|------|------|------|------|------|-----|-----|----|
| Code     | NRCWE 001 | NRCWE 002 | NRCWE 003 | NRCWE 004 | NM 101 | NM 110 | NM 111 | NM 300 |
| Characteristics and average size | Rutile, Neutral, 10 nm | Rutile, Positive, 10 nm | Rutile, Negative, 10 nm | Rutile, Neutral, 94 nm | Anatase, Neutral, 7 nm | Uncoated, 100 nm | Coated, 130 nm | <20 nm |
| Cytotoxicity (THP-1) | NS | NS | NS | NS | NS | 32 | 32 | 16 |
| Cytotoxicity (HUVECs) | 128 | 64 | NS | 128 | 32 | 32 | 32 | 64 |
| ROS (Acellular) | NS | 256 | NS | 256 | NS | NS | NS | NS |
| ROS (HUVECs) | NS | 256 | NS | 256 | NS | NS | NS | NS |
| ICAM-1 (HUVECs) | NS (1.5-fold at 64) | NS (1.3-fold at 64) | NS (1.3-fold at 64) | 32 (2.5-fold at 64) | 32 (3.9-fold at 64) | 8 (1.2-fold at 16) | 8 (1.2-fold at 16) | 16 (1.7-fold at 32) |
| VCAM-1 (HUVECs) | NS (1.7-fold at 64) | 64 (1.6-fold) | NS (1.8-fold at 64) | 64 (3.0-fold) | 32 (4.3-fold at 64) | 8 (1.1-fold at 16) | 8 (1.1-fold at 16) | 16 (2-fold at 32) |
| mRNA (THP-1) | CCL2 | 256 | 64 | 128 | 256 | 256 | NS | NS |
| | IL8 | 256 | 64 | 128 | 256 | 256 | NS | NS |
| | HMOXI | NS | NS | 256 | 64 | NS | 16 | NS |
| | TNF | NS | NS | 256 | 64 | NS | 16 | NS |
| | Cell adhesion | NS | NA | NA | NA | NA | NA | NA |

Interpretation with respect to the studied effects:
- Size: 94 nm induces a stronger effect than 10 nm (NRCWE 004/NRCWE 001)
- Crystal structure: anatase induces a stronger effect than rutile (NM 101/NRCWE 001)
- Coating: coating of ZnO NMs has limited importance (NM10/NMI11)

The table shows the lowest concentration in the concentration-range with a statistically significant increase in the endpoint measured. NS: non-significant finding; NA: not analyzed.

*At these concentrations (NM 101, 32 μg/ml) significant levels of cytotoxicity (WST-1 assay) were observed in HUVECs, but the data are included in the statistical analysis to keep a balanced design.

*bAt these concentrations (NM 300, 4 and 16 μg/ml) significant levels of cytotoxicity (WST-1 assay) were observed in THP-1 cells, but the data are included in the statistical analysis to keep a balanced design. Note that cytotoxicity was measured after 24 h exposure and gene expression after 3 h exposure.
monocytes (Ueda et al., 1997). The data on the proinflammatory cytokines CCL2 and IL8 showed that all the TiO2 NMs increased the mRNA expression after 3 h exposure, whereas TNF expression showed no response. The expression of the oxidative stress response gene HMOX1 was increased in cells exposed to negatively charged TiO2 (NRCWE 003) and the larger rutile TiO2 (NRCWE 004). Both ZnO NMs significantly increased the expression of IL8 and HMOX1, but only the coated ZnO (NM 111) significantly increased the expression of TNF in the THP-1 cells. Hepatocytes, exposed to the same panel of NMs as in this study, showed a concentration-dependent increase in the production of IL8, when exposed 24 h to TiO2, whereas the production of IL8 peaked around the LC50 levels, when exposed to ZnO NMs and Ag (Kermanizadeh et al., 2012b).

It was primarily the larger rutile TiO2 (NRCWE 004) and the anatase TiO2 (NM 101) that were able to induce VCAM-1 and ICAM-1 expression on the surface of HUVECs in a concentration-dependent manner compared to the less potent smaller rutile TiO2 (NRCWE 001). The same pattern was observed for the gene expression of IL8, TNF and HMOX1. There were statistically significant differences in gene expression between NRCWE 001, NRCWE 004 and NM 101, which could relate to either differences in particle size (NTA size measure: 108 nm (NRCWE 001) and 202 nm (NRCWE 004)) or crystal structure (anatase (NM 101) versus rutile (NRCWE 001)). A previous study showed that TiO2 with different sizes (12, 21 and 288 nm) increased the levels of ICAM-1 and VCAM-1 on the membrane of HUVECs, but without clear size or crystal structure dependency (Mikkelsen et al., 2013). A recent study of polymorph- and size-dependent uptake and toxicity of TiO2 in lung epithelial cells concluded that the induction of IL-8 and MCP-1 might be a size-related effect (Andersson et al., 2011). The crystal structure has been shown to be important for the biological effects in some studies. Nano-scale anatase TiO2 was shown in vitro to release more IL8 than nano-scale rutile TiO2 (Sayes et al., 2006), whereas in vivo studies have shown that rutile TiO2 was more inflammogenic than anatase TiO2 (Lu et al., 2009; Roursgaard et al., 2011). In acellular conditions, anatase TiO2 exhibited higher ROS production than similar sized rutile TiO2 (Jiang et al., 2008). Also surface properties, such as surface charge can have an impact on the measured biological outcome. In our study, one of the TiO2 (NRCWE 001) were functionalized using 3-aminopropyltriethoxysilane and succinic anhydride/tetrahydrofuran. The recovery products were a positively charged amino-TiO2 (NRCWE 002) and a negatively charged carboxy-TiO2 (NRCWE 003). We observed similar increases in ICAM-1 (approximately 1.5-fold) and VCAM-1 (approximately 1.6-fold) expression on HUVECs after exposure to the negatively charged (NRCWE003), positively charged (NRCWE 002) and neutral TiO2 (NRCWE 001). It has been shown that amino-functionalized
polystyrene nanoparticles, but not carboxyl-functionalized, induced inflammasome activation leading to mitochondrial damage, ROS and IL-1β production (Lunov et al., 2011). It is clear from these results that particle characteristics can play a significant role in toxicological responses. Still there is a paucity of toxicological studies that systematically examine the role of particle size or surface properties, such as crystal structure.

The co-culture experiment showed increased adhesion of THP-1 monocyte cells to HUVECs after exposure to Ag NMs. This interaction is an early event in atherosclerosis, although it is also a physiological response to tissue inflammation in the vicinity of blood vessels for recruitment of inflammatory cells. The Ag also significantly increased the levels of ICAM-1 and VCAM-1, but not the intracellular ROS production. It has been reported that dissolution of Ag ions from Ag NMs is an important mechanism for cytotoxicity, but it has also been shown that induction of ROS production and cell membrane damage was higher for Ag NMs than for Ag ions (Ivask et al., 2014). In a recent study, it was shown that Ag ions released from AgNO3 induced higher levels of cell death compared to Ag NMs, which showed low toxicity despite a higher cellular concentration of silver from the latter (Cronholm et al., 2013). In contrast, another study found that Ag NMs increased the cytotoxicity to a greater extent than AgNO3 (Piao et al., 2011). The Ag (NM 300) used in the present study showed less than 1% dissolution of Ag ions measured in deionized water or cell culture medium after 24 h incubation (Gaiser et al., 2013). Consequently, it is assumable that the effect of ions is negligible in this study.

Our data suggests major differences in cytotoxicity between the three overall types of NMs. The non-cytotoxic concentrations of ZnO NMs dramatically increased the expression levels of HMOX1 and IL8 (~80- to 175-fold) and this at a lower exposure concentration than for TiO2 NMs, where the increase was not higher than 5.5-fold. Also the Ag increased the expression levels of HMOX1 and IL8 (~92- and 22-fold). In contrast, the ICAM-1 and VCAM-1 expressions were higher in TiO2 NM-exposed HUVECs as compared to ZnO NMs and Ag NM at non-cytotoxic concentrations. The relationship between NM characteristics (coating, crystal structure, size and charge) and markers of toxicity are summarized in Table 2.

Conclusions
We found that there were no major effects of the coating of ZnO NMs. The TiO2 NMs data suggest that the anatase form (NM 101) was more potent than the rutile (NRCWE 001) in terms of ICAM-
1 and VCAM-1 expression. Related to size we found that the larger TiO₂ NM (NRCWE 004) were more potent than the smaller (NRCWE 001) for gene expression and ICAM-1 and VCAM-1 expression. The effect on the cardiovascular disease-relevant biomarkers depended on NM composition, size and crystal structure of TiO₂ NMs, whereas the charge of TiO₂ NMs and coating of ZnO NMs was not associated with differences in the toxicological profile.

Declaration of interest

The authors declare no conflicts of interest.

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References

Andersson PO, Lejon C, Ekstrand-Hammarskog B, Akfur C, Ahlinder L, Bucht A, Osterlund L. 2011. Polymorph- and size-dependent uptake and toxicity of TiO₂ nanoparticles in living lung epithelial cells. Small 7:514–23.
Brook RD, Rajagopalan S. 2010. Particulate matter air pollution and atherosclerosis. Curr Atheroscler Rep 12:291–300.
Carlos TM, Schwartz BR, Kovach NL, Yee E, Rosa M, Osborn L, et al. 1990. Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells. Blood 76: 965–70.
Christensen FM, Johnston HJ, Stone V, Aitken RJ, Hankin S, Peters S, Aschberger K. 2011. Nano-TiO₂ – feasibility and challenges for human health risk assessment based on open literature. Nanotoxicology 5:110–24.
Cronholm P, Karlsson HL, Hedberg J, Lowe TA, Winnberg L, Elinh K, et al. 2013. Intracellular uptake and toxicity of Ag and CuO nanoparticles: a comparison between nanoparticles and their corresponding metal ions. Small 9:970–82.
Danielsen PH, Loth S, Kochbach A, Schwarze PE, Moller P. 2009. Oxidative damage to DNA and repair induced by Norwegian wood smoke particles in human A549 and THP-1 cell lines. Mutat Res 674: 116–22.
Danielsen PH, Moller P, Jensen KA, Sharma AK, Wallin H, Bossi R, et al. 2011. Oxidative stress, DNA damage, and inflammation induced by ambient air and wood smoke particulate matter in human A549 and THP-1 cell lines. Chem Res Toxicol 24:168–84.
Dellino RJ, Sioutas C, Malik S. 2005. Potential role of ultrafine particles in associations between airborne particle mass and cardiovascular health. Environ Health Perspect 113:934–46.
Driscoll KE, Carter JM, Hassenbein DG, Howard B. 1997. Cytokines and particle-induced inflammatory cell recruitment. Environ Health Perspect 105:1159–64.
Elahi MM, Kong YX, Matata BM. 2009. Oxidative stress as a mediator of cardiovascular disease. Oxid Med Cell Longev 2:259–69.
Elahi MM, Matata BM. 2006. Free radicals in blood: evolving concepts in the mechanism of ischemic heart disease. Arch Biochem Biophys 450: 78–88.
Forchhammer L, Loth S, Roursgaard M, Cao Y, Ridderdvol VS, Sigsgaard T, Moller P. 2012. Expression of adhesion molecules, monocyte interactions and oxidative stress in human endothelial cells exposed to wood smoke and diesel exhaust particulate matter. Toxicol Lett 209:121–8.
Frikke-Schmidt H, Roursgaard M, Lykkefeldt J, Loth S, Neijgaard JK, Moller P. 2011. Effect of vitamin C and iron chelation on diesel exhaust particle and carbon black induced oxidative damage and cell adhesion molecule expression in human endothelial cells. Toxicol Lett 203:181–8.
Gaiser BK, Hirt S, Kermanizadeh A, Kanase N, Fytianos K, Wenk A, et al. 2013. Effects of silver nanoparticles on the liver and hepatocytes in vitro. Toxicol Sci 131:537–47.
Hansson GK, Libby P. 2006. The immune response in atherosclerosis: a double-edged sword. Nat Rev Immunol 6:508–19.
Hemmingens JG, Muller P, Neijgaard JK, Roursgaard M, Loth S. 2011. Oxidative stress, genotoxicity, and vascular cell adhesion molecule expression in cells exposed to particulate matter from combustion of conventional diesel and methyl ester biodiesel blends. Environ Sci Technol 45:8545–51.
Ivask A, Eldadawy A, Kaweetearawat C, Boren D, Fischer H, Ji Z, et al. 2014. Toxicity mechanisms in Escherichia coli vary for silver nanoparticles and differ from ionic silver. ACS Nano 8:374–86.
Jacobsen NR, Pogano G, Wallin H, Jensen KA. 2010. Nanomaterial dispersion protocol for toxicological studies in ENPRA. Internal ENPRA Project Report. Natl Res Centre Working Environ 6: Internal ENPRA report P1-P10.
Javaid R, Rahman A, Anwar KN, Frey RS, Minshall RD, Malik AB. 2003. Tumor necrosis factor-alpha induces early-onset endothelial adhesivity by protein kinase Zeta-dependent activation of intercellular adhesion molecule-1. Circ Res 92:1089–97.
Jiang J, Oberdorster G, Elder A, Gelein R, Mercer P, Biswas P. 2008. Does nanoparticle activity depend upon size and crystal phase? Nanotoxicology 2:33–42.
Johnston HJ, Hutchison GR, Christensen FM, Peters S, Hankin S, Stone V. 2009. Identification of the mechanisms that drive the toxicity of TiO₂ particles: the contribution of physicochemical characteristics. Part Fibre Toxicol 6:33.
Kermanizadeh A, Gaiser BK, Hutchison GR, Stone V. 2012a. An in vitro liver model – assessing oxidative stress and genotoxicity following exposure of hepatocytes to a panel of engineered nanomaterials. Part Fibre Toxicol 9:28.
Kermanizadeh A, Pogano G, Gaiser BK, Birkedal R, Bilanicoa D, Wallin H, et al. 2012b. In vitro assessment of engineered nanomaterials using a hepatocyte cell line: cytotoxicity, pro-inflammatory cytokines and functional markers. Nanotoxicology 7:301-13.
Kermanizadeh A, Vranic S, Boland S, Moreau K, Baeca-Squiban A, Gaiser BK, et al. 2013. An in vitro assessment of panel of engineered nanomaterials using a human renal cell line: cytotoxicity, pro-inflammatory response, oxidative stress and genotoxicity. BMC Nephrol 14:96.
Klingberg H, Oddershede LB, Loeschner K, Larsen ETH, Loth S, Moller P. 2014. Uptake of gold nanoparticles in primary human endothelial cells. Toxicol Res. DOI: 10.1039/c4tx00061g.
Libby P. 2002. Inflammation in atherosclerosis. Nature 420:868–74.
Lu S, Duffin R, Poland C, Daly P, Murphy F, Drost E, et al. 2009. Efficacy of simple short-term in vitro assays for predicting the potential of metal oxide nanoparticles to cause pulmonary inflammation. Environ Health Perspect 117:241–7.
Lunov O, Syrovets T, Loos C, Nienhaus GU, Mailander V, Landfester K, et al. 2011. Amino-functionalized polystyrene nanoparticles activate the NLRP3 inflammasome in human macrophages. ACS Nano 5: 9648–57.
Mikkelsen L, Jensen KA, Koponen IK, Saber AT, Wallin H, Loth S, et al. 2013. Cytotoxicity, oxidative stress and expression of stress-induced molecules in human umbilical vein endothelial cells exposed to dust paints with or without nanoparticles. Nanotoxicology 7:117–34.
Mikkelsen L, Sheykzhade M, Jensen KA, Saber AT, Jacobsen NR, Vogel U, et al. 2011. Modeist effect on plaque progression and vasodilatory function in atherosclerosis-prone mice exposed to nanosized TiO₂(2). Part Fibre Toxicol 8:32.
Mills NL, Donaldson K, Hadoke PW, Boon NA, MacNee W, Cassee FR, et al. 2009. Adverse cardiovascular effects of air pollution. Nat Clin Pract Cardiovasc Med 6:36–44.
Mills NL, Tornqvist H, Robinson SD, Gonzalez MC, Soderberg S, Sandstrom T et al. 2007. Air pollution and atherothrombosis. Inhal Toxicol 19:81–9.
Moller P, Mikkelsen L, Vesterdal LK, Folkmann JK, Forchhammer L, Roursgaard M, et al. 2011. Hazard identification of particulate matter on vasmotor dysfunction and progression of atherosclerosis. Crit Rev Toxicol 41:339–68.
Nel AE. 2013. Implementation of alternative test strategies for the safety assessment of engineered nanomaterials. J Intern Med 274: 561–77.
Piao MJ, Kang KA, Lee IK, Kim HS, Kim S, Choi JY, et al. 2011. Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. Toxicol Lett 201:92–100.
Qin Z. 2012. The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. Atherosclerosis 221:2–11.
Roebuck KA. 1999. Regulation of interleukin-8 gene expression. J Interferon Cytokine Res 19:429–38.
Roursgaard M, Jensen KA, Poulsen SS, Jensen NE, Poulsen LK, Hammer M, et al. 2011. Acute and subchronic airway inflammation after intratracheal instillation of quartz and titanium dioxide agglomerates in mice. Scientific World J 11:801–25.

Sayes CM, Wahi R, Kurian PA, Liu Y, West JL, Ausman KD, et al. 2006. Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. Toxicol Sci 92:174–85.

Schluesener JK, Schluesener HJ. 2013. Nanosilver: application and novel aspects of toxicology. Arch Toxicol 87:569–76.

Suzuki H, Toyooka T, Ibuik Y. 2007. Simple and easy method to evaluate uptake potential of nanoparticles in mammalian cells using a flow cytometric light scatter analysis. Environ Sci Technol 41:3018–24.

Ueda A, Ishigatsubo Y, Okubo T, Yoshimura T. 1997. Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-kappaB sites and NF-kappaB/Rel subunit specificity. J Biol Chem 272:31092–9.

Vandebriel RJ, de Jong WH. 2012. A review of mammalian toxicity of ZnO nanoparticles. Nanotechnol Sci Appl 5:61–71.

Vesterdal LK, Mikkelsen L, Folkmann JK, Sheykhzade M, Cao Y, Roursgaard M, et al. 2012. Carbon black nanoparticles and vascular dysfunction in cultured endothelial cells and artery segments. Toxicol Lett 214:19–26.

Vranic S, Boggetto N, Contremoulins V, Mornet S, Reinhardt N, Marano F, et al. 2013. Deciphering the mechanisms of cellular uptake of engineered nanoparticles by accurate evaluation of internalization using imaging flow cytometry. Part Fibre Toxicol 10:2.

Walkey CD, Chan WC. 2012. Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment. Chem Soc Rev 41:2780–99.

Wardman P. 2007. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. Free Radic Biol Med 43:995–1022.

Zucker RM, Daniel KM, Massaro EJ, Karafas SJ, Degn LL, Boyes WK. 2013. Detection of silver nanoparticles in cells by flow cytometry using light scatter and far-red fluorescence. Cytometry A 83:962-72.

Zucker RM, Massaro EJ, Sanders KM, Degn LL, Boyes WK. 2010. Detection of TiO$_2$ nanoparticles in cells by flow cytometry. Cytometry A 77:677–85.