Article details

Verschoor A.J., Harper S., Delmaar C.J.E., Park M.V.D.Z., Sips A.J.A.M., Vijver M.G. & Peijnenburg W.J.G.M. (2019), Systematic selection of a dose metric for metal-based nanoparticles, NanoImpact 13: 70-75.
Doi: 10.1016/j.impact.2019.01.002

NanoImpact 13 (2019) 70–75

Contents lists available at ScienceDirect

NanoImpact

journal homepage: www.elsevier.com/locate/nanoimpact

Systematic selection of a dose metric for metal-based nanoparticles

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ARTICLE INFO

Keywords:
Dose metrics
Risk assessment
Nanomaterials
Dose response
Dosimetry

ABSTRACT

Mass is traditionally the unique measure of the administered dose in toxicity studies with conventional chemical substances. Because of the variety of specific physical properties of nanoparticles, other dose metrics such as the number of particles, their size, shape, surface area or volume may be more appropriate. Here we applied a systematic, unbiased approach to derive the most appropriate dose metric for nanoparticles from experimental data. The approach was exemplified for copper, zinc oxide, and silver nanoparticles with different diameters, coatings and shapes, combining experiments with six aquatic organisms, two mammalian and two piscine liver cell lines from different research groups. The nanoparticle diameter appeared to be a powerful estimator of metal oxide nanoparticle effects. Since effect concentrations were related to size to the power 3, it is indicated that volume (mass) is the appropriate dose metric for all tested species and toxicological endpoints and all tested metal oxide nanoparticles within the tested size range (25–500 nm). The new method enables extrapolation of test results from one type of metal oxide nanomaterial to another, thereby offering a powerful tool to improved efficiency in risk research and risk assessment of nanomaterials.

1. Introduction

With nanotechnology facilitating the creation of complex structures of different sizes, a whole new array of materials has made its appearance. The various properties of nanoparticles (NPs) such as size, shape and surface chemistry, may all contribute to their toxic potential (Clift et al., 2010; Park et al., 2011; Schaeublin et al., 2011). The high surface area to volume ratio of nanoparticles results in highly reactive and physico-chemically dynamic materials in environmental media. Many transformations, e.g. reactions with biomacromolecules, redox reactions, aggregation, and dissolution, may occur in both environmental and biological systems. These transformations will alter the fate, transport, and toxicity of NPs (Lowry et al., 2012). The interpretation of dose-response data, but also the expression of the estimated exposure for risk assessment purposes, requires an unambiguous description of the dose, i.e. a dose metric. An adequate dose metric includes all characteristics that are necessary to explain differences between responses in experiments. Given the differences in size and shape of NPs, it is questionable if the administered mass of the chemical substance alone is sufficient to uniquely describe the dose of NPs.

Understanding the drivers of effects of nanomaterials (NMs) would improve the risk assessment of NPs and enhance opportunities to extrapolate experimental results to NPs with different characteristics, thus reducing the need for animal testing. Three major phenomena driving the toxicity of nanoparticles were reported to be: (i) dissolution of nanoparticles, (ii) cell type-dependent cellular uptake of NPs and (iii) induction of oxidative stress and consequent cellular damage (Ivask et al., 2014).

A common characteristic of the metal oxide nanoparticles included in the studies reported here is their relatively high dissolution rate under physiological conditions. As a consequence of dissolution, metal ions are formed that will contribute to the adverse effects imposed by the suspensions tested. A challenge in the search for a common dose metric for NPs is to distinguish the direct “nano” effects from the effects of the dissolved ions. When toxicity of a certain mass of a certain NP is different from an equal dose administered as a dissolved substance, kinetic processes may play a role (Harmon et al., 2014).

NPs interact in a different way with living cells as compared to dissolved molecules. Dissolved metal ions may cross the cell membranes by regulated uptake processes, with subsequent cellular effects.

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https://doi.org/10.1016/j.impact.2019.01.002
Received 14 November 2018; Received in revised form 9 January 2019; Accepted 11 January 2019
Available online 12 January 2019

The mode of action of NPs is still a topic of scientific debate. Ion release from NPs could also occur inside cells (following uptake of the intact particle), thereby circumventing the normal uptake control mechanisms that apply to ions (Park et al., 2010). For metal oxide NPs the dissolution and corresponding ion activity may blur the particle toxicity. A way to resolve the mode of action is by experimental studies such as genomics and cellular toxicology. An alternative is a mathematical approach to deduce the most optimal metric to relate to the mode of action. Size related metrics such as diameter (~d), surface area (~d^2) or volume (~d^3) are linked to the mode of action (Zhu et al., 2013). A relation between toxicity and particle size is indicative of the importance of cellular uptake mechanisms of the nanoparticle itself or of essential nutrients. For the uptake of nanoparticles, different mechanisms such as phagocytosis and pinocytosis are described, which are limited to a defined size range or specific surface properties (Kettiger et al., 2013). Moreover, it was demonstrated for example that gold nanoparticles in hippocampal neurons of mice caused physical blockage of potassium ion uptake channels (Salinas et al., 2014). A relation of surface area with toxic effects indicates the role of protein binding, oxidative stress or disturbance on the membrane potential of the NPs (von Moos and Slaveykova, 2014). NPs with a high surface reactivity are more likely to disturb protein functions on the cell membrane or inside the cell.

The identification of an appropriate dose metric for NPs is of utmost importance for the interpretation of results from toxicity experiments and for risk assessment purposes, but so far a systematic evaluation is lacking of what such a dose metric may be. Research on dose metrics has been performed only for isolated cases, using ad hoc methods, and is mainly focussed on inhalation toxicology (Sager and Castranova, 2009; Pauluhn, 2011; Pompa et al., 2011). As a consequence, conclusions cannot easily be compared or generalized to other materials and experimental systems. Several Quantitative Nanostructure-Activity Relationships (QNARs) have been proposed, including several types of descriptors: 1) structural morphological properties, such as size, shape and surface area, 2) physicochemical properties such as zeta-potential, surface charge, acidity coefficient and isoelectric point, 3) constitutional properties such as molecular weight and cation charge, and 4) electronic or thermodynamic properties such as electronegativity and ionization energy. A concise review of these properties and there use in QNARs is available (Ying et al., 2015). Certain particle properties influencing the response are uniquely related, such as particle size with surface area, reactivity and solubility, in such a way that only one parameter combining these properties needs to be included in the dose metric.

Risk assessment usually comprises several tiers, where each tier involves more efforts and costs but also reduces uncertainties and increases realism (Koelmans et al., 2015). For risk assessment purposes, easy to determine parameters are preferred. Structural morphological properties such as size, total number of particles, surface area or volume have been suggested as potential simplified dose metrics (Wittmaack, 2007; Griffitt et al., 2008; Klaine et al., 2008). Delmaar et al. introduced a mathematical approach to systematically select the most appropriate dose metric from dose-response data, and to assess the mathematical relation between this metric and NM toxicity (Delmaar et al., 2015). The method is focussed on properties linked to a particular NM with different size, surface area, volume and mass. The approach does not require a hypothesis of the adequate dose metric or underlying mode of toxic action a priori. As a proof of principle, the method was applied to a limited number of in vitro and in vivo experiments with spherical-shaped silver and silica NPs. The results indicated that the best dose response metric varied with test species and the type of endpoint considered. For example photosynthesis by *Raphidocelis subcapitata* showed a relation with total volume of the Ag nanoparticles (irrespective of their size within the range of 13–80 nm), whereas immobilization of *Chydorus sphaericus*, and morphology of *Danio rerio* and teratogenic effects could be related to the surface area of these Ag nanoparticles. The total nanoparticle surface area was also a good descriptor for metabolic activity in L929 fibroblasts and induction of reactive oxygen species in RAW264.7 macrophages.

In the current study we further elaborate on the method of Delmaar et al. The aim was to extend the applicability of the method to differently shaped NPs, for instance spheres, rods and cubes. The statistical approach to systematically select the most adequate dose metrics will support the search of the mechanistic background of effects of NPs, and provide practical rules for the risk assessment of NPs.

2. Materials and methods

2.1. Experimental data

We collected published data involving Cu, ZnO and phosphate coated Ag NPs of different sizes and shapes, comprising toxicity data of a variety of aquatic species (fish, cladoceran) and mammalian and fish cell lines. A primary key criterion for data selection was the availability of a set of toxicity data related to the same endpoint of toxicity for a set of NPs differing only with regard to their size and shape whilst all other particle characteristics like chemical composition and coating were the same. An overview of the data used in given in Table 1. As the dose metrics were developed to estimate toxicity of particles only, the contribution of metal ions toxicity was subtracted from the overall observed effect. This was done either by inclusion of a positive metal ion control or by using phosphate coated Ag particles. In the latter case, the contribution to toxicity of Ag^+-ions is assumed to be negligible given the extremely low dissolution rate of Ag from phosphate-coated NPs, given the solubility product constant of $8.89.10E^{-17}$ (Website Solubility of Things, 2016).

2.2. Modelling procedure

Dose-response experiments involve testing a range of NPs that vary both in particle number (N) and in NM properties (e.g. size, shape). Other potentially relevant dose metrics such as surface area and volume are related to size and shape. The classical metric in toxicology is mass concentration. Mass and volume of a particular metal oxide NP are interchangeable because they are related by the specific weight. For NPs which differ in metal component or structure (porosity) this is not the case.

Essentially, equal doses (specified in the appropriate metric) should give an equal response in an experimental system. For spherical NPs two NP characteristics are of interest; number of particles N and diameter d. The relation between N, diameter d and $EC_{x\%}$ can be visualized by a two-dimensional contour plot, that shows equi-response lines dependent on N and d (Delmaar et al., 2015). EC_{50} values were used in our study.

To find out which dose metric: number, size, surface or volume is most relevant, the following relation was fitted to the experimental data:

$$EC_{x\%} \sim N \times d^a \quad (1)$$

The value of a indicates which metric is the most appropriate one to describe differences in toxicity between differently sized NPs. If $a = 0$, the effect only depends on the number of particles, if $a = 1$ the effect depends on number of particles and the particle size, if $a = 2$ the effect is related to number of particles and the surface area, and if $a = 3$ the effect is related to the total volume of particles administered (Delmaar et al., 2015). The value of a can easily be estimated by linear regression after log-transformation of Eq. (1).

$$\log EC_{x\%} = \log N + a * \log d \quad (2)$$

where a is represented by the slope of the regression line, d is the particle radius (in nm), $\log N$ is indicative of the intrinsic toxicity of the particles tested and represents the number of particles with a diameter

Table 1
Toxicity data of nanoparticles used for modelling.

Species	Endpoint	Test duration	Substance	Nominal size (nm)	Actual size (average value of d - nm)	$EC_{50\text{-}NP}$ (mg/L)	Shape	Ref.
D. magna	Mortality	48 h	Cu-NP	25	48	0.103	Sphere	(Song et al., 2015)
				50	144	0.152		
				100	113	0.099		
				500	500	0.106		
D. pulex	Mortality	48 h	Cu-NP	25	48	0.007	Sphere	
				50	144	0.04		
				100	113	0.052		
				500	500	0.03		
D. galeata	Mortality	48 h	Cu-NP	25	48	0.01	Sphere	
				50	144	0.055		
				100	113	0.033		
				500	500	0.017		
C. dubia	Mortality	48 h	Cu-NP	25	48	0.002	Sphere	
				50	144	0.002		
				100	113	0.003		
				500	500	0.006		
C. sphaericus	Mortality	48 h	Cu-NP	25	48	0.052	Sphere	
				50	144	0.045		
				100	113	0.027		
				500	500	0.015		
H4IIE	Mitochondrial oxido-reductase activity	24 h	Cu-NP	25	48	9	Sphere	(Song et al., 2014)
				50	144	27		
				100	113	48		
				500	500	4		
HepG2	Mitochondrial oxido-reductase activity	24 h	Cu-NP	25	48	7	Sphere	
				50	144	28		
				100	113	33		
				500	500	9		
PLCH-1	Mitochondrial oxido-reductase activity	24 h	Cu-NP	25	48	22	Sphere	
				50	144	39		
				100	113	25		
				500	500	12		
RTH-149	Mitochondrial oxido-reductase activity	24 h	Cu-NP	25	48	30	Sphere	
				50	144	77		
				100	113	65		
				500	500	12		
D. rerio	Mortality	120 hpf	Cu-NP	25	20–40	0.58	Sphere	(Hua et al., 2014a)
				50	30–50	1.65		
				100	120–200	1.9		
				400	200–500	0.35		
	Mortality	120 hpf	ZnO-NP	43	27	12.5–16.2	Sphere	(Hua et al., 2014b)
				150	32 × 81	6.9–8.4	Stick	
				900	202	9.6–10.5	Cube	
D. rerio	Mortality	120 hpf	Ag-Pcoat		20.3	21.62	Sphere	(Tuttle, 2012)
					34.4	73.41		
					41.9	63.70		
					52.9	56.76		
					61.2	66.47		
					67.3	19.77		
					79.8	61.85		
					90.8	40.12		
					102.3	136.8		
					112.6	73.41		

of 1 nm per volume unit causing x % adverse effect, $EC_{x\%}$ is expressed in units of mg/L. The modelling assessment was performed by using the actual primary size, as determined by Tunnelling Electron Microscopy (TEM) in the experimental studies and by the researchers as described in the respective publications.

2.3. Dealing with non-spherical nano-particles

NPs of different shapes cannot be compared based on a single size metric; for spheres the diameter is a unique descriptor, for cubes the length of one side is a unique descriptor and for rods (cylinders) the diameter and the length are required to describe the dimensions. Taking the mean size of non-spherical particles for fitting may result in additional variation and uncertainty in the fitted slope a. Here we elaborate on the method of (Delmaar et al., 2015) and extend it to non-spherical nanoparticles. In order to determine if these differently shaped NPs fit in the proposed equi-response dose approach, three different options for normalisation of non-spherical NP sizes were tested: 1) an area based diameter, 2) a volume based diameter, and 3) a weight based diameter. An area-based diameter is the diameter of a particle that reflects the diameter of a spherical particle with the same surface area. Similarly, a volume-based diameter is the diameter of a particle that reflects the diameter of a spherical particle with the same volume. A weight-based diameter is the diameter of a particle that reflects the diameter of a spherical particle with the same weight. For particles of the same chemical composition and porosity, volume and weight are interchangeable, because they are uniquely related by the specific density. Dimensions for differently shaped NPs are presented in Table 2. The normalised size D can replace the sphere's diameter d in Eqs. (1) and (2), to derive the value of the exponent a. This will indicate which is the

Table 2
Dimensions of spherical, cylindrical and cuboidal NPs. A normalised size (D) is derived from volume and/or surface area to be able to compare the toxicity of differently shaped NPs in the same equi-response curve. d = diameter of a sphere, L = length.

		Spheres	Rods	Cubes
	Size (nm)	d	d, L	L
	Surface area (nm^2)	πd^2	$½\ \pi d^2 + \pi dL$	$6L^2$
	Volume (nm^3)	$^1/_6\ \pi d^3$	$¼\ \pi d^2 L$	L^3
Normalised size D				
Option 1	Area-based size	d	$\sqrt{\frac{Area_{rod}}{\pi}}$	$\sqrt{\frac{Area_{cube}}{\pi}}$
Option 2	Volume based size	d	$\sqrt[3]{\frac{6\times Volume_{rod}}{\pi}}$	$\sqrt[3]{\frac{6\times Volume_{cube}}{\pi}}$

most appropriate metric for differently shaped NPs.

For each bioassay, the reported critical dose was expressed in mg/L or µg/mL. The mass was subsequently transformed to the corresponding number of particles, using specific densities of 8.96 g Cu/cm^3, 5.606 g ZnO/cm^3 and 10.5 g Ag/cm^3. The number of particles corresponding to the EC_{50} on a mass basis is:

$$N50 = \frac{EC50 \cdot 10^{-6}}{Specific\ Density \cdot NPvolume} \quad (3)$$

where N_{50} is the equi-response dose expressing the number of particles causing 50% effect, EC_{50} is the mass based 50% effect concentration in µg/mL, *SpecificDensity* is the specific density of the nanoparticles in g/cm^3 and *NPvolume* is the actual volume of the nanoparticles in cm^3. Hence, a complete description of the dose is given by specifying the number of particles N and their size (i.e. a dose consists of N particles with size d). Every dose can be presented in an (N, d) plot. An equi-response curve was constructed by plotting different doses (N_i, d_i) that correspond to the same response. More details about equi-response dose curves are provided by (Delmaar et al., 2015). The experimental data were tested on outliers. Regression analysis was performed with and without outliers. Statistical analyses were performed with R Cran statistical software and GraphPad Prism 6.05.

3. Results and discussion

3.1. Spherical phosphate coated AgNP

The equi-response curve for the spherical phosphate-coated AgNP in zebrafish is shown in Fig. 1. The initial fit, using all data points, resulted in a slope a of -2.61 ± 0.29. For the final fit, the first data point (size

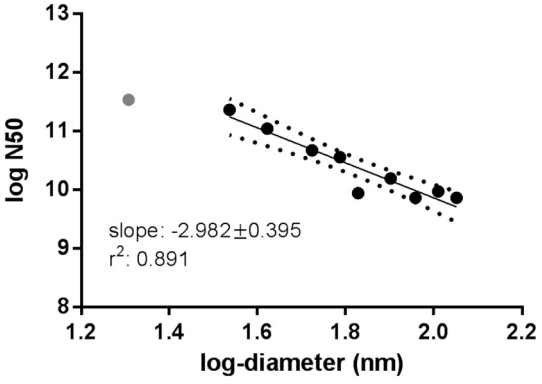

Fig. 1. Equi-response curve of spherical phosphate coated silver nanoparticles tested on zebra fish embryos. Log base = 10. The first datapoint (grey) was removed from the fit, due to high leverage.

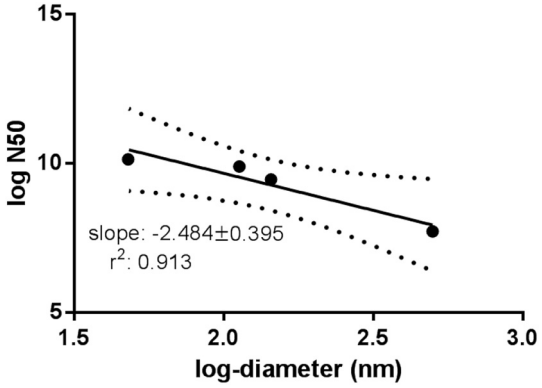

Fig. 2. Equi-response curve of spherical Cu nanoparticles tested on *Daphnia pulex*. Log-base = 10.

20.3 nm) was removed from the statistical analysis because of its high leverage, which implies that it would have an unjustified high impact on the outcome of the regression analysis (see Supplemental Information). This specific particle could be an occasional statistical outlier, but the response observed could also be due to a different mechanism of toxicity because it concerns the smallest particle. Fig. 1 basically shows that many small NPs have the same impact as a lower number of larger NPs. The final fit resulted in a slope of -2.98 ± 0.39. The 67.3 nm sized particle was outside the 95% confidence interval. Removal of this data point hardly changed the slope of the fit, although the confidence interval became narrower (-2.97 ± 0.20). As the slope of -3 is within the confidence interval of the best fit, it is to be concluded that the total volume of the nanoparticles is the most appropriate dose metric to explain differences in toxicity of differently sized spherical Ag nanoparticles to *D. rerio* embryos in this experiment.

3.2. Spherical CuNPs

The equi-response curve for the spherical CuNPs to *Daphnia pulex* is shown in Fig. 2. A complete overview of the graphical results of the other four crustaceans, fish and mammalian cell-lines is included in the Supplemental Information S2 and S3. The intercepts and slopes of the equi-response curves for all species and cell lines tested, are given in Table 3. The distribution of data on the x-axis is not very homogeneous. It appeared that except for the *Daphnia pulex* data, the leverage of the smallest and/or the largest nanoparticles was quite high, meaning that they may have had a disproportional influence on the regression. However, because of the limited number of data per species (n = 4) all the data were used for the regression. The slopes for the different species and cell lines varied from -2.48 to -3.51, and were not significantly different ($p = 0.73$). The mean slope was -3.08 ± 0.38, again implying volume as the most appropriate dose-response metric. The intercepts were significantly different ($p < 0.0001$). These results

Table 3
Results of fitting log (N50) versus log (diameter) for spherical Cu nanoparticles tested in several aquatic species and human and piscine liver cell lines.

Species or cell line	Intercept	Slope	se	*F*-statistic	*p*-Value	Adj. R^2
Danio rerio	17.80	−3.27	0.45	52.5	0.019	0.95
Daphnia magna	16.34	−2.98	0.14	427.2	0.002	0.99
Daphnia pulex	14.63	−2.48	0.54	20.9	0.045	0.87
Daphnia galeata	15.35	−2.84	0.53	28.3	0.034	0.90
Ceriodaphnia dubia	13.80	−2.54	0.19	180.9	0.005	0.98
Chydorus sphaericus	16.92	−3.51	0.20	319.0	0.003	0.99
H4IIE (rat)	19.54	−3.49	0.74	22.5	0.042	0.88
HepG2 (human)	18.51	−3.00	0.57	27.3	0.035	0.90
PLCH-1 (fish)	19.28	−3.28	0.30	123.1	0.008	0.98
RTH-149 (fish)	19.89	−3.47	0.53	43.4	0.022	0.93

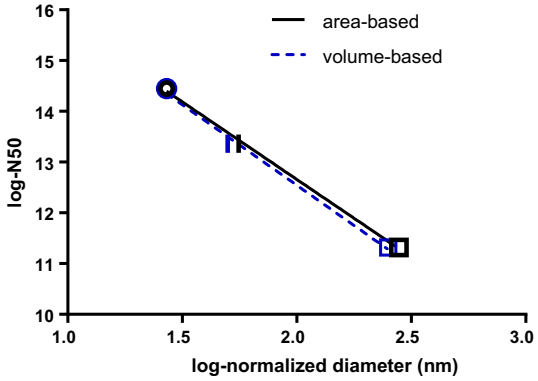

Fig. 3. Equi-response curve of differently shaped ZnO nanoparticles tested on zebrafish embryos. The shape of the particles is indicated by the markers. Log-base = 10.

indicate that intrinsic sensitivities (indicated by the intercept) between species are highly different, but dose metrics (indicated by the slopes) are similar. Results should be considered indicative, and more experiments with differently sized nanoparticles are required to decrease uncertainties in the fitted slopes and intercepts.

3.3. ZnO-NPs: cubes, rods and spheres

In order to plot differently shaped nanoparticles in an equi-response dose curve the size of the particles had to be normalised (Fig. 3). The equi-response dose fitting results are given in Table 4. Both the area-based and the volume-based size normalisation methods led to an acceptable fit of the equi-response dose fit of differently shaped ZnO NPs. The fit of area-based sizes versus equi-response dose was slightly better than the fit of volume-based sizes with equi-response dose, as is demonstrated by the higher residual standard error and higher *p*-value and the lower adjusted value of R^2 of the latter. The slopes of the corresponding equi-response curves were approximately 3, indicating that volume is the most appropriate dose metric to compare differently shaped ZnO nanoparticles. The slopes and intercepts are not significantly different, so it cannot be concluded which normalisation method is better. More data of differently shaped and sized NPs are required to improve the statistical power.

3.4. Recommendations for further experimental studies on dose metrics

The proposed approach has been applied in a number of examples. The examples serve mainly as an illustration of the approach and as proof of principle. To derive more rigorous conclusions, the experimental setup of the test systems used, could be improved in several ways.

Table 4
Results of normalisation of particle size for differently shaped ZnO NPs and fitting log (N50) versus log (normalised size) for ZnO NPs tested in zebrafish embryos.

	Actual size	Area based size	Volume based size
Sphere	d = 27 nm	27	27
Stick	d = 32 nm, L = 81 nm	56	50
Cube	L = 202 nm	279	250
Intercept		18.77	18.89
Slope		−3.06	−3.17
Residual standard error		0.09198	0.1759
p-Value		0.02606	0.04988
Adjusted R^2		0.9967	0.9877

Except for the Ag-NPs, equi-response curves were inferred on the basis of a relatively sparse number of equi-response levels, although each of the equi-response curves themselves was based on multiple experimental replicates (at least duplicates). The number of equi-response datapoints proved to be sufficient in most cases, as the method convincingly pointed to the existence of a simplified dose metric. However, in general, identification of an adequate dose metric, especially in more complex cases, will always benefit from including more equi-response data by investigating a larger range of NPs varying in one characteristic such as particle size, as well as improving the quality of the dose response curves of one specific NM by using more dose levels and replicates. More specifically, studies with at least three different variations in a NM characteristic (e.g. size) are required to verify whether the equi-response levels fit a straight equi-response curve.

Another issue to consider when designing experiments to analyze dose metrics is the fact that in practice, most NPs will consist of particles with a distribution of characteristics (for example, particle diameters) rather than having a single well-defined value for this characteristic. Preferably, for the purpose of studying dose metrics, distributions of particle characteristics within a NM should be as narrow as possible.

3.5. Implications for applying simplified dose metrics in risk assessment of nanoparticles

The approach to identify appropriate dose metrics applied in this paper provided a way to study the dose metric in a systematic way. The method is unequivocal and can be applied to any test system to directly determine the simplified dose metric from experimental data, if it exists. More specifically, it allowed for an unbiased assessment of whether specific scenarios (i.e. 'the response is determined by administered surface area/volume/number of particles') are to be accepted or rejected. Also, the approach does not require a detailed understanding of the complex and dynamic interactions of NPs with (biological) test systems a priori; such as aggregation, and heteroaggregation. Aggregation is a process that was demonstrated in the experiments from which we derived the EC50-values. It is shown that the sizes of the aggregates vary with concentration ranges of NP with identical primary sizes (Zhai et al., 2016). Currently it is not possible to mechanistically account for these processes in the evaluation of toxicity experiments.

Our data indicate that it is possible to adequately describe the dose of all the tested NPs by one and the same simplified dose metric for Ag, Cu, as well as Zn NPs, even when the nanoparticles are differently shaped. The slope of the log-log relation between the normalised size and the equi-response dose is in all cases close to 3, which indicates a relationship between total volume (or mass) and the effect. This relation was found for all species and cell lines tested, since the slopes for individual species were not significantly different. The results do indicate that in most of our in vitro and in vivo experiments a simplified dose metric was appropriate for NPs of the same chemical composition but with different diameters. A dose metric identified in this manner can be used to predict effect levels for NPs of the same chemical composition consisting of particles with different distributions in the parameters (for example, different sizes or size distributions), provided that these parameter distributions are within the range of distributions of the NPs that were tested to identify the dose metric.

As with any experimental findings with NPs, it needs to be realized that the dose metrics derived may only be valid under certain (experimental) conditions, and therefore, these conditions need to be clearly defined. Agglomeration of nanoparticles, which is very dependent on the experimental or environmental conditions, will affect the uptake and toxicity of NPs. For further use, systematic research into appropriate dose metrics of a wide variety of NPs as well as mechanistic understanding of the dose metrics and the impact of environmental conditions may help to understand the applicability and limitations of the dose metric.

The toxicity of Ag, Cu and ZnO NPs, is amongst others determined by the dissolution rates of metals inside and outside the cells. Dissolution rates are modified by coatings, such as the phosphate coating on the Ag NPs in this study. These effects, which involve the intrinsic sensitivity of species for a NPs, were reflected by the different intercepts resulting from the equidose-response fits. The current method does not provide a descriptor to a priori account for differences types of coated or uncoated metal oxide NPs.

In conclusion, identifying adequate dose metrics for NPs is highly relevant for interpreting results from toxicity studies, for risk assessment and regulatory purposes. The approach, first published by (Delmaar et al., 2015) to determine whether a simplified metric such as volume or surface area is appropriate to describe the dose of NPs in in vitro and in vivo studies, was very useful and indicated that volume was the most appropriate dose metric to describe the effects of phosphate-coated Ag, Cu and ZnO NPs of different size and shape in a variety of aquatic organisms and in mammalian and piscine cell lines.

Acknowledgements

The research described in this contribution was the result of the IRAN project, which is part of the strategic research program of RIVM.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.impact.2019.01.002.

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