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ZnO nanoparticle preparation route influences surface reactivity, dissolution and cytotoxicity

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Abstract

ZnO nanoparticles (nZnO) are commonly used in nanotechnology applications despite their demonstrated cytotoxicity against multiple cell types. This underscores the significant need to determine the physicochemical properties that influence nZnO cytotoxicity. In this study, we analyzed six similarly sized nZnO formulations, along with SiO₂-coated nZnO, bulk ZnO and ZnSO₄ as controls. Four of the nZnO samples were synthesized using various wet chemical methods, while three employed high-temperature flame spray pyrolysis (FSP) techniques. X-ray diffraction and optical analysis demonstrated the lattice parameters and electron band gap of the seven nZnO formulations were similar. However, electrophoretic mobility measures, hydrodynamic size, photocatalytic rate constants, dissolution potential, reactive oxygen species (ROS) production and, more importantly, the cytotoxicity of the variously synthesized nZnO towards Jurkat leukemic and primary CD4⁺ T cells displayed major differences. Surface structure analysis using FTIR, X-ray photoelectron spectroscopies (XPS) and dynamic light scattering (DLS) revealed significant differences in the surface-bound chemical groups and the agglomeration tendencies of the samples. The wet chemical nZnO, with higher cationic surface charge, faster photocatalytic rates, increased extracellular dissolution and ROS generation demonstrated greater cytotoxicity towards both cell types than those made with FSP techniques. Furthermore, principal component analysis (PCA) suggests that the synthesis procedure employed influences which physicochemical properties contribute more to the cytotoxic response. These results suggest that the synthesis approach results in unique surface chemistries and can be a determinant of cellular cytotoxicity and oxidative stress responses.

Abstract

The nZnO synthesis approach results in unique surface chemistries which influence agglomeration tendencies, dissolution potential, oxidative stress responses and NP-induced toxicity.

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Introduction

Zinc oxide nanoparticles (nZnO) are widely used in industrial processes, electronics and consumer products due to their unique optical, electronic, and structural properties that are absent in bulk ZnO (around one micron in size).¹⁻³ Given this, considerable research has centred around the inherent toxicity that nZnO demonstrates towards various organisms,^{4,5} as well as prokaryotic⁶⁻¹⁰ and eukaryotic cells.^{9,11-19} While many researchers have worked to engineer safer-by-design nanomaterials through material property modification,²⁰⁻²² others have sought to exploit this differential toxicity for potential use in nZnO-based therapeutics.^{9,11,12} Central to both of these long-term objectives are a comprehensive examination of nZnO-specific physicochemical properties and how material property modifications influence oxidative stress and nanoparticle (NP)-induced toxicity. Despite the wealth of available research, however, clear mechanistic determinants of these responses remain elusive. Among the proposed mechanisms is that intrinsic ROS production originating from redox reactions on the NP surface contributes directly to cellular membrane, organelle, or DNA damage.²³⁻²⁵ Alternatively, perturbations in intracellular zinc homeostasis from NP dissolution, pro-inflammatory responses and mitochondrial damage leading to ROS production from the cell death process have also been implicated.²⁶⁻³² Regardless, controlling reactivity at the NP-surface and nZnO dissolution is critical to influencing biological outcomes.

Most studies investigate changes in NP-induced toxic response as a function of a single material property modification in a specific nZnO formulation. For example, minor changes within the same synthesis method can alter both the crystal size and shape.²⁵ Likewise, surface modification via surface coating, chemical modification, or heat treatment has demonstrated efficacy in changing surface charge, surface structure and NP solubility.^{23-25,33,34} While this formulation controlled approach has been instrumental in identifying how specific physicochemical modifications influence cellular responses, it fails to explain how comparable studies using similar NP modifications and/or mammalian cell types, but differently synthesized nZnO samples, often result in differing cytotoxicity. These discrepancies have resulted in uncertainty as to how changes in material properties, within a very diverse set of nZnO formulations, influence the toxicity of nZnO.

Studies in bacterial models routinely employ step-wise synthesis method changes to improve nZnO photocatalytic activity and antimicrobial properties.^{6-8,10,35-40} However, similar studies in mammalian cells remain an underrepresented area in nZnO research. In previous work, our lab compared two spherical and similarly sized (~9 nm) pristine nZnO formulations fabricated using different wet-chemical methods.²⁵ Despite these similarities, the two formulations exhibited different (i) hydrodynamic size, (ii) electrophoretic mobility, (iii) isoelectric point (IEP), (iv) surface-adsorbed groups, (v) photocatalytic activity, and (vi) remarkably different cytotoxicity. This work illustrates the importance of investigating how changes to the synthesis method can dramatically alter the surface reactivity, and thus, NP-induced toxicity.

Additionally, there is still much debate as to the roles that extracellular,^{13,30} intracellular free,^{29,31} and total cell-associated zinc^{13,31} play in the mechanistic process of NP-induced toxicity. Once introduced into cell culture, nZnO will begin to dissolve into ionic zinc (shown as extracellular zinc in Scheme 1). Inevitably, some of this ionic Zn^{2+} will be taken up by cells to become intracellular free Zn^{2+} within the cytosol or endocytotic vesicles.^{32,41,42} Additionally, Zn^{2+} ions will interact with carbonate and phosphate anions to produce zinc carbonate or phosphate species.⁴³ While these species show negligible toxicity towards mammalian cells,^{44,45} they do interact with large agglomerates of intact nZnO (i.e., non-ionic zinc) to form amorphous complexes of encapsulated crystalline zinc that freely attach to cellular membranes or enter cells through various uptake mechanisms (designated as cell-associated Zn^{2+} in Scheme 1).^{17,31,41,45} Consequently, a thorough examination of these processes is critical in NP-toxicology studies.

In this work, we sought to expand upon our previous studies by evaluating a more diverse panel of nZnO formulations with differential toxicity to include four wet-chemical samples, two FSP prepared samples, and three control samples, specifically ZnSO_4 , bulk ZnO, and a SiO_2 -coated nZnO formulation. Careful consideration was used in choosing the nZnO samples for investigation. Wet chemical methods are ubiquitously used in research studies as they are easily synthesized using inexpensive, common laboratory equipment and chemicals, and can be readily modified with simple experimental changes. The four wet chemical methods chosen are among the most popular approaches utilized. All employ a forced hydrolysis synthesis reaction with variable precursors and solvent systems. FSP synthesis is favored in industrial settings because of its simplistic methodology with fewer process steps, scalability, easy collection systems, high yield, and purity of the final product.⁴⁶⁻⁵¹ Additionally, FSP methods employ high temperatures that well exceed any temperatures employed by most wet chemical methods. The specific FSP methods were selected based on differing precursor solutions and carrier gas mixtures. The use of nanomaterials bridging both research application and consumer product use should provide NP representation across multiple areas of nZnO usage. All the nanoscale samples were found to have average sizes in at least one dimension between 16 – 45 nm with similar bandgaps centred near 3.3 eV. Extensive characterizations were performed to evaluate the physicochemical differences present in the surface properties of the samples, and explore the roles that extracellular zinc, free intracellular zinc, and cell-associated zinc play in cellular toxicity. By looking at a broad panel of pristine nZnO with variable toxic potential, we have identified how NP-induced toxicity is influenced by both the dissolution potential and the surface reactivity of

nZnO. This understanding could provide critical insight into previously unexplained discrepancies in the wider body of research as well as define which material property interactions have the greatest potential to affect cellular toxicity.

Experimental

Synthesis of nZnO

Seven nZnO samples were used in this study. Four of the samples were synthesized using wet chemical methods. The DEG nZnO sample was produced using the forced hydrolysis of a zinc acetate dihydrate precursor in diethylene glycol (DEG). The solution was held at 150 °C for 90 minutes. Nanopure water was added to the solution at 80 °C to obtain the desired crystallite size.¹² Once cooled to room temperature, the nZnO were separated from solution via centrifugation and subsequent washings with ethanol.

The second wet chemical method, EtOH nZnO, was synthesized using a similar hydrolysis method with the same precursor, but denatured ethanol was used as the reaction solvent along with a strong base, LiOH, to maintain appropriate pH.²⁵ The solution was held at 80 °C for 90 minutes, cooled to room temperature, and aged with n-heptane for several hours. The NPs were removed from suspension via centrifugation, followed by subsequent washings with nanopure water and ethanol. After washings, both the DEG and EtOH nZnO precipitates were dried in an oven at 65 °C for >12 hours.

The third sample, EG nZnO, was synthesized using a wet chemical method followed by a thermal decomposition. Zinc acetate dehydrate was used as the precursor with polyvinylpyrrolidone (PVP) as a binding agent. The reaction was carried out by suspending the precursor and PVP in ethylene glycol (EG), and held at 180 °C for 90 minutes. After cooling to room temperature, the particles were collected by centrifugation, followed by ethanol washings. The sample pellet was dried overnight at 65 °C and then annealed at 350 °C for 2 hours to remove any residual organics.

The final wet chemical sample, NaOH nZnO, was synthesized by introducing a 0.5 M zinc nitrate $[\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}]$ precursor solution dropwise to an equal volume of 1.0 M NaOH heated to 80 °C.⁵² This solution was continuously stirred for two hours. The resulting precipitate was removed from solution via centrifugation and repeatedly washed with ethanol and nanopure water, and dried at 65 °C for >12 hours.

The three remaining nZnO samples were prepared using high-temperature flame spray pyrolysis (FSP). Rod-shaped FSP NPs (FSPR) and a silica-coated formulation of the FSPR sample (SiO_2 -FSPR) were prepared by collaborators at the Department of Environmental Health, Harvard University. Both samples employed a precursor solution of 0.5 M zinc naphthenate dissolved in ethanol for the formation of the nZnO core with the addition of hexamethyldisiloxane (HMDSO) utilized to form the silica coating. Specific synthesis details have been previously reported.^{21,22} A spherical FSP sample (FSPS) was synthesized by the Nanoscience Research Laboratory at Chiang Mai University in Thailand as outlined in Liewhiran et al. (2007).⁵³ For this formulation, zinc naphthenate was dissolved in a toluene /acetonitrile mixture with a ratio of 80/20 vol% at a precursor concentration of 0.5 M

ZnO. Lastly, a bulk ZnO sample was obtained by annealing a commercially available ZnO powder (Sigma Aldridge; St. Louis, MO) at 500 °C for 5 hours to ensure all nanosized particles less than 200 nm were removed from the sample.

Characterization

X-ray diffraction (XRD), transmission electron microscopy (TEM), dynamic light scattering (DLS), zeta potential, UV-vis spectrophotometry, and Fourier transform infrared spectroscopy (FTIR) were employed to evaluate chemical composition, crystal size, morphology, electrophoretic mobility and surface structure. Philips X'Pert and Rigaku Miniflex 600 X-ray diffractometers with a Cu K_α source ($\lambda = 1.5418 \text{ \AA}$) in Bragg-Brentano geometry were used to record room temperature XRD spectra. Rietveld refinement using Materials Analysis Using Diffraction (MAUD) software corrected for instrumental broadening was used to determine crystal size and lattice parameters.⁵⁴ An FEI Tecnai transmission electron microscope operating at 120 kV was employed to collect TEM images. High-resolution TEM analysis was performed on an FEI Titan microscope operating at 300 kV. Image processing was carried out using the Digital Micrograph software from Gatan (Pleasant, CA). The average particle size and the standard deviation of the nanopowders was determined by measuring 100 plus individual NPs within Image J software (NIH, Bethesda, MD).⁵⁵

The specific surface areas of the samples were determined via nitrogen gas physisorption at 77 K using a Quantachrome NOVA 2200e analyser. Prior to the analysis, all samples were vacuum degassed at 250 °C for 3.5 hours. The linear portion ($P/P_0 = 0.05\text{--}0.3$) of the Brunauer–Emmett–Teller (BET) model was used for the calculation of specific surface area with NovaWin Version 11.04 software.

Room-temperature (RT) ultraviolet and visible light optical absorbance spectra were obtained with a CARY 5000 spectrophotometer. Electrophoretic mobility and hydrodynamic sizes of nZnO dispersions created in nanopure water and cellular media were collected with a Malvern Zetasizer® NanoZS. Each formulation was measured three times using freshly prepared NP dispersions and a minimum of six replicates were collected for each measurement. FTIR spectra were measured using a Bruker Tensor 27 spectrometer.

Cell culture and cytotoxicity studies

Cells were cultured at 37 °C and 5% CO₂. For cell cytotoxicity assays, Jurkat leukemia T cells were cultured in supplemented Roswell Park Memorial Institute (RPMI)-1640 media (10% FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 1% penicillin-streptomycin) and maintained in log phase prior to treatment. Stock solutions for the nZnO samples were created by suspending the nanomaterial in nanopure water and sonicating for 30 minutes using a power of 1.05 W. These sonication parameters deliver a determined sonication energy⁵⁶ of 181 J/mL needed to obtain stable nZnO dispersions.⁵⁷ Working stock dilutions were prepared by introducing the NP stock to cellular media and vortex mixing for 30 seconds. The NPs were then added to cells (5×10^5 cells/mL) and cultured for 24 hours. ZnSO₄ was utilized as a positive ionic zinc control. Cell viability was determined using a metabolic assay in which alamar blue

(10% of total volume) was added to cells and incubated for an additional 4 hours. Fluorescence changes were measured with a Biotek Synergy MX[®] plate reader (Winooski, VT) using an excitation of 530 nm and emission collection at 590 nm.

Additional studies were performed using human primary CD4⁺T cells. Written informed consent was obtained from all blood donors and the study was approved by the Boise State University Institutional Review Board which maintains Federalwide Assurance of compliance with the Department of Health and Human Services (HHS) Office for Human Research Protections (OHRP; 45 CFR part 46, subpart A). Ficoll-Hypaque density centrifugation (Histopaque-1077, Sigma Aldridge; St. Louis, MO) was used to isolate peripheral blood mononuclear cells from normal human blood samples followed by negative immunomagnetic selection (Stem Cell Technologies; Vancouver, BC, Canada) to obtain CD4⁺ T cells.¹¹ Purified CD4⁺ T cells (>95% purity) were cultured at a concentration of 1×10^6 cells/mL in RPMI with 10% FBS. Cells (1×10^6 cells/ml) were treated with NPs for 24 hours. Cell were then stained with a fluorescein isothiocyanate (FITC)-labelled mouse anti-human HLA ABC antibody (Clone: G46-2.6; BD Bioscience; San Jose, CA) 0.04 µg/mL propidium iodide (PI) at 4 °C for 30 minutes. A BD FACSCalibur flow cytometer (BD Biosciences; San Jose, CA) was used to monitor the loss of cell membrane integrity, and light forward scatter (FSC) and side scatter (SSC) parameters were used to exclude any NPs from the analysis. Additionally, each sample was treated with 10 µL of fluorescent CountBright counting beads (Invitrogen; Carlsbad, CA) to enable determinations of absolute cell numbers.

Photocatalytic studies

The photocatalysis method employed was adapted with changes from Punnoose, et al. (2015).²⁵ Using the model fluorescent dye sulfo-Rhodamine B, nZnO/dye solutions were prepared by adding 2 mg of nanomaterial in 1 mL of nanopure water, sonicating for 15 minutes, and then introduced into 20 mL of 2.5×10^{-5} M solution of sulfo-Rhodamine B dye (excitation/emission maxima ~ 565/586 nm) The nZnO/dye solution was equilibrated in the dark at room temperature with continuous stirring for 15 minutes. After reaching equilibrium, an aliquot was removed for UV-Vis analysis. The nZnO/dye solution was then placed in a Rayonet RPR-100 UV reactor equipped with 2537 Å UV lamps to deliver approximately 3.2 mW/cm². Once the lamps were turned on, another sample was immediately extracted for analysis for the zero-minute time point. Aliquots were then removed every 15 minutes for a period of 120 minutes or until complete dye degradation. All liquid aliquots were centrifuged at $13,000 \times g$ for 90 seconds to separate NPs from the solution. The supernatant was then transferred to a quartz cuvette, and optical absorbance was measured using a Cary-5000 UV-vis spectrophotometer. The photocatalytic rate constant k is given by $kt = \ln(C_0/C)$, where t = time (minutes), C_0 = initial concentration of fluorescent dye molecules, and C = concentration of the fluorescent dye molecules following its illumination with the NP sample. As a control, the self-degradation of sulfo-Rhodamine B under UV irradiation without the presence of ZnO NPs was measured prior to nZnO studies and was found to be negligible

Extracellular dissolution of nZnO stock solutions

To simulate cellular assay conditions, aliquots of NP stocks were added to cellular media. These suspensions were placed in 25 mL culture flasks at a fluid depth of 6.6 mm to mimic the dispersion dynamics experienced by nZnO in the 96 well cellular assays and were kept in an incubator. Two mL aliquots were removed at 4 and 24 hours and centrifuged at $5,000 \times g$ for 20 minutes using an Amicon™ Ultra-4 Centrifugal Filter Unit with a 3-Kd molecular weight cutoff (0.1 nm pore size) to remove any undissolved nZnO while allowing free zinc ions to pass through. Control experiments were performed to demonstrate that 99% NP-derived zinc ions are recoverable using this methodology.³⁴ Quantitative analysis of the dissolved Zn^{2+} ions was conducted on a Thermo X Series 2 quadrupole inductively-coupled plasma mass spectrometer (ICP-MS) under normal operating conditions (i.e., no CCT) with the X_t cone set. Instrument performance was evaluated and optimized for each run. The instrument was calibrated using multi-element calibration standards containing Zn, Cr, Mn, Fe, Co and Ni in 2 % HNO_3 at concentrations of approximately 1, 10, 100 and 1000 ppb. Instrument drift was monitored and corrected using 20 ppb indium as an internal standard introduced online.

Intracellular Zn^{2+} determination

The amount of intracellular Zn^{2+} present in Jurkat cells post nZnO treatment was determined using the zinc specific dye FluoZin 3AM (Invitrogen; Eugene, OR). Jurkat cells were plated at 5×10^5 in 96-well plates and cultured with nZnO. At 4 and 24-hour time points, cells were harvested, rinsed twice with phosphate-buffered saline (PBS) and centrifuged at $500 \times g$ for 10 min. The cell pellets were re-suspended in phosphate-free Dulbecco's Modified Eagle's medium (DMEM), stained with 3.25 μM FluoZin 3AM dye and incubated at RT for 30 minutes in the dark. Cells were then rinsed with FACS buffer (PBS supplemented with 3% FBS and 0.02% sodium azide), centrifuged at $500 \times g$ for 7 min, re-suspended in FACS buffer, and stained with a FITC-labelled anti-HLA ABC antibody at RT for 30 min in the dark. Samples were then analysed using a BD FACSCalibur flow cytometer with unbound NPs excluded from analysis based on the absence of fluorescence signal and light forward scatter (FSC) and side scatter (SSC) characteristics.

Cell-associated Zn^{2+}

Cell-associated Zn^{2+} was determined via inductively coupled plasma mass spectrometry (ICP-MS). Jurkat cells (1×10^5 cells/ml) were treated with nZnO and incubated. At 4 and 24-hour time points, cells were washed twice in PBS, centrifuged at $500 \times g$ for 10 min, re-suspended in FACS buffer, stained with 10 μL of a FITC-labelled mouse anti-human HLA ABC antibody, and separated from any remaining extracellular nZnO via cell-sorting with a BD Influx cell sorter using Spigot software (version 6.1.4). Recovered live and dead intact cells were counted (>5 times) using a hemocytometer and a Luna – II™ automated cell counter (Logos Biosystems; Annandale, VA). Sample aliquots were resuspended in a 10 % HNO_3 (double distilled 15.8 N trace metal-grade) solution (pH ~3.0) containing 5×10^5 cells and were mechanically lysed with a probe-tip sonicator at 50W for 2 minutes using 30 seconds on/10 second off pulse cycles. The acidified, lysed aliquots were stored in acid-rinsed eppendorf tubes overnight to ensure complete dissolution of any undissolved nZnO.

The lysed aliquots were then filtered using a 10 kDa MW cut-off Eppendorf filter, Sartorius Vivaspin® 500 (Sartorius AG; Goettingen, Germany), to remove cellular debris prior to analysis. Control experiments were performed to demonstrate that .99% NP-derived zinc ions are recoverable using this methodology.³⁴ NP-only control samples demonstrated that the sorting procedure did not significantly affect the ionic zinc levels in the experimental samples. Quantitative analyses of the solutions were performed via ICP-MS.

Dosimetry

Two empirical methods, the Harvard Volume Centrifugation Method (VCM)^{58,59} and the *in vitro* sedimentation, diffusion and dosimetry (ISDD) computational model⁶⁰ were employed to determine the effective NP density of the agglomerates (ρ_e , g/cm³) and the amount of nanomaterial deposited within a cell culture scenario, respectively. The effective volume of NP pellets (V_{pellet}), was obtained by first creating a nZnO dispersion in cellular media as previously described.⁵⁷ The resulting dispersions were then centrifuged at $2,000 \times g$ for one hour in TPP (Techno Plastic Products, Trasadingen, Switzerland) packed cell volume tubes and the resulting NP pellets were measured utilizing the TPP “easy read” measuring device by the same manufacturer. The ρ_e of the NP agglomerates was calculated using the following equation (DeLoid *et al.*).⁵⁹

$$\rho_e = \rho_{media} + \left[\left(\frac{M_{ZnO} - M_{ZnOsol}}{V_{pellet} SF} \right) \left(1 - \frac{\rho_{media}}{\rho_{ZnO}} \right) \right] \quad (\text{Eq 1})$$

The ρ_e is determined as a function of the media density (ρ_{media}) and the material density of ZnO NPs (ρ_{ZnO}) of 5.606 g/cm³. To account for the solubility of ZnO, the mass of solubilized ZnO (M_{ZnOsol}) determined by ICP-MS (see “Extracellular Dissolution” section) was subtracted from the original mass of ZnO (M_{ZnO}). A theoretical stacking factor (SF) of 0.634 was utilized to approximate random close stacking employed by many dosimetry models.^{58,59} The ISDD model was provided by Justin G Teeguarden from the Pacific Northwest National Laboratory and is outlined in this paper.⁶¹

Zinc speciation determination

The molecular speciation into amorphous nZnO, zinc phosphate and zinc carbonate under cellular assay conditions was evaluated through FTIR, X-ray photoelectron spectroscopy (XPS) and TEM. For this assay, samples containing 32.4 µg/mL nanopure water-based nZnO stock solution were introduced to RPMI media and incubated. At 4 and 24 hours, the entire volume of one of the samples was centrifuged at $5000 \times g$ for 30 minutes to pellet any suspended nZnO or amorphous zinc complexes. The resulting pellet was rinsed with nanopure water, centrifuged and dried overnight at 60 °C. FTIR pellets were made by combining 0.2 g of anhydrous KBr with 1.5 mg of dried precipitate and then grinding into a fine powder. The powder was compressed with 8 tons of pressure for 4 minutes. Vibrational spectra were obtained with a Bruker Tensor 27 spectrometer and peak deconvolution performed using Opus 7.0.129 software. A Physics Electronics Versaprobe system outfitted with an Al K_α X-ray source delivering 25 W of power with a beam diameter of

approximately 100 μm was used to collect XPS spectra. Pass energy values of 117.5 eV and 23.65 eV were used to obtain survey and high-resolution core level scans, respectively. Peak deconvolution and integrated peak analysis were performed with OriginLabPro 2017 software. TEM samples were obtained by dispersing a ~ 0.5 mg of sample in EtOH followed by sonication for 10 minutes and deposition onto carbon grids. Image collection was performed using a JEOL JEM-2100 HR analytical TEM.

Reactive oxygen species (ROS) detection

To assess cellular ROS levels, Jurkat cells (1×10^5 cells/ml) were incubated with nZnO for 24 hours and then dually stained with 10 μL of an anti-HLA-ABC PE antibody and 2.5 μM of MitoSOXTM Red (Invitrogen; Eugene, OR). In the presence of mitochondrial superoxide, MitoSOX Red becomes oxidized and a fluorescent signal appears upon binding of the oxidized dye to nucleic acids.⁶² For a positive control, cells were treated with 0.2 nM antimycin-A to ensure that the cells were capable of producing ROS. Flow cytometry was used to measure mitochondrial superoxide levels with the percent positive MitoSOX cells designated as gated lymphocytes staining positive for both HLA-ABC and MitoSOX red.

Statistical analyses

Statistical analyses were performed using JMP Pro 12 software (SAS; Cary, NC). The cytotoxicity data were analysed using repeated measures of variance with post hoc comparisons and significance levels defined as $p < 0.05$ to determine statistical differences between the means and allow within-sample variation to be separated from between-sample variation. Data for Fig. 1-5 were analysed using a two-way analysis of variance (ANOVA) to test for statistical significance of the model, and post hoc comparisons were used to test for statistically significant effects of treatment on the experimental outcome ($p < 0.05$) with lowercase letters denoting statistical significance in the indicated figures. Linear least squares regression was employed to determine statistical significance between individual synthesis and control sample groups (Fig. 1-5). A linear regression was employed to determine the Pearson correlation coefficient (R^2) value for Fig. S3.

Principal component analysis (PCA) was used to reduce dimensionality of our multivariate data set to obtain canonical variables that are statistically independent of each other yet still representative of the experimental data.⁶³ Using the correlation method, PCA was performed with JMP Pro 12 software followed by common factor analysis using squared multiple correlation communality estimates and a varimax rotation method to obtain rotated principal components (PCs). A standard least squares regression model was used to evaluate the linearity of the measured IC_{50} values of the Jurkat cells against the obtained PCs to obtain a predictive model for NP-induced toxicity (Table S2 in the electronic supplementary information (ESI)).

Results

X-ray diffraction, XPS and TEM analysis for crystal structure, composition, and morphology

A diverse panel of nZnO was synthesized by both wet chemical and high temperature flame spray hydrolysis. Four of the formulations (DEG, EtOH, EG and NaOH) were produced using wet chemical forced hydrolysis synthesis (designated green in Table 1), while two nZnO samples, a spherical FSP (FSPS) and a rod-shaped FSP (FSPR), were prepared via flame spray pyrolysis (FSP; blue in Table 1). Finally, a nanosized silica-coated version of the FSPR sample (SiO₂-FSPR) and bulk ZnO (bulk) were employed as control samples (shown in dark grey in Table 1) given their reported tendencies to be biologically safer than most nanoscale ZnO formulations.^{21,22} Prior to the implementation of biological assays, all formulations were thoroughly characterized. The crystal structure, phase purity and compositional purity of the formulation panel were evaluated using XRD analysis and confirmed that all formulations were nZnO and displayed a wurtzite crystal phase without alternate crystal phases present (Fig. S1(a-b) in ESI) and XPS (Fig. S2). TEM (Fig. S1(c-j)) revealed that the nanoscale samples had average particle sizes in at least one dimension between 16 – 45 nm. The bulk ZnO sample was found to contain very large particles (mostly >500 nm) of mixed morphology (Fig. S1j). TEM images showed that the nZnO samples were spherical in morphology except for the NaOH, FSPR and SiO₂-FSPR samples which exhibited rod-like morphology (Fig. S1(f, h & i)).

Cellular toxicity and oxidative stress responses

To evaluate the toxicity and oxidative stress induced by the nZnO formulations, Jurkat leukemia T cells (Fig. 1(a)) and primary non-transformed CD4⁺ T cells (Fig. 1(b)) were treated with increasing concentrations of nZnO for 24 hours. The cytotoxic response of both cell types to the different NP formulations can be roughly divided into three groups. Nanoparticles prepared with wet chemical methods (DEG, EG, EtOH and NaOH) showed the greatest level of toxicity, followed by the FSP samples. Interestingly, the rod-shaped nZnO appeared less cytotoxic in both the wet chemical method group (NaOH) and the FSP group (FSPR) than the spherical NPs synthesized in a similar fashion (EtOH and FSPS, respectively); however, these differences were more pronounced in the primary cells. Consistent with the literature, the SiO₂-FSPR and bulk control samples were significantly less toxic than the nZnO formulations, whereas the positive ionic zinc ZnSO₄ control exhibited the most toxicity. Additionally, the IC₅₀ value obtained for SiO₂-FSPR is in line with those values obtained for silica NP of comparable size indicating an efficient and hermetic coating.^{64,65} Linear contrast analysis found the differences observed between three ZnO groupings, wet chemical, FSP and control samples to be statistically significant.

Intracellular ROS generation is considered a primary contributor to NP-induced toxicity in mammalian cells.^{11,12,16,66} ROS produced by structural or functional damage to mitochondria can be assessed through the measurement of mitochondrial superoxide.^{12,66,67} Figure 2 illustrates that the nZnO samples generated ROS in a formulation-dependent manner. Specifically, the most toxic formulation, DEG nZnO (Jurkat IC₅₀ = 20.7 ± 3.1 µg/mL), produced the highest percentage of ROS positive cells at 39.1 ± 2.5 %, while treatment

with the least toxic nanoscaled sample, FSPR ($86.4 \pm 4.7 \mu\text{g/mL}$), resulted in only $12.1 \pm 0.6 \%$ ROS producing cells. These findings directly correlated increases in NP-induced toxicity to corresponding increases in mitochondrial superoxide generation. The one exception, the SiO_2 -FSPR nZnO, produced only $6.1 \pm 0.2 \%$ of ROS positive cells despite showing significantly more toxicity than bulk ZnO, possibly due to complexation or shielding of the electron donor or acceptor sites needed for mitochondrial damage.^{23,68}

Surface characterization and reactivity

As different synthesis methods are expected to result in unique surface chemistry, several characterizations were performed to elucidate these differences. Surface bound species and hydroxylation were identified using FTIR analysis. Present in all formulations was the characteristic broad Zn-O vibrational mode (Fig. 3) centred between 451 cm^{-1} and 475 cm^{-1} , and the broad FTIR peak associated with surface adsorbed OH groups at 3410 cm^{-1} .⁶⁹⁻⁷² Notable differences present only in the wet chemically synthesized samples (Fig. 3(a)) included the O-H bending mode and the $\nu_s(\text{COO}^-)$ of carboxylic acid at 910 cm^{-1} at 1412 cm^{-1} , respectively.^{71,72} Additionally, the DEG sample displayed a $\nu(\text{C-OH})$ mode at 1076 cm^{-1} and the $\nu_{as}(\text{COO}^-)$ band at 1591 cm^{-1} .⁷⁰⁻⁷² The above mentioned peaks can be attributed to function group remnants of the synthesis precursor or the reaction solvent.²⁵ Finally, the SiO_2 coating was confirmed through the presence of the Si-O-Si bending and Si-O-Si stretching modes found at 800 and 1081 cm^{-1} , respectively.⁶⁹

The FTIR data confirmed that the NP formulations differed in the type and quantity of surface adsorbed functional groups and charged ions. These modifications, along with intrinsic surface defects are often conferred by the synthesis procedure, and alter the surface charge density and ionization potential of the nanocrystals resulting in the generation of an enhanced surface charge.²⁵ Zeta potential (ζ -potential) measurements (Table 1) verified these synthesis method-dependent differences. The positive ζ -potential values of the uncoated samples were higher for samples with more surface-adsorbed functional groups and hydroxylation. When the IC_{50} in Jurkat cells was plotted as a function of ζ -potential (Fig. S3 in ESI), a linear correlation ($R^2 = 0.72$; $p < 0.0001$) was observed for the uncoated nZnO samples. This correlation is interesting considering that all samples experienced similar electrophoretic mobility shifts when suspended in cellular media (Table 1). While this normalization of the NP surface charge in cellular media may be due in part to protein corona formation, these findings may also be skewed by free protein remaining in the media. Indeed, control experiments using a dried protein-coated DEG nZnO sample revealed a ζ -potential measurement of $-11.8 \pm 0.39 \text{ mV}$ as compared to the value of -6.80 ± 0.85 obtained for the wet sample.⁵⁷ This commonly observed surface charge normalization has prompted speculation that ζ -potential values measured in aqueous solutions contribute little to NP-induced toxicity,²³ however, our results clearly don't fit with this speculation. A possible explanation for this is intrinsic surface defects that contribute to the nZnO surface charge, may still be present in the interior of the nZnO crystals and contribute to their toxic response when internalized into cells. Additionally, the variably charged surfaces noted for the different formulations could result in differential attractive forces to organic and biological molecules present in the extracellular milieu and/or cellular environment. Indeed, Han *et al.* (2014), observed that differently charged nZnO experienced variable adsorption of humic

acid at circumneutral pH. In their study, a positively charged nZnO sample adsorbed higher amounts of the negatively charged humic acid along with concentration-dependent shifts in the measured zeta potential when compared to a negatively charged nZnO sample.⁷³

In addition to their ability to influence surface charge, intrinsic defects such as oxygen vacancies or interstitial Zn ions, increase the number of possible electron donor or receptor sites available to participate in abiotic redox reactions at the NP surface.^{25,74,75} Seen as an indirect measurement of redox potential, photocatalytic activity has been connected to synthesis modification and resulting biological outcomes.²⁵ The highly reactive valence band holes (h_{vb}^+) and conduction band electrons (e_{cb}^-) produced during UV irradiation participate in redox reactions at the NP surface to produce multiple ROS species, resulting in sulfo-rhodamine B fluorescent dye degradation.^{25,74,75} Figure 3(c), demonstrates this time-dependent degradation of the dye for the EG formulation. The average catalytic rate constants (k , min^{-1}) for the uncoated nZnO formulations varied from 0.076 min^{-1} (DEG) to 0.036 min^{-1} (FSPR) (Fig. 3(d)), while the SiO_2 -FSPR sample did not exhibit any significant photocatalytic activity. All reported k values are consistent with current literature.^{25,36,74} Additionally, rod-shaped NPs exhibited lower k values compared to spherical nZnO samples demonstrating a strong dependence of k values on NP shape.³⁶ The k values for the spherical nZnO plus the bulk control, (Inset; Fig. 3(d)), demonstrated a positive correlation with the observed cytotoxicity for both the cell types, and is consistent with our previous findings.²⁵ ZnSO_4 treatment did not result in any appreciable dye degradation with or without UV illumination thus eliminating ionic zinc as a contributor to the photocatalytic process.

Dissolution potential and zinc speciation

ZnO NPs are known to release Zn^{2+} in many biologically relevant solutions⁷⁶ and induce cellular damage both extracellularly and intracellularly.^{13,29-31} To determine if a relationship existed between these two dissolution measures, extracellular (bars in Fig. 4) and intracellular free Zn^{2+} (line graph in Fig. 4) dissolution assays were performed concurrently. As seen in Fig. 4, a clear trend exists between extracellular zinc release and the measured intracellular free Zn^{2+} for all samples. Additionally, the wet chemically synthesized NPs exhibited higher extracellular and intracellular free Zn^{2+} levels than the FSP or control samples with two notable exceptions. First, the EtOH sample was less soluble than the FSP nZnO despite being a wet chemical formulation and having a larger specific surface area (SSA) of $67.1 \text{ m}^2/\text{g}$. However, the increased hydrodynamic size of this sample (Table 1) reduces its surface area-to-volume ratio thereby decreasing its dissolution potential. Secondly, the SiO_2 -FSPR released relatively low amounts of Zn^{2+} , despite its small hydrodynamic size, due to the protective benefits of the silica coating.²² The same general trend was apparent for all samples at both time points, with modest increases between 1.5 and 3.9 % in the amount of Zn^{2+} released by 24 hours. Overall, these results agree with dissolution trends noted in the literature and are consistent with the kinetic profiles observed for these nZnO formulations in nanopure water (Fig. S4 in ESI).

One limitation of the extracellular dissolution and intracellular free Zn^{2+} assays discussed above is that they do not address zinc associated with cells as non-ionic complexed forms of nZnO.^{17,31,41,45} To address this, an assay was developed to isolate intact cells and

subsequently measure the total cell associated zinc including nanomaterial attached to the outer membrane and all forms of intracellular zinc. Following nZnO treatment, Jurkat cells were isolated from unbound nZnO by FACS sorting, and equivalent numbers of cells mechanically lysed and chemically digested to ensure that any zinc associated with the cells would be in an ionic form. Following filtration to remove cellular debris, zinc concentrations were determined by ICP-MS. Additionally, control experiments verified that the cell sorting procedure did not capture any un-associated nanomaterial. At 4 hours, the cell-associated zinc varied with no significant trends apparent amongst the synthesis methods or particle morphology (Fig. 5). By 24 hours, a few patterns emerged. First, within the wet chemical synthesis group, the amount of cell-associated zinc follows the inverse trend observed for cytotoxicity. Specifically, the most toxic NPs (DEG) contained the least cell-associated zinc while the least toxic formulation in that group (NaOH) had the most. This pattern was mirrored in nZnO samples created using high temperature conditions ($>1000\text{ }^{\circ}\text{C}$) including the FSPS, FSPR, SiO_2 -FSPR and bulk samples. Of interest are the observed inconsistencies between the intracellular free zinc data (Fig. 4) and cell-associated zinc experiments (Fig. 5). One might expect that NP formulations with higher amounts of intracellular free zinc would also contain higher amounts of cell-associated zinc; however, the opposite was found to be true.

It is evident from the extracellular, intracellular and cell-associated Zn^{2+} measurements, that there are other zinc species contributing to the cell-associated levels other than intracellular free zinc. Since nZnO readily aggregates into amorphous precipitates containing nZnO, mixed phosphate/carbonate phases and serum proteins within the extracellular environment,⁷⁷⁻⁷⁹ it is probable that large amounts of zinc will attach to or enter cells in non-ionic zinc forms. To characterize the nature and composition of these forms, FTIR and XPS analysis was employed. FTIR analysis of agglomerates isolated from cellular media demonstrated the time-dependent evolution of these amorphous precipitates (Fig. S5 in ESI and Fig. 6(a)) through the disappearance of peaks associated with the surface adsorbed groups and the appearance of the amide I vibrational mode at 1651 cm^{-1} indicative of bound serum proteins or crystalline water.⁸⁰⁻⁸² Furthermore, the development of zinc phosphate is evidenced by the broad band convolution of phosphate bending and stretching modes at 1031 cm^{-1} and phosphate shoulder peaks between 502 and 637 cm^{-1} ,^{44,80,83,84} whereas complexed carbonate phases are evidenced by the $\nu_3(\text{CO}_3^{2-})$ anion and the C-O stretching modes at 1405 cm^{-1} and 1535 cm^{-1} , respectively.^{43,85} XPS confirmed these compositional changes with the atomic additions of nitrogen and phosphorous and increases in the relative intensity of carbon and oxygen observed in the survey spectra (Fig. S7 in ESI). In addition, the single $\text{Zn}2\text{P}_{3/2}$ peak present at 1021.6 eV in the as-prepared samples was replaced by two peaks due to a chemical shift consistent with a Zn-P peak overlapping with the Zn-O peak with an approximate difference of $+2\text{-eV}$ (Fig. 7(d)).

To quantify the intensity variations observed between the different formulations, the ratio of crystalline nZnO to the amount of phosphate in the precipitates ($\text{ZnO}/\text{PO}_4^{3-}$) was determined by calculating the integrated area ratio of the nZnO peak to the broad phosphate band at 1031 cm^{-1} (Fig. 6). Since the broad peak from $\sim 350 - 700\text{ cm}^{-1}$ is no longer solely representative of pure ZnO vibrational modes, peak deconvolution was employed to separate the integrated area associated with phosphate species from that of pure nZnO vibrational

modes (Fig. 6(b & c), Fig. S6 and Table S1 in ESI). Interestingly, the area ratios (black spheres in Fig. 7(a & b)) increased as the amount of cell-associated zinc increased and mirrored the geometric trend established with the cell-associated zinc levels. As corroboration, calculated Zn/P ratios obtained from XPS survey spectra atomic concentrations, (white triangles in Fig. 7(a & b)) were generally consistent with the $\text{ZnO}/\text{PO}_4^{3-}$ values obtained from the FTIR experiment. A notable exception was the Zn/P ratio obtained for SiO_2 -FSPR nZnO sample which was negligible in comparison to the $\text{ZnO}/\text{PO}_4^{3-}$ ratio. Considering the slow dissolution kinetics of the SiO_2 -FSPR nZnO in cellular media, any agglomerates formed will likely have high protein content with lower zinc phosphate/carbonate formation thus resulting in the observed zinc and phosphate values below the detection limit (<0.1%).

Qualitative evidence of the FTIR and XPS results can be seen in the high resolution TEM images collected on the precipitates (Fig. S8 - Fig. S15 in ESI). Agglomerates of crystalline nZnO, confirmed by the lattice fringes observed in the high-resolution images, can be seen embedded in an organic matrix within all the nZnO and control samples. Furthermore, the more soluble nZnO formulations (DEG and EG) contained smaller, more porous nZnO aggregates with larger amounts of visible amorphous material compared to the denser ZnO agglomerates observed in less soluble samples. Additionally, the FSPR and SiO_2 -FSPR formulations retained much of their rod-shaped morphology even up to 24 hours. Taken with the $\text{ZnO}/\text{PO}_4^{3-}$ and Zn/P calculations, these observations suggest that large increases in cell-associated zinc can be attributed to non-ionic zinc complexes.

Dosimetry

Increasing nZnO agglomerate size results in many downstream effects including decreased dissolution, increased sedimentation, and changes in the overall dosimetry of the NPs.⁸⁶ The *in vitro* sedimentation, diffusion and dosimetry (ISDD) computational model⁶¹ was employed to determine the fraction of nanomaterial (in μg) that is deposited onto an adherent cell layer for a given time frame. Figure 8(a) demonstrates that formulation-specific differences exist in the amount of nZnO deposited over 30 hours with differences likely due to stronger sedimentation effects exerted by nZnO with large hydrodynamic diameters (Table 1 and Fig. 8(b)). The deposition differences at 24 hours when plotted with the hydrodynamic diameters of the NPs illustrated this connection (Fig. 8(b)). It is noteworthy that the geometric trend observed for these measurements was nearly identical to that observed for the cell-associated trend (Fig. 5 and Fig. 7). The SSA measurements obtained by BET were also plotted in Fig 8b to evaluate potential connection between dosimetry, hydrodynamic diameter and SSA. No clear patterns, however, were observed between these measures. While hydrodynamic diameter appears to correlate strongly to both dosimetry and cell-associated zinc in this study, there are cautions that need to be considered with the discussion of hydrodynamic size. One common artefact is the possible presence of very large agglomerates which may mask the detection of smaller agglomerates in the dispersion thus skewing the average hydrodynamic diameter. Additionally, agglomerates with a loosely packed open structure, allowing for exposed NP surface area within the agglomerates, may alter NP dissolution kinetics or mass transport in unpredictable ways. Despite these potential artefacts, hydrodynamic diameter appears to be a significant variable in NP-induced toxicity,

but should be considered as part of a broader characterization protocol including BET, TEM and dosimetry measurements.

Principal Component Analysis

Given the highly correlated and complex nature of our multivariate data set, 2D principal component analysis (PCA) was used to determine if the individual formulations could be quantitatively separated based on their material properties. The 2D analysis identified two statistically independent principal components (PCs) that accounted for 74 % of the observed experimental variability. From the table (Fig. 9(a)), the individual experimental factors appear to be grouped by their contribution to either oxidative stress responses (PC1; 51 %) or agglomeration potential (PC2; 23 %). At first glance, the inclusion of photocatalysis with PC2 appears anomalous; however, increasing aggregation behaviour decreases photon absorption leading to fewer electron-hole pairs and less ROS generation.⁸⁷ Qualitative groupings based on similar component scores (colored ellipses in Fig. 9(b)), revealed some interesting patterns. Not surprisingly, the bulk sample (purple ellipse) scored high for PC1 given its large hydrodynamic sizes and cell-associated zinc values. Whereas the SiO₂-FSPR formulation (pink ellipse) associated negatively with both PCs due to decreased dissolution potential, ROS production, aggregation potential and photocatalytic activity. Amongst the uncoated nanosized samples, the most toxic samples, DEG and EG (yellow ellipse), scored positively for both PCs and is likely a consequence of high intracellular Zn²⁺ levels, stronger surface reactivity and faster photocatalytic activity. In contrast, the other samples (blue ellipse), with lower toxicity, demonstrate less surface reactivity thus lowering their oxidative stress contributions.

Discussion

This work sought to expand upon previous results demonstrating the importance of synthesis method in modulating surface reactivity, NP-induced toxicity and oxidative stress.²⁵ The goal of this research was two-fold: 1) to thoroughly characterize the surface reactivity, and dissolution potential of the nZnO panel and 2) identify the relative contributions of these material properties in NP-induced toxicity and oxidative stress. Upon assessing the toxicity of the nZnO panel against Jurkat leukemic and primary CD4⁺ T cells, it was observed that NPs synthesized through wet chemical methods imparted greater toxicity to both cell types than those synthesized using FSP methods (Fig. 1). Several possible scenarios may explain these discrepancies.

First, the dissolution kinetics are remarkably different amongst the nZnO formulations. The more toxic samples demonstrate faster dissolution kinetics within the extracellular environment (Fig. 4). Consequently, these nZnO exhibit decreased agglomeration potential (Table 1) and increased intracellular zinc levels (Fig. 4). In comparison, as the toxicity of the nZnO formulations decreased, the kinetic balance shifted from extracellular dissolution towards more agglomerate formation and the cellular adherence of large amounts of non-ionic zinc as evidenced by the cell-associated zinc (Fig. 5 and 7) and dosimetry (Fig. 8) data. Ultimately, both extracellular and cell-associated zinc contribute to increased levels of intracellular free zinc and disruption of the zinc homeostasis of the cell.^{13,28,29,66} However,

identifying the source of intracellular free zinc levels can be difficult. One proposed mechanism is that large pools of extracellular ionic zinc that are liberated from the nZnO are taken up by the cell.^{32,41,42} This hypothesis is consistent with our extracellular/intracellular zinc data. While these results suggest that rapid increases in extracellular free zinc give rise to corresponding increases in intracellular free zinc, our experiments cannot definitively verify this correlation. Indeed, it is highly probable that some of the intracellular zinc levels observed may result from dissolution of non-ionic zinc intracellularly,^{17,18,28,29,31,88} which would occur more rapidly for smaller nZnO agglomerates. Alternatively, studies have also shown that increased intracellular zinc in cells treated with highly toxic nZnO^{13,28,89-91} results from disruption of cellular membranes by lipid peroxidation or by nZnO translocation into the cell^{13,28,80-82}

Secondly, the variable synthesis methods resulted in differing surface reactivity. The hydrolysis based synthesis methods used here are known to produce NPs with multiple defects such as oxygen vacancies and interstitial zinc and oxygen atoms.⁹² Additionally, singly and doubly ionized oxygen vacancies give rise to higher overall positive charge through increased ·OH surface absorption and excess Zn²⁺.^{25,93} The presence of these synthesis method induced defects is validated through the high positive surface charge (Table 1 and Fig. S3) and faster photocatalytic rates (Fig. 3) observed for the wet chemical formulations and the direct correlation of this surface reactivity to the toxicity and ROS findings. (Fig. 1 and 2).

Dissolution kinetics and surface reactivity are often viewed as competing mechanisms for NP-induced toxicity. We contend, however, that these processes work concurrently as suggested by the principal component analysis. The PCA reduced the complexity of this data into two statistically independent principal components (PC1 and PC2) (Fig. 9). The first PC represents the material properties that are direct contributors of oxidative stress; specifically, increased ζ -potentials, extracellular and intracellular dissolution, and cellular mitochondrial superoxide production. The second PC was more representative of agglomeration potential, with strong contributions coming from hydrodynamic size and cell-associated zinc. Regression analysis of the IC₅₀ values for Jurkat cells as a linear function of PC1 and PC2 revealed that both PCs contribute significantly to the model ($p < 0.05$; Table S2 in ESI).

As the model suggests, PC1 (oxidative stress contributors) increases the NP-induced toxicity; while PC2 (agglomeration potential) works to decrease these effects. Additionally, PC score associations also appear to be related to the synthesis method employed, as two of our most toxic wet-chemically synthesized formulations (yellow ellipse in Fig. 9(b)), aligned strongly with PC1 when compared to less toxic samples. The following mechanistic scenario described below highlights the synergistic contribution from the two PCs. In the more toxic samples (higher PC1 vs PC2 scores), it is possible that the higher positive surface charges (aligned with PC1) for the wet chemical samples results in increased electrostatic interactions with both anionic lipids and protein domains, potentially leading to increased nZnO uptake and increases in the total intracellular zinc. Additionally, nZnO with intrinsic surface defects have higher ROS generating capability abiotically allowing for increased lipid peroxidation and passive cellular uptake. Once internalized, interactions between a

more reactive formulation of nZnO and various cellular components would lead to heightened ROS generation and increased oxidation events within the cell. As for PC2 contributions, the smaller agglomerate sizes increase the extracellular dissolution potential and decrease non-ionic complex formation. A combination of zinc transporters and fluid phase pinocytosis pulling in larger amounts of free zinc, can elevate intracellular free zinc levels directly from liberated free zinc within the media.^{28-30,94} This scenario demonstrates that while these PCs are statistically independent, they are not mutually exclusive. Even the most toxic of the nZnO formulations exhibited agglomeration and sedimentation potential and measurable cell-associated zinc (PC2), however those variables associated with PC1 were more influential in the toxic response.

Conclusions

Identifying the physicochemical properties that most influence NP-induced toxicity has been a challenging endeavour in nanotoxicology. This investigation sought to resolve this question by evaluating a diverse panel of pristine nZnO samples. Through extensive analysis and mathematical modelling, two consistent sets of properties emerged as strong contributors to cytotoxicity. The first set of variables relating to surface reactivity, extracellular and intracellular Zn²⁺ dissolution, and oxidative stress responses has long been suggested as the primary mechanistic contributor to NP-induced toxicity. The second set (agglomeration tendencies, non-ionic zinc complexes and cell associated zinc levels) has also been implicated, but to a lesser degree. Collectively, our experimental results and the PCA model suggest that both sets of experimental variables contribute to nZnO cytotoxicity. Additionally, these findings suggest that the NP fabrication method employed results in differential material properties and resulting cellular responses. Wet chemically synthesized nanomaterials demonstrate increased surface reactivity and dissolution potential and decreased agglomeration potentially leading to the uptake of large amounts of non-ionic zinc complexes. Given that disruption of cellular zinc homeostasis and cell death will occur at a critical level of internalized zinc regardless of the NP formulation employed, other toxic mechanisms, such as surface reactivity, are likely responsible for the differential toxicity observed in our samples. This underscores the need for additional studies to evaluate how changes to specific NP physicochemical properties alter cellular uptake mechanisms, genotoxicity, apoptosis, and ROS-mediation pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Environmental Significance Statement

ZnO nanoparticles (nZnO) are widely used in consumer products because of their unique electronic and optical properties. Considering the impact that nZnO could have on public health and the environment, a thorough understanding of the material properties that influence nZnO-induced toxicity and a connection to observed cellular responses is required. In this original work, we investigated material property and NP-induced toxicity differences for a diverse set of variably synthesized nZnO samples. These differentially synthesized samples displayed significant differences in surface reactivity, dissolution potential and cytotoxicity towards cancerous and primary T cells. Additionally, mathematical modeling suggested that the synthesis procedure employed conferred unique material properties and can be a determinant of cellular cytotoxicity and oxidative stress responses.

Figure 1.

np-induced toxicity values for (a) jurkat leukemic cell and (b) normal primary $cd4^+$ t cell viability at 24 hours after treatment with the wet chemical method (green bars; wet), flame spray pyrolysis (blue bars; fsp) nzno formulations and the sio_2 -fspr and bulk controls (black bars; control). the white labels on the histogram bars depict the ic_{50} values obtained for the indicated sample. the histogram bars were ordered from lowest to highest ic_{50} for both cell types to depict the synthesis method trends observed for the np-induced toxicity. cultures were treated concurrently with varying concentrations of nzno dispersed in nanopure water/rpmi for 24 hours and cell viability was evaluated (means $\pm$ standard error, minimum of $n = 3$) using alamar blue staining (jurkat cells) or flow cytometry with pi staining ($cd4^+$ t cells). statistical analysis was performed using repeated measures analysis of variance and model-based means post hoc test ($p < 0.05$) with differing letters denoting statistical significance. linear contrast models were used to determine statistical significance between the wet chemical, flame spray pyrolysis and control samples.

Figure 2. nzno samples generated ros in a formulation-dependent manner. mitochondrial superoxide generation by all nzno at 24-hour post treatment with 32.4 $\mu\text{g/ml}$ zno using flow cytometry and mitosoxTM red staining. statistical analysis was performed using repeated measures analysis of variance and model-based means post hoc test ($p < 0.05$) with differing letters denoting statistical significance.

Figure 3. surface property characterization for the powdered samples ((a) and (b)). and catalytic activity plots ((c) and (d)) depicting the uv/vis monitored fluorescence of the model sulfo-rhodamine b dye and the average dye degradation kinetic values obtained for the evaluated treatment conditions. ftir spectra for (a) wet chemical synthesis methods, and (b) heat treatment methods illustrate the peaks observed and the corresponding wavenumber values. the graph in (c) is representative of the time-dependent plots obtained and demonstrate the photocatalytic decomposition of sulfo-rhodamine b dye in nanopure water after treatment with eg nps. the histogram in (d) depicts the average catalytic rate constants (k , min^{-1}) for the znso and control samples. statistical analysis in (d) was performed using repeated measures analysis of variance and model-based means post hoc test ($p < 0.05$) with differing letters denoting statistical significance. histogram bars represent $n=3$ replicates with error bars indicating s.e.

Figure 4.

nzno formulations display similar dissolution kinetic trends in cellular media at 4 and 24 hours. extracellular (ec) zn^{2+} concentrations ($\mu\text{g/ml}$) measured via icp-ms (bars and left y-axis) and intracellular zn^{2+} concentrations measured via flow cytometry and expressed as mean fluorescence intensity (mfi) of the zinc specific dye fluozin-3 am (line graphs and right y-axis) evaluated at 4 hours (top graph) and 24 hours (bottom graph). control samples, designated as the left most bar or line graph symbol were rpml-based cellular media (ec assay) and nt cells (intracellular (ic) assay). statistical analysis was performed for the extracellular zinc measurements (histogram bars) using repeated measures analysis of variance and model-based means post hoc test ($p < 0.05$) with differing letters denoting statistical significance. histogram bars or line graph circles represent the average of $n = 4$ replicates with error bars depicting s.e.

Figure 5. cell-associated (ca) Zn^{2+} concentrations ($\mu\text{g/l}$) measured at 4- and 24-hours via icp-ms. a control sample designated as the left most grey bar, were non-treated cells grown in cellular media. statistical analysis was performed using repeated measures analysis of variance and model-based means post hoc test ($p < 0.05$) with differing letters denoting statistical significance. histogram bars represent the average of $n = 4$ replicates with s.e. error bars.

Figure 6. representative samples highlighting the ftir region from 1800 to 350 cm^{-1} and the zno peak deconvolution. the figure includes (a) ftir spectra from 1800 to 350 cm^{-1} for the eg nzno sample at 4 hours (blue) and 24 hours (red) and peak deconvolution of the broad ftir band from 750-350 cm^{-1} for the eg nzno sample at (b) 4-hour and (c) 24-hour time points. samples were introduced to cellular media at a concentration of 32 $\mu\text{g}/\text{ml}$ and incubated for the indicated time points. after incubation, the dispersions were centrifuged and the precipitate retained and dried overnight at 60 $^{\circ}\text{C}$.

Figure 7.

the integrated area ratio of zn to po₄³⁻ from the ftir spectra and the atomic concentration ratio of zn/p from the xps survey spectra of the insoluble zinc amorphous precipitates isolated from nzno dispersions in cellular media post incubated for 4 and 24 hours. the left-hand side of the figure represents the integrated area ratio of zn to po₄³⁻ from the ftir spectra (line graphs with circles and first right y-axis) and the atomic concentration ratio of zn/p from the xps survey spectra (line graphs with triangles and second right y-axis) evaluated at (a) 4 hours and (b) 24 hours. the faded histogram bars represented the ca zinc results presented in fig. 5 and are included for reference. the right-hand side of the figure represents the xps spectra from 1027 ev to 1018.5 ev illustrating the deconvolution of the zn2p_{3/2} peak for the (c) eg nzno as prepared sample and the (d) eg nzno in cellular media at the 4 hour time point.

Figure 8.

the time-dependent deposition nZnO onto the bottom of a well in a 96-well culture plate as determined by isdd dosimetry modelling for the individual nZnO formulations. the total amount of nZnO introduced to the cellular media was 6.48 µg (32.4 µg/ml introduced into 0.2 ml of rpmi-based cellular media). the curves in (a) represent the calculated nZnO deposition over a 30-hour period. the histogram bars (left y-axis) in (b) demonstrate the modelled concentration of nZnO deposited at the 24-hour time point with the half white/half black circles (first right y-axis) correlating to hydrodynamic size and the red triangles (second right y-axis) representing the specific surface area (ssa) as measured by bet. error bars in (b) represent s.e. with n = 3 replicates.

Variable	Rotated Loadings	
	PC1	PC2
[IC Zn ²⁺] at 24 hours	90	
[EC Zn ²⁺] at 24 hours	90	
ROS % positive cells	86	
Zeta Potential	74	
[IC Zn ²⁺] at 4 hours	66	-56
Hydrosiize	-55	84
Photocatalysis		54
[CA Zn ²⁺] at 24 hours		57
[CA Zn ²⁺] at 4 hours		48
Eigenvalues	4.50	2.05
% of Variation	51	23

Figure 9.

pca model-generated values for both pc1 and pc2 and a graphical representation of the pc scores and jurkat cells ic₅₀ values for each evaluated sample. the table in (a) represents the loading values ($\times 100$) for each measured variable, eigenvalues and the percent variation explained for each pc. the indicated abbreviations refer to hydrodynamic size (hydrosiize), intracellular zinc [ic zn²⁺], cell-associated-zinc [ca zn²⁺], and extracellular zinc [ec zn²⁺] concentrations. the green cubes (wet chemical methods), blue spheres (fsp method) and black tetrahedrons (controls samples) depicted in the 3d graph in (b) represent pc1 scores versus ic₅₀ values versus pc2 scores. the colored ellipses denote sample grouping based on similar pc1 and/or pc2 scores.

Scheme 1. illustration depicting the nzno dissolution processes and interactions with cells after np treatment.

Physical properties of ZnO formulations measured via TEM, BET, UV-Vis, and DLS. Wet chemically and FSP synthesized formulations are shaded green and blue, respectively, while the control samples are indicated with the dark grey. The average size values are based on a minimum of 100 measured NPs on TEM images. The hydrodynamic diameters were determined by measuring the number of particles within each size bin in the histogram distribution.

Table 1

Formulation (Band Gap: eV)	Average Size (nm)	Specific Surface Area (m ² /g)	ζ-Potential (mV) ^a		Hydrodynamic Diameter (Media) (nm)
			Water	Media (4 h) (24 h)	
DEG (3.36)	33 ± 7	34.5	29.7 ± 0.19	-5.71 ± 0.49/-6.80 ± 0.85	221.7 ± 14.16
EG (3.36)	29 ± 5	22.5	27.6 ± 0.37	-5.58 ± 0.85 -6.68 ± 0.46	544.9 ± 120
EtOH (3.36)	16 ± 2	67.1	23.0 ± 0.25	-5.86 ± 0.90 -6.69 ± 0.48	630.4 ± 32.5
NaOH (3.31)	L: 79 ± 25 W: 21 ± 5	38	18.9 ± 0.18	-6.28 ± 0.34 -6.89 ± 0.11	623.2 ± 128
FSPS (3.38)	43 ± 10	25	18.9 ± 0.18	-6.11 ± 0.72 -6.53 ± 0.65	227.2 ± 15.6
FSPR (3.38)	L: 94 ± 24 W: 26 ± 5	41.0	17.8 ± 0.12	-6.29 ± 0.66 -6.36 ± 0.70	669.7 ± 119
SiO ₂ -FSPR (3.39)	L: 106 ± 19 W: 30 ± 8	55.0	-44.1 ± 0.23	-1.45 ± 0.46 -6.18 ± 0.50	148.0 ± 1.26
Bulk (3.39)	852 ± 350	1.25	7.23 ± 2.84	-6.67 ± 0.39 -6.30 ± 0.23	2039.5 ± 137.7

^aThe pH values of the nZnO dispersions varied from 7.5 – 7.7 for all dispersions used for ζ-Potential measurements.