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Review

A micro-sized model for the in vivo study of nanoparticle toxicity: what has *Caenorhabditis elegans* taught us?

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Environmental context. The ability of the soil nematode *Caenorhabditis elegans* to withstand a wide range of environmental conditions makes it an idea model for studying the bioavailability and effects of engineered nanomaterials. We critically review what has been learned about the environmental fate of engineered nanoparticles, their effects and their mechanisms of toxicity using this model organism. Future systematic manipulation of nanoparticle properties and environmental variables should elucidate how their interaction influences toxicity and increase the predictive power of nanomaterial toxicity studies.

Abstract. Recent years have seen a rapid increase in studies of nanoparticle toxicity. These are intended both to reduce the chances of unexpected toxicity to humans or ecosystems, and to inform a predictive framework that would improve the ability to design nanoparticles that are less likely to cause toxicity. Nanotoxicology research has been carried out using a wide range of model systems, including microbes, cells in culture, invertebrates, vertebrates, plants and complex assemblages of species in microcosms and mesocosms. These systems offer different strengths and have also resulted in somewhat different conclusions regarding nanoparticle bioavailability and toxicity. We review the advantages offered by the model organism *Caenorhabditis elegans*, summarise what has been learned about uptake, distribution and effects of nanoparticles in this organism and compare and contrast these results with those obtained in other organisms, such as daphnids, earthworms, fish and mammalian models.

Additional keywords: bioavailability, gene expression, mechanism of toxicity, uptake.

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The challenge of nanotoxicology

Nanotoxicological studies are of particular importance because of the possibility that manufactured nanosized particles may have unique biological effects, just as they have unique physical and chemical properties. Nanosized particles are produced in mass quantities anthropogenically and naturally. The focus in this review is on the first category, which is often referred to as 'manufactured' or 'engineered' nanoparticles. For simplicity, we will henceforth use the term 'NPs' to refer to all categories of manufactured NPs, including carbon-based as well as metal-based NPs. Past introductions of products with novel properties (e.g. the persistent organic pollutants addressed by the Stockholm Convention) have taught us toxicological lessons the hard way. Our current challenge is to gain critical insights about NP toxicity ahead of time.

Toxicological studies of NPs are complicated by their unique properties.[1] Chemical and toxicological paradigms are frequently not applicable. For example, oil–water partition coefficient (K_{ow}) values inform our understanding of environmental fate and transport as well as organismal uptake and distribution of organic molecules. However, there are experimental challenges in measuring K_{ow} for NPs, such as the distribution of NPs into the interface between octanol and water due to high surface activity. K_{ow} values for NPs have not been extensively linked with environmental fate or bioavailability.[2] Other considerations (e.g. acid dissociation constants, pK_a) may have some application but must be interpreted somewhat differently in the context of particles that may have a very large number of potentially unevenly distributed (among and between NPs) sites of protonation. Furthermore, we must incorporate additional consideration of physicochemical properties that are not often considered in the toxicology of discrete chemical species, such as particle size, shape, crystallinity, complex surface chemistry, aggregation state and inherent heterogeneity,

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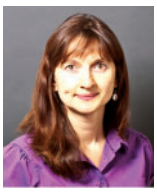

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variably stable coatings, impurities and, in some cases, dissolution.[3] Some of these are well studied in other fields (e.g. colloid science), but are not familiar to most toxicologists. Finally, nanotoxicological studies are complicated by some of the same factors that remain challenging in the fields of human health toxicology and ecotoxicology, including environmental variables (temperature, sunlight, presence or absence of other organisms, medium constitution including pH, salts, natural organic matter, sediments, etc.) and the potential for co-exposure to other stressors. Toxicologists must consider effects not just of pristine NPs, but also of environmentally modified NPs.

Nonetheless, some key toxicological concepts can still be employed, and may in fact be of more rather than less importance in the context of NPs. In particular, we are increasingly convinced that a fuller appreciation of the importance of the ADME (absorption, distribution, metabolism, excretion) paradigm for organismal toxicity[4] will be critical to a realistic evaluation of NP toxicity. We argue that because of their size, compared to chemicals, even the smallest NPs face very significant barriers to uptake in most living test systems, with the barriers being least significant for cells in culture because they are protected only by a cell membrane. All organisms have significant barriers to the environment: cell walls in the case of microbes, and cuticles, exoskeletons, epidermal layers, scales and so forth in the case of metazoans. However, even when NPs cannot pass through most of the organismal barriers, they can still bioassociate with the membranes and may cause toxicity due to such contact. Although cell membranes may have pores large enough to permit passage of smaller NPs, this is not generally true of the portions of free-living organisms that are in contact with the environment, with important exceptions such as gills, lungs, sensory organs, mucous membranes and gastrointestinal cells. Similarly, endocytosis results in ready uptake of NPs by cells in culture, but not across most epidermal barriers. Nonetheless, some studies have demonstrated penetration by NPs of some epidermal barriers, for instance through hair follicles and sweat glands.[5] The integrity of the skin barrier will also influence the uptake of NPs. The fact that life has evolved with constant exposure to naturally occurring NPs[6] further suggests that many organisms may have developed mechanisms to avoid or to adapt to the uptake of nanosized particles, although of course these putative defences may fail, depending on the exposure, and on the fact that the elemental content of the core and coatings of manufactured NPs differ from naturally occurring NPs. As a result, extrapolating data from cells in culture to an in vivo context is even more challenging than it is in traditional (chemical) toxicology, and careful analysis of uptake is even more important for NPs than for chemicals that may cross many biological barriers.

Extrapolation across biological levels and between models is also problematic in nanotoxicology. In vitro toxicity experiments are often conducted using only a few cell types. This approach does not take into consideration variability in sensitivity among different cell types and would also be unpredictive of emergent organismal responses (e.g. reproduction, behaviour). Results from in vitro studies are also not really applicable for ecotoxicological studies, where endpoints that are relevant to the population level responses (e.g. reproduction) should be selected. Thus, although in vitro (cell culture) experiments offer some strengths, it is critical to complement such work with nanotoxicological studies performed using whole organisms. Use of organisms with short generation times facilitates the ability to screen the effects of combinations of interactions between physicochemical properties inherent to the NPs and external environmental factors that are extrinsic to NP properties. For mechanistic studies, it is helpful to use well characterised organisms such as *Caenorhabditis elegans*, for which plentiful genomic information and functional genomic tools (mutant and transgenic strains and RNA interference (RNAi)) are available. *Caenorhabditis elegans* can also serve as a model for medium–high throughput screening (MTS-HTS) of NP toxicity (e.g. for mortality, growth and reproduction endpoints), as successfully shown previously with other toxicants.[7,8] In order to keep up with the rapid pace of innovation in nanotechnology, regulatory toxicity testing regimes will likely need at some point to also rely on MTS-HTS approaches.[9] In the context of these challenges, we propose that the nematode *C. elegans* is particularly suited to the study of nanotoxicology.

Advantages of *C. elegans* in nanotoxicological studies

Caenorhabditis elegans is a free-living, transparent nematode ~1 mm in length with a life cycle of ~3 days and an average life span of 2–3 weeks.[10] It was the first multicellular organism to have its genome completely sequenced,[11] and thus became one of the most important model organisms in various biological fields. Important biological phenomena such as apoptosis and RNAi[12,13] were first discovered in *C.elegans*. Its natural habitat and population biology, however, are less understood. Recent work has shown that *C. elegans* is often found in decaying plant material in nature[14] rather than being principally a soil nematode as frequently stated in earlier literature. *Caenorhabditis elegans* develops through four larval stages (i.e. L1–L4) before reaching the adult stage. 'Dauer' larvae are a stress resistant larval stage that develops in place of the 3rd larval stage under conditions of crowding, food depletion and high temperature.[15]

After several decades of rapid growth and success as a model organism in the fields of genetics and developmental biology, the use of *C. elegans* in toxicology has increased greatly in recent years.[16–22] In Table 1 we describe advantages and disadvantages of *C. elegans* in nanotoxicity studies, with comparisons to other important model organisms. Several attributes that make it particularly useful for toxicology are the short reproductive life cycle, large number of offspring and ease of maintenance, all of which make feasible systematic

Table 1. Advantages and disadvantages of *C. elegans* for nanotoxicity studies in comparison with other animal model organisms
MTS-HTS, medium–high throughput screening; NP, nanoparticle

Organisms	Advantages	Disadvantages
C. elegans	(i) Permits medium- to high-throughput toxicity experiments (MTS-HTS) and long-term multi-generational studies because of short generation time, ease of culturing, small size and large brood sizes	(i) poor system for toxicity detection in some organ systems, e.g. pulmonary
	(ii) Allows performance of aquatic and soil nanotoxicity experiments because of its ability to survive in both media; can live in media with low ionic strength, which is required when testing some NPs	(ii) less ecological relevance
	(iii) can provide guidance for ecotoxicity and human health studies with NPs because of high degree of molecular conservation	(ii) small size limits individual NP uptake and elimination studies
	(iv) offers most of the genetic power of single-celled systems in the context of the biological complexity of a multiple well developed organ systems	
	(v) provides high mechanistic power because of well established functional genomic tools (transgenic and mutant strains, RNAi)	
	(vi) RNAi is achieved easily through feeding	
Daphnia spp.	(i) suitable for rapid toxicity screening using mortality, reproduction and also for multigenerational experiments because of short life cycle and high number of offspring	(i) no functional genomic tools are available, limiting testing of mechanistic hypotheses
	(ii) toxicogenomic approaches available because of recently sequenced *Daphnia pulex* genome	
	(iii) high ecological relevance thus suitable for bioaccumulation and transfer of NPs in food chain studies	
Earthworms	(i) highly relevant for soil exposure	(i) functional genomic tools are not available
	(ii) larger size permits easier detection and analysis of internal NP distribution	(ii) mortality and reproduction toxicity experiments takes more than 10 times longer than in *C. elegans*, thus, not suitable for HTS
	(iii) better model for NP uptake and elimination assays	
Zebrafish and Japanese medaka	(i) excellent model for developmental toxicity assays	(i) not as easy and cheap to maintain as *C. elegans*
	(ii) ecotoxicological relevance of chorion, the barrier of embryos, for NP uptake studies.	(ii) nanotoxicity experiments are mainly performed on embryos because of larger adult sizes
		(iii) may need to remove chorion because of its impermeability to NPs
		(iv) limited functional genomic tools
Mammalian models	(i) rich literature and database of biological information	(i) do not reflect current trend of reducing animal use in toxicity studies
	(ii) high throughput screening power for in vitro models	(ii) limitations when extrapolating results between in vitro and in vivo models
	(iii) most realistic in vivo models for estimating risk and effects of NP exposure to humans	(iii) very high cost associated with maintenance and use of in vivo mammalian models
		(iv) limited sample sizes
		(v) limited functional genomic tools

investigations that may yield information permitting prediction of NP toxicity. *Caenorhabidits elegans* can be cultured either on solid or in liquid media, using either highly controlled media or more natural, complex media, such as soils[23,24] or sediments.[25] Although *C. elegans* is not principally found in soil, it can nonetheless be conveniently cultured in soil and exposed to environmental stressors such as NPs. Full life cycle effects including developmental and reproductive toxicity can be studied in a short period of time, and developmental nanotoxicity may be particularly well suited for analysis in *C. elegans* because of the normally invariant, and fully mapped, pattern of cell division that occurs in all somatic tissues. The ability to extrapolate results from *C. elegans* to ecotoxicology is improved by the ability to manipulate environmental variables including medium chemistry, temperature, pH, oxygen tension, etc. *Caenorhabditis elegans* is able to tolerate a wide range of environmental conditions, permitting analysis of the effects of environmental variables including temperature and chemical composition and pH of the medium.[26,27] *Caenorhabditis elegans* also offers the ability to relate mechanistic insights to human health, because of the high degree of molecular conservation and outstanding molecular, genetic and genomic tools.[12]

We highlight two particular strengths in the context of nanotoxicology. First, *C. elegans* permits the study of organismal uptake of NPs, and their distribution in whole organisms, because of its small size and transparency (Fig. 1). This allows efficient yet careful analysis of digestive tract absorption and subsequent distribution (there is currently no evidence for cross-cuticle uptake). For instance, as shown in Fig. 1, the distribution of Au in nematodes exposed to Au NPs can be observed using X-ray fluorescence microscopy; further, the composition of Au was confirmed as elemental Au by X-ray absorption near-edge spectroscopy (μXANES) indicating that the Au NPs were being taken up by the nematodes as intact particles.[28] Second,

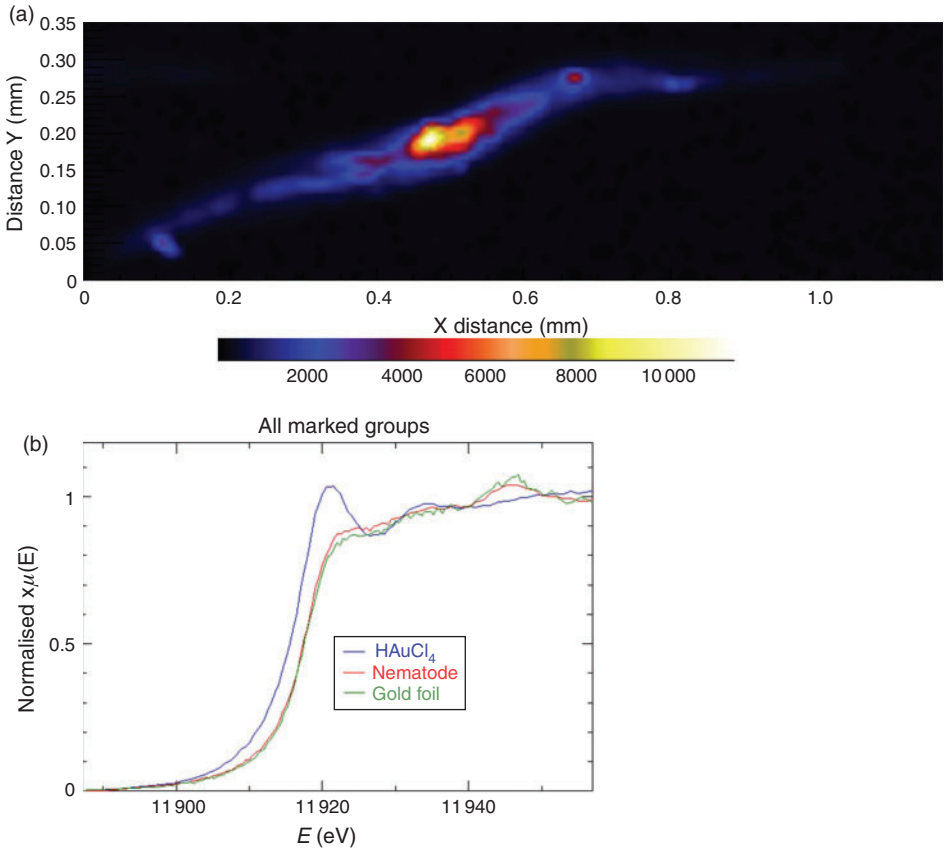

Fig. 1. Synchrotron X-ray fluorescence microprobe (μXRF) map of (a) Au at $Au_{L\alpha}$ in *C. elegans* exposed for 6 h to 20 mg L^{-1} of 4-nm citrate-coated Au in 50 % K-Medium. (b) Speciation for a pixel from the area of high Au abundance was determined with X-ray absorption near-edge spectroscopy (μXANES) as metallic Au^0 (adopted from Unrine et al.[28]) (*E*, energy).

C. elegans offers much of the genetic power of single-celled systems such as yeast or microbes in the context of the biological complexity of a metazoan with multiple well developed organ systems. The availability of two RNAi libraries and two mutant consortia that respectively cover ~90 and >30 % (and growing, in each case) of the genome permits a very powerful approach to mechanistic toxicity research.[29] For example, as described in more detail below, there is controversy regarding the role of oxidative stress in the toxicity of many NPs. Traditional approaches to testing for a role of oxidative stress, although readily employed in *C. elegans*, have significant shortcomings. Most oxidative stress-response genes lack specificity because they can be up-regulated by stressors other than oxidative stress; conversely, oxidative stress can up-regulate other global stress-responsive genes (e.g. p53 target genes).[30] Markers of oxidative damage are more reliable, but it can be challenging to determine whether the toxicity is the result of direct or indirect oxidative stress (i.e. whether the toxicity is caused by oxidative stress, or whether dysfunction results in oxidative damage). Pharmacological rescue experiments using chemical agents (to illustrate such a 'rescue' experiment: if co-exposure to an antioxidant such as vitamin E protects against toxicity, this supports the hypothesis that the mechanism of toxicity is oxidative stress) frequently lack specificity because the agents used typically have many effects, and the compounds used in these experiments can affect the properties of the NPs. Genetic approaches utilising RNAi simply through feeding,[31] transgenic (such as reporter green fluorescent protein, GFP) and mutant strains are a powerful complement to these traditional approaches (for protocols and description see http://www.workbook.org, accessed February 2014). For instance, if toxicity is exacerbated in vivo in the context of knockdown or knockout (using RNAi or a mutant strain) of a gene involved in a particular defensive pathway (e.g. an antioxidant protein), this strongly suggests that the exposure is causing toxicity by the associated stressor (e.g. oxidative stress). This approach has been termed 'functional toxicogenomics'[32] and has been successfully used to study and identify toxicity mechanisms of metals and complex environmental mixtures.[33–36] It has also been applied to nanotoxicity studies.[37–39] In those studies, NP-induced genes and pathways were selected based on toxicogenomics, and their physiological importance was investigated by observing organism level endpoints such as survival, growth or reproduction in wild-type nematodes compared to nematodes lacking specific protein functions due to mutations or RNAi knockdown.

Finally, we note that *C. elegans* studies, like research with other species, will always require complementary investigations in other systems; no single model organism is sufficient (Table 1). Physiological differences between *C. elegans* and other organisms are important; for example, *C. elegans* lacks lungs, and may therefore be a poor model for high aspect ratio nanomaterials (i.e. NPs with a very high height-to-width ratio) such as carbon nanotubes that might exhibit asbestos-like pulmonary toxicity.[40] Earthworms (e.g. *Eisenia fetida*) may be more suitable for some of the NP uptake and elimination studies because of their larger size, which allows work with

individual worms.[41,42] However, toxicity and reproduction studies can be performed much faster with *C. elegans*. Also, because of limited genomic information for organisms such as *E. fetida*, *C. elegans* remains preferable for mechanistic NP studies (i.e. those toxicology studies that attempt to identify not just a toxic effect, but the mechanism by which such toxicity occurs). Finally, despite a generally high conservation of signalling pathways, molecular and biochemical differences may limit some extrapolations. For example, *C. elegans* has phytochelatins that complement the function of its metallothionein proteins, lack the CYP1 family cytochrome P450 enzymes and possess an aryl hydrocarbon receptor homologue that lacks binding affinity for typical xenobiotic ligands of mammalian aryl hydrocarbon receptors.[43–45] In summary, *C. elegans* offers a bridge between very high-throughput systems (e.g. cell culture) that are hampered by low physiological and environmental relevance, and more physiologically complex organisms that offer more relevance to human and wildlife health, but less mechanistic power and lower throughput.

Here we review the state of the evidence based on nanotoxicity studies in *C. elegans* focussing on the following aspects:

- Factors influencing nanotoxicity in *C. elegans*
- Potential mechanism of NP uptake
- Potential mechanisms of NP toxicity in *C. elegans*
- Comparision of *C. elegans* with other model organisms in nanotoxicity studies

What are the factors that influence nanotoxicity?

We reviewed currently available published nano(eco)toxicological studies involving *C. elegans* (Table 2). Based on this information, it is possible to tentatively identify NPs that are highly toxic, harmful, non-toxic and even therapeutic in this organism. Among the NPs listed in Table 2, platinum NPs, for example, are tentatively defined as potentially therapeutic based on evidence of their antioxidant properties.[46] Silver NPs, in contrast, would rank as the most toxic NPs to *C. elegans*, because mortality, inhibition of growth and reproduction have been observed at much lower concentrations compared to the other NPs so far tested. This is also true for Ag NPs in other tested model organisms, including bacteria, algae, crustaceans, ciliates, fish and yeast.[47] Current literature suggests that the physicochemical attributes of NPs as well as various exposure conditions are critical parameters in determining the degree of nanotoxicity in *C. elegans*.

Physicochemical properties of NPs

Coatings

Coatings can significantly alter NP effects, frequently mitigating toxicity. For instance, we found that uncoated Ag NPs caused higher mortality (~10-fold) in *C. elegans* than polyvinylpyrrolidone (PVP)-coated Ag NPs.[48] Citrate-, PVP- and gum arabic (GA)-coated Ag NPs of very similar size ranges had dramatically different growth-inhibition effects, with the GA Ag NPs being nine times more toxic (based on growth inhibition) than PVP Ag NPs, whereas PVP Ag NPs were three times more toxic than citrate Ag NPs, apparently due in part to differences in dissolution.[49] In another comparative study on stability of citrate-, PVP- and polyethylene glycol (PEG)-coated Ag NPs in OECD standard media, PVP Ag NPs were the most stable in terms of concentration, shape, aggregation and dissolution[50]. In some cases, however, the toxicity of NPs with different coatings cannot be explained by dissolution alone, as we observed in *C. elegans* based on differences in transcriptomic responses between citrate and PVP-coated Ag NPs.[51]

Size

Although there is evidence from many studies that particle size and surface area can be important determinants of the toxicity of NPs,[52–54] many in vitro studies have failed to show any clear relationship between cytotoxicity and NP size.[55–57] In *C. elegans*, however, there is some evidence for size-dependent toxicity. When nematodes were exposed to the same concentration of different sizes of CeO_2 NPs (15 and 45 nm) and TiO_2 NPs (7 and 20 nm), the smaller NPs were more toxic based on survival, growth and reproduction in both cases.[58] It was also found that when comparing the toxicity of PVP Ag NPs with different sizes (i.e. 8 and 40 nm), smaller particles caused a higher level of accumulation of 8-OHdG, an oxidised DNA base, than did larger particles.[48] Thus, size seems to be an important variable in toxicity of NPs to *C. elegans*, and a smaller size typically results in greater uptake and thus toxicity. However, this is not universal for all Ag NPs in *C. elegans*,[49] and there is evidence that size-dependent differences in toxicity of NPs in general are typically observed only when the primary particle size is smaller than 10–20 nm.[59]

Release of metals

Many NPs can release metals by dissolution before, during and after their uptake in tissues (see Fig. 2). Different metal ions have varied and well studied mechanisms of toxicity.[60] Although a full discussion of those mechanisms is beyond the scope of this review, some progress has been made in understanding the extent to which dissolution as such drives the toxicity of specific nanomaterials in *C. elegans*. Qu et al.[61] found that release of toxic metals from quantum dots (QDs) was important in QD toxicity (reproduction) in *C. elegans*, and the use of metal-chelating deficient *C. elegans* strains as well as pharmacological chelators demonstrated that Ag NPs were toxic in part by Ag dissolution.[49,62,63] These approaches, however, do not clarify whether dissolution occurred internally or externally, and if the dissolution is internal, where it occurs. Further research progress on mechanisms of NP uptake will help inform our understanding of target tissues; it will also be critical to understand subcellular distribution. Ag NPs, for example, are likely to dissolve much better in the acidic environment of lysosomes than in most typical exposure medium conditions.[64]

Other physicochemical factors

Other NP properties that may be important for toxicity, such as shape and charge, influence uptake, toxicity or both in other organisms and in in vitro studies.[65,66] We are aware of one study describing how coatings with different surface charge (positively, negatively and neutral) of CeO_2 NPs affected their bioavailability and mortality in *C. elegans*.[67] In that case, positively charged CeO_2 NPs showed the highest toxicity and bioavailability. This result indicates that future similar studies examining the interactions between the NP charge and toxicity are warranted.

Exposure conditions

Exposure medium

One of the advantages of using *C. elegans* in toxicity testing is that both solid and liquid media can be easily used, which is particularly useful in the ecotoxicological context. The effect of

Table 2. Published nanotoxicity studies in *C. elegans*

Nanomaterials (size)	*C. elegans* strains and life stage of the exposure	Exposure media	Endpoints	References
CeO_2 (8.5 nm)	N2 L1 larva	Nematode growth agar medium (NGM) and CeO_2 mixed with broth	Life span, lipofuscin accumulation, oxidative stress (reactive oxygen species (ROS) fluorescence)	[113]
CeO_2 (nanostructured and amorphous materials, TX)	N2, *mtl-2*, *pcs-1*, *sod-3*	Moderately hard reconstituted water (MHRW)	Growth	[159]
CeO_2 (4-nm core size) with positively charged (diethylaminoethyl dextran, DEAE), negatively charged (carboxymethyl dextran, CM) and neutral (dextran, DEX) coatings	N2 L1 and L3	MHRW alone and with humic acid	Mortality, bioavailability	[67]
Nano-ZnO (60 ± 25 nm)	N2	K-medium	Lethality, lipid peroxidation Phototoxicity	[83]
ZnO (1.5 nm)	N2 and *mtl-2::gfp*	K-medium (acetate buffered or unbuffered)	Lethality, Reproduction, locomotion (behaviour), *mtl-2::gfp* expression	[63]
ZnO (20 nm), Al_2O_3 (60 nm), TiO_2 (50 nm)	N2 L1	Ultrapure water	Lethality, growth, reproduction	[79]
Al_2O_3 nanoparticle (NP) (60 nm)	N2 *phsp-16.2::gfp* L1, L4, young adult)	NGM	Intestinal auto-fluorescence, ROS production; stress response measured by heat shock protein expression	[125]
Al_2O_3 NP (60 nm)	*N2*, *sod-3*, *sod-2*, *Phsp-16.2::gfp and Psod-3-sod-3*	NGM (exposure solutions were added to NGM plates)	Locomotion behaviour with head thrash and body bend; imaging of glutamatergic, serotoninergic and dopaminergic systems; stress response (heat-shock) and oxidative stress response (ROS formation, superoxide dismutase (SOD) activity)	[124]
Al_2O_3 NP (60 nm)	N2 and *phsp-16.2::gfp* L1, L4 and young adult	Ultrapure water	Lethality, lipofuscin accumulation, stress response measured by heat shock protein expression	[160]
Dimercaptosuccinic acid (DMSA)-coated Fe_2O_3 NP (9 nm)	N2, *sod-2* and *sod-3* L1 and L4	K-medium	Lethality, development, reproduction, locomotion behaviour, pharyngeal pumping, defecation, intestinal autofluorescence and ROS production	[161]
CeO_2 NP (15 and 45 nm), TiO_2 NP (7 and 20 nm)	N2 (Young adult)	K-medium	Lethality, growth, reproduction, stress response gene expression	[58]
Fluorescent nanodiamond (FND) (120 nm)	N2, *daf-16::gfp*, *gcs-1::gfp*	NGM (feeding)	Brood size, life span, ROS production; stress response assay by *gfp* expression,	[114]
	L4 N2, *daf-16::gfp*, *gcs-1::gfp*	Microinjection of FND into gonads of the worm	NP uptake	
Uncoated Ag NP (<100 nm)	N2, *sod-3*, *daf-12*, *mtl-2* Young adult	K-medium	Uptake, global changes in gene expression determined with microarrays, lethality, growth, reproduction	[38]
Uncoated Ag NP (<100 nm)	N2, *pmk-1* Young adult	K-medium	ROS formation, Gene and protein expression Oxidative stress signaling	[109]
Uncoated Ag NP (<100 nm)	N2, *pmk-1*, *hif-1*, *egl-9*, *vhl-1*, *fmo-2 cyp35a2* Young adult	MHRW	Survival, ROS formation, Gene and protein expression	[37]
Ag NP (10 nm)	N2 L1, L2 larvae	Mixed in a ratio of 2 : 3 : 5 of milk to *C. elegans* habitation reagent (CeHR) to treatment solution in water (only water for control)	NP uptake, localisation, larval growth, morphology and oxidative DNA damage (8-OH guanine level)	[162]

(*Continued*)

Table 2. (Continued)

Nanomaterials (size)	*C. elegans* strains and life stage of the exposure	Exposure media	Endpoints	References
Uncoated Ag NP (3 and 8 nm) from Amepox	N2 Adult	Simulated soil pore H_2O with and without fulvic acid, M9 buffer	Reproduction	[26]
Ag NP (citrate (7 ± 11, 21 ± 17 nm) and polyvinylpyrrolidone (PVP) (75 ± 21 nm))	N2, *nth-1*, *sod-2*, *mtl-2*, *xpa-1*, *mev-1* All developmental stages started from L1	K-medium	Uptake, growth inhibition (toxicity), mechanism of toxicity by using different mutant models	[62]
Ag NP (1 and 28nm), coated with PVP	*pha-1*	K-medium containing cholesterol	Survival with and without presence of E*scherichia coli* as food source	[81]
Ag NP citrate (50.6 nm) from ABC Nanotech	N2	NGM with NP suspensions	Mortality, reproduction and biological surface interaction with Ag NPs by scanning electron microscopy	[68]
Platinum NP (2.4 ± 0.7 nm)	N2 L4	S-medium	Life span, lipofuscin accumulation, ROS, internalisation	[46]
Platinum NP (2.4 ± 0.7 nm)	N2 and *mev-1* L4	S-medium	Lethality, life span, lipofuscin, ROS	[155]
Silica NP fluorescent labelled) (50 nm)	N2	NP containing NGM plates	Translocation, life span, lipofuscin accumulation (4th and 10th day), reproduction (progeny production, vulva development defect)	[152]
Polystyrene NPs PS-YG, PS-YO, carboxy	L4 and young adult			
Fluorescent polystyrene nanoparticles (100 nm)	N2	Microinjected into the syncytial gonads of gravid hermaphrodite, recovery buffer (15min) and then in M9 buffer (1hr)	Particle-tracking intracellular microrheology	[163]
NaYF4:Yb,Tm NCs with strong NIR UC emission (28, 42 and 86 nm)	N2, *elt-2::gfp*, *lag2::gfp*	Colloid solution was dropped onto NGM agar plates	Life span	[115]
(used for bioimaging)	L4 and young adult		Egg production, viability and growth rate assays, *gfp* expression assay	
CdSe, ZnS core shell quantum dots (QDs) and CdTe QDs (10 nm)	N2 and *mtl-2:: gfp* L1- arrested and adult worms	NGM plates with or without QDs	Life span, brood size, larval developmental assay; imaging	[61]
(used in bioimaging)		M9 buffer treatment was used as negative control		
Graphite nano-platelets (GNPs) (mean lateral size of the flakes is 350 nm and the thickness measured through scanning electron microscopy is ~50 nm, with several graphene layers varying between 3 and 60	N2	GNPs suspended in distilled water, *Pseudomonas aeruginosa* was used as food source	Longevity and reproductive capability	[164]
Mercaptosuccinic acid (MSA)-capped QDs (QDs-MSA)	N2 L3 and L4	K-medium and H_2O	Growth (larvae to adults) and reproduction egg-laying defects and response to anticonvulsant ethosuximide and to neurotransmitter serotonin	[165]
Quantum dots, core (CdSe) (3.4-nm core diameter) and core–shell (CdSe, ZnS) (4.1-nm core diameter); hydrodynamic diameter of both QDs are ~17 nm	N2 L4	QDs in borate buer added to NGM agar plate with food (*E. coli*)	Lifespan and fertility, body length and locomotion	[166]

TiO_2 NPs (5 ± 1 nm (4 nm), 10 ± 1 nm (10 nm), 60 ± 9 nm (60 nm)	N2 Young adult	K-medium	Lethality, growth and locomotion behaviour, (ROS) production and *N*-acetylcysteine (NAC) treatment rescue, intestinal auto-fluorescence	[167]
TiO2 NP (<25 and <100 nm) ZnO NP(<25 and <100 nm	N2 L4	Ultrapure water	Mortality (24 h without food)	[78]
TiO_2 NPs, ZnO NPs and SiO_2 NPs with the same nanosize (30 nm)	N2 L1	K-medium	Lethality, growth, reproduction and locomotion behaviour, (ROS) production and NAC treatment rescue	[168]
TiO_2 NPs (10 nm)	N2 and *mtl-1*, *mtl-2*, *sod-1*, *sod-2*, *sod-3*, *sod-4*, *sod-5*, *mev-1*, *aak-2*, *xpa-1*, *pcm-1*, *hsp-16.48*, *hsp-16.2*, *gst-4*, *gst-8*, *gst-24*, *gst-5*, *gst-42* and *isp-1* Young adult	K-medium	Lethality, growth, reproduction and locomotion behaviour, Intestinal auto-fluorescence, ROS production and gene expression	[158]
TiO_2 NPs (10 ± 2 nm)	N2 and P*unc-47::gfp*, *lin-15* Young adult and L1 larvae	K-medium	Uptake, growth, locomotion behaviour, pharyngeal pumping and defecation, intestinal auto-fluorescence, ROS production, analysis of triglyceride content, TEM imaging for translocation	[169]
TiO_2 NPs (4 and 10 nm)	N2, P*sod-2-sod-2*, P*sod-3-sod-3* and P*dpy-30*-sod-2 L1 and young adults	K-medium in the presence of food	lethality, growth, reproduction, locomotion behaviour, intestinal autofluorescence and ROS production, gene expression (*clk-1*, *clk-2*, *ctl-1*, *ctl-2*, *ctl-3*, *gas-1*, *isp-1*, *mev-1*, *sod-1*, *sod-2*, *sod-3*, *sod-4*, *sod-5)*.	[170]
Au NPs	N2 and *chc-1*, *dyn-1*, *rme-2* and *pqn-5* L3 and Adult	K-medium	Mortality, global changes in gene expression determined from microarrays; mortality of mutants for endocytosis and UPR pathways	[39]
Hydroxylated fullerene, [fullerol, $C_{60}(OH)_{19-24}$] (4.7 and 40.1 nm)	*ced*-3, ced-*4* and *elt-2::gfp* L4 and adults	NGM plates with or without fullerene, mixed with OP50 cell pellet	Lifespan, reproduction, body length, defective digestion system and apoptosis (SYTO 12 staining and *ced-3* and *ced-4* expression)	[133]
Pristine single-walled carbon nanotubes (SWCNTs) (length 0.5–2.0 μm) and amide-modified SWCNTs (a-SWCNTs) (length 0.7–1.0 μm)	N2 and *daf-16:gfp* L1 larvae	NGM	Survival, growth, reproduction, endocytosis, oxygen consumption rate, DAF-16 nuclear translocation assay, genome-wide gene expression analysis (microarray and quantitative reverse-transcriptase real-time PCR, qRT–PCR)	[171]
Multi-walled carbon nanotubes (MWCNTs) and PEGylated modification in reducing MWCNTs	N2	K-medium	Translocation in targeted organs regulating the toxicity	[172]
Ag NPs Citrate-coated (CTL_7), PVP-coated (PVP_8 and PVP_{38}) and gum-arabic (GA)-coated (GA_5, GA_{22})	N2, VC433 (*sod-3* deletion), TM1748 (*pcs-1* deletion,) and TK22 (*mev-1*, mutation uncertain,)	MHRW	Mechanisms of toxicity by rescue with trolox and N-acetylcysteine, analysis of metal-sensitive and oxidative stress-sensitive mutants, growth assay	[49]
Pt NPs (2.5 ± 1 nm)	N2 (L4 and young adult for ROS detection)	Electrolysed-reduced water (ERW) medium	Life span assay and ROS detection	[157]
Pt NPs	N2 and *nuo-1* (LB25) (L4)	S-medium	Lethality, life span, lipofusion ROS measurement, NAD^+ and NADH assays	[156]
Au NPs surface modification for microscopic analysis (20 nm)	N2	Polyelectrolyte, NP coated worms were inoculated (100 μL) into NGM plates containing *E. Coli*	Viability and reproduction	[173]
Au NPs (10 nm) from Sigma Aldrich	N2	*E. coli* was added to LB Broth containing NPs and exposed *E. coli* were fed to *C. elegans*	Multigenerational effects on survival and reproduction over four generations.	[174]

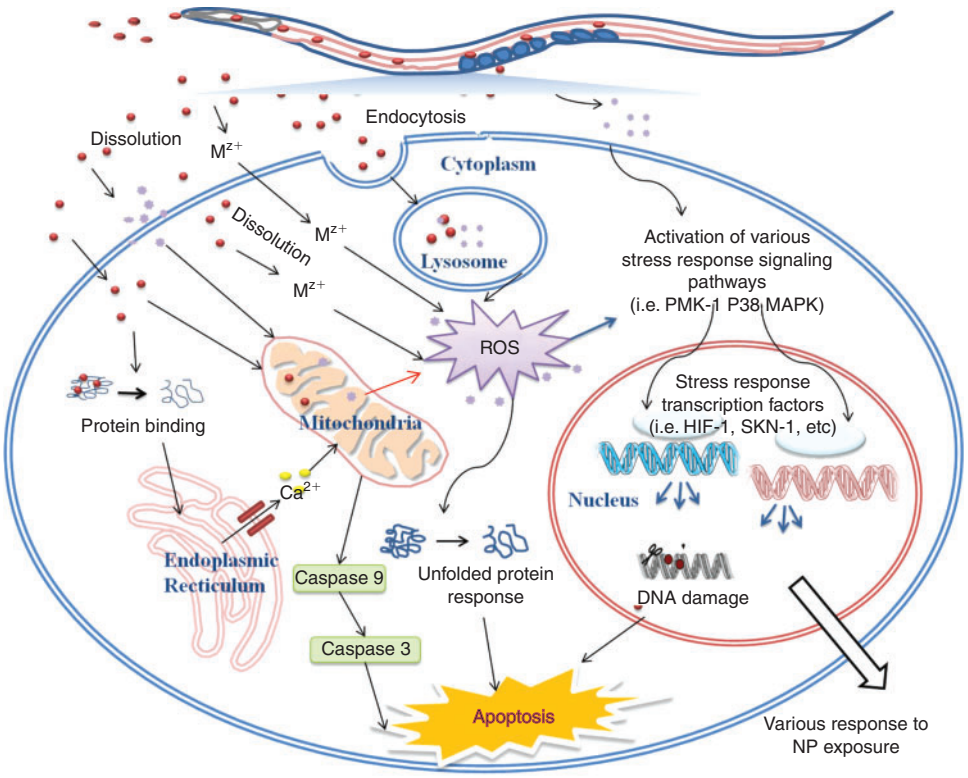

Fig. 2. Potential mechanisms for nanoparticle (NP) uptake and toxicity in *C. elegans* (M^+ are dissolved metallic ions released from nanoparticles; ROS, reactive oxygen species).

medium composition on the bioavailability of unstable NPs should be taken into account or tested before designing experiments. For instance, a recent study[68] used a suspension of Ag NPs embedded into solid nematode growth agar medium (NGM). The mortality of *C. elegans* in NGM could have been under-estimated in comparison with liquid medium because binding of Ag NPs to the agar probably limited their availability to *C. elegans*; in addition the particles could have undergone agar-mediated surface modification. The NP toxicity to *C. elegans* can also differ depending on the composition of liquid testing media. For instance, we and others have observed dramatically less toxicity after exposure of *C. elegans* to Ag NPs and Ag ions ($AgNO_3$) in K-medium (a standard medium for liquid *C. elegans* culture[69]) *v.* moderately hard reconstituted water (MHRW, for composition see Cressman and Williams[70] (Table 2[49]). This is likely a result of the high Cl^- concentration in the K-medium facilitating precipitation of highly insoluble AgCl, which would reduce or eliminate the toxicity from Ag ions. The Cl^- concentration is relatively low in MHRW (54 μM) compared to K-medium (32 mM KCl and 51 mM NaCl).

The effect of exposure medium may be less important in the cases of insoluble NPs. However, even in the case of poorly soluble NPs, a high ionic strength may cause NP aggregation, particularly in the presence of polyvalent cations. For instance, Au NPs are also very insoluble (1.4×10^{-5} % dissolution after 24-h exposure in 50 % K-medium[39]), but full strength K-medium caused aggregation of Au NPs, necessitating the use of 50 % strength K-medium for toxicity studies.[39]

Several toxicity studies have been conducted in *C. elegans* exposed to soil or sediment contaminated with metals or organic contaminants.[71–73] However, perhaps because of the complexity of the soil matrix, to our knowledge, there are no published nanotoxicity studies that have been conducted in soil/sediment exposure conditions. Studies evaluating the toxicity of NPs to *C. elegans* in different natural soils and also focussing on sensitive endpoints will be required to better understand the toxicity of NPs in soil media.

Developmental life stage and presence of food

Nematode developmental stage is a major factor influencing toxicity. In studies published so far that included earlier and later developmental stages, the first larva stage (L1) was the most vulnerable to NP exposure; early life stages have generally been observed to be more sensitive for many contaminants and many species.[74–77] For example, TiO_2 NPs with sizes between 25 and 100 nm in pure water were non-toxic for L4s[78] but toxic to L1s,[79] reducing survival, growth and reproduction.

Esherichia coli is typically used as a source of food in *C. elegans* toxicity experiments and the presence or lack of food during exposure significantly affects toxicity, possibly because of altered bioavailability and the effect that feeding can have on the physiological state of the nematodes. For instance, 24-h exposure to Ag NPs and Ag ions resulted in 50 % lethal concentration (LC_{50}) values at least 10-fold lower compared to the experiments performed without feeding.[80] However, the reverse results were found in another Ag NP study where higher *C. elegans* mortality was observed in fed *v.* unfed nematodes exposed for 24–72 h.[81] The opposite results in these two studies may be attributable to the fact that exposures were initiated at different *C. elegans* developmental stages (older L3 and young L2 nematodes). Limitation in food resources at *C. elegans* early developmental stages (L1 and L2) can result in developmental arrest of the nematodes at the dauer (resistant) larval stage, where nematodes do not feed and are characterised by extended lifespan, lower metabolism, increased fat storage and high levels of antioxidant enzymes.[82]

Environmental conditions and interactions

Solar irradiation and phototoxicity

Phototoxicity can be an important factor affecting the toxicity of NPs. In the environment, NPs may co-occur with the ultraviolet wavelengths associated with phototoxicity. Recently several *C. elegans* toxicity studies with metallic oxide NPs have considered the effects of phototoxicity. The toxicity (based on mortality) of ZnO NPs increased significantly with natural sunlight due to photocatalytic reactive oxygen species (ROS) generation by ZnO.[83] The phototoxicity of ZnO NPs was greater in the study of Ma et al.[83] than for bulk ZnO, even though the ZnO NP aggregates were similar in size to the bulk ZnO aggregates (respective average aggregate size of 2.8 and 2.4 μM), demonstrating that aggregation did not quench the photoreactivity of the particles and that primary particle size (10 *v.* 55 nm for ZnO NPs and bulk ZnO) rather than aggregate size dictated toxicity.[83]

Weathering

Once NPs are released into the environment, they will undergo modifications due to aging processes involving interactions with various organic and inorganic ligands, oxidation–reduction reactions and dissolution–precipitation reactions. A key environmental transformation for Ag NPs, especially in environmental compartments such as sediments and sewage sludge that contain reduced sulfur (sulfide), is the strong binding of Ag to sulfur (sulfidation), which might reduce toxicity of these sulfidised Ag NPs due to the very low solubility of Ag_2S.[84] We carried out experiments on the toxicity of Ag NPs with varying degrees of sulfidation including fully sulfidised particles, and found that sulfidation decreased mortality, and caused much less inhibition in growth of *C. elegans* when compared with those of the pristine Ag NPs.[85] Other particles are also likely to be altered by weathering, and for certain nanoparticles, weathering can increase their toxicity due to continued or accelerated release of metal ions, as has been shown for bacteria exposed to quantum dots as a result of their weathering under acidic or alkaline conditions.[86] Thus, understanding these processes will be critical to assessing the risk of NPs in the environment.

Organic matter

When NPs are released into the environment they will inevitably interact with natural organic matter (NOM). The presence of NOM in exposure solutions can significantly increase or decrease the toxicity of the NPs by several mechanisms including coating NP surfaces to reduce or augment interaction with biological receptors, and altering aggregation, charge or dissolution properties.[80] For example, fulvic acid significantly decreased the acute toxicity of both of Ag NPs and Ag ions to *C. elegans*, in some cases reducing mortality from 100 to 0 %.[80] When humic acid (HA) was added to the CeO_2 NPs exposure medium, Collin et al.[67] observed a significant decrease in mortality of *C. elegans*. The authors also demonstrated that the decreased bioavailability of CeO_2 NPs depended on the ratio of CeO_2 NPs to humic acid.

Potential mechanisms of entry for NPs

Uptake of NPs by ingestion and subsequent translocation into intestinal cells in particular, but also reproductive cells, has been observed in *C. elegans* with a variety of NPs (reviewed in Zhao et al[20]). There are several possible mechanisms, identified from in vitro studies, for how NPs can enter cells including direct diffusion of NPs across the plasma membrane without lipid bilayer disruption[87]; creation of pores in the membrane[88]; endocytosis[39,65,89–93] and by G-protein-coupled receptors.[94] Surface chemistry, size, shape and charge of NPs significantly influence their uptake. For instance, in an in vitro study with mouse dendritic cells, Au NPs coated with alternating anionic and hydrophobic groups were able to diffuse through the cell membrane without disrupting the lipid bilayer, whereas similar Au NPs that differ from the first ones only by random distribution of the same groups in the coating were endocytosed and trapped in endosomes.[87] Size dependence during NP uptake was observed by Chithrani and colleagues[65,89]: depending on the energetics of a single nanoparticle (such as bending and adhesion energy), its wrapping by a cell membrane and uptake through endocytosis occurs optimally when NPs are 20–50 nm, whereas smaller particles would reach the optimal size for endocytosis only by clustering.[89]

Xenobiotic uptake and distribution in *C. elegans* are not well studied. Nonetheless, and although the above-described mechanisms for NP entry into the cells were inferred from in vitro studies, we have some evidence that these mechanisms can also apply for NP uptake into *C. elegans* tissues and cells (as shown in Fig. 2). For instance, a study of toxicogenomic responses in *C. elegans* exposed to Au NPs identified clathrin-mediated endocytosis as one of the pathways activated by Au NPs.[39] In that study, two of the endocytosis mutants (*chc-1* and *rme-2*) were more resistant than wild type nematodes to Au NPs, thus providing evidence for the functional importance of this pathway to Au NP uptake and toxicity. In the same study, electron-dense particles with Au elemental composition were found only in the animal's gut lumen and microvilli, where endocytosis is plausible, and not near the cuticle surface, suggesting that the Au NPs are likely to be absorbed from the intestine.[39] However, the dermal route of exposure should not be entirely excluded for NPs. For instance, organically modified silica NPs were incorporated into cuticle and caused demelanisation in *Drosophila*.[95] In *C. elegans*, the cuticle has an evenly distributed net negative charge at neutral pH,[96] which can attract positively charged NPs. In addition, Ag NPs can cause damage to the cuticle of *C. elegans*,[68] although this did not result in detectably increased uptake.

How do NPs cause toxicity to *C. elegans*?

The toxicity of NPs may be mediated by multiple mechanisms or modes of action, depending on the physicochemical properties of the NPs as well as exposure conditions.[97,98] Many mechanistic studies conducted on NPs, mainly using in vitro systems, have reported that oxidative stress is associated with NP exposure.[20] However, the evidence for oxidative stress as a mechanism of toxicity in *C. elegans* exposed to various NPs is contradictory. Furthermore, it is unclear whether the lack of information on other mechanisms of toxicity is because of negative results that have been obtained, or from the fact that many researchers investigating toxicity of many NPs with *C. elegans* and other organisms have limited their mechanistic investigations to oxidative stress-related endpoints. Among other possible mechanisms of NP toxicity described in published studies are endoplasmic reticulum (ER) stress and protein toxicity resulting in an unfolded protein response (UPR), which was observed in *C. elegans* after Au NP exposure.[56] Below we review studies that provide evidence for oxidative stress and other mechanisms of NP toxicity (Fig. 2). Overall, although

some mechanisms shown in Fig. 2 are specific to *C. elegans*, many represent pathways of NP–cell interactions that have been determined for other organisms, including daphnid, zebrafish, medaka, earthworms and mammalian cells.[55,99–103]

Oxidative stress

In theory, NPs can induce oxidative stress, characterised by a pathologically high production of oxidants,[104] either directly by their intrinsic ability to generate ROS or indirectly by their interactions with biological systems. ROS can be generated by NPs as a result of the presence of transition metal impurities, the ability to release toxic metal ions, the presence of electronically active surface or photoactivation.[9,105,106] Direct generation of ROS by NPs may also result from exposure to an acidic environment, such as the intestine or lysosomes, either from the surface of the NPs or from leached ions.[107,108]

Although there are multiple studies with *C. elegans* providing support for oxidative stress as the mechanism of NP toxicity[38,49,83,109] (Table S1, Supplementary material), not all *C. elegans* studies support this conclusion. Whether the effects will be pro-oxidant or antioxidant may also depend on the concentrations used. For instance, CeO_2 NPs have been reported to have pro-oxidant effects at higher concentrations,[110–112] whereas at lower concentrations CeO_2 NPs have exhibited ROS scavenging and superoxide dismutase (SOD) mimetic activity.[113] Antioxidant activity has been also demonstrated for Pt NP compounds.[46] Other studies detected no ROS generation after *C. elegans* exposure to ZnO, Al_2O_3, or TiO_2 NPs,[79] perhaps because the exposures were performed in the dark. Nanodiamond particles did not significantly affect the ROS level in either germline or somatic cells of *C. elegans*, nor did they cause detectable changes in either brood size or longevity of the nematodes.[114,115] Meyer et al.[62] reported that toxicity caused by several types of Ag NPs could not be attributed to oxidative stress in *C. elegans* because oxidative stress-sensitive mutants were not more sensitive to the toxicity of the tested NPs, and Yang et al.[49] found that oxidative stress was one mechanism of toxicity, but was less important than dissolution resulting in metal ion toxicity, for a variety of Ag NPs.

Biological interactions of NPs could also contribute to ROS production. It is possible, therefore, that NPs devoid of intrinsic ROS generation capacity can also give rise to ROS generation by interaction with sub-organelles and biological systems. NPs can directly interact with organelles such as the mitochondria by destabilising the outer membrane, altering the mitochondrial membrane potential and disrupting the electron transport chain and oxidative phosphorylation,[116] which may increase production of ROS[117,118] (Fig. 2). NPs can also cause activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in cells of the immune system, such as macrophages and neutrophils, resulting in production of superoxide anions.[116,119,120] The interaction of NPs with cell surface receptors might lead to receptor activation and triggering of intracellular signalling cascades, such as MAPK, finally resulting in altered expression of stress response genes that may affect ROS production or quenching (Fig. 2). Accumulation of high intracellular calcium levels due to NP exposure might also act as an alternative mechanism for the induction of oxidative stress.[120,121] Finally, oxidative stress can be caused indirectly even by NPs that do not inherently produce ROS, as a result of depletion of antioxidants (e.g. reduced glutathione) that are consumed, bound or oxidised after exposure to dissolved metal ions from NPs.[122]

Thus, NPs may directly or indirectly induce a range of responses associated with increasing levels of oxidative stress. These responses have been characterised as induction of antioxidant defenses at lower levels, followed by inflammatory responses at intermediate levels and cytotoxic responses at high levels.[117,122] It should also be noted that very low levels of oxidative stress often serve important physiological functions, such that altered production or abnormal quenching can alter normal signalling pathways.[123]

Among the most studied NPs shown to induce oxidative stress in *C. elegans* are Ag NPs. A wide range of endpoints, from growth inhibition or mortality to stress response gene expression assays, have been used to examine oxidative stress by different groups (Tables 2, S1). Roh et al.[38] reported greater reproductive failure and increased expression of various stress-response genes due to Ag NP (<100 nm) exposure in a *sod-3* mutant strain (lacking a mitochondrial superoxide dismutase), compared to wild type nematodes (N2). A recent study showed that the antioxidant and metal chelator *N*-acetylcysteine (NAC) completely rescued the growth inhibition of *C. elegans* caused by Ag NPs with various coatings.[49] Two oxidant sensitive *C. elegans* mutant strains (*mev-1* and *sod-3*) demonstrated increased sensitivity to several (but not all) of the tested Ag NPs with different coatings but not to Ag ions, supporting the role of oxidative stress in the toxicity of those Ag NPs.[49] Lim et al.[109] found not only increased ROS formation, but also significant increase in the expression of p38 MAPK PMK-1 at both gene and protein levels after Ag NP exposure in wild type (N2) *C. elegans*. MAPK, which may be linked to oxidative stress or a response to UPR (discussed below) was one of the pathways induced after *C. elegans* exposure to Au NPs.[39] The hypoxia signalling pathway (*hif-1*) was also activated by Ag NPs in *C. elegans*.[109] Recently, Eom et al.[37] reported increased sensitivity to Ag NPs in strains with loss-of-function mutations in genes in the *hif-1* pathway, which was completely rescued by NAC treatment.

Exposure to ZnO (40–100 nm) and Al_2O_3 (60 nm) NPs caused photocatalytic ROS generation, intestinal lipofuscin accumulation, decreased SOD activity levels and decreased expression of *sod-2* and *sod-3* in N2 nematodes[83,124,125] (Table 2). Yu et al.[125] further confirmed that the accumulation of intestinal autofluorescence is largely due to ROS production in the intestines of Al_2O_3 NP-exposed nematodes. Li et al.[124] found that antioxidant treatment after chronic exposure to Al_2O_3 NPs suppressed oxidative stress. Zhang et al.[113] hypothesised that ROS accumulation and oxidative damage might be the cause of cyto- and genotoxicity of CeO_2 NPs in wild type *C. elegans*.

In summary, it would appear that oxidative stress is a common but not universal mechanism of toxicity of NPs in *C. elegans*.

Endoplasmic reticulum stress

Endoplasmic reticulum (ER) stress is another possible mechanism of NP toxicity. The ER is responsible for the biosynthesis and folding of multiple proteins, and also stores calcium. Under ER stress, protein denaturation, accumulation of misfolded proteins and changes in Ca^{2+} homeostasis take place[126] (Fig. 2). Protein denaturation has previously been suggested as one of the possible effects of Au NPs.[127] As a result of ER stress, or to respond to protein damage by helping with correct folding or degradation of damaged proteins, the adaptive pathway described as the 'unfolded protein response' (UPR) is activated.[128] In

response to 4-nm Au NPs, we found that *C. elegans* induced both canonical and non-canonical UPR pathways.[39] The canonical UPR comprised of up-regulation of molecular chaperones (heat shock proteins) including *hsp-4*, a marker of ER stress. The non-canonical (specific to *C. elegans*) UPR response included up-regulation of 25 genes from the *abu/pqn* families.[39] A strain with a mutation in one of these genes (*pqn-5*) showed much higher mortality than N2 after exposure to Au NPs, supporting a protective role for this gene.[39] The *abu/pqn* genes, controlled by the apoptotic receptor *ced-1*, have also been shown to activate after exposure of *C. elegans* to pathogens and are involved in regulation of innate immunity.[129] Another result of ER stress is destabilisation of Ca^{2+} homeostasis, which has also been observed in *C. elegans* after exposure to Au NPs.[39]

DNA damage and apoptosis

Despite a growing number of mechanistic nanotoxicology studies in *C. elegans*, there are few reports on possible genotoxic effects of NPs. Recently we found that Ag NPs caused both oxidative DNA damage and strand breaks to *C. elegans*, and induced the *hus-1* DNA damage checkpoint pathway and ultimately apoptosis. DNA damage intensity was higher in a *pmk-1* (the nematode p38 MAPK homologue, involved in apoptosis) mutant, and necrosis instead of apoptosis was observed in *pmk-1* mutants.[130] We also found oxidative DNA damage in *C. elegans* exposed to $AgNO_3$ and uncoated and PVP-coated Ag NPs.[48]

DNA damage, oxidative stress and ER stress can all eventually result in either apoptosis, necrosis or both (Fig. 2). In *C. elegans*, there are a fixed number of somatic cells in adults, and apoptotic events occur in two waves: during development (131 out of 1090 cells die) and in the germline of adults.[131,132] It is commonly accepted that toxicants can increase the rate of germ cell apoptosis (a basal level also occurs physiologically) but not the developmentally programmed apoptotic cell deaths.[132] However, to our knowledge, this question has not been investigated extensively, and the possibility of somatic cell apoptotic death should not be completely excluded. An increase in apoptotic germ cell corpses was observed in *C. elegans* exposed to fullerol NPs through food for 3 days.[133] In the same study *C. elegans* strains carrying mutations in genes functioning in the regulation of apoptosis, *ced-3* and *ced-4*, showed higher resistance to fullerol and a significant decrease in apoptotic body formations when compared to the wild type nematodes. In addition, apoptosis was one of the pathways identified in our transcriptomic analysis of genes induced in response to Au NP-exposed nematodes.[39] In the same study the authors also observed significant up-regulation of three genes (*ced-1*, *rab-7* and *dyn-1*) implicated in phagocytosis, suggesting that the processes associated with removal of necrotic cells might be also activated in response to Au NP exposure.

Other mechanisms

To our knowledge, other possible nanotoxicity mechanisms (e.g. receptor activation or antagonism, altered signalling, lysosomal destabilisation, mitochondrial toxicity, protein damage; Fig. 2) have not been well investigated in *C. elegans*. In addition, although molecular mechanisms and organism-level apical endpoints (e.g. growth, mortality, reproduction and behaviour) have been fairly well studied, cellular and tissue-level studies have been less common and are an important area for future investigation.

Comparison with other models

There are numerous studies published on NP toxicity using models other than *C. elegans*. It is beyond the scope of this review to address all of these. Rather, for comparative purposes, we focus on several model organisms that have been used frequently in nano(eco)toxicity studies, emphasising the similarity and differences with *C. elegans* in their responses to NP exposures (Table 1).

Daphnia species

Numerous nanotoxicity studies have been performed with species of *Daphnia*, fresh water free-swimming crustaceans that serve as indicator species for various environmental stressors, including exposure to NPs. *Daphnia* species, for example, have shown high sensitivity to Ag NPs with mortality LC_{50} of $40\,\mu g\,L^{-1}$ for *D. magna*[134] and half maximal effective concentration (EC_{50}) for reproduction of $121\,\mu g\,L^{-1}$ for *D. magna* and even lower ($9\,\mu g\,L^{-1}$) for *D. pulex*.[135] Similar to *C. elegans* studies with Ag NPs, particle- and ion-specific mechanisms of toxicity were identified in *D. magna* in response to exposure to PVP Ag NPs. These mechanisms were associated with abnormalities in mitochondrial activity,[136] and the authors suggested that the mechanisms of toxicity of the Ag ions and particles can be complementary, possibly resulting in enhanced toxicity. Although particle-specific effects were identified after exposure to Ag NPs in both *C. elegans* and *Daphnia*, the toxicity observed after ZnO NP exposure seems to be explained by ions in both model organisms (e.g. Ma et al.,[63] Adam et al.[137] and Zhu et al.[138]).

Like *C. elegans*, *Daphnia* species are also characterised by a short life cycle and high number of offspring, allowing rapid toxicity screening for mortality and reproduction. Multigenerational experiments have also been conducted for *Daphnia* with Ag NPs[135] and carbon nanomaterials.[139] Decrease in endpoints that may affect population levels (growth and reproduction) of three *Daphnia* species after exposure to Ag NPs for five consecutive generations were observed at Ag concentrations of $2.5–10\,\mu g\,L^{-1}$ Ag.

Daphnia pulex's genome has also been recently (in 2011) sequenced, so that a whole genome toxicogenomic approach is now available for studying the toxicity of NPs. Partial-genome toxicogenomic approaches with custom microarrays have been used to study the effects of Ag NPs on *D. magna*,[140] and revealed distinct patterns of altered gene expression for the NPs and ions. However, although the *D. pulex* genome has been sequenced, the genes are not functionally annotated yet to the same extent as for *C. elegans*, and functional genomic tools are not available yet for *Daphnia*. Thus, *C. elegans* is a better model species for testing mechanistic hypotheses for nanotoxicity. In contrast, it is important to note *Daphnia*'s ecological advantage over *C. elegans*. *Daphnia pulex* is a key species in freshwater ecosystems and its ecology is well studied[141] and thus, it is more suitable for bioaccumulation and transfer of NPs in food chain studies. For example, a study by Zhu et al.[142] demonstrated a transfer of TiO_2 NPs from *D. magna* to zebrafish through dietary exposure.

Earthworms

Important ecotoxicological data have been derived using earthworms (*Eisenia fetida*). Uptake and elimination,[41,42,143,144] mechanistic toxicity[103] and avoidance[145] studies have been performed with *E. fetida* exposed to metal and metallic NPs in

soils. One advantage of this species is that its larger size permits easier detection and analysis of internal NP distribution, and these studies demonstrated that earthworms can take up intact or oxidised NPs and distribute them within tissues. In contrast to *C. elegans* studies, different coatings (PVP and oleic acid) and sizes of Ag NPs did not result in differences in *E. fetida* toxicity.[41,42] It is important to note that these exposures were carried out in soil, and the particles were subject to transformations within the soil. Earthworm exposures to Ag NPs, similarly to *C. elegans*, have adversely affected reproduction but only at high concentrations, such as 700–800 mg kg^{-1}. Interestingly, earthworms avoided Ag NPs 48 h after the exposure at concentrations 100-fold lower than the concentrations where reproductive effects were observed.[145] Avoidance behaviour in response to NPs was also observed in *C. elegans*. For instance, Li et al.[124] (Table 2) observed decreased locomotive behaviour in *C. elegans* exposed to Al_2O_3 NPs at concentrations more than 10-fold lower than for bulk Al_2O_3, further supporting the hypothesis that behavioural response may serve as one of the most sensitive endpoints when studying NP toxicity.

Our study examining mechanisms of Ag NP toxicity in *E. fetida*[103] suggests that oxidative stress occurs with some delay (3 days) after exposure to Ag NPs as indicated by the increased level of protein carbonyls. In the same study similarity in expression levels of nine stress-response genes after exposure to both ions and particles suggested that Ag NP toxicity to earthworms is driven by ions. However, given that less than 15 % of Ag was oxidised in the soils that the earthworms were exposed to,[41] the dissolution of Ag NPs is likely occurring during their uptake, internally or both.[103] Down-regulation and decreased activity of catalase on day three suggest that increased levels of H_2O_2 could have promoted dissolution of Ag NPs internally within the short exposure period.[146,147] To our knowledge, Ag speciation for soil exposures with *C. elegans* has not been investigated, so the earthworm studies provide important complementary insight into Ag NP behaviour in soil. In *C. elegans* some of the toxicity can also be explained partially by dissolution that, given the nature of the aquatic exposures, probably occurs both internally and externally. However, mortality, dissolution and transcriptomic studies in response to Ag NPs in *C. elegans* suggest that the observed effects are due to both dissolution and particle-specific effects.[51]

Zebrafish and Japanese medaka

Other ecotoxicological models that have been used in NP toxicity research include zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). Studies on the toxicity of nC_{60} in *D. rerio* revealed adverse effects on embryo hatching, survival and development[148] and oxidative stress was proposed as a key mechanism of toxicity of nC_{60} in *D. rerio*.[100] Studies with metal oxide NPs (TiO_2, ZnO and Al_2O_3) in *D. rerio* showed toxicity (decrease in survival and malformations) only for ZnO NPs,[149] with similar toxicity resulting from exposure to ZnO and bulk material suggesting that the toxicity was likely due to dissolution. Similarly, in a *C. elegans* ZnO NP study there were no differences observed in toxicity between ZnO NPs and $ZnCl_2$.[63] Exposure of zebrafish fry to Ag and Cu NPs revealed LC_{50} values of less than 10 mg L^{-1}.[134] However, in contrast to results in *C. elegans*, the toxicities of Ag and Cu NPs were greater than those of their corresponding metals. Similarly, greater toxicity for Ag NPs than $AgNO_3$ was observed in medaka, at least at higher concentrations.[99] In the same study, gene expression patterns suggesting an activation of stress response pathways were documented for six stress related biomarkers after exposure to Ag ions and Ag NPs, as also observed for *C. elegans* exposed to $AgNO_3$ and Ag NPs.[94] Gene expression patterns in medaka suggested that the Ag NP toxicity was associated with oxidative stress, DNA damage and repair mechanisms and apoptosis.[99] Kashiwada et al.[101] observed changes in genes related to oxidative stress, growth regulation, embryogenesis and morphogenesis after exposing medaka to nano-colloidal Ag. These mechanisms and processes induced by Ag NPs in *D. rerio* and *O. latipes* show similarity to the toxicity mechanisms described above for *C. elegans* exposed to Ag and Au NPs, despite the significant differences in the organisms' physiology.

In fish, embryos are surrounded by a chorion (an acellular envelope), which can allow or delay passage of NPs. This barrier is of ecotoxicological relevance because it is important for many fish and other species, and it is therefore important to study it using the organisms that actually have such a barrier. Fortunately, the chorion can be easily removed to test whether it prevents NPs from passing to the embryo, as has been shown with single-walled carbon nanotubes (SWNTs) in *D. rerio*.[150] Lee et al.[151] examined transport of Ag NPs into the embryos of *D. rerio* and demonstrated that Ag NPs of 5–46 nm can pass through the chorion pore channels (0.5–0.7 μm in diameter) with some of the particles being trapped inside these channels. By comparison, QDs have been observed in the reproductive organs of *C. elegans* but not in the eggs,[61] whereas silica NPs[152] and Ag NPs[62] have been observed in *C. elegans* embryos. Movement into the gonads themselves may occur by passive diffusion, but the cuticle of *C. elegans* eggs is dense and appears to lack pores, suggesting that NPs probably reach embryos through a mechanism other than passive diffusion. It may be that NPs can be loaded into eggs during maternal loading of biomolecules such as vitellogenin; this may be facilitated in *C. elegans* by the fact that egg constituents are produced in intestinal cells (to which NPs have good access), and are then actively transported directly to the gonad.

Mammalian models

C. elegans can be used for both ecotoxicity and human health studies with NPs. Although a full review of the mammalian nanotoxicity literature is beyond the scope of this review, several studies indicate that molecular mechanisms in *C. elegans* have been generally similar to those obtained in mammalian systems. For example, a correlation was observed in the transcriptomic response in human cultured cells (HepG2 or Jurkat T-cells) and *C. elegans*.[93,153] Exposure to SiO_2 NPs resulted in significant changes in expression of oxidative stress related genes (i.e. catalase, Cu–Zn SOD), and DNA damage repair genes (i.e. Rad-51) in HepG2 cell and *C. elegans*. Interestingly, however, significant changes in expression of these genes was not observed in a mouse model,[130] and the reason for this discrepancy will be important to explore. ER stress, which is involved in Au NP-exposed *C. elegans* as described earlier,[39] was also identified as a primary response to Au NP-treated human leukaemia cells through proteomic and transcriptomic approaches.[102] A role for ER stress was also supported in an in vitro study with liver human cells exposed to Ag NPs.[154] Although there are some similarities in responses to NPs between *C. elegans* and mammalian systems, it is important to note that the bulk of the research in mammalian systems so far has been in cell culture rather than whole-organism studies,

and direct comparisons between *C. elegans* and mammalian cell cultures studies can only be made with caution.

Conclusion: 'what has *C. elegans* taught us so far about NP toxicity?'

Caenorhabdits elegans studies have yielded a wealth of insight into the relative toxicity of various NPs, their mechanisms of toxicity and the importance of physiological barriers in modulating their effects. So far, the general rank order of toxicity of different NPs, as well as the molecular-level mechanisms of toxicity, appear to extrapolate fairly well between *C. elegans* and other systems. However, the concentrations of NPs required to cause toxicity in *C. elegans* are higher than those reported in cell culture and are more comparable to those observed in other whole organism studies, highlighting the protective roles of physiological barriers when using in vivo models. Thus, *C. elegans* is poised to continue to serve a key role in bridging in vitro cell culture studies to whole-organism studies in more complex organisms.

We have also learned that interaction of NPs with environmental variables such as natural organic matter and aging can have dramatic effects on NP toxicity. In addition, the exposure conditions (composition of media, presence of food etc.) can also result in differences in toxicity, and enough studies have been conducted to work out some of the difficulties associated with performing controlled and well characterised exposures. However, because many experiments have been conducted using different conditions affecting toxicity (for instance, different exposure media) and also using different NPs or the same NPs with different surface chemistry, systematic comparison of the results is often difficult and therefore, our ability to draw clear conclusions or make generalisations about NP toxicity to *C. elegans* based on these experiments is limited. In this review we discussed the factors that may explain the differences in NP toxicity to *C. elegans* and provided recommendations for future nanotoxicity experiments emphasising a need for more studies that systematically vary NP properties in the exposure media to examine how these interactions affect NP toxicity.

Availability of RNAi, transgenic and mutant *C. elegans* strains along with toxicogenomic approaches allowed us to identify some of the mechanisms of NP toxicity to *C. elegans*, which are not limited to oxidative stress. Overall, although progress has been made in terms of understanding the role of NP characteristics in modulating toxicity in limited subsets of NPs, we are still far from true predictive capability. Important areas of future research are investigation of additional potential mechanisms of toxicity, further systematic probing of the effect of NP characteristics on toxicity to develop predictive models, extension of molecular-level mechanistic toxicity to an understanding of cellular, tissue and organism effects, and elucidation of multigenerational effects.

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