Zeta Potential and Solubility to Toxic Ions as Mechanisms of Lung Inflammation Caused by Metal/Metal Oxide Nanoparticles

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The toxicology of nanoparticles (NPs) is an area of intense investigation that would be greatly aided by improved understanding of the relationship between NP structure and inflammogenicity. To evaluate how their physicochemical parameters influence toxicity, we assembled a panel of 15 metal/metal oxide NPs and attempted to relate various physicochemical parameters, including zeta potential (ζP) and solubility, to lung inflammogenicity. The acute pulmonary inflammogenicity of the 15 NPs showed a significant correlation with one of two structural parameters—ζP and solubility, to toxic species for high-solubility NPs.

Key Words: metal oxide nanoparticles; zeta potential; solubility; hemolysis; lung inflammation; rat; QSAR.

The wide range of nanoparticles (NPs) and their modified derivatives mean that the use of in vitro testing approaches generates a considerable ethical problem and financial burden with respect to large-scale toxicology testing (Donaldson et al., 2009), which may hinder the industrial utilization of the products of the nanotechnologies. When considering workplace exposure to nanomaterials, the main route of exposure is inhalation of aerosols created during industrial processes (Christensen et al., 2010). Lung inflammation, the consequence of pulmonary deposition of pathogenic particles, can be seen as a rational priority toxicity endpoint because a spectrum of adverse pulmonary effects is to be anticipated (Cho et al., 2010; Li et al., 2008, 2010). In addition, inflammatory effects in the lungs generated by particle deposition could drive effects in secondary organs, such as the blood vessel wall and the brain (Li et al., 2010; Mills et al., 2009).

Along with others, we have already highlighted the shortcomings of in vitro tests for fully determining the likely effects of NP (Cho et al., 2010). This is in part because NPs are a heterogeneous group of materials that are likely to cause inflammation via different mechanism depending on their structure (Nel et al., 2009) and partly because NPs act variably in different cell types (Sohaebuddin et al., 2010). In addition, the structural characterization of NP is not as specific as it can be for chemicals where the latter can be based on specific chemical moieties or groups. For NP, the structural characteristics are less distinct and include such entities as size, surface charge, solubility, or oxidative potential (Sayes and Warheit, 2009).

Zeta potential (ζP) is the electric potential created between the charged groups associated with the surface of a particle and the suspension medium and can be used to derive information concerning the particle surface charge (Butt et al., 2004). As cell membranes are negatively charged, the degree of interaction between a particle and a cell surface or the membrane of an organelle may be influenced by the ζP of an NP in contact with it (McGuinnes et al., 2010). Surface-functionalized cationic NPs are known to be more cytotoxic than those of neutral or anionic NPs in vitro by causing lysosomal damage (Asati et al., 2010; Nel et al., 2009; Ruizendaal et al., 2009). The interaction between charged NP surfaces that are large in relation to mass (Rushton et al., 2010) and the internal face of
the phagolysosomal membrane may be a key event in lysosome destabilization by low-solubility NP.

For high-solubility NPs that are compartmentalized into the acid milieu of the lysosomes of macrophages, dissolution rate may be accelerated, leading to lysosomal damage and inflammation, dependent on the ions that are released (Cho et al., 2011). The rapid dissolution in lysosomes was therefore taken as a potentially key structural correlate of proinflammatory effects. We set out with a panel of 15 metal/metal oxide NPs and related the extent of the lung inflammation caused by the panel to various physicochemical parameters, including \( \text{P} \) and solubility.

As a first step, we equilibrated for surface area (SA) for all NPs because we have previously reported on the importance of SA as a driver of NP effects (Brown et al., 2001; Duffin et al., 2007; Montefierr et al., 2007). Our aim thereafter was to investigate the role of an equal SA dose in causing inflammation for a range of NPs and to relate any differences in inflammation that were caused to the physicochemistry of the NP (including \( \text{P} \) and solubility of toxic ions) as the explanatory variable.

**MATERIALS AND METHODS**

Details of all materials and methods described in brief here are presented in Supplementary material.

**Nanoparticles.** A panel of 15 metal/metal oxide NPs were purchased from several commercial sources (as detailed within the Supplementary methods) and consisted of Al\(_2\)O\(_3\)NP, AgNP, CeO\(_2\)NP\(_a\), CeO\(_2\)NP\(_b\), MgONP, NiONP, SiO\(_2\)NP, TiO\(_2\)NP\(_a\) (anatase form), TiO\(_2\)NP\(_b\) (rutile form), and ZnONP\(_a\): CeO\(_2\)NP\(_a\), CeO\(_2\)NP\(_b\), and ZnONP\(_b\); Cr\(_2\)O\(_3\)NP, CuONP\(_a\), MgONP, and TiO\(_2\)NP\(_a\). At 24 h postinstillation, rats were euthanized by i.p. injection of sodium pentobarbitone (200 mg). Lungs were lavaged four times with 8 ml of saline by cannulating the trachea with a luer port cannula (Portex, Kent, U.K.). The total number of cells in the bronchoalveolar lavage (BAL) was counted using a nucleocounter (Chemometec, Allerod, Denmark), and differential cell counts were performed with cytopsin slides by microscopic observation.

**Correlation test and statistical analysis.** To evaluate the parameters influencing hemolytic potential, each physicochemical parameter of the NP was plotted with the hemolytic potential of NPs at 300 cm\(^2\)/ml. Linear regression and Pearson correlation test were then applied to evaluate the correlation between \( \text{P}_{\text{acid}} \) and hemolytic potential. Likewise, each physicochemical parameter of the NP was plotted against the percentage of total granulocytes in the BAL to evaluate the parameters influencing in vivo inflammogenicity. Linear regression and Pearson correlation test were applied to evaluate the correlation between \( \text{P}_{\text{acid}} \) and acute lung inflammation. By plotting \( \text{P}_{\text{acid}} \) against hemolytic potential or acute lung inflammogenic potential, we found there is a threshold level triggering lysis of cellular membrane or causing lung inflammation. Therefore, nonhemolytic NPs from below the threshold with \( \text{P}_{\text{acid}} \leq 14 \text{ mV} \) were excluded for linear regression. For the same reason, namely that they were below the threshold, we ruled out the NPs having \( \text{P}_{\text{acid}} < 0 \text{ mV} \) in the plot \( \text{P}_{\text{acid}} \) versus percent granulocytes. Although Cr\(_2\)O\(_3\)NP, TiO\(_2\)NP\(_a\), and TiO\(_2\)NP\(_b\) were not significantly inflammogenic, they were included in the linear regression because they were in the trend line of significantly inflammogenic NPs. Data were analyzed and plotted with GraphPad Prism software (Version 5; GraphPad Software, Inc., La Jolla, CA). To compare each treatment group, one-way ANOVA with post hoc Tukey’s pairwise comparisons was applied. A value of \( p < 0.05 \) was considered to be statistically significant.

**RESULTS**

**Physicochemical Characterization**

Table 1 summarizes the physicochemical properties of the panel of NPs. The hydrodynamic sizes of NPs exceeded the primary particle sizes, showing that there were small agglomerates except for AgNP and Cr\(_2\)O\(_3\)NP (Table 1). NP
agglomerates are typically found in workplaces where NPs are made (Brouwer, 2010) and so represent the size of agglomerates that lung cells would encounter following aerosolization exposure, inhalation, and deposition. To investigate the role of oxidative stress, EPR was used to evaluate the intrinsic oxygen–centered free radical generation of NP under cell-free conditions. The EPR signal for oxidation of the spin trap Tempone-H was significantly increased by AgNP, CeO2NPa, Cr2O3NP, CuONPb, NiONP, and TiO2NPb, whereas others were comparable with vehicle control (Table 1).

### Solubility Test

Solubility has been proposed as another important factor influencing the toxicity of NP. The solubility of NPs was tested at two different pH levels for artificial lysosomal fluid (pH 5.5) and presented by percentage of soluble ions compared with initial mass. *Significance versus vehicle control p < 0.001.

### Zeta Potential Measurement

When respirable NPs are inhaled, they “splash down” into LLF, a lipid/protein mixture, which has pH 6.9 (Nielson et al., 1981). NPs in biological fluid such as LLF and serum attract molecules with an opposite charge to the surface charge, which reduces the overall charge. Particles are then taken up by alveolar macrophages where they are localized into the phagosomes, which fuse with lysosomes to form acidic (pH 5.5) phagolysosomes (Nyberg et al., 1992). The ζP of particles is known to be influenced by different properties of the dispersion media, in particular pH (Berg et al., 2009) and the presence of molecules such as lipoproteins (Doorley and Payne, 2011). We therefore measured the ζP of the panel of NPs in various media using the following nomenclature:

\[ ζP_{\text{physiological saline (pH 5.5)}} = ζP_{\text{acid}} \]
\[ ζP_{\text{in 10% PBS (pH 7.4)}} = ζP_{\text{basic}} \]
\[ ζP_{\text{in saline with serum}} = ζP_{\text{surfactant corona}} \]
\[ ζP_{\text{in saline with LLF}} = ζP_{\text{serum corona}} \]

The ζP of the NP panel was heterogeneous but predominantly positive, as expected because of the acidic nature of saline (Fig. 1). The ζP values of the NPs gave entirely negative values, resulting from neutralization of positively charged metal ions by anionic species in the medium. The ζP values were closely similar and generally negative but less negative than ζPbasic.

### Acute Pulmonary Inflammation

The acute inflammatory effects of NP were evaluated by determining the percentage of total granulocytes (neutrophils,
eicosinophils, and basophils) in the BAL 24 h after instillation into the lungs of rats, a measure that has been used previously to summarize inflammatory potential of NPs (Rushon et al., 2010). The percentage of granulocytes in the BAL was significantly increased by instillation of Al₂O₃NP, CeO₂NP, CeO₂NPb, Co₃O₄NP, CuONP, CuONPb, NiONP, ZnONP, and ZnONPb, whereas instillation of the other NPs produced levels of BAL granulocytes not significantly different from the vehicle control (Supplementary fig. 2).

**Hemolysis Assay and Enzymatic Digestion of Protein Corona**

We utilized the erythrocyte membrane, in a hemolysis assay, as a simple surrogate for the lysosomal membrane to investigate the likely effects of NP \( \zeta P \) on its integrity. The hemolysis assay was carried out under protein-free conditions in physiological saline, i.e., when the \( \zeta P_{\text{acid}} \) was effective and under these conditions, Al₂O₃NP, CeO₂NP, CeO₂NPb, Co₃O₄NP, CuONP, CuONPb, NiONP, ZnONP, and ZnONPb were significantly hemolytic compared with vehicle control (Fig. 2A). ZnONPs were excluded from the assay because they adsorb hemoglobin, which confounds the assay (Supplementary fig. 3). When NPs were coated with a corona of 5% FBS or LLF, the hemolytic potential of NPs was abolished (Fig. 2A). However, enzymatic digestion of the corona using PLA₂ and proteinase K and testing in the hemolysis assay, where the \( \zeta P_{\text{acid}} \) is expressed, partially restored hemolytic potentials except for CuONPb (Fig. 2B).

**The Relationship Between \( \zeta P_{\text{acid}} \) and Hemolytic Potential**

The physicochemical parameters in Table 1 and Supplementary table 1 were then plotted against the hemolytic potential of NPs. Of the parameters, \( \zeta P_{\text{acid}} \) showed the best correlation with hemolytic potential with NPs having \( \zeta P_{\text{acid}} \) less than +14 mV showing no hemolytic potential, whereas the hemolytic potential of NP with \( \zeta P_{\text{acid}} \) greater than +14 mV showed a linear relationship with hemolysis (linear regression:

\[
R^2 = 0.81, \ p < 0.0001; \text{Pearson correlation test: } R^2 = 0.94, \ p = 0.0058, \text{95% confidence interval [CI] = 0.62–0.99} \text{ (Fig. 3A). Other parameters (primary size, hydrodynamic size, mass dose, EPR intensity, solubility, } \zeta P_{\text{basic}}, \zeta P_{\text{serum corona}}, \text{ and } \zeta P_{\text{surfactant corona}} \text{) showed poor correlations with hemolytic potential (Supplementary figs. 4 and 5).}
\]

**The Relationship Between \( \zeta P_{\text{acid}} \) and Acute Lung Inflammogenicity**

For each NP, the physicochemical parameters were related to inflammation by plotting their values against the percentage of granulocytes in the BAL. NPs having positive \( \zeta P_{\text{acid}} \) showed a significant linear correlation with the percentage of granulocytes (linear regression: \( R^2 = 0.79, \ p < 0.0001; \) Pearson correlation test: \( R^2 = 0.86, \ p = 0.0009, \text{95% CI} = 0.64–0.99 \) (Fig. 3B). NPs with a \( \zeta P \) of 10 or greater showed significantly more inflammation than the vehicle control. This linear correlation developed the equation for acute lung inflammation as follows:

\[
\text{Percentage of granulocytes} = 6.227 \cdot \zeta P_{\text{acid}}(mV) - 29.24. \quad (1)
\]

Dividing the percentage of granulocytes by \( \zeta P_{\text{acid}} \) units and SA units (% of granulocytes/\( \zeta P_{\text{acid}} \) unit/cm²) substantially collapsed the data, confirming that the value of \( \zeta P_{\text{acid}} \) was the explanatory variable for inflammation (Fig. 3C). Plotting of hemolytic potential against lung inflammation showed an essentially “all or nothing” pattern (Supplementary fig. 6). All NPs having 10% hemolytic potential recruited more than 75% of granulocytes in the BAL; however, there was no further increase in inflammation as the hemolytic potential increased.

**Correlation of the Inflammogenicity of High-Solubility NPs**

Regarding the high-solubility NPs, the inflammogenicity of CuONPs and ZnONPs was derived from their soluble ions (Cu²⁺ and Zn²⁺), which are known to be toxic (Nel et al., 2009; Studer et al., 2010), whereas MgONP showed no inflammation because they release nontoxic Mg²⁺ (Flink et al., 1992) (Fig. 3D). Other parameters (primary size, hydrodynamic size, mass dose, EPR intensity, \( \zeta P_{\text{basic}}, \zeta P_{\text{serum corona}}, \) and \( \zeta P_{\text{surfactant corona}} \)) showed a poor correlation with inflammation of low-solubility or high-solubility particle types (Supplementary figs. 7 and 8).

**Relationship Between Primary Particle Size and \( \zeta P_{\text{acid}} \)**

The primary size of NPs was plotted against the \( \zeta P_{\text{acid}} \) using the same compositional NPs (CeO₂NP, CuONP, TiO₂NP, and ZnONP) (Supplementary fig. 9). Two NPs (CeO₂NP and CuONP) showed dramatic increases in \( \zeta P_{\text{acid}} \) as the size increases, one (TiO₂) showed a very modest increase in \( \zeta P_{\text{acid}} \) with the larger particles, whereas ZnONP showed a marked decrease in \( \zeta P_{\text{acid}} \) with larger particles. Thus, there is no simple relationship between \( \zeta P_{\text{acid}} \) and NP size within composition.
In this study, the acute pulmonary inflammogenicity of 15 metal/metal oxide NPs showed an excellent correlation with one of two structural parameters—f_{Pac} for low-solubility NPs and solubility to toxic species for high-solubility NPs (Fig. 4 presents the hypothetical mechanisms for this in diagrammatic form). Other physicochemical parameters except for f_{Pac} were not the main parameters influencing the in vivo lung inflammogenicity as evidenced by their lack of correlation with inflammation.

f_{Pac} of the NP panel was heterogeneous for insoluble NPs but predominantly positive (−15 mV), as expected because of the acidic nature of saline (pH 5.6), which has a similar pH as the phagolysosomal fluid of alveolar macrophages (Nyberg et al., 1992). In pH-buffered PBS (pH 7.4), the f_{Pbasic} values of the metal/metal oxide NPs were entirely negative (−50 to −18 mV), resulting from neutralization of positively charged metal ions by anionic species in the medium. When NPs encounter biological fluids containing macromolecules, they will naturally attract the oppositely charged ones and form f_{Pserum corona} or f_{Psurfactant corona}. The change of the charge is based on the adsorption of those molecules and proteins thereby reducing the overall charge of the NP (Lundqvist et al., 2008; Xia et al., 2006). Furthermore, when particles enter the acidic lysosomal milieu, enzymatic digestion of the corona by lysosomal enzymes (Wallace et al., 1992) may cause the negative f_{Pserum corona} or f_{Psurfactant corona} to be converted to f_{Pacid} values, which are predominately positive. The positively charged NPs, as measured by f_{Pacid}, may then actively interact with the negatively charged internal face of the lysosomal membrane leading to lysosomal destabilization. Rupture of this lysosomal membrane can trigger an inflammation cascade in the lung (Hornung et al., 2008; Yazdi et al., 2010). Within the same composition, NPs showed no consistent relationship between the size and the f_{Pacid}.

As a marker for acute lung inflammation, we used percentage of granulocytes in the BAL rather than the number of the granulocytes. The process of BAL does not reach the interstitium but samples only the airspaces and so interstitial inflammation such as perivascular and peribronchial inflammatory cell infiltration might not be well monitored by BAL; this was found in our previous studies to be especially true for ZnONP-induced inflammation. When the inflammogenicity of ZnONPs was measured by the total number of BAL granulocytes, only mild inflammogenicity was detected although treatment caused severe perivascular and peribronchial inflammation (Cho et al., 2010). However, when inflammation was evaluated by the percentage of granulocytes, the BAL analysis better mirrored the actual inflammogenicity, suggesting that the percentage of granulocytes might be more reliable than the number of granulocytes for comparing between different NP types that have their inflammatory effects differentially compartmentalized between the interstitium and the airspaces.

We used the hemolysis assay as a surrogate for the lysosomal membrane to determine the effects of high and low f_{P} surfaces in damaging the phagolysosomal membrane. The hemolytic potential of NPs was abolished when NPs were dispersed in serum protein or LLF and so had a corona. However, enzymatic digestion using PLA2 and proteinase K, similar to enzymes found in the lysosomes, restored the hemolytic potential. This suggests that the acid and proteolytic conditions inside the lysosome might enhance the toxicity of NP, which possessed a high f_{Pacid} that had been passivated by adsorption of an LLF corona at the point of deposition. In support of this contention, a previous study with quartz particles revealed that their coating of dipalmitoyl lecithin was removed by lysosomal enzymes inside cells of a macrophage cell line (Hill et al., 1995). The same group also showed for quartz what we have shown for NP in the present paper.
namely that the hemolytic activity of quartz particles was reduced by binding with LLF, whereas digestion of the surfactant coated on particles using PLA2 restored the hemolytic potential (Wallace et al., 1992).

In contrast, the hemolytic potential of CuONP could not be restored by enzymatic digestion. Copper has higher binding affinity with proteins compared with other metals such as nickel and cobalt (Thompson et al., 2011), enabling its use in the protein detection assays (Wilkinson-White and Easterbrook-Smith, 2008). This high binding affinity might interfere with the stripping off of the corona by enzymes. However, the high protein binding affinity of CuONP has limited meaning on the toxicity of CuONP because CuONP are likely to be rapidly dissolved in the acidic lysosomal milieu.

In our previous study, hemolysis assay predicted pulmonary inflammogenicity of 12 of 13 NPs tested, which included a high proportion of metal oxide NPs (Lu et al., 2009). The only NP showing a false positive was CeO2NP, which was hemolytic but not inflammogenic in the lung. However, recently, we modified the dispersion methods and instilled better-dispersed CeO2NP into the lungs of rats and provoked acute inflammation (Cho et al., 2010) at much lower SA dose than was used in previous study (Lu et al., 2009). This further supports the idea that the hemolysis assay models a key crucial

![FIG. 3. Relationship between zeta potential and hemolytic potential, lung inflammogenicity, and solubility.](image)
cellular interaction, namely the interaction between insoluble NPs and the lysosomal membrane and so holds promise as a predictive test for inflammatory potential of low-solubility NPs.

CuONPs, MgONP, and ZnONPs underwent rapid dissolution in the artificial lysosomal fluid (pH 5.5), but no particles underwent dissolution at pH 7.4, the pH of interstitial fluid. These data suggest that the low-solubility NPs remain intact until they encounter the milieu of the phagolysosomes where acid conditions will cause rapid dissolution with soluble metal ion accumulation. When the ions are toxic, as in case of Cu²⁺ (Studer et al., 2010) and Zn²⁺ (Nel et al., 2009), this may elicit destabilization of the lysosomal membrane, triggering inflammation. When the soluble ions are nontoxic, as in the case of Mg²⁺ ions (Flink et al., 1992) released by MgONP, then no lysosomal damage would be anticipated. Therefore, the inflammogenicity of five high-solubility NPs (CuONPa, CuONPb, MgONP, ZnONPa, and ZnONPb) showed a good correlation with the toxicity of their compositional ions.

The solubility of NPs mainly depends on the composition, and CuONP, MgONP, and ZnONP were found to be highly acid soluble, whereas others were not. Although we found that MgONP was of low toxicity, it is important to note that MgO differs dramatically from other metal oxides in that MgO has high chemical reactivity in water where some of the MgO exothermically combines with it to form Mg(OH)₂, which might have different physicochemical properties (Tai et al., 2007). The high reactivity of MgONP with water is very different behavior from the other metal oxide NP and so more research is warranted on the behavior of MgONP in biological systems. However, our studies clearly show that the MgONPs were both acid soluble and that the products of the solubility reaction are not toxic, and this is borne out by another study showing MgONP possess low toxic potency to cells even at a high dose in vitro (Lai et al., 2008), which we have also found (data not shown). Therefore, the chemical reactivity of MgONP with water is not critical in this study because MgONP fits with the “dissolution to harmless ions” part of the paradigm. ZnONP was present in two sizes 10.7 and 137 nm, and we found that both dissolved rapidly within a few seconds under acid conditions, with no real difference in rate. It is unlikely that all NPs will be similarly acid soluble and the results here are likely to be applicable only to rapidly dissolving NP, and a different mechanism may emerge for less readily soluble NPs and should be the topic of further research.

We recognize that our experiments are based on only 15 NPs, but the evidence we present makes it worthy of further study and validation. In this study, we found the two novel parameters, which can underlie NP toxicity. These parameters may provide impetus for research toward future quantitative structure activity relationship (QSAR) modeling of NPs. We recognize that individuals are exposed to NPs by inhalation, whereas we exposed our rats by instillation. Instillation is recognized to be a methodology whose limitations for risk assessment are well recognized but that allows comparison between particles and hazard (Driscoll et al., 2000; Henderson et al., 1995).
We do not propose that $\zeta_{\text{acid}}$ is necessarily a structure that drives the inflammogenicity of all insoluble NPs because, for example, high-aspect ratio NPs are known to have a different relationship between their structure and their toxicity (Donaldson et al., 2010). Finally, our data provide an impetus for more research toward $\zeta_{\text{acid}}$ and solubility as additional toxicity paradigms that may be added to the fiber pathogenicity paradigm (Donaldson et al., 2010) and the oxidative stress paradigm (Rushton et al., 2010) that seek to explain how the NP hazard is translated into “harmful dose” at the cellular level. In addition, we contend that our findings significantly contribute to the area of QSAR studies, an important strategy for prediction of NP toxicity.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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