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Published in:
Toxicology in Vitro

Link to article, DOI:
10.1016/j.tiv.2018.12.016

Publication date:
2019

Document Version
Peer reviewed version

Citation (APA):
Da Silva, E., Kembouche, Y., Tegner, U., Baun, A., & Jensen, K. A. (2019). Interaction of biologically relevant proteins with ZnO nanomaterials: A confounding factor for in vitro toxicity endpoints. Toxicology in Vitro, 56, 41-51. https://doi.org/10.1016/j.tiv.2018.12.016
Interaction of biologically relevant proteins with ZnO nanomaterials: a confounding factor for in vitro toxicity endpoints.

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Abbreviations: ANOVA, analysis of variance; BET, Brunauer Emmett Teller; BSA, bovine serum albumin; DLS, dynamic light scattering; DMEM, Dulbecco’s modified eagle’s medium; ECD, equivalent circular diameter; FBS, fetal bovine serum; ICP-MS, inductively coupled plasma mass spectrometry; ILs, interleukins; LDH, lactate dehydrogenase; MNs, manufactured nanomaterials; OD, optical density; OECD, organisation for economic co-operation and development; RCF, relative centrifugal force; RPMI, Roswell park memorial institute; SDR, sensor dish reader;
Abstract

The results of in vitro toxicological studies for manufactured nanomaterials (MNs) are often contradictory and not reproducible. Interference of the MNs with assays has been suggested. However, understanding for which materials and how these artefacts occur remains a major challenge. This study investigated interactions between two well-characterized ZnO MNs (NM-110 and NM-111) and lactate dehydrogenase (LDH), and two interleukins (IL-6 and IL-8). Particles (10 to 640 µg/mL) and proteins were incubated for 24 hours in routine in vitro assays test conditions. LDH activity (OD_{LDH}), but not interleukins concentrations, decreased sharply in a dose-dependent manner within an hour after exposure (OD_{LDH} < 60% of OD_{ref} for both MNs at 10 µg/mL). A Freundlich adsorption isotherm was successfully applied, indicating multilayer adsorption of LDH. ZnO MNs and LDH had neutral to slightly negative surface charges in dispersion, precluding electrostatic attachment. Particle sedimentation was not a limiting factor. Fast dissolution of ZnO MNs was shown and Zn^{2+} could play a role in the OD_{LDH} drop. To summarize, ZnO MNs quickly reduced OD_{LDH} due to concentration-dependent adsorption and LDH inhibition by interaction with dissolved Zn. The control of particle interference in toxicological in vitro assays should become mandatory to avoid misleading interpretation of results.

Keywords: artefact, adsorption, lactate dehydrogenase, interleukin, dissolution, pH.
**Introduction**

Nanotechnology remains one of the fastest growing markets worldwide with potential use in a large number of sectors. The continuous development of chemically modified- or new manufactured nanomaterials (MNs) and an ever growing number of applications make the ability to perform cost-efficient risk assessment of these MNs a priority. Concurrently, the interest in *in vitro* toxicity assays to perform hazard screening and for regulatory use is rising due to the requirement of the 3Rs to replace, reduce and refine the use of animals. However, the results from *in vitro* studies are often contradictory, not reproducible and/or erroneous.

The lack of verification of the test material characteristics combined with the lack of control for artefacts, caused by the interaction between test components and MNs, are observed in many published manuscripts and must be taken into account. Interference of MNs with routine cytotoxicity assays has already been demonstrated with carbon-based MNs as well as metal oxide MNs. Adsorption of cytokines, including interleukins IL-6 and IL-8 has also been demonstrated for some MNs. Although these artefacts are known, and several ELISA kits for protein measurements specify that a control for interaction is needed to identify whether the test material in the medium could interfere with the assay, such tests are only reported in a few toxicological studies. In their review, Ong et al. reported that, in 2012, among papers using colorimetric or fluorescence tests, 90% did not control for the occurrence of interference. Proposed mechanisms for interaction include optical interference, interaction by adsorption of the target molecule onto the test material and chemical reactions with the assay components. These types of interaction could either lower or increase the signal, thereby leading to false interpretation of the results.

It is essential to ensure that *in vitro* toxicity assays produce reliable and reproducible data if results will be used for hazard screening leading to decision making for regulatory purposes. Cytotoxicity and inflammatory response are two of the most commonly investigated endpoints. Cytotoxicity is often assessed and quantified by measuring the concentration of the cytosolic enzyme lactate dehydrogenase (LDH) that is released to the medium from damaged cells. In this assay, LDH first catalyzes the conversion of lactate to pyruvate via reduction of NAD$^+$ to NADH. Then, NADH reduces a tetrazolium salt to a red formazan product. This product can be detected by spectrophotometric absorbance. Similarly, pro-inflammatory cytokines IL-6 and...
IL-8 are commonly used for investigating and quantifying the inflammatory potential of MNs. While IL-6 is a mediator of acute phase response and promotes differentiation of T cells, IL-8 is involved in chemoattraction of cells, mainly neutrophils. The amount of interleukins in the media is directly measured using antibodies and spectrophotometric detection.

Serum- or isolated proteins are often used as a means to disperse MNs in the test medium for toxicological studies both in vitro and in vivo. The proteins facilitate an electrosteric or polymeric stabilization, preventing fast agglomeration at the high MNs concentrations normally required in batch dispersions protocols. However, even though serum- or isolated proteins are usually biocompatible, it is important to investigate whether their presence can modulate the response or be a source of artefacts in a specific test.

As an example, a difference has been observed in genotoxicity results in vivo on day 1, but not later, after intratracheal instillation of different MNs dispersed in different media. Differences were also observed depending on the timing of fetal bovine serum addition to nutrient mixtures used for cell incubation.

Understanding the MNs characteristics and the test parameters leading to the observed interferences and the mechanism of interaction remains a major challenge in the field of in vitro nanotoxicology. It is often stated that proteins attach to particles due to electrostatic, steric or combined electrosteric interaction. Nonetheless, the proteins may also transform or degrade due to changes in chemical conditions in the medium or interactions with medium constituents or at the particle-protein interface. It is presently unclear how specific particle properties such as particle curvature, specific surface area, morphology, surface charge in the medium, particle sedimentation, and dissolution influence toxicity assays in vitro. For example, the role of released Zn during dissolution of ZnO MNs has been proposed to trigger inhibition of LDH activity.

Identifying the mechanisms of interaction between MNs and proteins in in vitro assays is also particularly challenging due to the dynamic nature of the test. Indeed, the MNs are first dispersed in a batch dispersion medium (a water- or water-based nutrient mixture that may or may not contain serum- or isolated proteins), and then they are dosed into the test media in which they likely sediment at different rates and/or release ions through leaching and dissolution. Interaction between the MNs and the medium constituents is expected from the first entry into the dispersion medium and throughout the entire in vitro test.
A key objective of this study was to investigate the interactions occurring in the typical conditions for in vitro toxicological studies. We followed the guidance for test item preparation defined in the large EU FP7 NANoREG project where a major effort was made to harmonize the test procedures used to increase interlaboratory compatibility of the initial in vitro exposure characteristics. The experiments reported here were designed to identify the interaction of LDH, IL-6 and IL-8 with well-characterized uncoated (NM-110) and coated (NM-111) ZnO MNs. The ZnO MNs were selected for study due to initial results from screening studies and literature data on potential interaction between ZnO and proteins. ZnO is also a widely used material that can lead to human pulmonary and cardiovascular health effects and a large number of studies report on their toxicity in vivo as well as in vitro.
Experimental section

Test materials

The ZnO MNs (NM-110 and NM-111) studied are representative of industrial MNs, which were donated to the past Sponsorship Programme for the Testing of Manufactured Nanomaterials organized by the OECD Working Party on Manufactured Nanomaterials. A batch of each of the materials was homogenized and subsampled into 2 g emerald colored sample vials by the Fraunhofer Institute (Munich, Germany) and stored under an inert Argon atmosphere. Later subsamples of NM-110 and NM-111 materials have been made by the Joint Research Centre (Ispra, Italy) and are called JRCNM01100a and JRCNM01101a, respectively.

NM-110 and NM-111 have been thoroughly characterized in previous work. Table 1 gives a detailed summary of their physicochemical characteristics. In brief, NM-110 and NM-111 are highly pure synthetic zincite. Whereas NM-110 is uncoated, NM-111 is coated with approximately 1% wt. triethoxycaprylsilane (C_{16}H_{34}O_{4}Si). Rietveld refinement of X-ray diffraction data have yielded average crystallite sizes of 71 to >100 nm and 58 to 93 nm for NM-110 and NM-111, respectively. Qualitative electron microscopy analysis showed that ZnO in both materials has two main types of morphologies with low and high aspect ratios and a wide size range. Quantitative size analysis by transmission electron microscopy, showed that the average Feret min/average Feret max diameter size distribution of well dispersed primary particles and their agglomerates and aggregates were 106±111 nm/178±175 nm (NM-110) and 101±76 nm/170±126 (NM-111) respectively. The median equivalent circular diameter (ECD) and the corresponding median Feret min sizes were both below 100 nm. The specific surface areas as determined by the Brunauer Emmett Teller (BET) nitrogen adsorption method (degassed at 300 °C) were 12.4±0.6 m^2/g and 15.1±0.6 m^2/g for NM-110 and NM-111, respectively. Considering a theoretical relative density of 5.99 g/cm^3, these surface areas correspond to volume specific surface areas of 74.3 (NM-110) and 90.4 g/cm^3 (NM-111) which, in contrast to the average particle size distributions and in agreement with median size distribution measurements, classify both materials as nanomaterials. Based on the listed physicochemical characteristics, the materials are relatively similar, except from a slightly higher specific surface area and coating on NM-111.
Table 1: Key physicochemical characteristics of the NM-110 and NM-111 ZnO test materials.

| Characteristics | NM-110 (alias JRCNM01100a) | NM-111 (alias JRCNM01101a) | Source in report |
|----------------|----------------------------|----------------------------|------------------|
| Phase          | Zincite (Space Group: P 6\textsubscript{3}mc) | Zincite (Space Group: P 6\textsubscript{3}mc) | NRCWE\textsuperscript{a}, JRC\textsuperscript{b} |
| Morphologies   | Euhedral to subhedral rectangular, tabular, rod, and fiber shapes | Euhedral to subhedral rectangular, tabular, rod, fiber, and tetrapod shapes | UNIVE\textsuperscript{c}, CODA CERVA\textsuperscript{d}, JRC |
| Coating        | None | Triethoxycaprylsilane (C\textsubscript{16}H\textsubscript{34}O\textsubscript{4}Si) | Supplier |
| Mass-loss by TGA\textsuperscript{e} (wt.\%) | N.D. | ~1 (at 250-400 °C) | NRCWE\textsuperscript{a} |
| Crystallite size by XRD\textsuperscript{f} (nm) | 71 to > 100 | 58 to 93 nm | NRCWE\textsuperscript{a} |
| Average ECD\textsuperscript{g} (nm) | 114±97 | 106±69 | CODA CERVA\textsuperscript{d} |
| Median ECD\textsuperscript{h} (Q1/Q3) (nm) | 94 (63/144) | 93 (54/142) | CODA CERVA\textsuperscript{d} |
| Average Feret min (nm) | 106±111 | 101±76 | CODA CERVA\textsuperscript{d} |
| Median Feret min (Q1/Q3) (nm) | 79 (50/133) | 89 (49/138) | CODA CERVA\textsuperscript{d} |
| Average Feret max (nm) | 178±175 | 170±126 | CODA CERVA\textsuperscript{d} |
| Median Feret max (Q1/Q3) (nm) | 135 (93/214) | 143 (82/119) | CODA CERVA\textsuperscript{d} |
| Mean aspect ratio | 1.6±0.6 | 1.8±0.7 | CODA CERVA\textsuperscript{d} |
| Median aspect ratio (Q1/Q3) | 1.7 (1.4 / 1.9) | 1.6 (1.4 / 2.0) | CODA CERVA\textsuperscript{d} |
| Specific surface area | 12.4±0.6 | 15.1±0.6 | NRCWE\(^a\) |
|-----------------------|----------|----------|-------------|

\(^a\) National Research Center for the Working Environment (Copenhagen, Denmark); \(^b\) Joint Research Center (Ispra, Italy); \(^c\) Ca’ Foscari University of Venice (Italy); \(^d\) CODA-CERVA has changed name to Sciensano (Brussels, Belgium); \(^e\) Thermogravimetric Analysis; \(^f\) X-ray diffraction (TOPAS Rietveld refinement of powder X-ray diffraction data obtained on a Bruker D8 diffractometer (Cu\(K_\alpha\), 40 kV; 40 mA; stepping mode: 0.02°/20 per second) with Bragg-Brentano setup); \(^g\) Equivalent Circular Diameter of well-dispersed primary particles and their aggregates and agglomerates; \(^h\) Q1 and Q3 denotes the 25 and 75 percentile around the median value.

### Preparation of Ham’s F12 nutrient mixture and ZnO MNs dispersions

The complete test medium (cHam’s F12) was prepared by mixing Ham’s F12 nutrient mixture (ThermoFisher Scientific, Hvidovre, Denmark) with 1% v/v penicillin/streptomycin (10000 U/mL and 10 mg/mL respectively; In Vitro A/S, Fredensborg, Denmark) and 10% v/v of inactivated Fetal Bovine Serum (USA grade; In Vitro A/S, Fredensborg, Denmark). The medium was filtered through a 0.8 μm filter mounted on a syringe. Fifty μL IL-6 standard (National Institute for Biological Standards and Controls, Hertforshire, UK; final concentration = 500 pg/mL), 400 μL IL-8 standard (National Institute for Biological Standards and Controls, Hertforshire, UK; final concentration = 4000 pg/mL), and 2 μL rabbit muscle LDH (Sigma-Aldrich, Brøndby, Denmark; final concentration = 100 ng/mL) were then added to constitute the final medium. This medium was stored in the refrigerator for up to a week.

### Preparation of particle batch dispersions

MNPs batch dispersions were produced following the NANOGENOTOX batch dispersion protocol \(^{25}\). Briefly, for preparing a 6 mL dispersion at 2.56 mg/mL, 15.36 mg of MNPs were pre-wetted in 30 μL ethanol and dispersed in 0.05% sterile filtered BSA in Nanopure Diamond UV water by sonication for 16 min at 400 W and 10% amplitude using a 13 mm disruptor horn (Branson Sonifier S-450D, Branson Ultrasonics Corp., Danbury, CT, USA). Dispersions were prepared immediately prior to the experiments and checked each time for dispersion quality using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, United Kingdom).

### Evaluation of proteins interaction with ZnO MNs
Particle dispersions, prepared as mentioned above, were incubated with BSA solution for 1 h at 37 °C and 5% CO₂ to reach a final BSA concentration of 500 µg/mL. After incubation, dispersions were centrifuged (20,000 × Relative Centrifugal Force – RCF – for 30 min) and the supernatant analyzed with the Pierce™ BCA Protein Assay kit (ThermoScientific, Rockford, USA).

Evaluation of LDH, IL-6 and IL-8 interaction with ZnO MNs

NM-110 and NM-111 were incubated with cHam’s F12 containing interleukins and LDH in 24-well plates for 24 h in a cell incubator (CelCulture® CO₂ Incubator, ESCO Medical, Egaa, Denmark) maintaining a temperature of 37 °C and a 5% CO₂ atmosphere with a 95% relative humidity in the dark without shaking. Particle concentrations ranged from 10 to 640 µg/mL. After incubation, suspensions were collected by flushing the wells, and centrifuged at 20,000 × RCF for 30 min. LDH absorbance and cytokines concentrations were measured in the supernatant. Three independent experiments with two replicates for each test were conducted. The quantification of LDH absorbance was performed using LDH Cytotoxicity Detection Kit’s instructions (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) which relies on tetrazolium salt reduction by NADH/H⁺ in the presence of active LDH. LDH absorbance was measured in the supernatant after centrifugation by a single measurement at one time point with a spectrophotometric microplate reader (ELISA reader). The amount of proinflammatory IL-6 and IL-8 was measured by ELISA (BD Pharmingen kit, Cat. No. 555220 and 555244 respectively; BD Biosciences, Lyngby, Denmark).

To evaluate the kinetics for the interaction between LDH and ZnO MNs, the quantification of LDH was performed over time 5, 10, 20, 40, 60, 90 and 120 min after 24 h incubation with 640 µg/mL NM-110. Experiments were performed at increasing initial LDH concentrations: 50, 100, 200 and 1000 ng/mL. Three experiments with two replicates each were performed for each LDH concentration.

Calculation of adsorption isotherms for LDH on ZnO MNs
The interaction between LDH and ZnO MNs was quantified in terms of adsorption isotherms. Adsorption isotherm experiments were conducted at 37 °C after 24 h incubation at initial concentration of ZnO MNs ranging from 10 to 640 µg/mL and initial LDH concentration ranging from 20 to 1000 ng/mL as described above. A control study without ZnO MNs was included. The amount of LDH adsorbed, \( q \) (mg/m\(^2\)) was estimated from the mass balance shown in equation (1):

\[
q_e = \frac{(C_o - C_e)V}{A \times M}
\]

In this equation, \( C_o \) (mg/L) is OD\(_{\text{ref}}\) (initial LDH absorbance), \( C_e \) (mg/L) is OD\(_{\text{LDH}}\) (defined as the optical density measured at 490 nm) at time \( t \), \( V \) (mL) is the solution volume, \( A \) (m\(^2\)/g) is the particle specific surface area per unit mass and \( M \) (g) is the particle mass.

**Measurement of ZnO MNs zeta potential and LDH isoelectric point**

The hydrodynamic size distribution and zeta potential of dispersed ZnO MNs, and LDH were measured using dynamic light scattering analysis (DLS, Zetasize Nano ZS, Malvern Instruments Ltd., Malvern, United Kingdom). Data recording and treatments were made using the Zetasizer software v. 7.11 and 7.12 (Malvern Instruments Ltd., Malvern, United Kingdom). Measurements conditions were established following the automated optimization for measurement and position, attenuation and sample runs calculated by the software.

Zeta potential measurements in both the batch dispersions and cHam’s F12 were performed at 37 °C using the standard viscosity (0.6864 cP) as well as optical and electrical properties of water. The absorbance and refractive indices for ZnO were set to 0.400 and 2.020 respectively.

LDH isoelectric point was determined from zeta potential measurements in the pH range 1.23±0.01 to 11.9±0.00 by titration with HCl (1 M) and NaOH (1 M) and dosing using the MPT-2 Titrator (Malvern Instruments Ltd., Malvern, United Kingdom).

Before each measurement, the samples were thermally equilibrated for 2 min to minimize changes in viscosity during measurements.
Sedimentation of ZnO MNs during incubation

The sedimentation behavior of both NM-110 and NM-111 was studied over time using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, United Kingdom). Experiments were conducted at a ZnO concentration of 320 µg/mL reached by adding 125 µL batch dispersion to 875 µL cHam’s F12 in a DLS cuvette (10×10×45 mm, Sarstedt AG&Co, Nümbrecht, Germany). The resulting 1 mL volume occupies a 1 cm vertical path length in the DLS cuvette, which resembles the liquid height in the 24-well Sensor Dish Reader® and testing plates for in vitro testing.

Experiments were performed at 37 °C and data were recorded over 6 h. After 5 min thermal equilibration, ten measurements were collected with automatic measurement optimization. After the ten first measurements, the measurement position, the attenuator settings and number of sub-runs were fixed and data recording continued.

The agglomeration and sedimentation behaviors were then assessed from the evolution over time in hydrodynamic size and derived count rate. It is worth noting that the detector was placed 3 mm above the base of the DLS cuvette. Thus, the recorded signal describes the accumulation and particle size changes that would happen in the nutrient mixture close to the cells growing at the bottom of the 24-well plates in the case of a cell assay.

Evaluation of ZnO MNs dissolution and LDH interaction with dissolved Zn during incubation

The dissolution behavior and 24 h solubility limit were determined for NM-110 and NM-111 in cHam’s F12 under simulated in vitro test conditions. The potential influence of dissolved Zn was further investigated at different concentrations using ZnCl₂ (Merck, EMSURE® ACS,Reag. Ph Eur, catalogue no. 108816) dissolved in water.

The ZnO dissolution experiments were conducted at 320 µg/mL using the 24-well plate SensorDish® Reader system (SDR, PreSens Precision Sensing GmbH, Germany), which allows simultaneous time-resolved...
monitoring of the evolution of temperature, pH and dissolved O$_2$. The pH (HydroDish®) measurements were made using optical-chemical sensors mounted at the bottom of the wells.

The SensorDish® with 875 µL cHam’s F12 added to each well were placed on the plate readers in the incubation chamber. After thermal equilibration to 37 ºC, 125 µL ZnO batch dispersion or control dispersion medium was added to the wells. Medium samples were collected either at 0.25, 1, 2, 4 and 24 h into the test or after 24 h with no sampling. After collection of the medium samples by pipette, the samples were immediately added to 3 kDa centrifugal filter tubes and centrifuged at 4000 × RCF for 30 min. Filtered mediums were sampled and added 500 µL 2% ultrapure HNO$_3$. Vials and liquid masses were weighed for subsequent quantification. Liquids were stored in darkness until Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis was performed by Eurofins Miljø A/S (Vejen, Denmark) as a commercial service. The ICP-MS method has a minimum detection limit of 5 µg Zn/L and an expanded method uncertainty of 30%.

In order to test whether decrease in LDH absorbance was due to interaction with dissolved Zn, LDH absorbance was measured following incubation with 1.75 to 640 µg/mL Zn$^{2+}$ ions in the form of ZnCl$_2$. ZnCl$_2$ was dissolved in 0.05% BSA water (2.56 mg/mL) and added to the test medium for 24 h at 37 ºC, following the same protocol as for NM-110 and NM-111. After centrifugation at 20,000 × RCF for 30 min, LDH absorbance was measured in the supernatant as described above. Three independent experiments with two replicates each were conducted.

**Investigating the role of pH on LDH**

We observed that ZnO dissolution resulted in changes in the pH in cHam’s F12. Therefore, a detailed study was made to further investigate the temporal evolution of pH for both NM-110 and NM-111. These studies were conducted using the SensorDish® Reader system with incubation in the cell incubator at 37 ºC, 95% relative humidity, and 5% CO$_2$ atmosphere. ZnO MNs were dispersed in the test medium and dispensed in 24-well plates on the SensorDish® Readers in the incubator immediately after sonication. The system was then calibrated at 37 ºC using the single measurement command three times. Dissolved oxygen and pH were
measured every 5 min for 2 h because we know that interaction between LDH and ZnO MNs occurs within 60 min (see Results section). The experiments were conducted with 320 µg ZnO/mL. Measuring range for pH was 6.0-8.5 with a resolution of ±0.05 at pH 7.

**Thermodynamic chemical reaction modeling**

The dissolved Zn concentration, pH evolution, and chemical species distribution during addition of either ZnO or dissolved ZnCl₂ to Ham’s F12 was assessed using the React program and plotted using the Gtplot Apps in Geochemist Workbench® v. 11.0 ⁴². A simplified saline Ham’s F12 composition was used in the calculations. This was due to lack of thermodynamic data for especially sugars, organic and amino acids, as well as vitamins in the Geochemist Workbench® database. Table 2 lists the general composition of Ham’s F12 nutrient mixture and indication of the compounds considered in the calculations. The most important omissions by molarity were D-glucose (6.06%), amino acids (2.32%), and vitamins (0.13%). The compounds included in the modelling were converted to anions and cations and comprised 90.27% of the total mixture by molarity and were by far dominated by NaCl (79.41%). The starting conditions for the calculations were set to pH 7.4, fO₂(g) = 0.2 (to balance with O₂(aq)). The HCO₃⁻ concentration was set to a starting concentration of 14 mM and set free to vary.

Table 2. List of main compounds and their concentrations in Ham’s F12 and indication (X) of compounds used for thermodynamic chemical reaction modelling in Geochemist Workbench® (GWB).

| Component       | Concentration | GWB |
|-----------------|---------------|-----|
|                 | (mg/L) | (mM) |     |
| CaCl₂           | 33.22   | 0.299 | X   |
| CuSO₄·5H₂O      | 2.5e-3  | 1e-5  | X   |
| FeSO₄·7H₂O      | 0.834   | 3e-3  | X   |
| KCl             | 223.6   | 2.98  | X   |
| MgCl₂           | 57.22   | 0.6   | X   |
| NaCl            | 7599    | 131   | X   |
| NaHCO₃         | 1176    | 14    | X   |
| Compound          | Value  | Error | Significance |
|-------------------|--------|-------|--------------|
| Na₂HPO₄           | 142    | 1     | X            |
| ZnSO₄·7H₂O        | 0.863  | 3e-3  | X            |
| D-glucose         | 1802   | 10    |              |
| Hypoxanthine-Na   | 4.77   | 0.03  |              |
| Linoleic acid     | 0.084  | 3e-4  |              |
| Lipoic acid       | 0.21   | 9.71e-4 |          |
| Phenol red        | 1.2    | 3e-3  |              |
| Putrescine-2HCl   | 0.161  | 1e-3  |              |
| Na-pyrovate       | 110    | 1     |              |
| Thymidine         | 0.7    | 3e-3  |              |
| Amino acids       | 614.08 | 3.8288|              |
| Vitamins          | 35.6403| 0.2063|              |

**Statistical analysis**

Statistical analysis, tables and graphs were made using R (version 3.4.3). Statistical significance was set at \( p \)-value < 0.05. Each treatment was repeated in duplicate and each experiment replicated three times, unless stated otherwise.
**Results and discussion**

The interaction of LDH, IL-6, and IL-8 with NM-110 and NM-111 was investigated under experimental conditions for routine *in vitro* toxicological studies using complete Ham’s F12 test medium (cHam’s F12 containing 1% v/v penicillin/streptomycin and 10% v/v inactivated Fetal Bovine Serum - FBS) with addition of 100 ng LDH/mL, 500 µg IL-6/mL and 4000 µg IL-8/mL. The applied cHam’s F12 recipe is often used for *in vitro* toxicological testing and the tested levels of LDH and interleukins correspond to moderate levels of particle induced cytotoxicity and inflammation in human lung epithelial cell line A549. The interaction of LDH with ZnO MNs was described using two adsorption isotherm models. Additionally, the kinetics of the observed effects, the role of particle sedimentation, pH, and particle dissolution was examined. All tests were conducted in a cell incubator at 37 °C with a 5% CO₂ atmosphere and a relative humidity of 95%.

**Evaluation of protein interaction with ZnO MNs**

**State of the test materials after batch dispersion**

To understand the starting conditions of the LDH, IL-6, and IL-8 interactions tests, we investigated the extent of BSA interaction with NM-110 and NM-111. The batch dispersions were allowed to rest for 1 h before sampling to mimic the recommended maximum storage time before use of the dispersions. The results showed a significant decrease in BSA levels in the ZnO MNs dispersions as compared with control: 490.4±21.2 µg/mL (control), 151.8±27.0 µg/mL (NM-110) and 292.8±41.5 µg/mL (NM-111). NM-110 adsorbed significantly more BSA than NM-111 (ANOVA, p-value < 0.001). Hence, the coating of ZnO MNs with triethoxycaprylsilane resulted in a lower level of interaction with BSA. However, the surface charges of NM-110 and NM-111 were both close to neutral in the batch dispersion (-0.16±0.34 mV and 0.01±0.27 mV after 60 min respectively; Table 1 in Ref51). This is important because previous studies have shown that surface charge and adsorption of serum proteins from the dispersion medium on to MNs can affect their fate and interaction with specific proteins in the assay. Even though the FBS concentration in cHam’s F12 was considerably higher than the BSA concentration used for making the batch dispersions (10% and
0.05% respectively), the adsorbed, as well as excess BSA, could either a protective of facilitating role in the MNs interactions with FBS, interleukins and LDH in our tests.

**Protein-particle interactions**

To test the protein-particle interactions, 10 to 640 µg/mL (NM-110 and NM-111) were incubated in cHam’s F12 with LDH, IL-6, and IL-8 for 24 h. We observed that only the LDH levels were significantly reduced in the test medium (Figure 1 and Table 2 in Ref51). The amounts of IL-6 and IL-8 decreased with less than 5% at all ZnO MNs concentrations (not statistically significant). However, with 10 µg ZnO/mL, the average OD_{LDH} were reduced by 59.5% (NM-110) and 52.8% (NM-111) and increased in a dose-dependent manner to 73.5% (NM-110) and 74.6% (NM-111) at 640 µg/mL. As the reduction in LDH levels was similar for the two ZnO MNs, we conclude that the presence of the triethoxycaprylsilane coating on NM-111 and the lower level of BSA adsorption on NM-111 in the initial batch dispersion do not influence the interaction with LDH. Lastly, a test was conducted for investigation of the interaction of ZnO MNs with FBS present in cHam’s F12 after 24 h incubation. No detectable interaction was observed (data not shown).

Figure 1: Decrease in LDH content in cHam’s F12 as a function of MNs concentration after incubation for 24 h at 37 °C with NM-110 and NM-111.
The observed interaction between LDH and ZnO MNs, but not between IL-6 and ZnO MNs nor IL-8 and ZnO MNs, is in agreement with results from Kroll et al. who detected a specific interference of ZnO MNs with the LDH ELISA assay among a set of 24 MNs of diverse natures and physicochemical properties. Furthermore, evidence was provided that IL-8 measurements were strongly affected by TiO₂ MNs but not by ZnO MNs. This is in agreement with other studies demonstrating a decrease in IL-8 levels after incubation with TiO₂ MNs. As the LDH assay is often used to screen and control for cytotoxicity, it is important to further our understanding of the kinetics of the LDH interaction and investigate whether determinant physicochemical characteristics can be identified. The kinetics of the interaction can play a role in for example in vitro genotoxicity studies, where early time points are used to understand initial effects on DNA and signaling.

**Kinetics of LDH interaction**

Repeated measurements of LDH absorbance levels were made during 120 min incubation with 640 μg/mL NM-110, dosing 50, 100, 200, and 1000 ng LDH/mL Ham's F12 (Figure 2 and Table 3). The OD₅₄₀ reduction rate (initial slope) increased with increasing initial LDH concentration. Statistically significant differences were observed (p-value < 0.05) between 50 and 200 ng/mL, 50 and 1000 ng/mL and 100 and 1000 ng/mL. In all tests, a plateau was reached after approximately 60 min (borderline for 1000 ng/mL) where the LDH levels were inversely proportional to the initial LDH concentration (statistically significant difference, except between 200 and 1000 ng/mL, Tukey pairwise comparison, p-value < 0.001), i.e., the more LDH that was initially present in the test medium, the more had interacted with the NM-110.
Figure 2: Decrease in LDH levels as a function of time after incubation of 640 μg NM-110/mL cHam’s F12 at 37 °C with four initial LDH concentrations. Separate linear models were fitted from 0 to 60 min and from 60 to 120 min.

Table 3: Initial slope and plateau values describing the kinetics of the interaction between LDH and NM-110 at different initial LDH concentrations.

| Initial LDH concentration (ng/mL) | Initial slope | Plateau |
|----------------------------------|--------------|---------|
|                                  | Rate constant | R² | LDH level (% of OD<sub>ref</sub>) |
| 50                               | -0.7±0.1      | 0.62 | 54.9±5.1<sup>**</sup> |
| 100                              | -1.2±0.2      | 0.56 | 38.5±5.7<sup>**</sup> |
| 200                              | -1.5±0.2<sup>a</sup> | 0.64 | 25.0±3.6<sup>**</sup> |
| 1000                             | -1.4±0.1<sup>b,c</sup> | 0.71 | 22.4±14.0 |

Statistically significant difference of the initial slope between 50 and 200 ng/mL (<sup>a</sup>), 50 and 1000 ng/mL (<sup>b</sup>) and 100 and 1000 ng/mL.<n>.<sup>c</sup>. LDH levels in the plateau were significantly different (***, Tukey multiple pairwise comparison, p-value < 0.001) except between 200 and 1000 ng/mL. OD<sub>ref</sub> is defined as the optical density of the control.
These results show that the interaction between LDH and NM-110 was completed within the first hour of incubation. The fast onset of LDH disappearance from the test medium was also observed after exposure to soot and oxidized soot particles. The occurrence of a plateau after 60 min incubation regardless of the initial LDH concentrations suggests that a material-related phenomenon occurs in the test medium, which prevents further interaction between ZnO MNs and LDH after 60 min. In addition to saturation of ZnO MNs adsorption sites, the physicochemical conditions in the dispersions, including particle sedimentation and/or dissolution may play a role. The timing of measurement in interaction tests appears to be highly important.

Thereby, correct interpretations of in vitro toxicological results can only be made if the interaction studies are conducted so they match the timing of sampling in the in vitro toxicological tests. However, even then, correct measurements and interpretations of the scale and kinetics of a biological in vitro response may be challenging if it occurs at time periods where the interaction kinetics play a role.

**Adsorption isotherms assuming LDH adsorption on to ZnO MNs**

Interference by adsorption on to ZnO MNs is the simplest mechanism by which the LDH can disappear from the test medium. The coverage and adsorption mechanisms can be determined based on adsorption isotherm models in which the amount of LDH adsorbed on to NM-110 and NM-111 per MNs surface unit (qₑ, mg/m²) is calculated and plotted against the LDH concentration in the medium. Two models, namely the Langmuir isotherm (Equation 1) and the Freundlich isotherm (Equation 2) were used for assessment.

1. \( \frac{C_e}{q_e} = \frac{1}{Q_m k} + \frac{C_e}{Q_m} \)
2. \( \ln(q_e) = \ln(K_F) + \frac{1}{n} \ln(C_e) \)

where \( C_e \) is the LDH concentration at equilibrium (mg/L); \( Q_m \) (mg/m²) and \( k \) (L/mg) are Langmuir constants representing the maximum amount of LDH adsorbed onto ZnO MNs and the adsorption constant respectively; Freundlich constants \( K_F \) (mg/g) and \( 1/n \) (dimensionless) describe the adsorption capacity and the adsorption intensity respectively. The Langmuir model assumes adsorption in monolayer where all adsorption sites are equally probable and without interactions between adsorbed molecules. The Freundlich
model assumes adsorption of molecules in multilayer with heterogeneous adsorption energies and interaction between adsorbed molecules. The results for the calculations are plotted in Figure 3 while the constants obtained from applying the models to each set of independent experiment (each conducted in duplicate) and the correlation coefficients $R^2$ are listed in Table 4.

Figure 3: Experimental $q_e$ (black) and theoretical $q_e$ (lines) using the Langmuir (blue line) and Freundlich (red line) adsorption isotherm models for NM-110 and NM-111.

| Isotherms | Parameter | NM-110 | NM-111 |
|-----------|-----------|--------|--------|
|           |           | #1    | #2    | #3    | #4    | #5    | #6    |
| Langmuir  | $K$ (L/mg)| -1.69 | -1.32 | -2.55 | -1.69 | -1.19 | -1.17 |
#1-6 designates independent experiments.

The correlation coefficients (Table 4) and the graph (Figure 3) show that the adsorption data fit better to the Freundlich model, which indicates that the LDH interaction may be due to multilayer adsorption of LDH on to ZnO MNs without saturation of adsorption sites. Similar adsorption pattern was observed by Sasidharan et al. 56 for interaction of BSA with ZnO MNs.

While LDH adsorption can be governed by electrostatic mechanisms or physical properties, the negative correlation between MNs concentration and adsorbed LDH suggests that other phenomena in the test medium play a role. It is likely that the increase in MNs concentration leads to an increase in particle sedimentation rate and particle-particle interaction which could result in increased MNs agglomeration altogether reducing the surface area available for adsorption. Besides, ZnO MNs are known to be partially soluble in test medium 57. On one hand, particle dissolution will release Zn ions, which have previously been proposed to interact with LDH 6. On the other hand, dissolution will further reduce the surface area available for interaction. Finally, the test medium pH could change during dissolution of ZnO MNs and affect LDH in the medium. The role of these phenomena in the interaction between ZnO MNs and LDH is assessed below.

**Parameters of potential influence**

**Zeta potential of LDH and ZnO MNs in chHam’s F12**

The interaction between LDH and ZnO MNs can be governed by several mechanisms, including electrostatic interactions. The particle surface charge in a given medium varies with pH. Therefore, to elucidate whether electrostatic forces were responsible for the observed interaction between LDH and ZnO MNs, we
investigated the zeta potential and the pH evolution during incubation of the two ZnO MNs as well as the isoelectric point of LDH alone in Ham’s F12.

The zeta potential and pH measurements of the particles were made with 10, 320, and 640 µg ZnO/mL of Ham’s F12 and conducted at 37 °C (Table 1 in Ref51). The results showed that the zeta potential was neutral (NM-110: -0.01±1.13 mV) to slightly negative (NM-111: -13.18±1.22 mV) with negligible changes over 1 h and different ZnO MNs doses. The pH values remained close to neutral (NM-110: 7.0±0.3; NM-111: 6.8±0.3).

For measurement of the surface charge and isoelectrical point of LDH in Ham’s F12 (NB: without serum), the LDH concentration was increased to 10000 ng/mL and titrated with HCl (1 M) and NaOH (1 M) for acidic and basic conditions, respectively. The analysis showed that the isoelectric point of LDH in Ham’s F12 occurred at pH 4.67 (triplicates). The zeta potential became more negative at higher pH values reaching -10.00±1.22 mV at pH 8.16±0.01 and -12.03±0.06 mV at pH 9.92±0.00 (Figure 1 in Ref51). Consequently, electrostatic forces do not seem to drive the interaction between ZnO MNs and LDH.

**Temporal sedimentation**

Particle agglomeration and sedimentation in a given dispersion medium are governed by surface charge or steric stabilization but also by the particle concentration58. Due to the neutral to slightly negative zeta potential of the ZnO MNs (reported above), we expected that the ZnO MNs could have relatively fast sedimentation rates in Ham’s F12 in our experiments. This was confirmed by testing the stability of the two ZnO MNs dispersions at 320 µg/mL in Ham’s F12 over 6 h at 37 °C. Sedimentation plots were obtained using the variation in the scattered light intensity (I, detected as mean count rate) relative to the initial value (I₀) of the dispersion measured by dynamic light scattering (DLS) (Figure 4). Full sedimentation (drop in the I/I₀ ratio) was reached within 200 (NM-110) to 250 (NM-111) min with approximately 25% of the I/I₀ intensity ratio being lost after 60 min. Even though sedimentation occurred rapidly, its duration extended considerably beyond the period when the interaction between LDH and ZnO MNs occurred. Thus, loss of
MNs from the suspension by sedimentation does not appear to be a major causal factor in the observed interaction artefact.

Figure 4: Temporal evolution of mean count rate ratio ($I/I_0$, red curve) for NM-110 and NM-111 at 320 µg/mL and dpH (difference between pH measured and pH in control wells containing medium only) measured at the bottom of the wells (blue curve). The large gap in dpH for NM-110 is due to a pH over the detection limit (pH>9).
pH and dissolution of ZnO MNs and interaction with LDH

Solubility of ZnO MNs in Ham’s F12 nutrient mixture

ZnO is known to be partially soluble in water as well as in different media used for in vitro toxicological testing and Zn has been proposed to interfere with the LDH due to binding of zinc ions to histidine tails of LDH.

Results from thermodynamic chemical reaction modeling using the React program in the Geochemist Workbench® show that ZnO is thermodynamically unstable in a Ham’s F12 nutrient mixture of simplified composition. The calculations also indicated that the pH could increase by more than 2 pH units during dissolution of only 10 µg ZnO/mL (Figure 2-A in Ref51) and that more than 99.3% (by weight) of the dissolved Zn species would be Zn\(^{2+}\). The other species are strongly dominated by ZnCl\(_2\). We demonstrate that several parameters determinant for LDH interaction can vary during incubation.

The full composition of the Ham’s F12 nutrient mixture is, however, chemically more complex than the one that could be used for modeling based on available chemicals in the thermodynamic database. The full Ham’s F12 nutrient mixture also contains various additional organic compounds, amino acids, nutrients and vitamins, as well as the 1% v/v penicillin/streptomycin and 10% v/v FBS. In addition, the test medium with the ZnO MNs was incubated in a 5% CO\(_2\) enriched atmosphere, which can also influence the system. Finally, due to sedimentation, there may also be temporal differences between the chemical microenvironment at the bottom of the wells, where the ZnO MNs accumulate, compared to the volume above, which is progressively being depleted of MNs over time. This could be important when cells are attached at the bottom of the wells, which is the case for e.g., lung epithelial cells.

Therefore, we investigated the pH reactivity and release of Zn during dissolution of ZnO MNs using the SensorDish™ Reader (SDR) method. The results showed that the pH values rapidly exceeded 9 (the upper limit of detection of the SDR sensors) (Figure 4). The strongest and most prolonged effect was observed for NM-110 where the pH was above the detection limit between 170 and 520 min.
Similar to the rapid increase in pH, a fast dissolution was also observed for both NM-110 and NM-111. Most of the dissolution appeared to have already occurred after 0.25 h incubation resulting in 5802.3±67.5 (NM-110) and 5177.4±148.9 (NM-111) µg Zn/L and reached 7431.1±373.5 (NM-110) and 8265.2±178.0 (NM-111) µg Zn/L after 24 h (Table 5). These Zn concentrations are in the same range as reported previously for ZnO MNs and bulk particles (10 and 100 mg/L) dissolved in different media (DMEM, RPMI, hard water, Nanopure™ water) (exceeding 7000 µg/L of Zn^{2+} after 810 h^{57}). The total Zn concentrations in the sample exceeded by far the Zn concentrations already present in the nutrient mixture (41.1±7.5 to 49.4±14.6 µg/L; Table 5).

Table 5: Temporal evolution in the total Zn concentration during dissolution of NM-110 (n=3) and NM-111 (n=3) in cHam’s F12 and the average Zn concentration in nutrient mixture controls (n=4).

| Sampling time (h) | Zn (total, µg/L) |
|------------------|------------------|
|                  | NM-110 | σ    | NM-111 | σ    |
| 0*               | 872.6  | 22.6 | 864.3  | 19.0 |
| 0.25             | 5802.3 | 67.5 | 5177.4 | 148.9|
| 1                | 5978.4 | 162.2| 5595.2 | 22.0 |
| 2                | 5956.5 | 101.3| 5827.5 | 139.4|
| 4                | 6154.7 | 131.4| 6056.2 | 242.0|
| 24               | 7431.1 | 373.5| 8265.2 | 178.0|
| cHam’s F12       | 49.4   | 14.6 | 41.1   | 7.5  |

* estimated concentration at t = 0 h.

Our experimental results were in agreement with the chemical reaction modeling suggesting a pH increase and ZnO dissolution in the cHAM’s F12 (Figures 4-A and B in Ref^{51}). The maximum pH could not be determined due to experimental limitations of the sensors, but Geochemist Workbench® modeling of the pH evolution considering the measured dissolved Zn concentrations showed that the pH could reach a maximum around pH 9.5 (Figure 2-C and D in Ref^{51}).
The different tests results show that both the physical and chemical conditions in the test wells during *in vitro* incubation with ZnO MNs are dynamic and complex. Some of the observed variations can also influence on each other. For example, an increase in pH of the test medium can (*i*) lower ZnO dissolution\(^6^0\). (*ii*) promote the conversion of lactate to pyruvate\(^6^1\), disturbing the LDH assay, (*iii*) lead to differences in ZnO MNs and LDH surface charge and conformational changes of LDH\(^6^2\) with (dis)appearance of available adsorption sites for interaction with ZnO MNs, and (*iv*) enhance, or reduce, ionic interaction with charged ZnO MNs. Last but not least, the observed marked increase in pH during ZnO dissolution can directly affect cell viability and be a different mechanism of ZnO MNs inducted cytotoxicity\(^6^3,6^4\).

**Evaluation of interaction between released Zn and LDH**

Based on the dissolution results, a specific study to investigate the potential role of dissolved Zn on the interaction with LDH was conducted. A marked decrease in LDH absorbance was observed with increasing Zn concentration starting at 10 µg Zn/mL (Figure 5). The effect of dissolved ZnCl\(_2\) on the LDH was comparable to what was observed after incubation with ZnO MNs. However, the effect was observed at much higher Zn concentrations than was reached during dissolution testing of the two ZnO MNs (7 to 8 µg/mL after 24 h, Table 5). The decrease in LDH absorbance following dosing and incubation with 1.75 to 10 µg dissolved Zn/mL was not significant. Consequently, the previously reported inhibition of LDH due to dissolved Zn does not appear to be relevant at the Zn concentrations achieved by ZnO dissolution in cHam’s F12. However, it should be noted that full simulation of the chemical changes during ZnO dosing cannot be mimicked by dosing ZnCl\(_2\). Whereas the Zn speciation remains strongly dominated by Zn\(^{2+}\) (>95.8% by weight), calculation of the pH evolution suggests a slight reduction in pH with increased ZnCl\(_2\) dose (Figure 2-C and D in Ref\(^5^1\)).
Figure 5: Decrease in LDH levels following incubation with ZnCl\(_2\) solution for 24 h at 37 °C at concentrations over 7 μg/mL.

**Overall interpretations and recommendations**

Our study of the interaction between LDH and ZnO MNs has covered several potential mechanisms of interaction at both relevant and exceedingly high doses for consideration in *in vitro* toxicological studies. We observed a clear reduction in LDH levels (OD\(_{LDH}\)) with increasing ZnO MNs concentration. In addition, the higher the initial LDH concentration, the more LDH interacted with the ZnO MNs. Zeta potential measurements showed that the surface charge of NM-110, NM-111 and LDH were neutral to slightly negative at neutral pH in cHam’s F12. Besides, the sedimentation of ZnO MNs in cHam’s F12 was fast in our experimental conditions but the interaction between LDH and ZnO MNs was faster and complete in approximately 1 h. Therefore, the extent of LDH interaction does not appear to be affected or controlled by the ZnO MNs sedimentation rate in cHam’s F12. Furthermore, we observed that the ZnO MNs partially dissolved in cHam’s F12. However, the resulting dissolved Zn concentrations could not directly explain the observed effects on the LDH levels. Finally, the pH of the test medium temporarily exceeded 9 in the presence of both ZnO MNs. The effect was more pronounced for NM-110 than for NM-111. Rise in the pH causes greater negative surface charge of the LDH (Figure 1 in Ref\(^{51}\)) and, thereby, further increases
repulsive forces between LDH and ZnO MNs. Chemical reaction modeling suggests that an increase in dissolved Zn in cHAM’s F12 can lead to a slight drop in pH. Lowering of the pH would reduce the negative surface charge towards neutral as the isoelectrical point of LDH was found to be at pH 4.67.

None of the investigated physicochemical parameters could directly justify the observed interaction between LDH and ZnO MNs. Even though dosing with dissolved Zn did not immediately explain the LDH interaction, this may still be an explanation. Indeed, one can expect the dissolved Zn concentration to be higher at the interface and in the surroundings of dissolved ZnO MNs than the average concentrations in the mediums, which is what we measured. Our chemical reaction modeling suggested that the dissolved Zn concentration can be higher than the 24-hour solubility limit measured in our test. However, high dissolved Zn concentration at the ZnO interface does not explain that more LDH interacts when more LDH is added to the cHam’s F12 test medium. In this regard, the reasonable fit of the data with the Freundlich isotherm model suggests that the observed interaction between LDH and ZnO MNs could be driven by multilayer adsorption of LDH without saturation of adsorption sites. In summary, we suggest that the observed interferences are mainly due to concentration-dependent physisorption on to ZnO MNs potentially assisted by inhibition of LDH by interaction with dissolved Zn.

Based on the results obtained in this study and findings in scientific literature, we propose that a number of controls should be mandatory while conducting in vitro toxicological testing. It is important to account and, if necessary, adjust for:

(i) interaction of the MNs with BSA, FBS or any other agents used as dispersants in batch dispersions 24,25,

(ii) activation or inhibition of biological processes and responses due to presence of BSA, FBS or any other agents used in batch dispersions and as additions to nutrient mixtures,

(iii) interaction between the MNs and measured biomolecules, e.g. interleukins, LDH, lipids, and reactive oxygen species, which could lead to artefacts in the identification and quantification of biological response,
(iv) the kinetics of the interactions potentially observed,
(v) the physicochemical conditions in the dispersions, including particle sedimentation, surface charge, pH and dissolution to understand the mechanisms for the interaction and derive relevant conclusions.

If interactions are observed, tests for adjustment should be made using the exact exposure concentrations and time points used in the toxicological assay. Correct interpretations of results can only be made if all assays match in terms of time and exposure concentrations. Other suitable in vitro toxicological assays should always be considered in order to limit artefacts and erroneous conclusions regarding the toxicity of a test material. However, if other methods are not available, it is recommended to use correction factors to remove, or at least limit, the effect of an interaction. Recommendations for controlling particle interference in the optical analysis in colorimetric assays are insufficient. While standardized methods are needed for dispersing, testing and quantifying toxicological effects, a case-by-case evaluation of the interaction of MNs with the assay is strongly recommended to obtain reliable results.
Summary and conclusion

The interaction of LDH, IL-6, IL-8, and FBS with NM-110 (uncoated) and NM-111 (coated) was studied in cHam’s F12 (Ham’s F12 nutrient mixture with 1% v/v penicillin/streptomycin and 10% v/v FBS) under typical in vitro test conditions. The MNs were added to cHam’s F12 after dispersion in BSA water following the NANOGENOTOX batch dispersion protocol. When dosed in cHam’s F12, the ZnO MNs were, at least partially, coated with BSA and had neutral to slightly negative zeta potential. LDH interacted with the ZnO MNs and the LDH levels in cHam’s F12 decreased linearly to a minimum plateau within one hour. Moreover, LDH reduction could be augmented with increasing initial LDH concentrations. No interaction was observed with IL-6, IL-8, and FBS. Full sedimentation of ZnO MNs occurred after about 3 h and is hence not a limiting factor for LDH interaction.

Addition of 10 to 20 mg Zn/mL (ZnCl₂ dissolved in Nanopure™ water) caused a steep reduction in the measured LDH level. However, Zn concentrations only reached 5-6 µg Zn/mL after 1 h dissolution of NM-110 and NM-111 and 7-8 µg Zn/mL after 24 h. In spite of partial dissolution, the LDH loss could be explained using a Freundlich adsorption isotherm model. Electrostatic interaction was ruled out since the zeta potential of ZnO MNs, LDH and FBS in cHam’s F12 were all neutral to slightly negative. We conclude that the observed LDH interaction with ZnO MNs in cHam’s F12 is mainly governed by physisorption. It is possible that high concentrations of Zn ions in microenvironments around dissolving ZnO also play a role in lowering the level of measured LDH due to binding of Zn ions to histidine tails of LDH.

We demonstrate that LDH levels measured in in vitro toxicological tests of ZnO MNs cannot be used directly for interpretation. If not controlled for, artefacts in toxicological assays can lead to erroneous estimation of particles toxicity. We highlight the need for careful considerations and thorough characterization of the test system when conducting in vitro toxicological assays with particulate materials. The current study only covers the interaction between ZnO nanoparticles and LDH and it remains to be tested if other enzyme based assays are prone to similar confounding factors.
Acknowledgements

This study was conducted as part of the NANoREG project: "A common European approach to the regulatory testing of nanomaterials", funded by the European Union's 7th Framework Programme, under grant agreement N° 3105846 and co-funded by the National Research Centre for the Working Environment (Copenhagen, Denmark). Emilie Da Silva received additional funding from the Technical University of Denmark (Lyngby, Denmark). Renie Birkedal, formerly employed at the NRCWE, is acknowledged for her X-ray diffraction analysis and Rietveld refinement of NM-110 and NM-111.

Supporting information

Supporting Information Available: state of the test materials after batch dispersion (Table S1), protein-particle interaction (Table S2), LDH zeta potential (Figure S1), chemical reaction modeling (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.
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