AN ABSTRACT OF THE THESIS OF

Zitao Zhou for the degree of Master of Science in Chemical Engineering presented on June 9, 2015.

Title: An *in silico* Approach to Determine the Influence of Surface Chemical Properties on the Toxicity of Engineered Zinc Oxide Nanoparticles.

Abstract approved:

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Stacey L. Harper

Nanotechnology products have long since made their way to markets around the world increasing the concerns about whether nanomaterials pose a risk to our environment or health. It has been suggested that engineered nanomaterial (ENM) with broad applications and rapid commercialization need better risk assessment and regulation. However, the refinement of regulations to deal with ENMs is limited by the time consuming and costly nature of *in vivo* and *in vitro* toxicity testing. *In silico* methods offer an inexpensive and rapid mechanism to integrate data from *in vitro* and *in vivo* testing and to ultimately predict their toxicity without the need for toxicological evaluations. Quantitative structure activity relationships (QSARs) can be developed to correlate descriptors of chemical compounds with their biological activities to inform risk assessments. As one of the most widely used additives in

paints, sunscreens and electronic devices, zinc oxide nanoparticles (NP) are expected to increase in our environment. Some computational models have been established for simple bare metal NPs; however, none to date have focused on surface modified ZnO NPs. The goal of this project was to use NP toxic response data and determine if the inherent NP surface modification has a predictable effect on toxicity. To assess for hazardous effects caused by ZnO NPs, embryonic zebrafish were selected as vertebrate test species as their transparent tissues allow for easy visual assessment of multiple developmental malformations and their short life span allows for rapid assessments. The physicochemical properties of NP surface modifications were calculated with consideration of fish water pH and electrolyte concentrations. Principal component analysis (PCA) and ordinary kriging (OK) methods were applied to develop our model. To test our model for prediction of more complicated ZnO NPs, we selected 2 additional ZnO NPs that were doped with Fe_2O_3 or Al_2O_3, and determined if they matched our toxicity estimations. Based on this strategy, ENM toxicity could be rapidly estimated from label information and wide range of kriging maps with increasing support from our publically available knowledgebase and global collaborations.

An *in silico* Approach to Determine the Influence of Surface Chemical Properties on the Toxicity of Engineered Zinc Oxide Nanoparticles

by

Zitao Zhou

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 9, 2015
Commencement June 2015

Master of Science thesis of Zitao Zhou presented on June 9, 2015

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Zitao Zhou, Author

ACKNOWLEDGEMENTS

I wish to express my appreciation to everyone who has helped me throughout my master program.

I am grateful for God's eternal economy that leads me through all circumstances. And of course my parents' ultimate understanding and unlimited support.

I also want to express my appreciation to my advisor Dr. Stacey Harper for her continuous encouragement and wise advice. Thanks to Bryan Harper's great patience and care during my data analysis and writing. For the former and current lab members: Dr. Josie Bonventre, Zheng Zhou, Fan Wu, Alicea Clendaniel, Dr. Jino Son, Lindsay Denluck, Lauren Crandon.

CONTRIBUTION OF AUTHORS

Zitao Zhou established the data process design, conducted the statistical analyses and wrote the manuscript. Dr. Jino Son assisted in the design and interpretation of the data. Bryan Harper assisted in the interpretation of the data and was involved with manuscript preparation. Zheng Zhou was involved with the design of data analysis. Dr. Stacey Harper collected the zebrafish data, assisted in the interpretation of the data, and assisted with writing and editing.

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Chapter 1 Introduction

Since Richard Feynman gave his famous lecture “There's plenty of room at the bottom” in 1959, which showed us his vision that atoms or molecules manipulated in nanometer (10^{-9} m) scale can create novel materials [1]. Since then, this novel material field has become the focal point of present academic and industrial research and development. National Science Foundation (NSF) funding for nanoscale science and engineering (NSE) related projects increased from $3 million in 1991 to more than $460 million in 2012 [2]. This rapid development of NSE is boosting interdisciplinary development on nanotechnology which is estimated to occupies semiconductor industry and half of the pharmaceutics and nanostructured catalysis in 2020 [3]. Based on the dimension differences: ENMs include one dimensional nanofilm, two dimensional nanotube and three dimensional nanoparticle (NP). According to a survey focused on global engineered nanomaterial (ENM) industrial production, the top three ENMs are SiO_2 (5500 t/year), TiO_2 (3000 t/year) and ZnO (550 t/year) [4]. For eco-toxicity to *B. subtilis* and *E. coli* among these three NPs, ZnO has a higher growth inhibition than SiO_2 and TiO_2 [5]. Also, ZnO NPs’ value as UV-protects [6], self-cleaning surfaces [7], sensors [8] and catalysts [9] are much closer to us in daily contact. It is expected that production of ZnO NPs will only increase with future market demand. This kind of widely used NPs will also lead to more environmental releases and a higher risk for human exposure. In current Europe regulation of nanomaterial chemical compositions, so called REACH (Registration, Evaluation, Authorization of Chemicals), other factors such as size, shape and surface modifications that may contribute to NPs toxicity are being captured but the question still remains as to what those changes do to the NP’s toxicity [1]. As such, investigation on ZnO NPs’ features which affect their risks to humans and/or the environment is necessary and important [10]. Although various NPs have been noticed their toxicity to target species are size-dependent [11-14], very few studies to date have investigated a wide-range of engineered ZnO NPs to investigate how surface chemical modifications alter toxicity. Due to lacking of guidance for ENM-specific risk assessment information and given the fast nanoscale products commercialization, *in silico* methods are an appropriate solution to better understanding and regulating ENM.

In silico test, an inexpensive and time saving method, is widely used to identify the potential hazard of drugs and predict their toxicity. Quantitative structure activity relationship

(QSAR) are starting to be estimated for nanomaterial through global collaborations that have developed computational tools [15]. To correlate descriptors of chemical compounds with their biological activities [16]. These effects are grounded in health risk assessment data based on OECD guideline on risk assessment of manufactured nanomaterial [17]. There are quite a few methods that have been developed or applied to establish predictive models of NP behavior [18]. For manufactured NPs, not only the traditional chemical properties like solubility and reactivity, but also the NPs properties such as primary size (TEM size) and particle size distribution are recommended to be taken into consideration [17].

The toxicity of ZnO NPs to a wide range of species has been well-established in literature from *in vivo* to *in vitro* studies. Previous studies have shown that apoptosis of Jurkat cells induced by ZnO NP is mainly driven by intracellular zinc ion from dissolved ZnO rather than reactive oxidative species (ROS) [19]. ZnO NP exposed to embryo-larval stages of zebrafish generated excessive ROS then induced DNA damage, altered the activities of the defense enzymes, and increased lipid peroxidation in zebrafish larvae [20]. *Daphnia magna* reproduction rates were decreased when exposed to ZnO NP or $ZnCl_2$. due to the zinc ions released from NPs [21]. For feeding rate of *Daphnia magna* measured by exposing them to ZnO NPs, ZnO NP form was found to be more determinant than zinc ion form and ZnO NP as the zinc ion source may affect changes of *Daphnia magna* reproduction [22]. Bare ZnO NPs (lacking surface ligands) are known to cause delayed embryo hatching, developmental abnormalities [23] through dissolution and release of ionic zinc [24-25] as well as induction of DNA damage through generation of ROS [23, 26]. ZnO NPs are often coated with a variety of capping agents or surface ligands with differing chemical properties to functionalize the surface and improve stability against agglomeration and dispersibility in a given medium [27]. These surface alterations have the potential to alter their toxicity as a result of differences in the release of Zn^{2+} ions and ROS production compared to bare ZnO NPs [28-29]. In addition, the behavior of surface functionalized ZnO NPs may vary compared to non-functionalized (bare) ZnO NPs by altering stability and/or agglomeration, potentially altering bioavailability and toxicity to aquatic organisms [29-32].

As ZnO is a soluble metal oxide with an increasing dissolution in smaller size [34], the NPs' surface chemical modifications are closely related to their fate and effects [29-30, 34]. Thus, we expect that the surface chemical properties of ZnO NPs can be employed as descriptors

to model their toxicity to test species. The development of such relationships between a set of intrinsic properties of surface chemistries with their biological responses could help in building nanomaterial specific structure–activity relationships (nanoSARs) [35-36]. Several nanoSAR models have been developed to predict: uptake of NPs with same metal core but different surface modifications in PaCa2 cancer cells [37]; bare metal oxide NPs' toxicity probability to RAW 264.7 and BEAS-2B cell line [38]; magnetofluorescent engineered nanoparticles uptake in pancreatic cancer cells [39]; cytotoxicity of metal oxide to bacteria *Escherichia Coli* cells[36, 40]. However, there remains limited understanding of the relationship between different nanoparticle surface chemistries and their fate and effects in whole animals. Little has been done to determine if surface modification properties can be used to develop predictive models useful in design and manufacturing of safer ZnO nanomaterial [10].

As there are multiple factors that influence the behavior of NPs (described in Chapter 2), dimension reduction is necessary to simplify factors in our problem. To investigate and assess the toxicity of NPs, principal component analysis (PCA) [41] a well-known technique [42], was initially applied to reduce dimensionality in multivariate analysis [43], and then used to analyze the *in vitro* toxicity data of NPs [44]. By applying PCA, the two most dominant principal components (PCs) were selected to represent the properties of NPs. And due to the transparent nature of zebrafish (*Danio rerio*), the responses of test animals can be easily observed through microscope [14]. The most significant endpoint — mortality is used in following ordinary kriging (OK) method as z direction response to estimate the pattern of variation of mortality in a given coordinate system, which is build with the selected PCs.

After this estimation, we assumed that doped ZnO NPs were evenly diffused on the NP surface in Chapter 3. Then we simply used the weighted fraction to adjust the surface chemistry property descriptors appropriately. To test the prediction of OK, the new descriptors were standardized and converted through previous PCA coefficient parameter and relocated on kriging map. The test results show that our model overestimated the mortality because the doped and bare ZnO NPs clustered very closely and the bare ZnO NPs were highly toxic.

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Chapter 2 Manuscript 1

Influence of surface chemical properties on the toxicity of engineered zinc oxide nanoparticles to embryonic zebrafish

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Beilstein Journal of Nanotechnology

Beilstein-Institut zur Förderung der Chemischen Wissenschaften

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Influence of surface chemical properties on the toxicity of engineered zinc oxide nanoparticles to embryonic zebrafish

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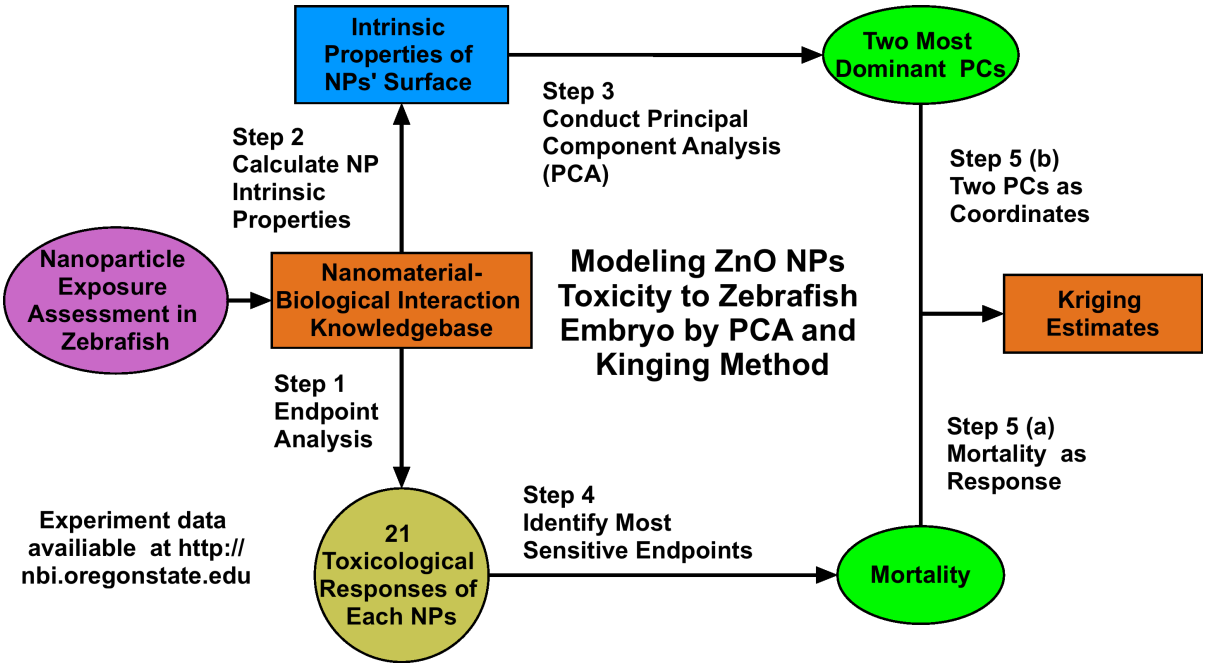
Abstract

Zinc oxide nanoparticles (ZnO NPs) are widely used in a variety of products, thus understanding their health and environmental impacts is necessary to appropriately manage their risks. To keep pace with the rapid increase in products utilizing engineered ZnO NPs, rapid *in silico* toxicity test methods based on knowledge of comprehensive *in vivo* and *in vitro* toxic responses are beneficial in determining potential nanoparticle impacts. To achieve or enhance their desired function, chemical modifications are often performed on the NPs surface; however, the roles of these alterations play in determining the toxicity of ZnO NPs are still not well understood. As such, we investigated the toxicity of 17 diverse ZnO NPs varying in both size and surface chemistry to developing zebrafish (exposure concentrations ranging from 0.016 to 250 mg/L). Despite assessing a suite of 19 different developmental, behavioural and morphological endpoints in addition to mortality in this study, mortality was the most common endpoint observed for all of the ZnO NP types tested. ZnO NPs with surface chemical modification, regardless of the type, resulted in mortality at 24 hours post-fertilization (hpf) while uncoated particles did not induce significant mortality until 120 hpf. Using eight intrinsic chemical properties that relate to the outermost surface chemistry of the engineered ZnO nanoparticles, the highly dimensional toxicity data were converted to a 2-dimensional data set through principal component analysis (PCA). Euclidean distance was used to partition different NPs into several groups based on converted data (score) which were directly related to changes in the outermost surface chemistry. Kriging estimations were then used to develop a contour map based on mortality data as a response. This study illustrates how the intrinsic properties of NPs, including surface chemical modifications and capping agents, are useful to separate and identify ZnO NP toxicity to zebrafish (*Danio rerio*).

Graphic abstract: Data Process for model development



Keywords

Kriging estimation, modelling, nanomaterials, nanotechnology, toxicology

Introduction

Accelerated advancements in nanotechnology and nanoscience have found applications in a variety of scientific fields, leading to a rapid increase in the types of engineered nanoparticles on the market. In particular, zinc oxide nanoparticles (ZnO NPs) are the third highest production volume nanoparticles at roughly 550 tons per year [1]. Given their value as UV-protects [2], self-cleaning surfaces [3], sensors [4] and catalysts [5], it is expected that the use of engineered ZnO NPs will continue to increase with the increasing market demand. Such widespread use will also inevitably result in increased environmental release and a higher potential for human exposure [6]. As such, understanding which features of ZnO NPs increase their risks to humans and/or the environment is of paramount importance [7]. Despite this fact, very few studies to date have looked across a wide-range of engineered ZnO nanoparticle types to investigate how surface chemical modifications alter toxicity.

The toxicity of ZnO NPs to a wide range of species can be found elsewhere in literature from *in vitro* [8-9] to *in vivo* studies [10-11]. Bare ZnO NPs (lacking surface ligands) are known to cause delayed embryo hatching, developmental abnormalities [12] through dissolution and release of ionic zinc [13-14] as well as induction of DNA damage through generation of reactive oxidative species (ROS) [12, 15]. ZnO NPs are often coated with a variety of capping agents or surface ligands with differing chemical properties to functionalize the surface and improve stability against agglomeration and dispersibility in a given medium [16]. These surface alterations have the potential to alter their toxicity as a result of differences in the release of Zn^{2+} ions and ROS production compared to bare ZnO NPs [17-18]. In addition, the behavior of surface functionalized ZnO NPs may vary compared to non-functionalized (bare) ZnO NPs by altering stability and/or agglomeration, potentially altering bioavailability and toxicity to aquatic organisms [18-21].

Surface chemical ligands and capping agents are more closely related to the fate and effects of ZnO NPs than the core composition alone [18-19, 22]. Thus, it is expected that surface chemical properties can be employed as descriptors to model the toxicity of various types of engineered ZnO NPs. The development of such relationships between a set of intrinsic properties

of ligands and/or capping agents with their biological effects could serve as the basis of nanomaterial structure–activity relationships (nanoSARs) [23-24]. However, there is a limited understanding of how to link different nanoparticle surface chemistries directly to the fate and effects of ZnO NPs in organisms, and whether these properties can be used to develop predictive models useful in the development of safer engineered ZnO materials [7].

The main objective of this study were 1) to investigate whether the intrinsic properties of different capping agents or surface ligands of engineered ZnO NPs alter their toxicity and 2) to determine if these features can be used to model the developmental toxicity of ZnO nanoparticles to embryonic zebrafish (*Danio rerio*) (Figure 1). Zebrafish embryos were selected as vertebrate test species as their transparent tissues allow for easy visual assessment of multiple developmental malformations and their rapid development makes them ideal for studies of numerous types of NPs [25-26]. Due to the agglomeration of ZnO NPs in fish water, the chorionic membrane can serve as a barrier to the direct interaction of NPs or dissolved oxygen with the developing embryo, thus we chose to remove this barrier in our study. The removal also allows for the visual analysis of the developing embryo, which can be hampered when the chorion is intact and coated with nanoparticles [25, 27]. To achieve these objectives, we conducted zebrafish embryo toxicity testing for 17 different types and sizes of ZnO NPs with differing surface chemistries. Then, using bare and surface modified NP toxicity data and eight intrinsic chemical properties related to the outermost surface chemistry, we conducted principal component analysis (PCA) to extract descriptors useful as coordinates to develop a model of how surface chemistry impacts ZnO NP toxicity.

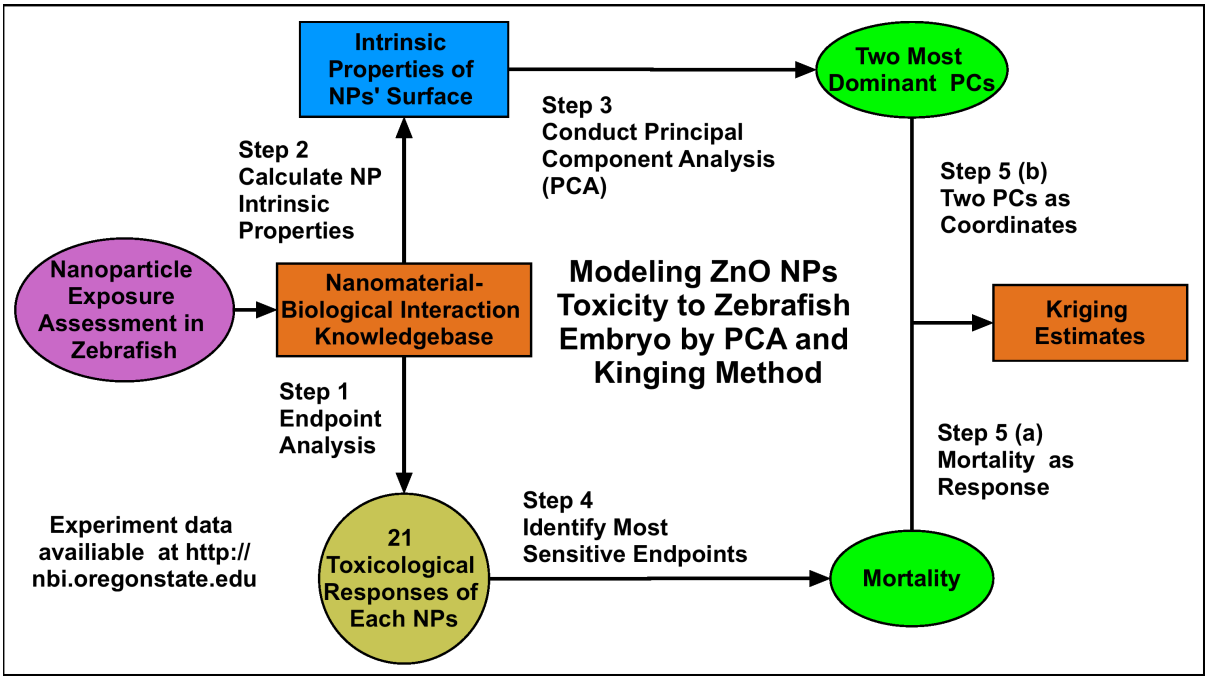


Figure 1: Data processing for model development.

Selected surface features used in the PCA were those deemed likely to influence biological interactions with the NP surface. Size (SZ) was chosen as it has been reported by others to influence NP toxicity [11, 28]. Hydrophobicity was selected as the Log P (partitioning coefficient) of NPs has been found to be related to toxic responses in other organisms [29]; however, since ZnO NPs can release zinc ions [30] and Log P is pH-independent [31], distribution coefficient (Log D) was also considered for both ionic and non-ionic forms. Polarizability was selected (PL) as a factor to describe the molecules electronic properties and its ability to change with external fields in biochemical reactions [32]. Polar surface area (PS) represents the area formed by the polar areas of the molecule and has been used to predict drug intestinal absorption in humans, thus it may be a useful predictor of other biological interactions [33]. Van Der Waals (VDW) surface area calculated by VDW radius, is associated with the likelihood of NP agglomeration [34]. Solvent accessible surface area (SASA) can be used to estimate the protein-ligand binding free energy [35], and molar refractivity (RF) represents the energy required to polarize one mole of the substance and is associated with receptor binding affinity [36]. Dreiding energy (DE) will be used to predict the binding affinity of organic molecules with Zn and membrane proteins [37]. Although zeta potential is known to be crucial to biological response [38]; it's dependent on the environment in which it is measured and thus is

not an intrinsic feature of the NP and thus was omitted from the model.

Following PCA, the ordinary kriging (OK) method was applied to estimate the pattern of variation of mortality in a given coordinate system. We hypothesized that surface chemical modifications would result in significant alterations in toxicity that would depend on the type of surface chemical modification performed.

Results

Estimation of Intrinsic Capping Agent Properties

The 17 ZnO NPs (Table 1) had 6 different surface chemistries including bare ZnO, oleic acid, octanoic acid, para-nitrobenzoic acid, cyclohexanecaboxylic acid and benzoic acid (Figure 2). The average primary particle sizes in this study ranged from 4 to 70 nm (Table 1). Table 2 provides the values calculated for the intrinsic features of the 6 surface chemistries. The calculated distribution coefficient (Log D) had the least variance of all the parameters ranging from -1.22 to 5.62. Van der Waal surface area is the surface of the union of the spherical atomic surfaces defined by the van der Waals radius of each component atom in the molecule. Van der Waal surface area values for bare ZnO were 50.3 $Å^2$ and ranged from 173 to 560.40 $Å^2$ for other surface chemistries. These values had the highest variance in our estimations.

Table 1: Description of zinc oxide nanoparticles included in this study (17 in total).

NBI record	Particle Descriptor	Manufacturer	Surface Group	Size (nm)
nbi_085	ZnO + Oleic Acid	Voxtel	oleic acid	62
nbi_086	ZnO + Oleic Acid	Voxtel	oleic acid	26
nbi_087	ZnO	Sigma-Aldrich	---	62
nbi_088	ZnO	Voxtel	---	26
nbi_089	ZnO + Octanoic Acid	Voxtel	octanoic acid	62
nbi_090	ZnO + Octanoic Acid	Voxtel	octanoic acid	26
nbi_091	ZnO + para-Nitrobenzoic Acid	Voxtel	para-nitrobenzoic acid	62
nbi_092	ZnO + para-Nitrobenzoic Acid	Voxtel	para-nitrobenzoic acid	26
nbi_093	ZnO + Cyclohexane Carboxilic Acid	Voxtel	cyclohexane carboxilic acid	62
nbi_094	ZnO + Cyclohexane Carboxilic Acid	Voxtel	cyclohexane carboxilic acid	26
nbi_095	ZnO + Benzoic Acid	Voxtel	benzoic acid	62
nbi_096	ZnO + Benzoic Acid	Voxtel	benzoic acid	26
nbi_136	ZnO	Boise State University	---	14.6
nbi_137	ZnO	Boise State University	---	33.6
nbi_138	ZnO	Boise State University	---	4.5
nbi_139	ZnO	Boise State University	---	10.2
nbi_187	NanoGard ZnO (NGZ)	Alfa Aesar, NanoGard, Prod.#44898, lot#D28X017	---	70

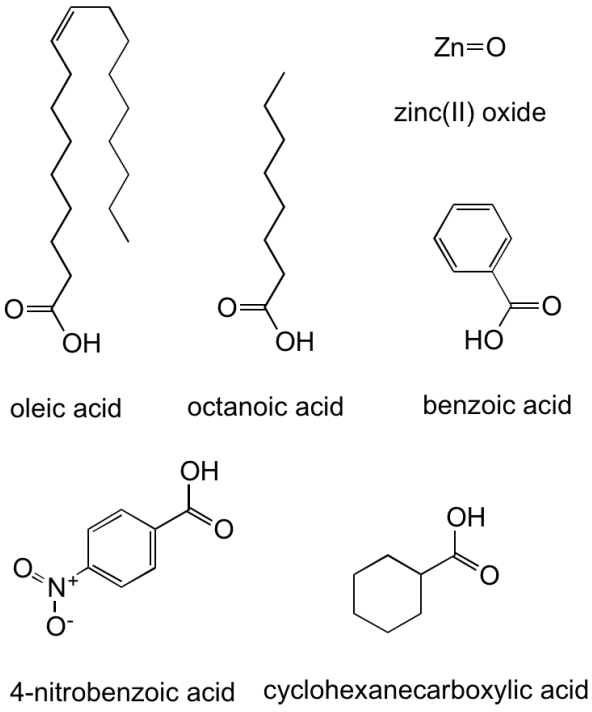


Figure 2: Chemical structures used to calculate the surface properties

Table 2: Intrinsic properties of different surface chemistries

Intrinsic Descriptor	Oleic Acid	Octanoic Acid	4-Nitrobenzoic Acid	Cyclohexane Carboxylic Acid	Benzoic Acid	Zinc Oxide
Log D	5.62	0.53	-1.22	-0.43	-1.08	-0.20
Polarizability ($Å^3$)	34.5	16.1	15.8	13.4	13.2	1.00
Polar Surface Area ($Å^2$)	37.3	37.3	83.1	37.3	37.3	17.1
VDW Surface Area ($Å^2$)	560	283	211	221	173	50.3
Solvent-accessible Surface Area ($Å^2$)	689	403	330	260	284	156
Molar Refractivity (cm^3/mol)	87.1	40.7	39.7	39.7	33.2	1.44
Dreiding Energy (kcal/mol)	35.7	12.1	23.1	24.8	16.6	0.00

ZnO Nanoparticle Toxicity

Embryonic zebrafish mortality was concentration dependent and varied with different types of bare and surface engineered ZnO NPs as expected. Mortality for the bare and surface modified ZnO NPs as a function of exposure concentration is shown in Figure 3. Surface modified ZnO particles caused significant mortality at 24 hpf, in some cases at exposure concentrations as low as 0.08 mg/L; however, despite the exposures continuing until 120 hpf, no significant mortality or developmental problems were noted after 24 hpf (Figure 3A). Bare ZnO NPs showed similar results with 2 out of 7 displaying no visible signs of toxicity at the highest concentration tested (Figure 3B). In contrast to the surface engineered particles, the toxicity of bare particles occurred more frequently at 120 hpf (3 out of 7 materials, Supporting Information File 2). Bare NanoGard ZnO (NGZ) showed the highest 120 hpf mortality of all the tested particles (bare and surface modified) with 100% mortality (n=24 embryos) at 50 mg/L. In addition, NGZ was the only ZnO particle tested (bare or surface modified) that resulted in any significant sublethal responses, eliciting swim bladder malformations at 10 mg/L and notochord malformations at the highest exposure concentration. (See Supporting Information File 1). The results of the endpoint analysis using the Fisher's exact test for all tested NPs are provided in Supporting Information File 2. Detailed raw toxicity data for each individual exposure is also available online from the Nanomaterial-Biological Interactions knowledgebase (nbi.oregonstate.edu) [39].

Analysis of the 5 pairs of surface modified particles, with the same surface chemistries and differing average particle sizes, showed no clear trend related to the primary particle size (Figure

3A). Smaller oleic acid coated ZnO NPs (26 nm) caused significant mortality at the highest test concentration that did not occur for the larger (62 nm) oleic acid functionalized particles. In contrast, the larger octanoic acid coated ZnO NPs caused significant mortality at 0.4 mg/L while the smaller 26 nm particles did not induce toxicity until exposure concentrations reached 50 mg/L. Similarly, the ZnO NPs coated with cyclohexane carboxylic acid had a significantly different mortality rate between sizes, with the larger particles being more toxic than the smaller version ($p = 0.009$, 0.234 respectively).

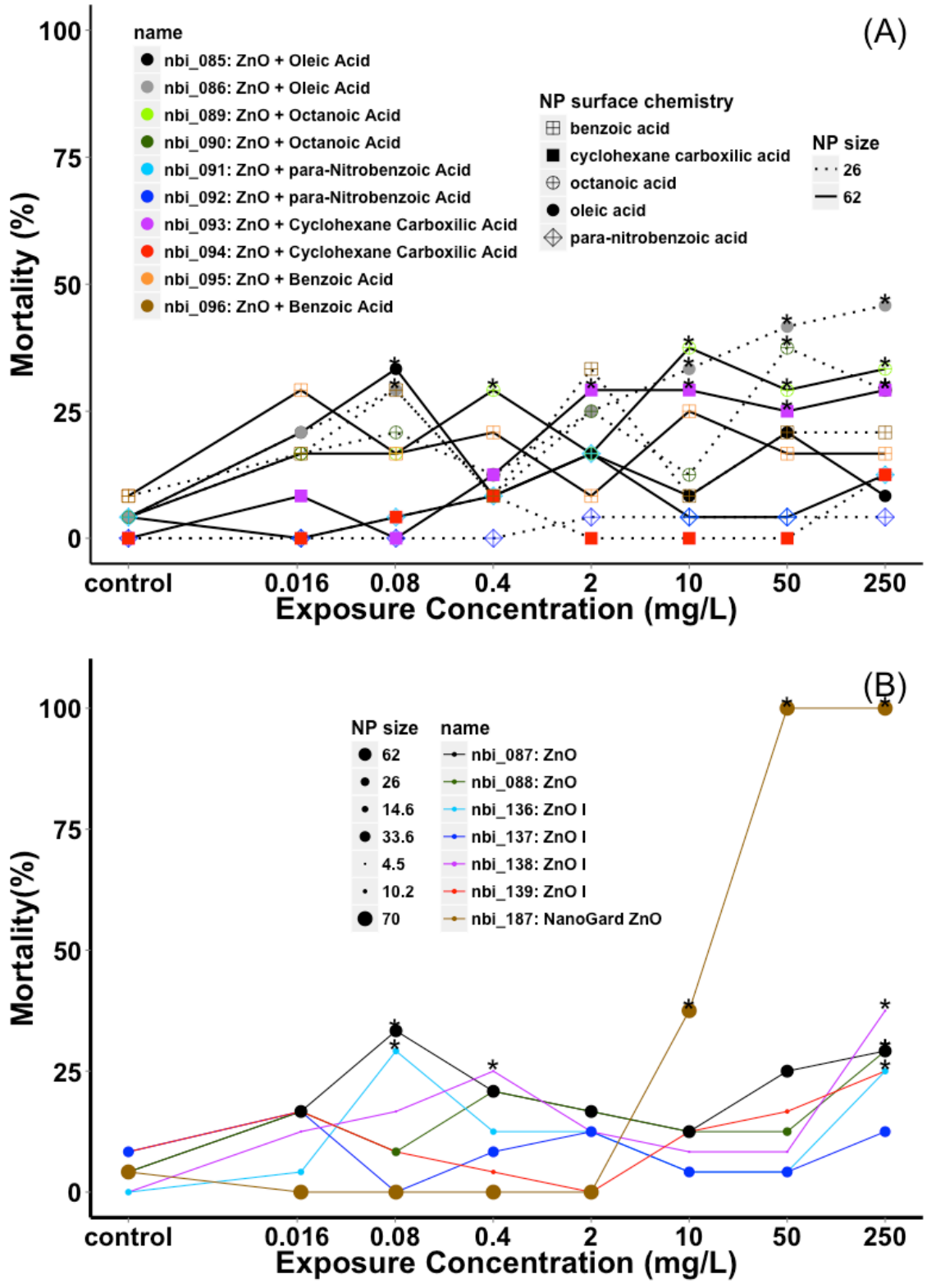


Figure 3: Zebrafish mortality at 120 hpf following exposure to: (A) ZnO NPs with and (B) without surface modification.

Principal Components Analysis

By selecting the most dominant components to explain the majority of data variance, PCA effectively reduced the dimensions of the dataset with keeping most information. It eliminated the correlation between different independent variables by creating different linear combinations, which are independent of each other [40]. PCA was conducted on the database that consists of 8 property descriptors: size (SZ), Log D, polarizability (PL), polar surface area (PS), Van del Walls surface (VS), solvent-accessible surface area (SASA), molar refractivity (RF) and Dreiding energy (DE) with 10 surface modified and 7 bare ZnO NPs (17 ZnO NP datasets × 8 properties). Each individual NP exposure dataset is comprised of results from experiments conducted at 8 exposure concentrations, thus the final matrix of the database was comprised of 136 rows and 8 columns (17 materials×8 concentrations×8 surface chemical properties).

The first two principle components (PCs), whose standard deviations both were greater than 1, explained 87.3% of the total variance of the matrix. As the linear combinations (or weights) of these two PCs was calculated based on all of the input data, they represent all of the particle information. As such, these two PCs were determined to be appropriate to represent the variability in this dataset (Figure 4). These two PCs were selected as the new independent variables, reducing the independent variables' dimensions from 8 to 2.

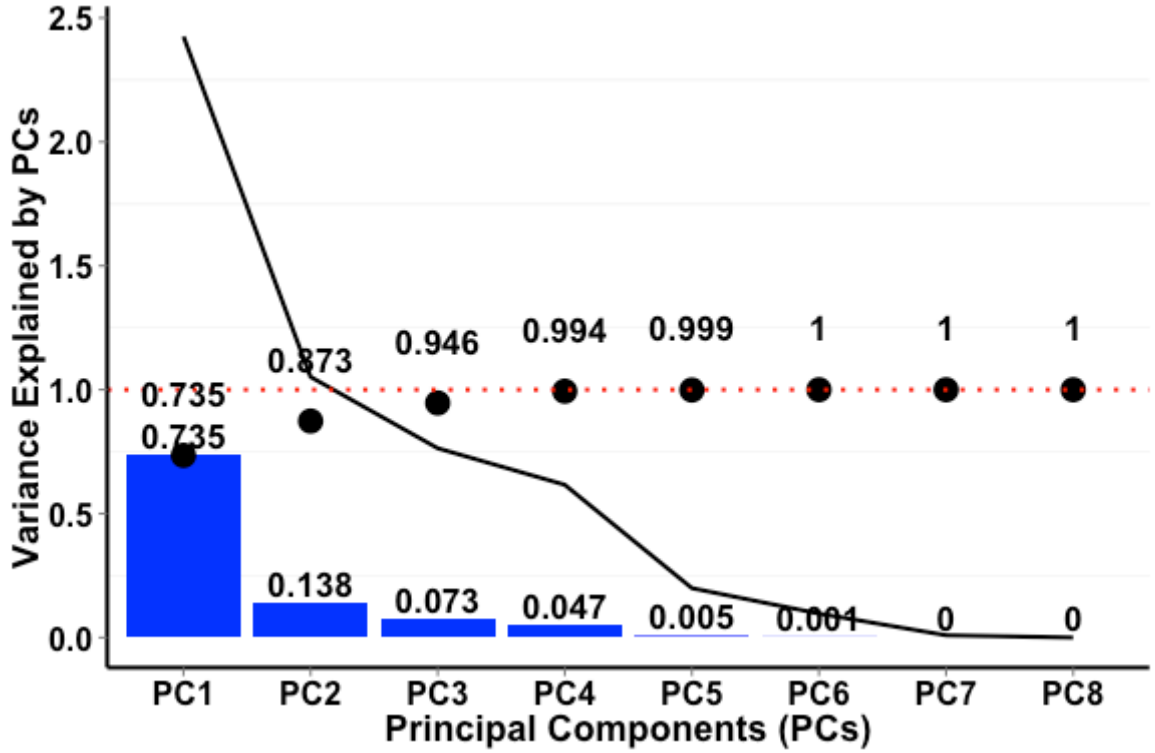


Figure 4: Individual variance for each of the principal components (PCs). Black dots represent the accumulated variance explained by each PC, while the solid line shows the Eigenvalue.

Table 3 shows the 8 descriptors all have moderately similar weights in PC1, but Log D, PS and SZ have outstanding weights in PC2. The variable coefficients in the PC1 linear combination all have the same sign, suggesting these parameters have similar effects on the model. In contrast, the sign of the variable coefficients for SZ and PS in PC2 are opposite to the other parameters suggesting these variables help separate the particles. Graphing the PCA scores for PC1 versus PC2 allows for the use of Euclidean distance to identify clusters of similar NPs with respect to their toxicity to embryonic zebrafish. As predicted, the various surface modifications to ZnO NPs resulted in distinct groupings based on these capping agent properties (Figure 5). When partitioned into three clusters, the plot shows a clear separation as: (Group 1) oleic acid; (Group 2) octanoic acid, para-nitrobenzoic acid, cyclohexane carboxylic acid and benzoic acid; (Group 3) bare ZnO with blank control responses (Figure 5). Similar analysis using either four or five clusters shows minor differences compared to the use of three clusters, namely the coated 26 nm NPs (except octanoic acid) separated out of Group 3 in the four cluster calculation and the blank control point separated out of Group 1 in the five clusters calculation in addition to 62 and 70 nm bare ZnO NP separating out of Group 3 (See Supporting Information File 3).

Table 3: Rotation of PCA (weighting of each property).

Property	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
SZ[a]	0.188	0.669	0.711	0.072	-0.077	-0.027	0.001	0.000
PS[b]	0.270	0.497	-0.610	0.454	-0.262	0.100	0.063	0.139
SASA[c]	0.404	-0.025	-0.002	0.173	0.844	0.196	-0.090	0.218
RF[d]	0.407	-0.058	-0.063	-0.205	-0.182	-0.320	-0.803	0.062
DE[e]	0.378	-0.001	-0.039	-0.634	-0.222	0.531	0.217	0.274
Log D[f]	0.292	-0.535	0.339	0.538	-0.359	0.142	0.069	0.266
VS[g]	0.410	-0.099	-0.015	0.053	-0.020	0.191	0.063	-0.882
PL[h]	0.408	-0.070	-0.051	-0.150	0.037	-0.714	0.536	0.072

[a]size; [b]polar surface; [c]solvent-accessible surface area; [d]molar refractivity; [e]dreiding energy; [f]distribution coefficient; [g]Van der Waal surface; [h]polarizability.

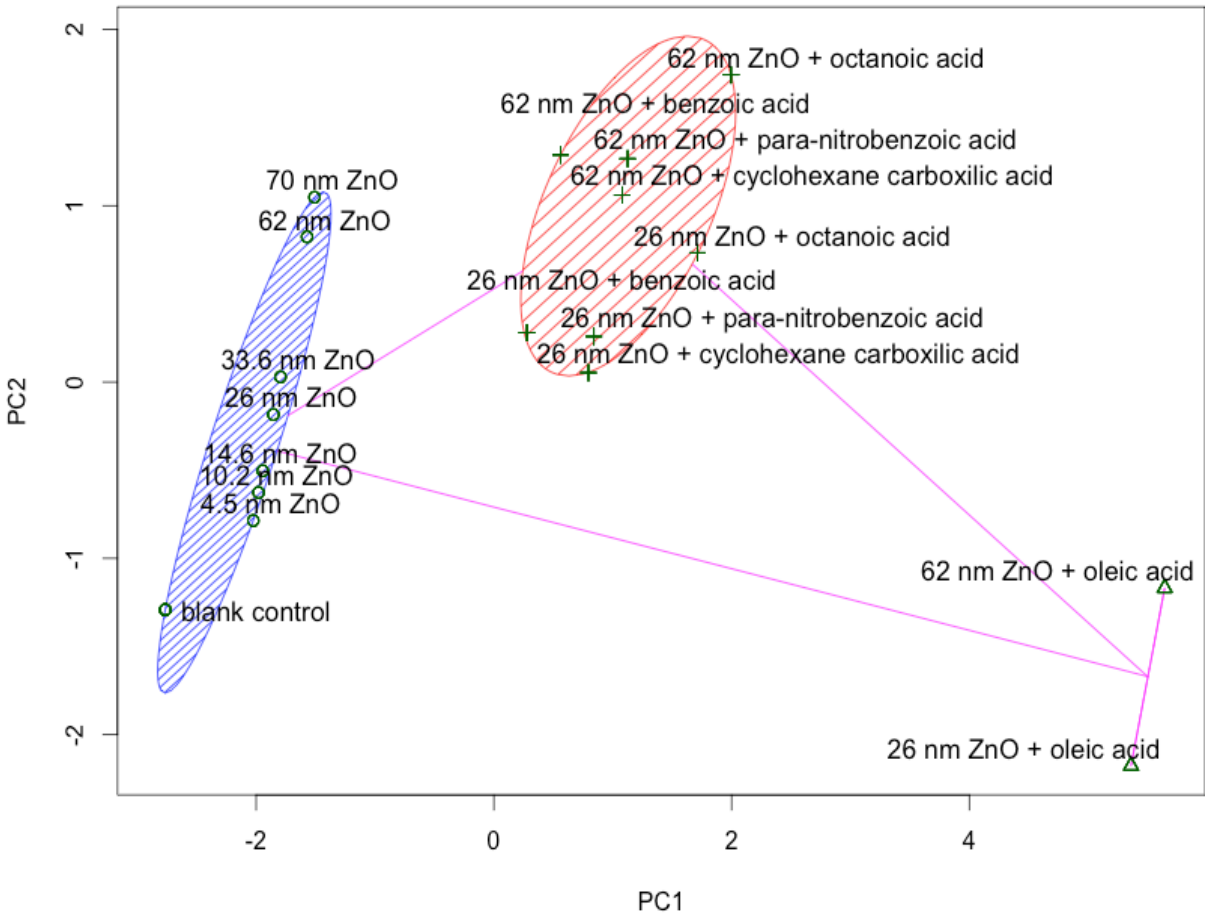


Figure 5: Clustering analysis based on Euclidian distance for ZnO NPs partitioned into 3 clusters. Shown on the left (blue hash marks) are the bare ZnO NPs with the blank control point. In the middle (tan hash marks) are ZnO NPs with 4 different surface chemistries and on the right are the oleic acid modified particles.

Estimation of Toxicity by Ordinary Kriging Method

By using the two most dominant PCs identified earlier as coordinates (X~Y direction) and mortality data as the response (Z-direction), we calculated the kriging estimation of mortality. The ordinary kriging method, based on the spherical model, was used to model the mortality of zebrafish embryos at each of the different exposure concentrations for each of the 17 tested NPs. The resulting contour map for the highest exposure concentration (250 mg/L) is shown in Figure 6 and the contour maps for other exposure concentrations can be found in Supporting Information File 4. The coefficient of determination was calculated to determine how well the estimation fit the original data. Similar coefficients of determination were found at each concentration (0.702 – 0.778).

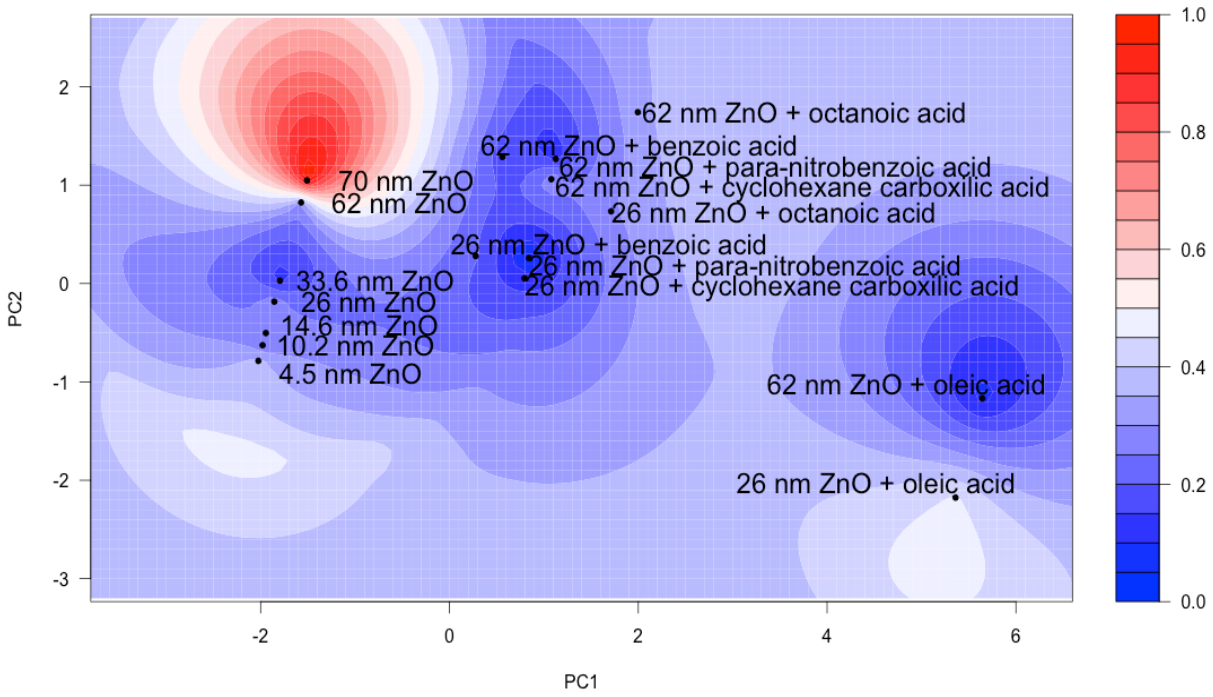


Figure 6: Kriging estimation contour map for embryonic zebrafish exposed to 250 mg/L of each type of zinc oxide nanoparticle using the first two surface chemistry-based principal components as the coordinates and 120 hpf total mortality as response. The coefficient of determination was found to be 0.702.

Discussion

ZnO NP Toxicity to Embryonic Zebrafish

Of the numerous sub-lethal endpoints evaluated in our study, most of the significant toxicity resulting from exposure to ZnO NPs was associated with mortality, regardless of the type of surface chemistry found on the nanoparticle. Interestingly, when mortality occurred in the surface functionalized ZnO NPs, it was always within the first 16-18 hours of exposure (observed at the 24 hpf evaluation). Embryos surviving exposure to surface coated ZnO NPs after this initial period had almost 100% survival and no significant developmental abnormalities (See Supporting Information File 1 & 5). In contrast, the bare ZnO particles resulted in mortality at both 24 and 120 hpf for some materials and a complete lack of toxicity in others. This result supports the hypothesis that outermost surface chemistry is a primary driver of biological interactions, even more than core composition. This finding has been supported in other studies investigating a wide range of NP types [27, 41-42].

Given that dissolution and the resulting release of zinc ions and ROS are the primary cause of ZnO NP toxicity [8], it is possible that the lack of late-onset mortality in coated particles is the result of decreased dissolution of these particles [7, 21]. It has been reported that the release of zinc ion from ZnO NPs coated with organic molecule can be slower than uncoated ZnO NPs by up to 10 days, due to the protective effect of the surface coating [43]. The idea that coated particles were more benign overall is also supported by the most toxic response being noted for a bare particle (NGZ, Figure 3). In addition, the observed mortality at 24 hpf for some of the surface functionalized particles could have been due to either residual impurities or zinc ions, as any dissolved zinc would have remained in the exposure media due to the static nature of these experiments. The delayed mortality response in the bare ZnO particles could also relate to the onset of mouth-gaping behavior during fish development that led to increased uptake over the exposure period; however, this would likely have occurred with the coated particles as well unless this was specific to zinc ion uptake or direct impacts of generated ROS.

Only one ZnO NP (NGZ) caused any significant sublethal impacts in the developing fish with notochord malformations as well as significant malformations of the swim bladder. Despite

NGZ being an uncoated ZnO NP, its unique toxicity relative to the other non-coated ZnO NPs suggests some other features, such as crystal morphology, may be contributing to the observed differential toxicity. It is known that ZnO NPs with sharper angles have been noted to contribute to lower viability in cell culture studies with A549 and HT29 cells [30]. Similar morphology effects on toxicity have been observed in studies of manganese oxide, where the sharp points and edges were found to generate more ROS than smooth surfaces [44]. We tested this hypothesis by comparing X-ray Diffraction (XRD) results for NGZ relative to a representative sample of the other bare ZnO NPs (Sigma-Aldrich, 63nm, NBI_0215) using a Bruker-AXS D8 Discover XRD instrument (Karlsruhe, Germany and Madison, WI). No differences in the lattice parameters were identified, thus other intrinsic factors must be contributing to the unique toxicity of this commercial ZnO NP (See Supporting Information File 6).

Since size of the ZnO NP did not elicit any general trends in the toxic responses observed, it is likely that surface features of the particle impacting interactions with biological membranes may drive toxicity more than size of the particle itself. NP agglomeration in aquatic environments often occurs and can be influenced by particle surface physicochemical properties and environmental factors affecting the zeta potential [27, 45-46]. Thus, it is possible that agglomeration of the particles in the fish water media could be indirectly affecting dissolution or interactions with the developing embryo. Previous studies have found that uncoated ZnO NPs form smaller aggregates on the surface of bacteria than are formed in suspension [47], and this type of surface aggregation cannot be ruled out as a contributing factor in our results. Previous studies with the freshwater crustacean *Daphnia magna* based on 30, 80-100 and 200 nm ZnO NPs found that toxicity was not dependent on primary particle size [11]. This is similar to what we found for the bare ZnO NPs in our study that range from 4 to 70nm.

Overall, the toxicity results suggest that surface features do impact ZnO NP toxicity. In addition, the evaluation or mortality at multiple time points during development is useful in modeling nanoparticle-biological interactions using zebrafish [45].

PCA

PCA combines as much information as possible to provide an overview of the known and unknown relationships between inherent NP features and developmental toxicity. The eight original intrinsic properties descriptors were correlated with each other based on similarities in value of PC1 weights, however more separation was gained using the weighting of PC2 (Table 3). The latent factor suggested by PC2 is the Log D, which plays a different role in the ZnO NPs toxicity compare to size and polar surface effects. The unique clustering of both sizes of oleic acid functionalized particles suggests the properties of this ligand are somewhat unique relative to the others, perhaps due to the long chain length (Figure 2) and high hydrophobicity of oleic acid (Table 2). Oleic acid coated ZnO NPs that have the highest hydrophobicity (Log D 5.62), showed the smaller size one was more toxic and separated from the remainder of the coated particles in the PCA. In contrast, the remaining surface functionalized particles all had much lower log D values (Table 2) and clustered together in our analysis. The Log D calculations can be affected by electrolyte concentration, however in our study this was too small (Cl- 0.0174 mol/L and Na+, K+ 0.0165 mol/L) to affect its value relative to water, thus these inherent properties value are expected to reflect the true properties in fish water. This suggests that future studies should continue to investigate surface features impacting the hydrophobicity of the particle as potential contributors to toxicity. However, this result depends on our assumption that the coating chemicals dominate the hydrophobicity of the metal oxide NP [22]. Even when surface chemistry is constant among ZnO NPs, differential particle morphology and variations in the suspension media will likely affect dissolution and alter the hydrophobicity in comparison to theoretical values of Log D [30].

Other intrinsic properties not considered, such as the proportional amount of ligand coverage on the surface of the nanoparticle, may improve model performance further. Unfortunately this level of detailed characterization of the surface chemistry is often unavailable from manufacturers and is cost- and time-intensive to determine for a wide range of surface chemistries. Further refinement of the model could likely also be achieved by including more complex calculation of intrinsic values that are based on the actual ligand-nanoparticle structure rather than surface ligand structure alone (in the absence of consideration of bonding with the NP). In studies of multiple engineered nanoparticles, it is nearly impossible to set single variable

control groups due to correlated descriptors and constraints in characterizing NPs in the experiment conditions. However, we have shown that PCA can be used as a valuable alternative method to estimate the relative effects of multiple inherent properties simultaneously to support the development of predictive models that will allow for the development of safer ZnO materials.

Based on the large differences in molecular properties between the organic surface coatings and the bare zinc oxide properties (Table 2), it was expected that each group would separate during clustering analysis, as was the case with this data (Figure 5). Identified clusters suggest that a set of appropriate intrinsic properties of surface chemistry can be used to partition NPs into different groups. The 17 ZnO NPs partitioned into clusters that were fairly easy to identify using only capping agent properties. However, with more complex surface structures, overlap between clusters might happen making determination of the cluster number the first concern. Although there are several algorithms to decide the cluster number, the lack of robust data sets such as this preclude a current understanding of which algorithm may be appropriate [48].

Kriging Estimation

Based on the two most dominant PCs that explained 87.3% of the variance in the toxicity data, we performed the Kriging estimation at each of the exposure concentrations. Interestingly, the exposure concentrations had little influence on the coefficients of determination with similar values being determined at each concentration (Figure 6, Supporting Information File 4). Kriging estimation further elucidated the impacts of NP size. Based on Figure 6, we can see that the largest bare particle (NGZ) also has the highest mortality (Figure 3B) and the cluster 2 surface modified 26 nm particles were predicted to have overall lower toxicity than the larger versions of the same particle. This trend does not hold for the oleic acid functionalized particles however as the smaller particles are predicted to be higher in toxicity. Thus, outermost surface chemistry continues to play a more important role in determining toxicity.

Conclusions

The observed toxic responses of developing zebrafish embryos to ZnO NP exposure varied with surface chemical modification and were only minimally impacted by particle size. Only NGZ, a bare ZnO NP, had relatively high toxicity, suggesting specific product features of bare ZnO NPs drive toxicity. This work has shown that large databases of similar NPs with varying surface features studied under identical experimental design protocols, are invaluable in the development of models of nanoparticle-biological interactions. We have shown that intrinsic features of NPs, particularly those encompassing the outermost surface chemistry, are useful in the classification and clustering of NP toxicity data. Our finding that hydrophobicity was the strongest determinant of toxicity of the many surface features we investigated will contribute to the development of predictive models of ZnO NP-biological interactions. We have found that PCA is a useful tool for reducing numerous surface molecular properties to fewer dimensions. Future development of highly accurate predictive models will depend on detailed information provided by *in silico* modeling and analysis of the outermost surface of the nanoparticle. Overall, identification of specific material features, such as outermost surface chemistry, that drive biological interactions appears feasible and models such as this should continue to be tested and refined to achieve safer design principles for the manufacture of ZnO NPs.

Experimental

Nanomaterials

The ZnO NPs with different capping agents and sizes were obtained from a variety of commercial and research laboratories (Table 1). More detailed characterization of the nanomaterials are also available on the open-source Nanomaterial-Biological Interactions Knowledgebase [39] provided by Oregon State University.

Estimation of Surface Chemical Parameters

The eight surface chemical descriptors we utilized were size, hydrophobicity (Log D),

polarizability, polar surface area, Van der Waals surface area, solvent accessible surface area, molar refractivity and Dreiding energy (Table 2). Except for the primary particle sizes (which were provided by manufacturers), the seven other intrinsic properties of capping agents were calculated by software (Table 2). Log D is calculated using Advanced Chemistry Development (ACD/Labs) Software version 11.02. PL is retrieved from ChemSpider (Mar. 2014), which was predicted by ACD/Labs Percepta Platform - PhysChem Module. VDW surface (VS), PS, SASA, RF and DE were calculated in Marvin Beans (version 6.2.2, Cambridge, MA). All inherent chemical properties were calculated based on the pH used in zebrafish toxicity test.

Embryonic Zebrafish Assay

Wild-type 5D zebrafish (*Danio rerio*) embryos were obtained from group spawns of adult fish housed at the Sinnhuber Aquatic Research Laboratory at Oregon State University (Corvallis, OR). All NP dilutions and exposures were conducted in fish water (FW). The FW was prepared with 0.26g/L Instant Ocean salts (Aquatic Ecosystem, Apopka, FL) combined with approximately 0.01g $NaHCO_3$ pH buffer in reverse osmosis water (pH 7.0-7.4, conductivity 450-600μS). Embryos were collected at 6 hours post-fertilization (hpf) and maintained at 27° C under 14/10 light and dark cycle. Embryos were exposed individually in 96-well plates to 7 different concentrations (0.016 to 250 mg/L) of each type of ZnO NP suspended in FW. Prior to exposure, embryos were dechorionated at 6 hours post-fertilization (hpf) with pronase (Sigma-Aldrich) and then rinsed several times with FW [25]. The control groups are FW alone without NPs present. A total of 21 endpoints were observed during development at 24 and 120 hpf that included mortality as well as morphological, behavioral and developmental endpoints in sub-lethal exposures [49]. The 19 sub-lethal endpoint include: developmental progression (DP), spontaneous movement (SP), notochord (N), yolk sac edema (Y), axis (A), eye (E), snout (Sn), jaw (J), otic (O), heart (H), brain (B), somite (So), pectoral fin (PF), caudal fin (CF), pigment (P), circulation (C), trunk (T), swim bladder (SB), touch response (TR).

Statistical Analysis

Due to the non-parametric nature of the data and the small sample size (<30 embryos for each exposure concentration), the Fisher's exact test (Sigma Plot v12.0, San Jose, CA) was used to analyze individual endpoints recorded at 24 and 120 hpf [50]. P-value was calculated based on two-tailed test and a $p \leq 0.05$ significance level was maintained for all analyses. Mortality data was compared between NPs with the same capping agent but different sizes using two-way analysis of variance (R, version 3.1.0, Vienna, Austria).

Principal component analysis (PCA) was conducted in R using the primary particle size and seven intrinsic properties of NPs' surface chemistry shown in Tables 1 and 2, respectively. To include control groups (blank group) in the analysis, all of the intrinsic NP properties are set to 0 for the blank groups. The same intrinsic properties were used for all exposure concentrations (0.016 mg/L to 250 mg/L) for a given particle type. The normalization process was conducted on the dataset as a matrix in PCA, with the mean of normalized data equal to 0 and standard deviation equal to 1. Then 8 different linear combinations consisting of 8 independent variables and their coefficients (also called "rotation" in R) were generated as new vectors, called principal components (PCs). The converted value, called score (stored as "x" in R), was used to model the toxic responses. The ordinary kriging was conducted in R using the additional "Kriging" and "gstat" packages.

Acknowledgments

We thank the staff of Sinnhuber Aquatic Research Laboratory for providing the embryos used in these studies. These studies were supported with funding from #ES017552-01A2; #P30ES03850; #ES0166896-01; #FA8650-05-1-15041; #P30E5000210.

Supporting Information

Supporting Information File 1:

File Name: S1.xlsx

File Format: .xlsx

Title: **Table S1:** Zebrafish malformation and behavioural data. The 19 sub-lethal EPs are developmental progression (DP), spontaneous movement (SP), notochord (N), yolk sac edema (Y), axis (A), eye (E), snout (Sn), jaw (J), otic (O), heart (H), brain (B), somite (So), pectoral fin (PF), caudal fin (CF), pigment (P), circulation (C), trunk (T), swim bladder (SB), touch response (TR).

comp.	Size	Conc.(ppm)	DP	SM	N	Y	A	E	Sn	J	O	H	B	So	PF	CF	P	C	T	SB	TR
nbi_085: Aldrich ZnO+Oleic Acid (TLAD25A)	62	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	6%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_086: Voxtel ZnO + Oleic Acid (TLAD25)	26	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	5%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_087: Aldrich ZnO (TLAD24A)	62	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	0%	0%	0%	0%	6%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_088: Voxtel ZnO (TLAD24)	26	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	5%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	5%	0%	0%	5%	10%	5%	5%	5%	5%	5%	5%	5%	5%	5%	0%	5%	5%	5%	5%
		10	5%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_089: Aldrich ZnO + Octanoic Acid (TLAD27A)	62	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	10%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	7%	7%	7%	7%	7%	0%	7%	7%	0%	0%	7%	0%	0%	0%	0%	0%
		50	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_090: Voxtel ZnO + Octanoic Acid (TLAD27)	26	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	5%	0%	0%	0%	0%	0%	0%	0%	0%	5%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	6%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	6%	0%
		10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_091: Aldrich ZnO + para-Nitrobenzoic Acid (TLAD35A)	62	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	5%	0%	0%	0%	0%	5%	0%

nbi_092: Voxtel ZnO + para-Nitrobenzoic Acid (TLAD35)	26	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	4%	0%	0%	4%	4%	4%	0%	4%	0%	4%	4%	0%	4%	4%	0%	4%	4%	4%	4%
		50	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	4%	0%	0%	4%	0%	0%	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_093: Aldrich ZnO + Cyclohexane Carboxilic Acid (TLAD31A)	62	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	5%	5%	5%	5%	5%	0%	5%	5%	0%	5%	5%	0%	0%	5%	5%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_094: Voxtel ZnO + Cyclohexane Carboxilic Acid (TLAD31)	26	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	4%	4%	4%	4%	4%	0%	4%	4%	4%	4%	4%	0%	4%	4%	0%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	5%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_095: Aldrich ZnO + Benzoic Acid (TLAD33A)	62	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	5%	0%	0%	0%	5%	0%	0%	0%	0%	0%	0%	0%	5%	0%	0%	0%	0%	5%	0%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	5%	0%	0%	0%	6%	0%	0%	0%	0%	0%	0%	0%	6%	0%	0%	0%	0%	6%	0%
		50	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	5%	0%	0%	0%	5%	0%	0%	0%	0%	0%	0%	0%	5%	0%	0%	0%	0%	5%	0%
nbi_096: Voxtel ZnO + Benzoic Acid (TLAD33)	26	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_136: ZnO I (NI001)	12.6-16.6	0	0%	0%	0%	0%	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	4%	0%	0%	0%	4%	0%	0%	0%	0%	0%	0%	0%	0%	4%	0%	0%	0%	0%	0%
		0.08	5%	5%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	4%	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	0%	0%	0%	0%	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_137: ZnO I (NI002)	18.3-48.9	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	0%	4%	0%	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	4%	0%
		0.4	0%	0%	0%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%
		2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		50	0%	0%	0%	4%	4%	4%	4%	4%	4%	4%	4%	4%	4%	4%	4%	4%	4%	4%	4%
		250	5%	0%	0%	5%	0%	0%	5%	5%	0%	5%	5%	0%	0%	0%	0%	0%	0%	0%	0%
		0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

nbi_138: ZnO I (NI003)	4.0-5.0	0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	5%	0%	5%	5%	5%	0%	5%	0%	0%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	5%	5%	5%	5%	0%	0%	0%	5%	0%	0%	0%	0%	0%	0%	0%
		10	0%	0%	0%	9%	9%	9%	9%	9%	0%	9%	9%	0%	5%	5%	0%	0%	0%	0%
		50	5%	0%	0%	9%	9%	9%	9%	9%	0%	9%	9%	0%	5%	9%	0%	0%	5%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_139: ZnO I (NI005)	8.7-11.7	0.016	0%	0%	0%	5%	5%	5%	5%	5%	0%	5%	0%	0%	0%	0%	0%	0%	0%	0%
		0.08	0%	0%	0%	9%	9%	9%	5%	5%	0%	0%	5%	5%	0%	0%	0%	0%	0%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		2	0%	0%	0%	4%	4%	4%	4%	4%	0%	4%	4%	0%	0%	0%	0%	0%	4%	0%
		10	0%	0%	0%	10%	10%	10%	10%	10%	10%	10%	10%	0%	0%	0%	0%	0%	10%	5%
		50	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		250	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
nbi_187: NanoGard ZnO	40-100	0.016	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	8%	0%
		0.08	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	4%	0%
		0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	4%	0%	0%	0%	0%	4%	0%	4%	0%
		2	0%	0%	0%	17%	4%	0%	0%	0%	0%	13%	0%	0%	8%	0%	4%	0%	17%	0%
		10	0%	0%	0%	13%	7%	0%	0%	0%	0%	20%	0%	0%	7%	0%	0%	0%	27%	0%
		50	0%	0%	0%	all dead — no data availiable														
		250	0%	0%	18%															

Supporting Information File 2:

File Name: S2.xlsx

File Format: .xlsx

Title: **Table S2:** Fisher's exact test p-value. The 19 sub-lethal EPs are developmental progression (DP), spontaneous movement (SP), notochord (N), yolk sac edema (Y), axis (A), eye (E), snout (Sn), jaw (J), otic (O), heart (H), brain (B), somite (So), pectoral fin (PF), caudal fin (CF), pigment (P), circulation (C), trunk (T), swim bladder (SB), touch response (TR). Include 3 mortality (M) EPs are at 24 and 120 hours post fertilization after ZnO NP exposure and sum of two M.

(b) ZnO without capping agent P-value data

composition	Size	dose(ppm)	M(24hpf)	DP	SM	N	M(120hpf)	Y	A	E	Sn	J	O	H	B	So	PF	CF	P	C	T	SB	TR	M(total)
nbi_087: Aldrich ZnO (TLAD24A)	62	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	0.348	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.348
		0.08	0.048	1	1	1	0.425	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.023
		0.4	0.188	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.188
		2	0.348	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.348
		10	0.609	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.609
		50	0.097	1	1	1	1	1	0.439	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.097
		250	0.097	1	1	1	0.439	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.048
nbi_088: Voxtel ZnO (TLAD24)	26	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	0.348	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.348
		0.08	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.4	0.609	0.477	1	1	0.222	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.188
		2	0.348	0.465	1	1	1	0.465	0.21	0.465	0.465	0.465	0.465	0.465	0.465	0.465	0.465	0.465	1	0.465	0.465	0.465	0.465	0.348
		10	1	0.489	1	1	0.489	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.609
		50	0.609	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.609
		250	0.048	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.048
nbi_136: ZnO I (NI001)	2.6-16.6	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.489	1	1	1	1	1	1
		0.08	0.234	0.477	0.477	1	0.04	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.009
		0.4	0.234	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.234
		2	1	1	1	1	0.234	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.234
		10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		250	0.05	1	1	1	0.442	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.022
nbi_137: ZnO I (NI002)	8.3-48.9	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	1	1	1	1	0.223	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.666
		0.08	0.489	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.489
		0.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		250	1	1	1	1	1	0.488	1	1	0.488	0.488	1	0.488	0.488	1	1	1	1	1	1	1	1	1
nbi_138: ZnO I (NI003)	4.0-5.0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.234
		0.08	1	1	1	1	0.467	0.465	1	0.465	0.465	0.465	1	0.465	1	1	1	1	1	1	1	1	1	0.109
		0.4	1	1	1	1	0.234	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.022
		2	1	1	1	1	1	0.477	0.467	0.477	0.477	1	1	1	0.477	1	1	1	1	1	1	1	1	0.234
		10	1	1	1	1	0.489	0.223	0.223	0.223	0.223	0.223	1	0.223	0.223	1	0.478	0.478	1	1	1	1	1	0.489
		50	1	0.478	1	1	1	0.223	0.223	0.223	0.223	0.223	1	0.223	0.223	1	0.478	0.223	1	1	0.478	1	1	0.489
		250	1	1	1	1	0.0032	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.002
nbi_139: ZnO I (NI005)	8.7-11.7	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	1	1	1	1	0.488	0.476	0.476	0.476	0.476	0.476	1	0.476	1	1	1	1	1	1	1	1	1	0.666
		0.08	1	1	1	1	1	0.223	0.223	0.488	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.489
		10	1	1	1	1	1	0.233	0.233	0.233	0.233	0.233	0.233	0.233	0.233	1	1	1	1	1	1	0.233	0.488	1
		50	0.666	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.666
		250	0.245	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.245
nbi_187: NanoGard ZnO (NGZ)	40-100	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.489	1	1
		0.08	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2	1	1	1	1	1	0.109	1	1	1	1	1	0.234	1	1	0.489	1	1	1	1	0.109	1	1
		10	1	1	1	1	0.0016	0.489	0.395	1	1	1	1	0.054	1	1	0.395	1	1	1	1	0.018	1	0.01
		50	1	1	1	1	0.001	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.001
		250	1	1	1	0.049	0.001	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.001

significant in fisher's exact test

blank control data

(a) ZnO with capping agents' P-value data

composition	Size	dose(ppm)	M(24hpf)	DP	SM	N	M(120hpf)	Y	A	E	Sn	J	O	H	B	So	PF	CF	P	C	T	SB	TR	M(total)
nbi_085: Aldrich ZnO+Oleic Acid (TLAD25A)	62	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	0.348	1	1	1	0.465	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.188
		0.08	0.023	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.41	1	0.023
		0.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2	0.348	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.348
		10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		50	0.188	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.188
		250	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
nbi_086: Voxtel ZnO + Oleic Acid (TLAD25)	26	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	0.348	1	1	1	0.465	1	0.452	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.188
		0.08	0.048	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.048
		0.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2	0.097	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.097
		10	0.023	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.023
		50	0.001	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.004
		250	0.001	1	1	1	0.395	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.002
nbi_089: Aldrich ZnO + Octanoic Acid (TLAD27A)	62	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	1	1	1	1	0.233	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.348
		0.08	0.609	1	1	1	1	1	0.21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.348
		0.4	0.048	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.048
		2	0.348	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.348
		10	0.097	1	1	1	0.077	0.395	0.395	0.395	0.395	0.395	1	0.395	0.395	1	1	0.395	1	1	1	1	1	0.01
		50	0.048	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.048
		250	0.023	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.023
nbi_090: Voxtel ZnO + Octanoic Acid (TLAD27)	26	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	0.348	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.348
		0.08	0.348	1	1	1	0.465	1	1	1	1	1	1	0.452	1	1	1	1	1	1	1	1	0.452	0.188
		0.4	0.609	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.609
		2	0.097	1	1	1	1	0.439	1	1	1	1	1	1	1	1	1	1	1	1	1	0.439	1	0.097
		10	0.609	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.609
		50	0.01	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.01
		250	0.048	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.048
nbi_091: Aldrich ZnO + para-Nitrobenzoic Acid (TLAD35A)	62	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.08	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2	0.348	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.348
		10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		250	1	1	1	1	0.489	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.609
nbi_092: Voxtel ZnO + para-Nitrobenzoic Acid (TLAD35)	26	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.08	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		10	1	0.489	1	1	1	1	0.489	0.489	1	0.489	1	0.489	0.489	1	0.489	0.489	1	0.489	0.489	0.489	0.489	1
		50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		250	1	1	1	1	1	1	1	1	0.489	1	1	0.489	1	1	1	1	1	1	1	1	1	1
nbi_093: Aldrich ZnO + Cyclohexane Carboxilic Acid (TLAD31A)	62	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	0.489	1	1	1	1	0.489	0.478	0.478	0.478	0.478	1	0.478	0.478	1	0.478	0.478	1	1	0.478	0.478	1	0.489
		0.08	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.4	0.234	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.234
		2	0.009	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.009
		10	0.022	1	1	1	0.439	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.009
		50	0.022	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.022
		250	0.009	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.009
nbi_094: Voxtel ZnO + Cyclohexane Carboxilic Acid (TLAD31)	26	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.08	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.4	0.489	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.489
		2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		250	0.234	0.477	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.234
nbi_095: Aldrich ZnO + Benzoic Acid (TLAD33A)	62	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	0.416	1	1	1	0.209	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.137
		0.08	1	1	1	1	0.45	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.666
		0.4	0.416	0.463	1	1	1	1	0.463	1	1	1	1	1	1	1	0.463	1	1	1	1	0.463	1	0.416
		2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		10	0.416	0.463	1	1	1	1	0.45	1	1	1	1	1	1	1	0.45	1	1	1	1	0.45	1	0.245
		50	0.666	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.666
		250	1	0.488	1	1	0.45	1	0.476	1	1	1	1	1	1	1	0.476	1	1	1	1	0.476	1	0.666
nbi_096: Voxtel ZnO + Benzoic Acid (TLAD33)	26	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		0.016	1	1	1	1	0.45	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.666
		0.08	0.245	1	1	1	0.209	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.137
		0.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2	0.072	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.072
		10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		50	0.416	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.416
		250	1	1	1	1	0.233	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.416

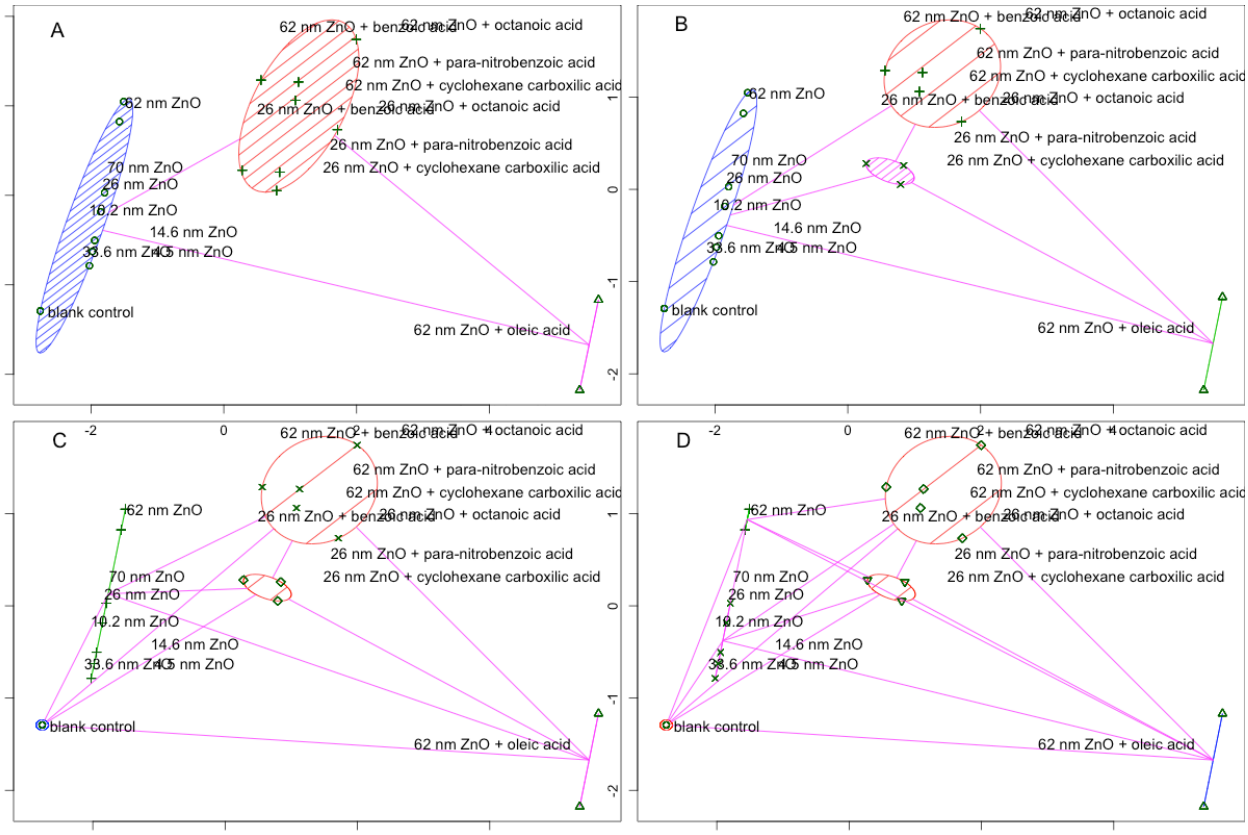
significant in fisher's exact test

blank control data

Supporting Information File 3:

File Name: S3.png

File Format: .png



Title: **Figure S3:** Cluster analysis of converted data using Euclidean distance to partition into A) 3, B) 4, C) 5, D) 6 clusters.

Supporting Information File 4:

File Name: S4.png

File Format: .png

Title: **Figure S4:** Kriging estimations of zebrafish mortality data at A) 0.016 ppm, B) 0.08 ppm, C) 0.4 ppm, D) 2 ppm, E) 10 ppm, F) 50 ppm.

Supporting Information File 5:

File Name: S5.xlsx

File Format: .xlsx

Title: **Table S5:** Embryonic zebrafish mortality at 24 and 120 hours post fertilization after ZnO NP exposure.

(a) ZnO with capping agents' Mortality data

composition	Size	dosage	24M	120M	Total M
		ppm	%	%	%
nbi_085: Aldrich ZnO+Oleic Acid (TLAD25A)	62	0	4.17%	0.00%	4.17%
		0.016	16.67%	5.00%	20.83%
		0.08	33.33%	0.00%	33.33%
		0.4	8.33%	0.00%	8.33%
		2	16.67%	0.00%	16.67%
		10	8.33%	0.00%	8.33%
		50	20.83%	0.00%	20.83%
		250	8.33%	0.00%	8.33%
nbi_086: Voxtel ZnO + Oleic Acid (TLAD25)	26	0	4.17%	0.00%	4.17%
		0.016	16.67%	5.00%	20.83%
		0.08	29.17%	0.00%	29.17%
		0.4	4.17%	4.35%	8.33%
		2	25.00%	0.00%	25.00%
		10	33.33%	0.00%	33.33%
		50	41.67%	0.00%	41.67%
		250	41.67%	7.14%	45.83%
nbi_089: Aldrich ZnO + Octanoic Acid (TLAD27A)	62	0	4.17%	0.00%	4.17%
		0.016	8.33%	9.09%	16.67%
		0.08	12.50%	4.76%	16.67%
		0.4	29.17%	0.00%	29.17%
		2	16.67%	0.00%	16.67%
		10	25.00%	16.67%	37.50%
		50	29.17%	0.00%	29.17%
		250	33.33%	0.00%	33.33%
nbi_090: Voxtel ZnO + Octanoic Acid (TLAD27)	26	0	4.17%	0.00%	4.17%
		0.016	16.67%	0.00%	16.67%
		0.08	16.67%	5.00%	20.83%
		0.4	12.50%	0.00%	12.50%
		2	25.00%	0.00%	25.00%
		10	12.50%	0.00%	12.50%
		50	37.50%	0.00%	37.50%
		250	29.17%	0.00%	29.17%
nbi_091: Aldrich ZnO + para-Nitrobenzoic Acid (TLAD35A)	62	0	4.17%	0.00%	4.17%
		0.016	0.00%	0.00%	0.00%
		0.08	0.00%	4.17%	4.17%
		0.4	4.17%	4.35%	8.33%
		2	16.67%	0.00%	16.67%
		10	4.17%	0.00%	4.17%
		50	4.17%	0.00%	4.17%
		250	4.17%	8.70%	12.50%
nbi_092: Voxtel ZnO + para-Nitrobenzoic Acid (TLAD35)	26	0	0.00%	0.00%	0.00%
		0.016	0.00%	0.00%	0.00%
		0.08	0.00%	0.00%	0.00%
		0.4	0.00%	0.00%	0.00%
		2	0.00%	4.17%	4.17%
		10	4.17%	0.00%	4.17%
		50	0.00%	4.17%	4.17%

(b) ZnO without capping agent Mortality data

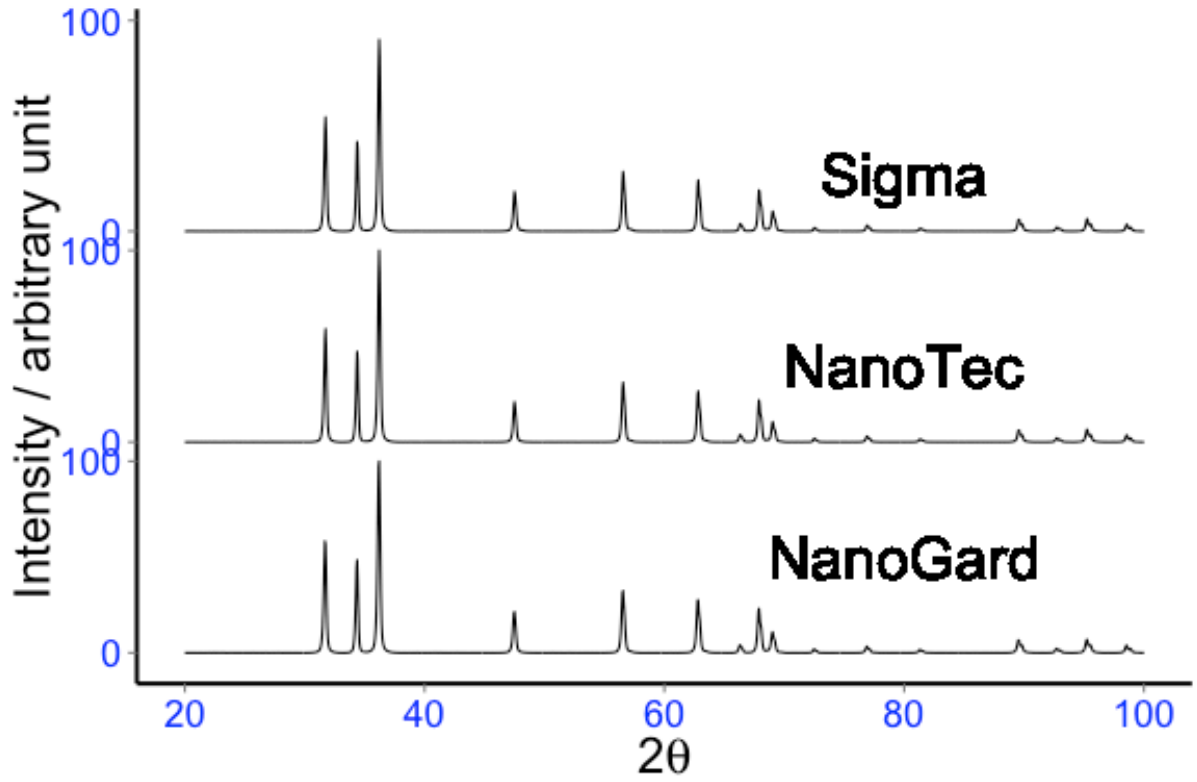
composition	Size	dosage	24M	120M	Total M
		ppm	%	%	%
nbi_087: Aldrich ZnO (TLAD24A)	62	0	4.17%	0.00%	4.17%
		0.016	16.67%	0.00%	16.67%
		0.08	29.17%	5.88%	33.33%
		0.4	20.83%	0.00%	20.83%
		2	16.67%	0.00%	16.67%
		10	12.50%	0.00%	12.50%
		50	25.00%	0.00%	25.00%
		250	25.00%	5.56%	29.17%
nbi_088: Voxtel ZnO (TLAD24)	26	0	4.17%	0.00%	4.17%
		0.016	16.67%	0.00%	16.67%
		0.08	8.33%	0.00%	8.33%
		0.4	12.50%	9.52%	20.83%
		2	16.67%	0.00%	16.67%
		10	8.33%	4.55%	12.50%
		50	12.50%	0.00%	12.50%
		250	29.17%	0.00%	29.17%
nbi_136: ZnO I (NI001)	12.6-16.6	0	0.00%	0.00%	0.00%
		0.016	0.00%	4.17%	4.17%
		0.08	12.50%	19.05%	29.17%
		0.4	12.50%	0.00%	12.50%
		2	0.00%	12.50%	12.50%
		10	0.00%	4.17%	4.17%
		50	4.17%	0.00%	4.17%
		250	20.83%	5.26%	25.00%
nbi_137: ZnO I (NI002)	18.3-48.9	0	8.33%	0.00%	8.33%
		0.016	8.33%	9.09%	16.67%
		0.08	0.00%	0.00%	0.00%
		0.4	4.17%	4.35%	8.33%
		2	12.50%	0.00%	12.50%
		10	0.00%	4.17%	4.17%
		50	0.00%	4.17%	4.17%
		250	8.33%	4.55%	12.50%
nbi_138: ZnO I (NI003)	4.0-5.0	0	0.00%	0.00%	0.00%
		0.016	12.50%	0.00%	12.50%
		0.08	12.50%	4.76%	16.67%
		0.4	12.50%	14.29%	25.00%
		2	12.50%	0.00%	12.50%
		10	4.17%	4.35%	8.33%
		50	8.33%	0.00%	8.33%
		250	8.33%	31.82%	37.50%
nbi_139: ZnO I (NI005)	8.7-11.7	0	8.33%	0.00%	8.33%
		0.016	12.50%	4.76%	16.67%
		0.08	8.33%	0.00%	8.33%
		0.4	0.00%	4.17%	4.17%
		2	0.00%	0.00%	0.00%
		10	12.50%	0.00%	12.50%
		50	16.67%	0.00%	16.67%

nbi_093: Aldrich ZnO + Cyclohexane Carboxilic Acid (TLAD31A)	62	250	0.00%	4.17%	4.17%	nbi_187: NanoGard ZnO (NGZ)	40-100	250	25.00%	0.00%	25.00%
		0	0.00%	0.00%	0.00%			0	4.17%	0.00%	4.17%
		0.016	8.33%	0.00%	8.33%			0.016	0.00%	0.00%	0.00%
		0.08	0.00%	0.00%	0.00%			0.08	0.00%	0.00%	0.00%
		0.4	12.50%	0.00%	12.50%			0.4	0.00%	0.00%	0.00%
		2	29.17%	0.00%	29.17%			2	0.00%	0.00%	0.00%
		10	25.00%	5.56%	29.17%			10	0.00%	37.50%	37.50%
		50	25.00%	0.00%	25.00%			50	0.00%	100.00%	100.00%
		250	29.17%	0.00%	29.17%			250	8.33%	100.00%	100.00%
nbi_094: Voxtel ZnO + Cyclohexane Carboxilic Acid (TLAD31)	26	0	0.00%	0.00%	0.00%	nbi_096: Voxtel ZnO + Benzoic Acid (TLAD33)	26	0	8.33%	0.00%	8.33%
		0.016	0.00%	0.00%	0.00%			0.016	12.50%	4.76%	16.67%
		0.08	4.17%	0.00%	4.17%			0.08	25.00%	5.56%	29.17%
		0.4	8.33%	0.00%	8.33%			0.4	8.33%	0.00%	8.33%
		2	0.00%	0.00%	0.00%			2	33.33%	0.00%	33.33%
		10	0.00%	0.00%	0.00%			10	8.33%	0.00%	8.33%
		50	0.00%	0.00%	0.00%			50	20.83%	0.00%	20.83%
		250	12.50%	0.00%	12.50%			250	8.33%	13.64%	20.83%
nbi_095: Aldrich ZnO + Benzoic Acid (TLAD33A)	62	0	8.33%	0.00%	8.33%						
		0.016	20.83%	10.53%	29.17%						
		0.08	12.50%	4.76%	16.67%						
		0.4	20.83%	0.00%	20.83%						
		2	4.17%	4.35%	8.33%						
		10	20.83%	5.26%	25.00%						
		50	16.67%	0.00%	16.67%						
		250	12.50%	4.76%	16.67%						

Supporting Information File 6:

File Name: S6.png

File Format: .png



Title: **Figure S6:** XRD analysis of three different ZnO NPs.

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Chapter 3 Manuscript 2

Short Communication:

Application of Kriging estimation model for ZnO NPs to predict the toxicity of doped ZnO NPs.

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Due to industry and customer demands, more and more nanoparticles (NPs) are being developed and applied in our daily living. Based on project on emerging nanotechnologies' (PEN) consumer product inventory, over 1600 manufacturer-identified nanotechnology products have been introduced to the market [1]. While the applications for new materials have grown exponentially, testing of these materials for potential hazards has fallen behind NP R&D. This raises concerns about the risks of these new technologies for our environmental and human health [2-6]. Based on the market investigation, most of manufactured NMs are metal oxides: SiO_2, TiO_2 and ZnO [7]. To advance the development of NM risk and hazard assessment, diverse *in vivo, in vitro* and *in silico* tests have been conducted [3-6, 8-9]. *In silico* testing is an inexpensive method, which promises to accelerate scientific research that support NP safety policies and inform manufacturing management decisions. The rapid growth of NMs already on the market and the paucity of information on potential hazards associated with these materials; there is an urgent need to take action to protect our community and the environment. Currently, several models have been demonstrated to predict NP toxicity to whole animals or cells based on various descriptors of NMs [9-14]. These quantitative structure activity relationship (QSAR) models have been designed to specific: use metal oxide NP band gap to predict their toxicity in mouse lung [10]; model uptake of NPs with same metal core but different surface modifications in PaCa2 cancer cells [11]; estimate bare metal oxide NP toxicity probability to RAW 264.7 and BEAS-2B cell lines [13]; magnetofluorescent engineered nanoparticles uptake in pancreatic cancer cells [15]; cytotoxicity of metal oxide to bacteria *Escherichia coli* [12, 16]. Most of these studies have focused solely on simple metal oxides without surface modification, which makes it possible to make comparison among different core materials as to their potential hazard. With the finding that NPs with different capping agents or surface ligands functionalized on the surface can improve stability against agglomeration [17], comparison among NPs with same core material but different surface modifications is also important to investigate.

To investigate the different effects of surface modification on same core material, a new approach has been applied on ZnO NPs with and without surface coatings. NP responses to embryonic zebrafish have been recorded and stored in a publically available online knowledgebase at nbi.oregonstate.edu. The responses include mortality and 19 different sublethal endpoints: developmental progression (DP), spontaneous movement (SP), notochord (N), yolk sac edema (Y), axis (A), eye (E), snout (Sn), jaw (J), otic (O), heart (H), brain (B),

somite (So), pectoral fin (PF), caudal fin (CF), pigment (P), circulation (C), trunk (T), swim bladder (SB), touch response (TR). The 8 NP descriptors we previously identified (in Chapter 2) as predictive of ZnO NP toxicity are: primary particle size, distribution coefficient Log D, polarizability, polar surface area, Van Der Waals surface area, solvent accessible surface area, molar refractivity and Dreiding energy. Distribution coefficient Log D was used to consider both ionic and non-ionic forms. Polarizability was used to describe the molecule's ability to change with external fields in biochemical reactions and its electronic properties [18]. Polar surface area represents the area formed by the polar areas of the molecule. Van Der Waals surface area calculated by VDW radius, which is associated with the trend of NP agglomeration [19]. Solvent accessible surface area was used to estimate the protein-ligand binding free energy [20], and molar refractivity represents the energy required to polarize one mole of the substance. Dreiding energy (DE) was used to describe the binding affinity of organic molecules with Zn and membrane proteins [21].

Principal component analysis (PCA) was used to reduce data dimensionality. As a result of PCA, the two most dominant principal components (PCs) were used to represent NPs information. For toxicity data, mortality was found to be the most significant endpoint in our embryonic zebrafish assay. Thus, two PCs were used as coordination system to generate kriging estimations with mortality rate as the response.

In previous work (Chapter 2), a kriging estimation model was developed for diverse ZnO NPs' toxicity to zebrafish embryos (Figure 1A Zhou et al, 2015). Because the model was limited to simple NPs with ZnO cores and various surface chemistries, it was unclear whether this model would be predictive of ZnO NP behavior once doped with common dopants such as Al_2O_3 or Fe_2O_3. Two ZnO NPs doped with Fe_2O_3 or Al_2O_3 with similar primary sizes to previous bare ZnO NPs used to build the model, were tested through the same data processing — multiple PCA's (Principal Component Analysis) loadings (coefficient) to generate new score (converted values). Based on the new PC location, an estimation of toxicity was generated from previous kriging results (Figure 1B). The results show the mortality observed for the doped ZnO NP to embryonic zebrafish doesn't follow our estimation (Figure 1D). The comparison of bare and doped ZnO NPs mortality to embryonic zebrafish shows the estimated value of doped ZnO still

follow the trend of bare ZnO (Figure 1C & D); however, toxicological testing of the doped NPs showed much lower toxicity compared with bare ones.

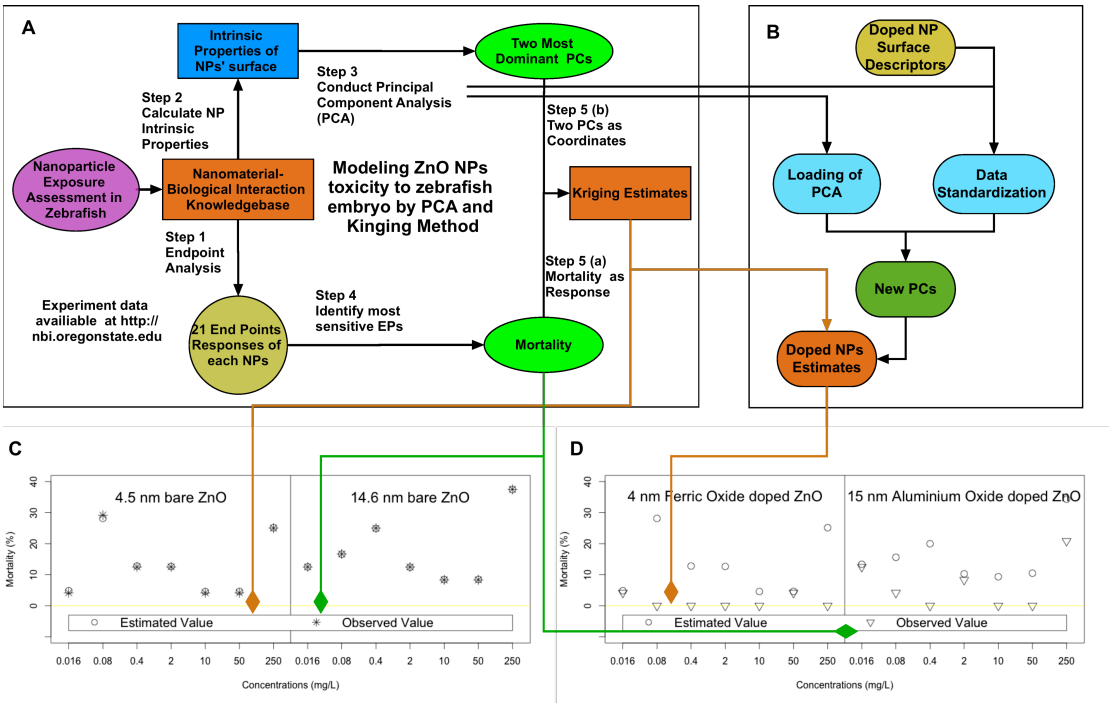

Figure 1: Data processing: (A) The estimation processes of various ZnO NPs toxicity to embryonic zebrafish; (B) The estimation processes of metal oxides doped ZnO NPs toxicity to embryonic zebrafish, the new descriptors were calculated based on calculated molar percentage of doping chemicals; (C) estimated and observed mortality for 4.5 and 14.6 nm bare ZnO; (D) estimated and observed mortality for 4 and 14 nm doped ZnO.

To investigate the differences between doped and bare ZnO NPs toxicity, delayed hatching and DNA damage have been detected in zebrafish development due to Zn^{2+} and reactive oxygen species (ROS) generated by bare ZnO NPs [22]. Based on the fact that Fe doping of ZnO NP decreases its dissolution in cell culture medium without cells [23] and that Fe_2O_3 and Al_2O_3 have nearly 0% dissolution compared to that of ZnO NPs at about 30% [10], the altered dissolution of the ZnO NPs once doped could be the underlying cause for the differences observes in toxicity. So for metal oxide NPs, surface modification and doping elements should be taken into account when developing further models [24-25]. To optimize our NP descriptor selection, dissolution rate should also be considered for doped NPs. To improve upon current QSAR models, the following considerations occur:

(1) A potential solution is to include the dissolution rate of NPs in the data metrics, but the problem is that it is hard to estimate the dissolution rate of dissolvable metal oxides due to their modified surfaces by other organic molecules [25] and size effects by core materials. (2) An alternative way is to treat the dopant as a surface coating and use their molecular descriptors to partition the clusters based on PCA, but this will also result in the overlap of coating and doping NPs data. (3) The most suitable way is to consider the composition (surface ligand, doping element, core and shell element) of NPs as concentration-independent descriptors and released ion (can also include hydrodynamic diameter, agglomeration state and zeta potential) as concentration-dependent descriptors. (4) For further improvement, mortality used in our method can be replaced with other endpoints such as sublethal responses, particularly for materials classes in which numerous sublethal effects were observed.

The purpose of this article is to attract and stimulate the discussion and investigation on NP descriptor selection to refine future nanoQSAR models. Although the idea that combination of concentration-independent properties of NP surface modifications as variables is helpful for dimension reduction and cluster analysis, a widely applicable model still needs more information on concentration-dependent properties. More work is needed to investigate the properties of NPs for a better reflecting the physicochemical properties important in driving a toxicological response.

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Chapter 4 Conclusion

In manuscript 1, we investigated whether inherent properties of ZnO NP surface chemicals could be used to estimate NPs toxicity to embryonic zebrafish. Firstly, based on Fisher's exact test for all dosage and endpoints we observed, mortality was found to be the most significant endpoint in our embryonic zebrafish assay for ZnO NPs. Interestingly significant mortality occurred at 24 hours post fertilization (hpf) for coated ZnO NPs, but significant mortality was not observed for the uncoated ZnO NPs until 120 hpf. To represent the inherent properties of NP surface modifications with same ZnO as core material, we calculated 8 intrinsic properties that should be constant at different NP concentrations in our fish water. PCA was applied to reduce the NP descriptor dimension and two most dominant PCs were selected. Finally, an ordinary Kriging method was used to link the two PCs with mortality. Based on the physicochemical properties we selected, NPs can be partitioned into clear clusters after PCA and based on Euclidean distance. This classification process has also been useful to other QSAR modeling efforts.

In manuscript 2, we tried to expand our model range from ZnO NP with various surface modifications to ZnO NP doped with different atoms. New property values were adjusted based on the percentage of dopants (Fe_2O_3 and Al_2O_3) in ZnO NPs. Then we applied same standardization process on the properties data to calculate PCs. Finally, we found new PC locations on the previous coordination generated using kringing estimation. Unfortunately, the results showed that the estimations of doped ZnO NPs did not match our observations, which may be because the dopants altered the ZnO NP dissolution behavior.

From our study, the observed ZnO NP toxic responses combined with NP surface chemical properties are promising to be applied to estimate NP with same core material and similar surface modifications. Although our expanded test showed that the doped ZnO NP toxicity to zebrafish embryo could not be estimated based on our original model, model refinements could be incorporated to achieve better predictability for future models. This study has shown that data on similar NP core materials, with various surface modifications studied under identical experimental design protocols, are worthwhile in the development of models of nanoparticle-biological interactions. Based on our finding that the distribution coefficient log D of ZnO NP surface modifications was a stronger determinant of toxicity than any other surface features we investigated, determination of ZnO NP hydrophobicity characterization and its alteration by surface chemicals is of importance. More work concentrating on modified NP surfaces instead of bare ones will be helpful to accelerate the development of predictive models of ZnO NP-biological interactions. This study has shown that PCA is a useful tool for data dimension reduction, which can convert numerous surface molecular properties into fewer dimensions. Future investigation on more accurate predictive models will depend on detailed information provided for *in silico* modeling and comprehensive understanding of the outermost surface of the NPs.

As we discussed in manuscript 2, the inherent properties we chose are concentration-independent, which make it difficult to integrate across different concentration regimes. In order to combine different dosage estimations as an integrated one, the external properties affected by concentration needed to be introduced into our data preparation process and then represented in our model. But this kind of information certainly needs additional work on dissolution or other NP parameter measurement, which leads it a bit far away from a rapid estimation method with

only label information. Overall, identification of specific material features, such as outermost surface chemistry, which drive biological interactions appear feasible and models such as this should continue to be tested and refined to achieve safer design principles for the manufacture of ZnO NPs.