Caenorhabditis elegans: Evaluation of Nanoparticle Toxicity

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Abstract

The relevance of *Caenorhabditis elegans* (*C. elegans*) as an in vivo model organism in the study of nanoparticle/biological interactions and nanotoxicology has gained popularity recently. This is attributed to its short life cycle, a high degree of homology with higher organisms, and cost-effective maintenance. The ability of worms to self-fertilize and generate large numbers of progeny aided with the presence of complex tissue systems is ideal for nanotoxicological multiple endpoint study both in terms of mechanistic and high-throughput screening approaches. Nanoparticle-mediated toxicity in *C. elegans* can be assessed using different standard methods and protocols. For example, assays that determine worm growth, mortality rate, reproductive capability, and locomotion changes can provide accurate measurements and predictability when applied to higher mammalian systems. The use of reporter gene analysis such as green fluorescence protein (GFP) in transgenic strains and microRNAs studies in *C. elegans* has led to the discovery of different biomarkers for toxicity studies. Thus, researches on *C. elegans* model have contributed immensely to our realms of knowledge in nanoparticle-based toxicity, and this has allowed for elucidation of alterations at the cellular and molecular levels. In this chapter, discussions are directed toward our general outlook of *C. elegans* as a model organism to study nanoparticle-mediated toxicity and the different approaches and assays employed regularly in the measurement of nanotoxicity. Special emphasis is taken considering significances of different biomarkers and molecular responses involved in the process (e.g., oxidative stress, DNA damage, and apoptosis, endoplasmic reticulum stress). Finally, based on recent evidence, the roles of common and important signaling pathways in regulations of nanotoxicity formation in *C. elegans* (p38

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MAPK signaling, insulin signaling, programmed cell death, and TGF-β signaling pathway) are discussed.

Keywords
Nanoparticles · *Caenorhabditis elegans* · Toxicity · Neurotoxicity · Apoptosis · Immunotoxicity · Insulin signaling

17.1 Introduction

Recent advancement in nanotechnology has encompassed across various disciplines as seen with its applications in different areas such as pharmaceutical, healthcare, transportation, and energy. Indeed, in our day-to-day life, we encounter many of the nanoparticle products ranging from gold nanoparticles (AuNPs) in our facial creams (Guix et al. 2008), silver nanoparticles in preservatives (Kokura et al. 2010), to titanium dioxide and zinc oxide nanoparticles in colorants and sunscreens for skin protection (Gulson et al. 2010). The bigger question is how safe regular exposure to nanoparticle is.

"Nanoparticles are generally defined as those microscopic particles which are having one structural dimension less than 100 nm, intentionally designed engineered nanoparticles having a similar physicochemical characteristic" (Gonzalez et al. 2008). Due to its characteristic physicochemical properties such as nano-size and large surface area per unit volume, it can elicit functions that can have wide applications. On the contrary, amidst its popularity and the wide spectrum of usability, the same inherent characteristic nature of nanoparticles or engineered nanomaterials (ENMs) predisposes biological systems such as biomolecules and organelles as an obvious target of unwanted and long-term toxicity. They can readily pass through the lipid bilayer of the cell membrane and other biological barriers, and this may lead to unwanted interactions that can alter homeostasis and normal cellular functions (Xia et al. 2008; Brar et al. 2010).

According to the latest studies, it is highlighted that human beings are in constant exposure to nanoparticles in their daily life through inhalation of airborne ultrafine/nanoparticles (respiratory tract), direct and indirect skin contact, ingestion through the oral route, and most significantly through injection by any means to blood circulation (Nel 2006). Nonetheless, physicochemical and biological interactions are poorly understood (Buzea et al. 2007). On the practical front, experimentations utilizing in vivo whole animal model are expected to throw lights to the unknown nanoparticle/biological interface. Similarly, in model organisms, the availability of defined cells, tissues, and organ systems as exposure routes of study may provide realistic cases on the different unknown effects of nanoparticles, and this can be correlated to that of a higher mammalian system such as a human. Fundamental insights obtained from these organisms can be adopted for the safety designs and development of future nanoparticles and in the judicious usage and control applications of the current notable toxic nano-based products.

17.2 Why Choose *C. elegans* for Nanotoxicology?

Experimental approaches utilizing various in vitro, cell-based, computational approaches and different in vivo models have undoubtedly enhanced our understanding of the process and precision requirements of toxicological science. Nowadays, a free-living *Caenorhabditis elegans* (*C. elegans*) model has become a trend for biosafety assessments of nanoparticles especially in ecotoxicological studies (Leung et al. 2008; Zhao et al. 2013; Qiao et al. 2014). Inherently similar to higher mammalian system, this model organism has provided useful information both in terms of the mechanistic and molecular basis of toxicity besides its scope in the field of modern predictive toxicology mentioned in Table 17.1. The answer to a bigger question of how these tiny little worms can contribute so much to

Table 17.1 General comparisons of *C. elegans* model in nanotoxicological studies

Advantages	Disadvantages
• Short life cycle—life span of 2–3 weeks multigeneration toxicity studies can be done in a couple of weeks rather than years • Easy, simple, and inexpensive cultivation with cooling incubator requirement • Small size and large number of offsprings. Can be cultured in 96 and 386 well plates • Transparent body allows for direct visualization, fluorescence monitoring, ontogenetic approach for different physiological studies • Whole genome sequence completed and availability of mutants or GFP transgenic strains for visualization at the tissue, cellular, and subcellular levels after nanoparticle exposure • Behavioral studies can be done through fully mapped body, and neuronal plans allow for rapid morphology and physiological and behavioral assessment • No restriction of use derived from bioethical regulations • *C. elegans* has well-functioning and characterized innate immune system for nanotoxicity studies • Conserved alimentary features and architectures make *C. elegans* a good oral nanotoxicity model • Accessible online resources and research community	• *C. elegans* lacks specific tissues such as the eyes, lungs, heart, kidney, and liver • Lack of circulatory system • Limited information about toxicity • Lack of adaptive immunity • Temperature-dependent development • *C. elegans* is not a good absorption model due to its tough cuticle • pH range is wide but still limited, and liquid culture testing requires soluble test compounds • Small changes in temperature, nutrient, salt can give inaccurate result

toxicological sciences and in particular to nanotoxicology is mentioned in the description below.

17.2.1 General Experimental Considerations, Parameters, and Techniques

C. elegans represents many fascinating characteristics as an in vivo model shown in Fig. 17.1. The first and the most significant characteristic of these nematodes is their short life cycle and self-propagation ability, which allow for large brood size progeny to be produced, whereby an individual animal can populate a plate. This unique short life cycle and its capacity to generate numbers of progeny in a short span of

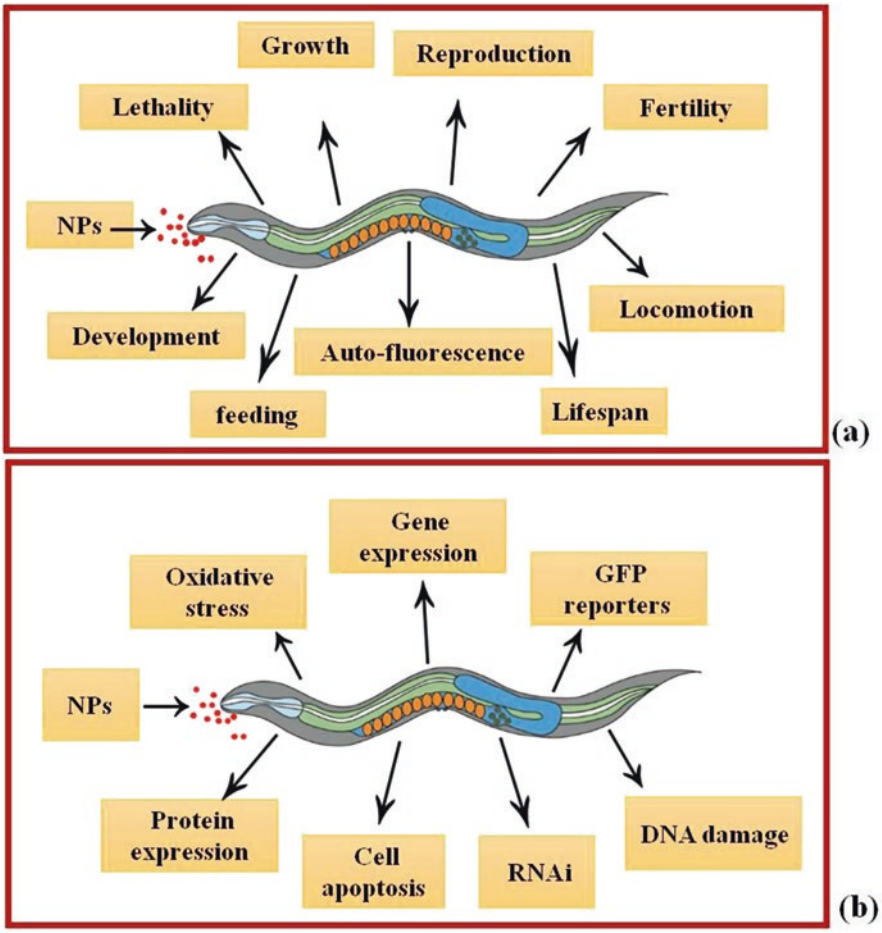

Fig. 17.1 *Caenorhabditis elegans* in nanotoxicity assessment and its endpoints (**a**) biological parameter and (**b**) molecular marker

time are ideal for experimenting with lots of animals as required for high-throughput screens without any ethical constraints (Fig. 17.1). The next important feature is ease of cultivation which requires a simple composition of nematode growth media (NGM) and a cooling incubator. Thus, it compensates for the financial expense and highly skilled professionals required. Third, because of its small size which is around 1 mm, it can be cultured easily on a Petri dish and 96 and 584 well plates, and it can be preserved for long-term at −80 °C. Worms can be synchronized easily using available standardized protocols, and this enables for uniform and age-dependent toxicological studies when needed.

With being simple, transparent, and multicellular, it can be used to elucidate the nanotoxicological process step by step such as uptake, translocation, distribution, and metabolism in certain targeted organs. *C. elegans* can also be useful for the assessment of different nanoparticles for sublethal endpoints. Its feasibility for high-throughput nanoparticle toxicity screening allows for the fast and easy identification of barren or toxic nanoparticles either engineered or synthesized. With whole-genome sequenced, *C. elegans* model is also ideal for different specialized and sensitivity studies. Different mutant strains can be generated easily through self-propagation with fast propagation time, and the feasibility of worms targeting gene inhibition by simple RNAi feed has further accelerated the process. These mutants allow easy assessment and identification of a particular gene or pathways which are involved in toxicity. The temperature-dependent growth of worms allows controlling the rate of growth, and temperature-sensitive strains can be used in toxicity studies. Another desirable feature which is not usually mentioned is that it is harmless to humans because worms cannot grow on the existing temperature of the human body (Table 17.1).

17.2.2 *C. elegans* Capacity for High-Throughput Nanotoxicity Screening

In terms of efficient productivity and predictivity that often involved in preclinical toxicity studies, evaluation in more than one mammalian model organisms is advantageous (Olson et al. 2000). Additionally, to minimize the unwanted cost and time involved in safety design and development of synthesized or engineered nanomaterials (ENMs), high-throughput-based toxicity screening is the current leading archetype. Previously, *C. elegans* toxicity assays were used only for small-scale validation studies to predict the harmful effects of compounds in genetic and chemical screens in mammalian species (Leung et al. 2008). Recently, Jung et al. (2015) presented with the first successful large-scale multi-endpoint and high-throughput screening design for nanomaterials. This method employed a whole animal *C. elegans* model system and has been applied to examine 20 ENMs including carbon-based ENMs. Most importantly, it utilizes and quantifies many important sublethal and lethal endpoints of *C. elegans* population including its growth, locomotion, fitness, and life span. Thus, it demonstrates the potential of *C. elegans* for high-throughput micro-techniques in different nanotoxicological studies. Its capacity in

generating cohesive dataset at high speed can have translational applicability for environmental and human health safety precautions prior to the applications of nanoparticles. Most significantly, this information is also publicly available at www.QuantWorm.org/nano (Jung et al. 2015).

17.2.3 Homologous Genes and Concordant Pathways

The reproducibility of *C. elegans* model in nanotoxicology relies heavily on the availability of homologs and orthologs genes, and this has been estimated for 60–80% of the human genes (Sonnhammer and Durbin 1997; Harris et al. 2004; Kim et al. 2017b). Similarly, counterparts for genes linked with many human diseases have been identified in *C. elegans* genomes (Kaletta and Hengartner 2006; Markaki and Tavernarakis 2010). Because of its well-conserved apoptotic pathway to human, the same fullerenol nanoparticles that are widely used in the medical field are found to enhance apoptotic cell death in Bristol N2 and mutant *C. elegans* strains as reported in different multicellular organisms (Vaux et al. 1992; Cha et al. 2012). Similarity is also observed in elements of the insulin and IGF-1 (IIS) signaling pathway with identical modes in regulations of metabolism, growth, and life span in *C. elegans* to that of mammals IGF-1 signaling pathway (Hunt et al. 2012). Zhao et al. (2016) highlighted the significance of IIS pathway in the control of graphene oxide (GO) toxicity in *C. elegans*, where mutations of *daf-2*, *akt-1*, *age-1*, or *akt-2* gene enhanced resistance of worms to GO toxicity. On the contrary, enhanced toxicity and susceptibility of worms that carry a mutation of the only *daf-16* gene to GO exposure was also reported. These findings provide evidence that GO can modulate the functions of *daf-2* (encodes the only homolog of IGF-1 mammalian receptor), AGE-1 (phosphatidylinositol 3-kinase), AKT-1, AKT-2-mediated kinase cascade, and DAF-16 (FOXO) transcription factor. These findings described on how different *C. elegans* strains can contribute to our knowledge of understanding on the roles of this signaling pathway in nanotoxicity formation in vivo (Zhao et al. 2016; Ma et al. 2009).

In terms of neurotoxicology, the nervous system of the worms represents another important target system where its neural fitness and neuromuscular defects in the forms of altered locomotion, reduced fecundity, and impaired olfaction can be used as parameters for nanotoxicity screening. Interestingly, most of the important human neurotransmitter systems employed for neuronal signaling and transmissions perform the same function in the worms (Kaletta and Hengartner 2006; Peterson et al. 2008). For example, serotonin and dopamine's significant roles in the movement are also required for the locomotion of worms (Vidal-Gadea et al. 2011). Worms have been pioneered as a model organism for various human pathologies including Alzheimer's disease (AD) (Levitan et al. 1996; Braungart et al. 2004), diabetes (Ogg et al. 1997), and human infections (Markaki and Tavernarakis 2010). Scharf et al. (2016) identified the neurotoxic effects of silica nanoparticles through widespread protein aggregations and activated amyloid fibrillation in *C. elegans* (Scharf et al. 2016).

17.2.4 *C. elegans* Mutant and Transgenic Strains in Nanotoxicology

The active involvement of researchers in the worm community aided with the output from *C. elegans* deletion mutant consortium has led to the creation of different mutant and transgenic strains. At present, more than 20,377 protein-coding genes in *C. elegans* and 6764 genes with associated molecular lesions through deletions or null mutations are available (WormBase WS220). Thus, mutants with predicted sensitivity for a distinct type of nanoparticles can be utilized to elucidate a distinct mechanism and signaling pathways involved in nanoparticle-mediated toxicity. One good example of such a mutation is in the genes involved in the antioxidant defense mechanism. Mutations of manganese/superoxide dismutase's encoding *sod-2* and *sod-3* genes heightened the sensitivity of worms to nanoparticles in comparison with wild-type N2 Bristol (Li et al. 2012). Wu et al. reported that exposure of *C. elegans* to DMSA-coated iron oxide nanoparticles, there were increased ROS productions with altered locomotion in *sod-2* and *sod-3* mutants in comparison with the wild-type worms, and this effect is much more pronounced in worms with double mutation of *sod-2* or *sod-3* genes (Wu et al. 2012). In another study, metallothionein-2 (MTL-2) protein that is encoded by *mtl-2* genes in *C. elegans* is observed to have protective effects against nanotoxicity by scavenging the enhanced ROS generation from metals exposure and also by binding with released metal ion nanoparticles (Table 17.3).

In another development, taking advantage of *C. elegans* transparent body combined with the availability of functional genetics tools, the upregulation and downregulation of target genes can be easily visualized using transgenic *C. elegans* which carries GFP reporter gene fused with DNA construct (Kaletta and Hengartner 2006). Thus, the gene of interest can be monitored and analyzed, and the fluorescence intensity quantified gives accurate information about the biological effect of a particular nanoparticle (Ma et al. 2009; Wu et al. 2012). For example, mutant strain CL2122, which carries *mtl* gene fused with the GFP reporter, has been used to detect the uptake of silver nanoparticle (Kim et al. 2017b). In another study, that demonstrated the similarities of toxicity mechanisms in *C. elegans*, both ZnO and $ZnCl_2$ nanoparticles could enhance the *mtl-2*::GFP expressions in transgenic *C. elegans*. This allows for speculations of the similarity in the process of intracellular biotransformation of both the nanoparticles to mediate the toxic effect observed (Ma et al. 2009).

Another commonly used markers for stress response genes are *daf-16* and *gcs-1* where GFP is fused to the either the C terminus of *daf-16* or promoter region of *gcs-1*. Responses to stress can be monitored by DAF16-GFP localization, which under optimal condition are located in the cytoplasm and translocation into the nuclei under different stressful conditions. In the case of GCS-1, GFP expression in worm intestine increases dramatically on exposure to toxic stress such as arsenic toxicity (Mohan et al. 2010). In monitoring different kinds of stresses, PMK-1, GST-4, and HSP-16.2 GFP carrying strains are often used to measure stress responses of nematodes exposed to different environmental toxicants or

Table 17.2 Examples of selected *C. elegans* transgenic strains commonly employed in nanotoxicology

Nanoparticles (NPs)	*C. elegans* transgene	Gene description	Findings	References
TiO_2 NPs	*sod-3::gfp*	Superoxide anion radical scavenger, protect against oxidative stress	Declined locomotion, intestinal ROS overproduction. Enhanced oxidative stress reporter in combination with nanopolystyrene particles	Dong et al. (2018)
MWCNTs	*let-7::gfp*	It is micro-RNA which exhibits mRNA 3′-UTR binding activity, involved in molting cycle, can regulate signaling macromolecule metabolic pathways	Decreased GFP expression in the body and intestine of a nematode. Dysregulation of development-timing transition, which is controlled by the let-7 gene, enhances intestinal ROS production and locomotion deficits	Zhao et al. (2017)
AgNPs	*mtl-2*::*gfp*	Gene encoding GFP fused with metallothionein 3 promoter. Gives protection against metal toxicity	The fluorescence signal of the AgNPs-exposed worms enhanced by fourfold in comparison with the nonexposed worms	Kim et al. (2017b)
Al_2O_3 NPs	*hsp16.2::gfp*	Encodes heat shock protein fused with GFP, it is involved in defensive response to heat, and localization is in the cytoplasm	Accumulation of intestinal lipofuscin in L1 larvae stage increased stress response and decreased in survival	Wu et al. (2011a, b)
CuO NPs	*hsp-16.2::gfp*	Gene-encoding GFP reporter driven by an hsp-16.2 promoter. Response to heat and unfolding protein function	Enhanced fluorescence intensity in hsp-16.2 transgenic strain upon nanoparticle exposure in comparison with untreated nematodes	Mashock et al. (2016)

(continued)

Table 17.2 (continued)

Nanoparticles (NPs)	*C. elegans* transgene	Gene description	Findings	References
ZnO NPs	*mtl-2::gfp*	Gene encoding GFP under the control of metallothionein 2 promoter, gives protection against metal toxicity	Nanoparticle exposure enhanced transgene expression in the mutant worms	Ma et al. (2009)
CeO_2 NPs	*gst-4::gfp*	It exhibits glutathione transferase activity	Enhanced ROS production levels, increased GST-4 fluorescence intensity on nanoparticle exposure	Rogers et al. (2015)
CeO_2 NPs	*hsp-4::gfp*	Orthologs of human HSP-70 have RNA polymerase II transcription factor binding activity, involved in ER stress response	Increased HSP-driven fluorescence intensity upon nanoparticle exposure, and this is dependent on dosage and exposure time	Rogers et al. (2015)
Fluorescent nano-diamond	*gcs-1::gfp*	Encodes GFP under the control of the *gcs-1* promoter, plays a role in resistance to arsenite and oxidative stress	Nanoparticle (0.5 mg/ml) exposure does not induce changes in GCS-1 and DAF-16 expressions	Mohan et al. (2010)
Ag NPs	*tph-1::DsRed*	Orthologs of human tryptophan hydroxylase 1; involved in axon regeneration, entry in dauer stage and adult life span regulation	Enhanced aggregations of tph-1::DsRed in ADF neurons, serotonergic neurons are a more sensitive target for Ag NPs	Piechulek and von Mikecz (2018)

nanoparticles (Wu et al. 2012). For example, exposure to Al_2O_3-nanoparticles was reported to have increased HSP-16.2 expression in nematodes (Yu et al. 2011) (Table 17.2). Overall, the analysis of gene expressions from transgenic worms can be easily reproducible with minimal variability unlike endpoints such as worm motility which measures lethality (Roh et al. 2006). Some examples of *C. elegans* mutant and transgenic strains commonly employed in nanotoxicology are mentioned in Tables 17.2 and 17.3.

Table 17.3 Examples of selected *C. elegans* mutant strains commonly employed in nanotoxicology

Nanoparticles (NPs)	*C. elegans* mutants	Findings	References
GO	*daf-16(mu86)*	Mutants strains exhibit reduced life span in comparison with wild-type nematodes	Zhao et al. (2016)
GO	*lys-1(ok2445)*	Enhanced the susceptibility to GO toxicity on the functions of both the primarily targeted organs and the secondary targeted organs	Ren et al. (2017)
GO	*daf-18(ok480)*	Worm carrying mutation exhibited susceptibility to GO toxicity as evidenced with defective in locomotion behavior and life span reduction	Zhao et al. (2016)
MWCNTs	*let-7(mg279)*	Worms carrying this mutation showed increased resistance to MWCNT toxicity upon exposure. Thus, the levels of intestinal ROS production and defective locomotion behavior are reduced in comparison with control	Zhao et al. (2017)
Ag-NPs	*pmk-1(km25)*	The increased levels of ROS formation and declining reproductivity observed were counteracted in comparison with wild type. Involvement of ROS and innate immune pathway PMK-1 p38 MAPK	Lim et al. (2012)
GO	*daf-2(e1370)*	Mutants showed resistance upon exposure to GO with enhanced head thrashing and the body bending capacity. Have a longer life span as compared with wild-type nematodes	Zhao et al. (2016)
MWCNTs	*hbl-1* or *lin-41*	Highly sensitive to MWCNTs, higher intestinal ROS production, and locomotion behavior is affected significantly	Zhao et al. (2017)
GO	*fat-5(tm420) nhr-49(nr2041)*	Mutant strain-dependent toxicity. Enhanced intestinal fat accumulation and shortened life span in *fat-5(tm420)* mutants. Enhanced life span in *nhr-49(nr2041)* mutants	Kim et al. (2019)
Ag-NPs	*pmk-1(km25)*	Ag-nanoparticle modulation of HIF-1, glutathione *S*-transferase (GST) enzyme activity, and reduced reproduction ability in wild type (N2) but not in a mutant strain of *C. elegans*	Lim et al. (2012)
Ag-NPs	*sod-3(gk235)*	Mutants exhibit dramatic enhancement in expression of different genes involved in MAPK signaling pathways as compared with the wild-type N2 worms	Roh et al. (2012)

17.2.5 *C. elegans* Lethal and Sublethal Endpoints

There are several endpoints which have been proposed for the assessment of nanomaterials using *C. elegans*, and these endpoints are classified under two categories: lethal endpoints and sublethal endpoints (Dhawan et al. 1999) as mentioned in Fig. 17.1. On the one hand, lethality is the basic endpoint for nanotoxicological

studies, and it can be measured by killing (mortality) assay, a manual method of scoring dead or live worms after exposing worms to nanoparticles. However, this manual counting increases the errors and can affect the accuracy in experimentation. Sublethal endpoints, on the other hand, are classified into morphological, behavioral, reproductive, developmental, and enzymatic endpoints (Jiang et al. 2016). In recent years, the importance of "3 Rs" (replacement, reduction, and refinement) is commonly referred for animal studies (Burden et al. 2015; Singh 2012), and *C. elegans* with its unique features has met with the 3R demands. Different toxicity ranking screens have repeatedly shown that *C. elegans* has predictive endpoints as that of the rat and mouse LD_{50} ranking by different researchers (Hunt et al. 2012). In some reports, there were suggestions that toxicity can also be easily assessed or equated by the survivability and mortality assays in *C. elegans* using its lethal endpoint. Williams and Dusenbery (1988) highlighted that mortality in adult *C. elegans* worms is comparable with that of higher mammals such as the rat and mouse LD_{50} ranking, and the same experiment in *C. elegans* can be done at a cheaper cost (Williams and Dusenbery 1988) (Fig. 17.1).

To monitor different sensitivities and on track changes, assessment of sublethal endpoints in worms has been used regularly for in vivo assessment and safety evaluation of different ENMs (Zhao et al. 2013; Charão et al. 2015). The credibility of these assessments has come from years of research on this model organism. Thus, evaluations of different sublethal endpoints can be conducted in a well-established and fairly systematic manner. These endpoints mainly include the rate of worm's survival and life span monitoring (Barsyte et al. 2001; Harada et al. 2007); growth inhibition and development (Anderson et al. 2001; Swain et al. 2004); increased cell death and germ-line apoptosis (Kim and Sharma 2004); changes in reproduction, progeny production, or phenotypes (Wang and Yang 2007; Wang et al. 2007); and changes in pharyngeal pumping rate, body motion, behavior, and feeding behavior (Wang and Xing 2009; Chen et al. 2013) (Fig. 17.1).

Based on its specificity and sensitivity, sublethal endpoints for different toxicants are manifested differently. Thus, morphological endpoints can be carried for a fast and more sensitive indicator of toxicity. Briefly, morphological endpoints can be assessed in terms of body width and length measurements of the nematodes. These measurements can be generally done manually with capturing images of worms by light microscope and its further analysis with image software. Different behavioral endpoints can also be assessed where changes in behavior can serve as an indirect measurement of internal physiological state or response to external stimuli. Thus, changes that affect the locomotion, body bend frequency (turning frequency), head thrash frequency, and pharyngeal pumping are often informative (Jiang et al. 2016). Scharf et al. observed that *C. elegans* on exposure to silica nanoparticles and its accumulation in the pharynx and vulva often resulted in an altered organ function, reduced pharyngeal pumping, and increased egg-laying capability (Scharf et al. 2013) (Fig. 17.1).

Sublethal endpoints are also associated with oxidative stress, and this is routinely monitored using transgenic/mutant strains fused with anti-oxidative enzyme and stress response genes (Li et al. 2012). Furthermore, parameters ranging from the

innate immune response, nervous system and neuronal functions, intestinal morphology, and epidermal vulnerability have been employed and raised (Wang and Wang 2008; Ma et al. 2009; Yang et al. 2015; Zhao et al. 2016a). For example, exposure to CeO_2 nanoparticle aggregates even at higher concentration is not linked with mortality/lethality endpoints but is associated with organism stress markers with sublethal endpoints in the form of higher levels of ROS, HSP-4, and declined fertility rate (Rogers et al. 2015). Using light microscopy and complex object parametric analyzer and sorter (COPAS), it was shown that cadmium can reduce the intestinal diameter and opacity of the worms. This provides a clue that identification of other similar intestinal toxicants using high-end optogenetic devices and high-throughput microfluidics techniques in *C. elegans* can make identification of classifications of various nano-toxins easier (Hunt et al. 2012) (Fig. 17.1).

17.2.6 Predictive Nanotoxicology Using *C. elegans*

Predictive toxicology forms a part of the modern pharmaceutical approach in drug discovery, it is proposed to have simplified the drug development process, and this is applicable too for fast and accurate assessment of nanoparticle toxicities in the near future. Recently, *C. elegans* in vivo assays have been used successfully in predictive toxicology testing (Hunt 2017). Interesting findings were also reported by Yang and his colleagues (2017) on the toxicity of zero-valent nanoparticles, wherein a toxicity-based-toxicokinetic (TBTK)/toxicodynamic (TD) modeling of different endpoints of *C. elegans* model has been formulated, a reiteration on the significance of this organism in the field of predictive toxicology. Empirical data obtained from this bioaccumulation experiments and nanotoxicity studies on fertility, locomotion, and development of *C. elegans* after Fe_0 nanoparticle exposure have been used to investigate environmental and health risks of Fe_0 nanoparticles and to regulate eco health with controlled applications of Fe_0NPs for environmental remediation and sustainability (Yang et al. 2017). Thus, *C. elegans* biomarker-based risk model, although in its early stage of development, is most likely to have a huge application in the field of modern predictive nanotoxicological sciences.

17.3 Biomarkers and Molecular Response to Nanoparticle Toxicity

At the cellular and molecular levels, nanoparticle toxicity can be initiated and responded by several mechanisms, and this is dictated directly by the physicochemical properties of nanoparticles and exposure conditions mentioned in Fig. 17.2. Many mechanistic studies on nanoparticle toxicity have highlighted the involvement of overwhelming oxidative stress level for nanoparticle-mediated toxic effects (Thomas et al. 2011; Handy et al. 2012) shown in Fig. 17.4. Its indirect involvement in the mechanism of nanoparticle toxicity has been proposed; for example, Hussain and his colleagues observed that Au-nanoparticle exposure leads to endoplasmic

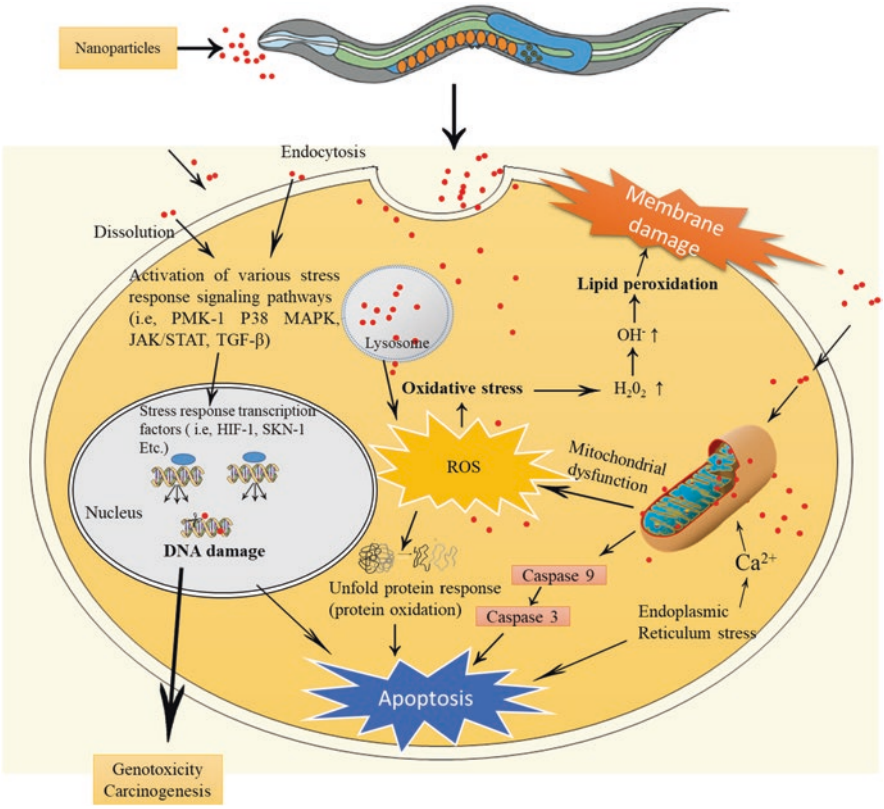

Fig. 17.2 Common mechanisms of nanoparticle-mediated toxicity in *Caenorhabditis elegans*

reticulum (ER) stress and an unfolded protein response (UPR) in *C. elegans* (Hussain et al. 2005) (Fig. 17.3).

17.3.1 Oxidative Stress

The high concentration of reactive oxygen species (ROS) or free radicals can exaggerate oxidative stress, and this is mainly due to imbalances between prooxidant and antioxidant enzyme level in an organism (Jat and Nahar 2010; Tan et al. 2018). ROS could be overproduced either directly by intrinsic ability (i.e., direct generation of ROS by nanoparticles due to acidic surrounding such as intestine or lysosomes either from leached ion or from the surface of the nanoparticles) or indirectly by nano-biological interactions (Choi et al. 2014) (Fig. 17.3). Chemically, it was proposed that on exposure to nanoparticles, an overproduction of ROS can occur due to the availability of an electronically active surface or photoactivation, transition metal impurities, and due to toxic metal ions (Ma 2010; Ludwig et al. 2007; Li et al. 2008; Damoiseaux et al. 2011). Wu and his colleagues have shown that TiO_2,

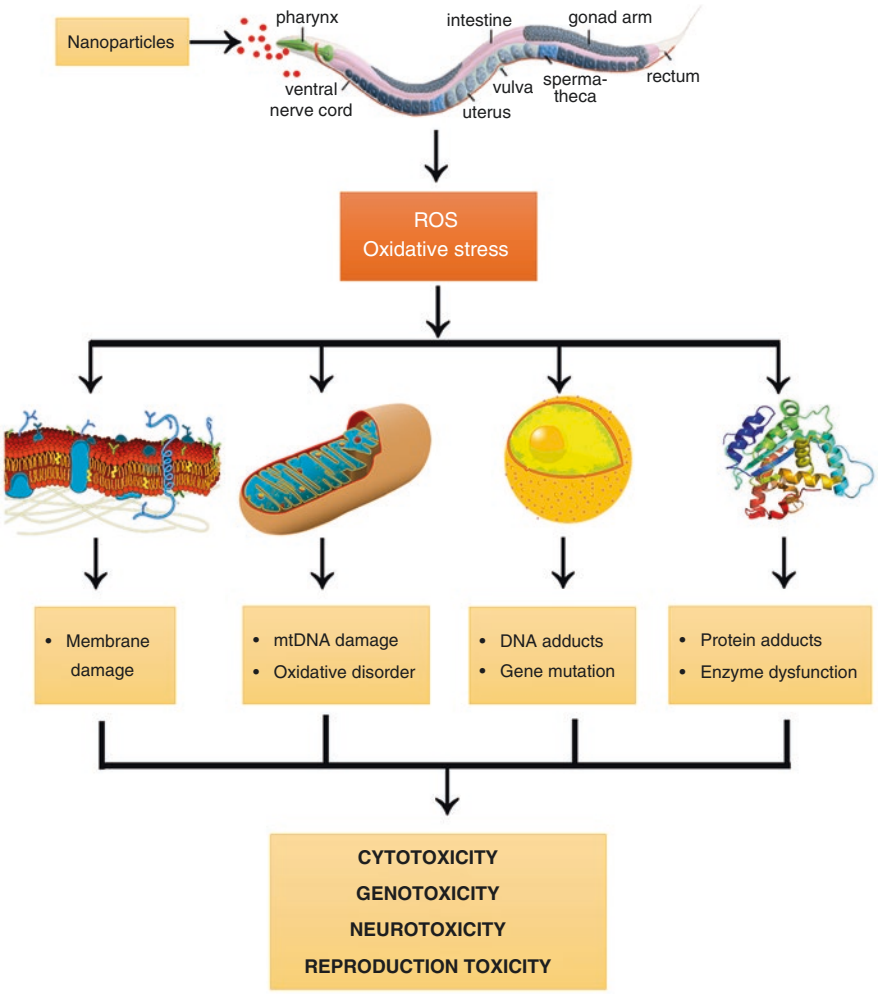

Fig. 17.3 Common cellular and molecular events of nanoparticle-based toxicity mediated through enhanced ROS generation and oxidative stress

ZnO, and SiO_2 nanoparticles enhance the production of ROS, and their toxicities can be correlated with different worms endpoints such as mortality, locomotion, development, and reproduction (Wu et al. 2013). Furthermore, among all the three nanoparticles studied, TiO_2 nanoparticles were reported to be more toxic as observed with significant decline in head thrash and body-bending movement in mutant strains of *sod-2*, *sod-3*, *mtl-2*, and *hsp-16* strains compared with Bristol N2 type (Wu et al. 2014).

The chemical properties of the nanoparticles directly dictate the levels of ROS that can be generated, and what kind of existing nano-biological interactions exists could orchestrate the whole process. Thus, it is possible that some

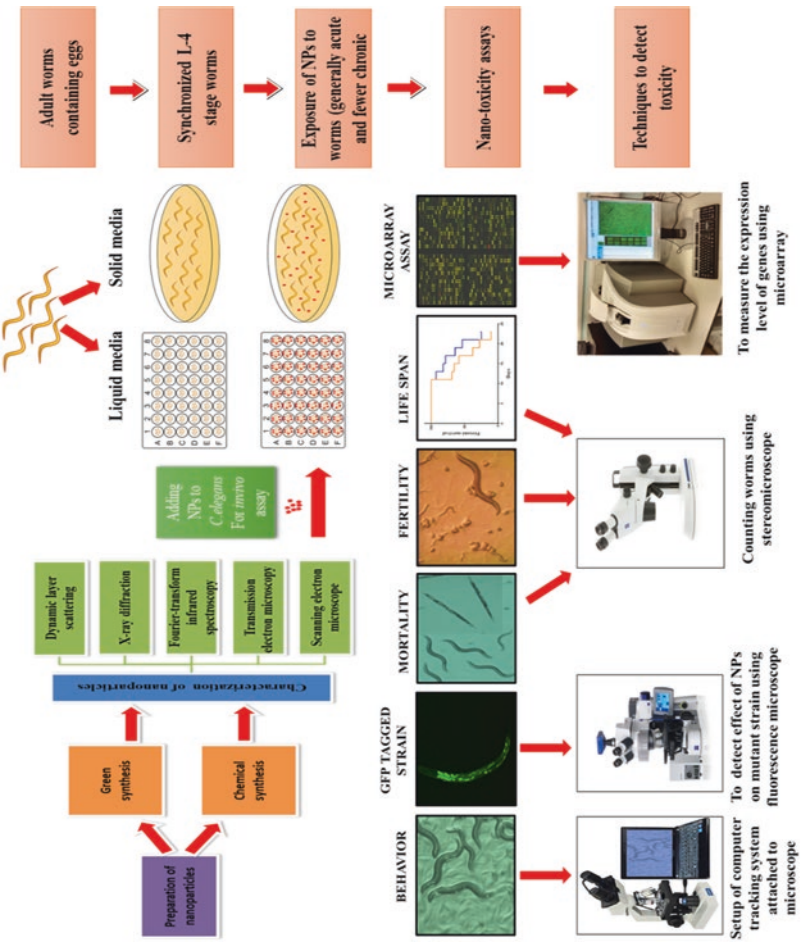

Fig. 17.4 Summarized and simplified diagram depicting the common utilities and methods involved in nanotoxicological studies using *C. elegans* model organism

nanoparticles which lack the intrinsic ability of ROS production can also generate ROS via interaction with the biological system and organelles. For example, nanoparticle direct interaction with organelle-like mitochondria could initially begin with disruption of cell cytosolic membrane, followed by changes in membrane potential and interrupted functions of the electron transport chain and oxidative phosphorylation. This will finally end up with overwhelming ROS levels (Xia et al. 2006; Meyer et al. 2013). Choi et al. have indicated that binding of nanoparticles to membrane receptor can activate the receptor present, and the amplification of intracellular cascades such as MAPK changes the expression levels of stress response genes that could influence ROS production (Choi et al. 2014). Other mechanism reported to have been involved in enhanced oxidative stress levels on nanoparticle exposure is the increased accumulation of high calcium level (Marano et al. 2011; Soenen et al. 2011).

Some types of nanoparticles can have a catalytic activity which is due to photoactivation. Kim et al. (2017a) examined the adverse effect of TiO_2 nanoparticles on the nematode *C. elegans* with or without UV activation, and they observed that UV-activated TiO_2 nanoparticles significantly reduced the reproduction potential of the worms via oxidative stress mechanism (Kim et al. 2017a). Exposure to TiO_2 nanoparticles is reported to enhance levels of intestinal ROS, brood size reduction, and retarded locomotory behavior in worms (Li et al. 2012; Wu et al. 2013). In another interesting study, Dong et al. (2018) have emphasized on the role-combined effects, wherein nano-polystyrene particles further exaggerate TiO_2 nanoparticle-mediated toxicity as seen with the impaired motor neuron and change in locomotion behavior in SOD-3 mutant worms. This enhanced toxicity which could not be produced by nano-polystyrene alone is linked to enhanced activation of intestinal ROS and accumulation of oxidative stress (Dong et al. 2018).

Among the nanoparticles, Ag-nanoparticles are the most studied in *C. elegans*, and this is based purely on their antimicrobial properties and different potential biomedical applications. On the contrary, there were reports suggesting that the release of Ag^+ ions from the Ag-nanoparticles could catalyze the production of free radicals (Chávez-Andrade et al. 2017). Lim et al. (2012) have shown that exposure of Ag-nanoparticles to wild-type *C. elegans* (N2) enhanced ROS formation through significant upregulation of PMK-1 of p38 MAPK pathway at both gene and protein levels. Additionally, Ag-nanoparticle modulation of HIF-1, glutathione *S*-transferase (GST) enzyme activity, and reduced reproduction ability in wild-type (N2) but not in a mutant strain of *C. elegans pmk-1* (km25) were also reported (Lim et al. 2012). Similarly, CeO_2-nanoparticle exposure was linked to enhanced oxidative stress, inflammation, and genetic damage, whereby the redox cycle and oxidation state between Ce^{3+} and Ce^{4+} are thought to magnify the production of free radicals. In *C. elegans*, CeO_2 nanoparticle exposure is linked to a short life span, inhibition of growth and development, decrease fertility, and thermo-intolerance (Rogers et al. 2015).

17.3.2 Genotoxic, Apoptosis, DNA Damage, and Repair

Apoptosis or programmed cell death which is under extreme regulations is part of normal animal development. Similarly, in *C. elegans*, the numbers of genes are linked with the apoptotic process during embryonic development, wherein apoptosis process is involved in the removal of 113 cells during the normal development process of adult hermaphrodite, while in the larval stages, the death of 18 cells occurred due to apoptosis (Sulston and Horvitz 1981; Sulston et al. 1983). In *C. elegans*, options for both developmental cell death (programmed cell death) and necrotic-like cell death during extensive cell injury have been reported too. Hence, to protect the somatic cells of adult *C. elegans* which are mainly post-mitotic in nature, it is crucial that the structures, numbers, and fidelity of DNA replication are at their best constantly.

At the cellular level, the process of replication of DNA and the different mechanisms required for repair is reported to be conserved between *C. elegans* and other higher mammals (Leung et al. 2008). Two accepted paradigms for nanoparticle toxicity arises because of their involvement in excess production of ROS and other proinflammatory markers. These excess markers which are interconnected at different levels in their targets can overwhelm the cell components, thereby causing irreparable damage if not properly controlled. Thus, with DNA considered to be susceptible to enhanced oxidative stress, the most accepted hypotheses to explain nanoparticle roles in DNA damage rely directly on ROS levels. Overproduction of ROS influenced by nanoparticles can oxidatively modify DNA, leading to strand breaks, unspecific base pairing, and formation of abasic sites which in return induces mutation, tumorigenesis, and aging-related diseases (Valko et al. 2006) (Fig. 17.4). Chatterjee et al. observed that Ag-nanoparticle exposure to *C. elegans* can produce oxidative modifications of DNA and strand break which triggers the *hus-1* components of DNA damage checkpoint pathway and finally programmed cell death. In the *pmk-1* mutant (homologue of p38 MAPK and function in apoptosis), the DNA damage level was reported to be higher, and instead of apoptosis, necrosis occurred in *pmk-1* mutant (Chatterjee et al. 2014). In another comparative and evidence-based toxicity study, it was found that Ag-nanoparticles and $AgNO_3$ nanoparticle exposure in *C. elegans* enhanced the levels of a specific biomarker of oxidative damage and mutagenesis, 8-OHdG (8-hydroxy-2′-deoxyguanosine), a clue for the cause toxic effect observed (Ahn et al. 2014).

The exposure of an organic-based nanoparticles and hydroxylated fullerene nanoparticles in *C. elegans* was also reported to have induced its programmed cell death pathway (PCD) (Cha et al. 2012). This is also linked with ROS exaggerations on nanoparticle exposure that ultimately proceeds with either necrosis, PCD, or both (Stergiou and Hengartner 2004; Lant and Derry 2013). Khare et al. (2014) provide evidence that ZnO nanoparticle apoptosis activation occurred in *C. elegans* with upregulated expressions of different proapoptotic genes such as *ced-3*, *cep-1*, *ced-13*, *ced-4*, and *egl-1* observed in ZnO nanoparticles exposed worms in comparison with untreated control. Similarly, based on the size, a 21 nm ZnO nanoparticles could downregulate important antiapoptotic gene *ced-9* (Khare et al. 2015).

According to nucleotide sequence homology, proteins which function in DNA repair mechanism are highly conserved between *C. elegans*, *Mus musculus*, and *Homo sapiens*. Similarly, the use of mutant strains deficient in DNA repair (e.g., *nth-1*, *xpa-1*) and germline DNA damage checkpoints mutant strains (e.g., *mrt-2*, *hus-1*, and *rad-5*) has been significant in understanding biologically relevant mechanisms involved in the toxicity of nanoparticles. This has wide applications with respect to DNA damage, genomic instability, and in testing the anticancer activities of different NPs (Stergiou and Hengartner 2004). Hunter et al. reported that efficiency of base excision repair (BER) in *C. elegans* found to be similar with that observed in mammals in repairing in vivo of alkylating and oxidatively modified mtDNA, nuclear DNA that is damaged after H_2O_2 pretreatment (Hunter et al. 2012). Thus, *C. elegans* has an enormous potential in the investigating field of DNA damage and repair processes and in understanding the roles of different nucleic acid toxicants.

17.3.3 ER Stress and Heat Shock Proteins

Endoplasmic reticulum (ER) is known as an organelle required for proper protein formation, leading into their native conformations besides its role as a storehouse of calcium. Normally, proteins are synthesized from ribosome which is bound to rough ER, and improperly folded proteins are detected and modified to their native conformations by molecular chaperones and enzymes which are present in the ER lumen. On the contrary, under numerous unwanted conditions, accumulation of misfolded/truncated proteins followed with disturbance in Ca^{2+} homeostasis predisposed to a cell to ER stress. It was reported that Au-nanoparticle exposure can lead to protein denaturation and improper folding (Nel et al. 2009). Under stressful conditions, ER loses its functions, and this causes further accumulation of misfolded proteins and protein denaturation. Added with changes in calcium homeostasis, this can lead to enhancing the activity of the unfolded protein response (UPR) pathway. This activation of UPR pathway is required for the degradation of protein via apoptosis (Lai et al. 2007; Nel et al. 2009; Xu and Park 2018). In *C. elegans*, the UPR pathway is triggered by both canonical and noncanonical pathway. The canonical pathway consists of upregulated molecular chaperones (heat shock protein). The noncanonical pathway comprises UPR response, and it includes 25 genes upregulated from abu/pqn families (Haskins et al. 2008).

Tsyusko et al. (2012) reported that Au-NPs (4 nm) have the ability to gain access inside *C. elegans* cell through clathrin-mediated endocytosis, and this entry enhances the formation of improperly folded/unfolded proteins, followed by mediation of ER stress, and the commence activity of both canonical and noncanonical UPR pathways. Thus, irreversible and accelerated cell death will occur that is significant in the case of *C. elegans* postmitotic tissues. Additionally, Au-NPs exposure to worms is reported to have interfered with Ca signaling and amyloid processing pathways, that would result in the accumulation of Ca^{2+} intracellularly and the ultimate promotion of non-caspase proteases events and calpain/cathepsin axis activation leading to

cell necrosis and destruction (Tsyusko et al. 2012). The role of p38 MAPK in giving protection against nanopolystyrene particle toxicity by activating XBP-1-mediated ER-UPR in *C. elegans* intestine was also reported (Qu et al. 2019).

Molecular chaperones are a highly conserved and ubiquitous class of folding modulators expressed in all subcellular compartments that have a crucial role in preventing nonnative conformations and stabilization of various proteins. Molecular chaperones also known as heat shock proteins (HSPs) are classified according to their molecular mass and different families of genes. On exposure to Au-NPs, 26 pqn/abu genes of the noncanonical unfolded protein response (UPR) pathway are reported to be upregulated besides molecular chaperones (*hsp-16.1*, *hsp-70*, *hsp-3*, and *hsp-4*), and this further confirmed ER stress involvement. Additionally, the heightened sensitivity to Au-NPs in a mutant strain of worms (*pqn-5*) is an indication for the direct involvement of this pathway in amelioration of Au-NPs toxicity (Tsyusko et al. 2012). Furthermore, on chronic exposure of worms to CeO_2 nanoparticle, aggregation of proteins is linked to the enhancement of ROS generation and HSP-4 expression, but not mortality (Rogers et al. 2015).

17.4 *C. elegans*-Based Assays for Nanoparticle Toxicity Studies

17.4.1 Survival Assay

Among the different assays available in the worm model, the notable survival assay has been used regularly prior to in-depth identification of novel genetic factors, molecules, or signaling pathways involved (Hae-Eun et al. 2017). As part of its application, exact environmental conditions for different survival conditions (life span assays, abiotic stress resistance assays, and pathogen resistance assay) should be standardized first based on the sensitivity and features of a typical survival assay to be employed (Amrit et al. 2014; Keith et al. 2014). Survival assay can be carried out with synchronized isogenic populations in both solid and liquid media, fed with *E. coli* OP5O as their food source. With the exception of some temperature-sensitive mutants, worms can be grown at different temperatures (15–25 °C), with 20 °C considered to be ideal for survival assay. After nanoparticle exposure, counting of live and dead worms by simple light microscope can be done at regular intervals (e.g., hours or days). To facilitate counting and to differentiate between dead and live worms, fluorescent dyes, such as SYTOX, can be used to measure the viability of *C. elegans* cells differentiated with fluorescent signals of dead worms (Gill et al. 2003). Subsequently, for survival analysis, two widely used curves are simple survival curves/mortality rate with Kaplan/Meier survival plots to illustrate the percentage of animals alive at different time scales (Kaplan and Meier 1958). Additionally, log-rank test and Fisher's exact test and other statistical methods are also employed for analyzing survival curves (Fisher 1990; Mantel 1966).

17.4.2 Biochemical and Oxidative Stress Assays

Different standardized biochemical assays and protocols are available to evaluate changes in the biochemistry of *C. elegans* upon nanoparticle exposure. The metabolic activity can be measured by monitoring oxygen consumption level (polarographically using Clark-type electrodes), carbon dioxide generation (gas respirometry), and heat production (by microcalorimetry). Carbon dioxide generation is reported to reduce drastically by about 50% in 12 days in comparison with a 6-day-old worm; hence, proper references should be utilized (Braeckman et al. 2002; Van Voorhies and Ward 1999). Changes in the energy status of the worms after nanoparticle exposure can be done with ATP measurement of worm fractions using enzymatic or luciferase-based reactions. Biochemical changes in activity levels of different enzymes such as acetylcholinesterase (AChE), alkaline phosphatase (ALP), catalase (CAT), and SOD enzymes (Wang and Wang 2008; Roh and Choi 2008) and relevant molecular and genetic endpoints such as HSP and MTL are other markers that are routinely monitored (Swain et al. 2004; Shashikumar and Rajini 2010).

Emphasizing on the significance of enhanced ROS and oxidative levels in nanotoxicology, different sensitive methods and protocols are available for its measurement. Oxidative stress resistance assay is employed to measure the sensitivity and resistivity of the worms. These include exposure to t-BOOH, H_2O_2, and paraquat treatment (Castello et al. 2007; Keith et al. 2014). Most of these assays enhanced ROS production levels and can be used to check to the resistance of the worms prior to or after nanoparticle exposure. Detection of carbonylated proteins by DNPH and lipid peroxidation adduct measurements can be done spectrophotometrically to measure oxidative damage after exposure. Staining techniques commonly employed include fluorescent markers such as CM-H_2DCFDA (5-6,chloromethyl-2,7-dichlorodihydrofluorescein diacetate), MitoSOX, propidium iodide, and Sytox to measure cellular viability (Hunt et al. 2012; Roth et al. 1997). Nile red and BODIPY-labeled fatty acids stain for lipid (Ashrafi et al. 2003; Mak et al. 2006) can be performed to get a wholesome idea of nanoparticle toxicity whether in larvae or adult worms. Antibody-based histochemical stains and protein expression studies such as Western blot and Elisa (enzyme-linked immune-sorbent assay) with sensitive detection systems have also been applied with high accuracy in *C. elegans* (Duerr 2006). Other commonly used methods are transmission electron microscopy (TEM) for visualization of body morphology, the TUNEL (terminal transferase dUTP nick end labeling) for detection of DNA fragmentation and apoptosis, SYTO dyes, and 4,6-diamidino-2-phenylindole (DAPI) to assess nuclear morphology and dead cells. Numbers of apoptotic cells can be visualized using green fluorescent protein (GFP) transgenic nematodes strains fused with genes of different components of PCD pathway (Chalfie et al. 1994).

17.4.3 Toxicogenomic Studies and Genomic Assays

To monitor the effect of tested nanoparticles at the genetic level routinely, visualization of fluorescence intensity of *C. elegans* using the reporter genes of GFP and β-GAL (LacZ) by fluorescence microscopy is employed routinely (Fire et al. 1990). Characteristics of fragmented DNA of apoptotic cells or germline can be visualized using the fluorescent dye acridine orange (AO) dye (Kelly et al. 2000). Microarray, ChIP-seq with histone modification-specific antibodies and qRT-PCR are another recent and more sensitive techniques that have been proved to be useful for genetic studies in *C. elegans* (Hall et al. 2010). Genome-scale RNAi screen to elucidate target gene expression during exposure conditions has been reported too (Wang et al. 2009). To provide in-depth information into the activating functions of different genes within different tissues, determination of the spatiotemporal pattern of gene expressions in *C. elegans* has also been reported. Unique transcriptional expression patterns for the effect of a particular nanoparticle can be determined in *C. elegans* model on a large-scale by the application of serial analysis of gene expression techniques (SAGE) (Ruzanov and Riddle 2010; Velculescu et al. 1995).

17.4.4 Reproductive Assays

In *C. elegans*, the development of reproductive organs can be assessed with different assays to measure reproductive toxicity of a particular nanoparticle. These assays include brood size, the number of transgenerational progeny (beyond the egg stage), number of oocytes, embryonic lethality, and male formation assay (Zhao et al. 2016). The size of gonads can be a good prediction of reproductive toxicity potential of a tested nanoparticle (Wu et al. 2011a, b). Toxic effects can also be determined by the decline in egg-laying capacity, disturbed egg-laying pattern, and the decline in number of viable progenies that are reproduced at different time points (Gomez-Eyles et al. 2009; Smith et al. 2013). Multigeneration reproductive toxicity of nanoparticles can also be evaluated for different worm generations.

17.4.5 Nervous Tissues Toxicity Assay

In *C. elegans*, primary evaluation of the toxic effects of different nanoparticles on the nervous system can be carried out by simple tracking of the locomotion behavior and movement speed, an estimation of motor neurons functions in nematodes. Specific fluorescently tagged neurons can be visualized by microscopy. Example are DAergic neurons through dopamine transporter (*dat-1*::GFP reporter) (Helmcke et al. 2010; Vanduyn et al. 2010), serotonergic neurons through tryptophan hydroxylase (*tph-1*::GFP reporter) (Sze et al. 2000; Nass et al. 2002), GABAergic neurons through glutamic acid decarboxylase (GAD) (*unc-25*::GFP) (Cinar et al. 2005),

cholinergic neurons through *unc-1*::GFP (a close homolog of mammalian protein stomatin) (Winnier et al. 1999; Nass et al. 2002), and glutamatergic neuron through *eat-4*::GFP (Lee et al. 1999; Earls et al. 2010). Additionally, analysis of axonal degeneration and loss of neuronal contact, analysis of certain neurons types such as the AVL and the DVB neurons, thermotaxis learning assays, paralysis, neurotransmitter, and enzyme assays such as AChE levels are often carried out. Indeed, with an expanding *C. elegans* toolkit and the availability of new technologies in the optogenetics field, the transparent body and well-defined nervous system of worms are ideal for evaluation of nanoparticle toxicity.

17.4.6 Growth, Development, and Life Span Assays

Measuring the synchronized nematode body length is a common method used for determining the developmental effects of different nanoparticles. Using standard references, measurement of the length (dorsal/ventral; tip of head to tail) and width (ventral to posterior end of the vulva) can be done using simple microscope and image analyzer (Boyd et al. 2010; Cha et al. 2012; Rudel et al. 2013; Wu et al. 2013; Zhuang et al. 2014). Applications of high-tech equipment such as COPAS Biosort, microfluidic devices with automated sorting, counting devices, and morphological detection systems have made the process faster with accuracy. Dauer formation assay can also be used to investigate the possible effect of nanoparticles on development (Wang et al. 2010).

For life span assay, healthy synchronized L4 stage worms can be grown in the presence of 5-fluorodeoxyuridine (FUDR) to prevent progeny production, and the parent plate is transferred into a fresh plate every 3 days where counting of dead or live worms is scored (Shen et al. 2009; Wang et al. 2010; Zhuang et al. 2014). Lipofuscin measurement is an autofluorescent marker of oxidative degeneration of cellular components that usually increased in levels with the aging of organisms (Brunk and Terman 2002). Lipofuscin levels can be measured spectrofluorimetry using a band filter of 525 nm, and the intensity can be determined using the mean or net pixel of the whole body/intestine of each animal to determine the effect of a particular nanoparticle on the age of the worms. Other parameters that can be useful indicators of normal and accelerated aging include pharyngeal pumping rate, body movement, chemotaxis response, defecation pattern, lipids, and enzyme activity levels (Klass 1977; Garigan et al. 2002).

17.5 Major Signaling Pathways and Their Roles in Nanotoxicity in *C. elegans*

17.5.1 p38 MAPK Signaling Pathway Roles in Nanotoxicity

Stress-associated mitogen-activated protein kinase (MAPK) pathway responses to a variety of stressors, thereby serving a transducer role to convert extracellular

signals into various intracellular outputs. In *C. elegans*, the p38 MAPK signaling pathway is highly conserved with orthologs *pmk-1*, *sek-1*, and *nsy-1* available. On receiving signals from the extracellular cell surface, p38 isoforms transduce the signals into the nucleus to modulate the activities of numbers of transcription factors. For example, as part of its response and regulation to arsenite stress, *pmk-1* of MAPK transactivates transcription factor *skn-1* gene. The SKN-1 protein, in turn, can translocate into the nucleus which activates *aip-1* gene (which encodes for a protein carrying the ring finger domain) to protect cells from the overwhelming arsenite toxicity (Inoue et al. 2005). Inhibition of *pmk-1* gene by RNAi was also found to enhance the worm's susceptibility to pathogens, which suggests that PMK-1, NSY-1, and SEK-1 proteins are crucial players in the defense response of the worms (Kim et al. 2002).

On the one hand, Roh et al. reported that Ag-nanoparticle exposure in worms can upregulate genes involved in MAPK pathway in wild-type *C. elegans* as seen with activation of *sod-3* gene expression (Roh et al. 2012). On the other end, it was found that Ag-nanoparticle exposure increased ROS production in wild-type *C. elegans* which was rescued from *pmk-1* (km25) strain, a direct indication that Ag-nanoparticle modes of toxicity are through oxidative stress (Lim et al. 2012). In addition, SKN-1/Nrf is thought to play additional roles through phase II detoxification involving glutathione *S*-transferase 4 (GST-4) which is present in the pharynx, hypodermis, and intestine of the worms. Studies have shown that intestinal knockdown of GST-4 by RNAi enhanced the susceptibility of worms to GO toxicity manifested in the form of decreased life span and enhanced intestinal ROS levels.

17.5.2 Insulin Signaling Pathway Roles in Nanotoxicity

In *C. elegans*, the transcriptionally active DAF-2/IGF-1 signaling pathway regulates many genes which are important for *C. elegans* longevity, growth, and metabolism. The activation of this signaling pathway has been linked to various processes such as fat storage and immune and stress responses (Zhao et al. 2016b). Its significant roles required for maintaining the longevity of worm intestine and neurons have been reported based on tissue-specific activity assay (Libina et al. 2003). When a ligand molecule insulin binds to DAF-2/IGF-1 receptor, it transmits signals through tyrosine kinase domain of the receptor to stimulate several kinases that includes phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase (PDK-1/3), serine/threonine kinase (AKT-1/2/Akt/PKB), and serine or threonine-protein kinase (SGK-1) (Gami and Wolkow 2006). Further phosphorylation of AKT and SGK-1 leads to downregulation of DAF-16/FOXO transcription factor which inactivates its target gene such as *sod-3* (Gami and Wolkow 2006; Yang et al. 2015).

In normal physiological condition, DAF-2 activation and the ultimate DAF-16 phosphorylation are necessary to keep it sequestered in the cytoplasm for regulating the normal life span of the worm (Antebi 2007). On the contrary, inactivation of DAF-2 by different capable stimuli can free DAF-16 from phosphorylation, and thus, it can translocate to the nuclei to transactivate or initiate the expression of a

series of genes that include defense and stress-related genes (Baumeister et al. 2006). ZnO-nanoparticle exposure is reported to have modulatory roles in the insulin signaling pathway in a dose-dependent manner (Khare et al. 2015). On the one hand, multi-walled carbon nanotube (MWCNT)-mediated toxicity is reported to have repressed the expression levels of *daf-16* and *daf-18* genes (Zhao et al. 2016a). Nano-polystyrene, on the other hand, has been linked with downregulation, and *daf-2*, *age-1*, and *akt-1* transcriptional factors (Shao et al. 2019) of the insulin signaling pathway were also reported.

17.5.3 The Programmed Cell Death (PCD) and DNA Damage Pathway

The programmed cell death is the evolutionarily conserved program for self-destruction and is important for the development and homeostasis of the functional organ (Lettre and Hengartner 2006). The core PCD pathway in *C. elegans* is similar to the human pathway, and this includes *egl-1* (*BH3*-only gene), *ced-9* (*bcl-2*), *ced-4* (*apaf-1*), and the terminal *ced-3* (caspase) (Conradt and Horvitz 1998; Hengartner and Horvitz 1994; Hengartner et al. 1992). For the execution of apoptosis, CED-3 is required, and it requires autocatalytic cleavage which is initiated by *ced-4*, acting as an initiator caspase. In living cells, interactions of CED-9 are required for sequestration of CED-4, and hence its affinity for CED-3 protein to remain on the outer surface of mitochondria will inhibit the cells to undergo PCD (Chen et al. 2000). In cells that are destined to undergo PCD, conformational changes of CED-9 take place through interactions with EGL-1, and these changes relieve CED-4 from the regulation of CED-9 (Yan et al. 2004). Other candidates include EGL-1 (apoptosis upstream activator and checkpoint), CEP-1 (ortholog of human tumor suppressor p53), CLK-2 (ortholog of telomere length-regulating protein Tel2p), and checkpoint protein HUS-1.

In *C. elegans*, ZnO nanoparticle is reported to have modulatory activity on the expression level of *cep-1*, which causes germ-line cell death through cep-1/p53-dependent signaling pathway (O'Donnell et al. 2017). The CEP-1 activation triggers the activation of *egl-1* and *ced-13* genes. EGL-1 and CED-13 interact with CED-9 homolog of BCL-2 (antiapoptotic protein) that cause inhibition of its transcription, activates and induces the release of CED-4, and therefore activates CED-3 (caspase) which initiates apoptosis. Upregulation of retinoblastoma protein-coding gene *lin-35* which inhibits ced-9 has also been reported, and this can further promote physiological apoptosis. In addition, *efl-2* and *dpl-1* encode for transcription factor E2F, and the cooperative work of DPL-1, RB, and E2F increases the expression levels of caspases which promotes the germ cell apoptosis (O'Donnell et al. 2017). Recent reports have also shown that *C. elegans* carrying either mutation of *ced-3* or *ced-4* can reverse germline apoptosis that is activated by GO exposure; whereas, mutation of *ced-9* accelerates germline apoptosis upon GO prolonged exposure (Conradt and Horvitz 1999).

17.5.4 TGF-Beta Signaling Pathway

The intercellular signaling molecule, transforming growth factor-β ligands (TGF-β), plays a significant role in communication between cells in eukaryotic organisms. TGF-β ligand functional and molecular mechanisms are highly conserved, with regulations and components of the TGF-β signaling pathway in *C. elegans* found to be similar to higher organisms. Genes which encode for five members of TGF-β ligands (*dbl-1*, *daf-7*, *unc-129*, *tig-2*, and *tig-3*) have been identified in *C. elegans* genome. From these ligands, DBL-1 and DAF-7 interact and play their role by canonical receptor-*smad* signaling mechanism, while UNC-129 interacts and functions through the noncanonical signaling pathway. TIG-2 and TIG-3 members of TGF-β ligand function are not well described, and *daf-1*, *daf-3*, *daf-5*, *daf-8*, *daf-12*, and *daf-14* are the essential components in TGF-β pathway in *C. elegans* (Savage-Dunn and Padgett 2017). In 2017, Kim et al. described a comparative analysis in which *C. elegans* response was observed underexposure of TiO_2 nanoparticles and UV-activated TiO_2 nanoparticles using mutant strains which clearly showed that TiO_2 nanoparticles induce toxicity through JAK/STAT pathway while UV-activated TiO_2 nanoparticles could induce toxicity through TGF-β pathway. The activation of both JAK/STAT and TGF-β pathway across TiO_2NPs leads to phototoxicity and reproductive failure in *C. elegans* (Kim et al. 2017a).

17.5.5 Other Signaling Pathways

Levels of important molecules involved in the innate immune system of the worms change drastically on exposure to nanoparticles. There were instances of significantly increased and decreased expressions of different isoforms of lysozymes, saponin-like proteins, etc. (*lys-1*, *dod-6*, *F55G11.4*, *lys-8*, and spp-1), in *C. elegans*, which is presumed to be dependent on types of nanoparticles and exposure time. Similarly, mutations of any lysozyme genes (*lys-1* and *lys*-8) and RNAi knockdown of different antimicrobial genes could accelerate GO toxicity as observed with increased intestinal ROS production and declined locomotive behavior (Ren et al. 2017). These findings pinpoint to the fact that innate immune response signaling pathways have a defined role to play in the process of nanotoxicology.

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway represent a signaling pathway that can integrate a multitude of signals for development and homeostasis in animals. Transcriptomic studies suggested that exposed TiO_2 nanoparticles cause deregulation of JAK/STAT pathway, a pathway known for its involvement in development and reproduction process. Also, TiO_2 NPs exposure can lead to downregulation of glutathione (GPx) which is an antioxidant enzyme required to counteract with enhanced oxidative stress levels generated from JAK/STAT signaling pathway (Kim et al. 2017a).

17.6 Systemic Approaches and Evidence of Nanotoxicity in *C. elegans*

17.6.1 Effect of Nanoparticles on *C. elegans* Nervous System (Neurotoxicity)

In *C. elegans*, the nervous system contains 302 neuronal cells along with 56 glial cells (Hobert 2010). These 302 neurons belong to two different subtypes of the nervous system, the major somatic system comprises of 282 neurons and the remaining 20 neurons belong to the small pharyngeal system. Within the nematode nervous system, different behaviors and sensory functions, with associative and non-associative learning, are observed. Worms use all major neurotransmitters (White et al. 1986; Susman et al. 2016). The locomotion behavior is controlled by serotonin, dopamine, and glutamate neurotransmitters in *C. elegans* (Yu et al. 2015). The neuronal network within this model organism is fully elucidated, and almost all genes necessary for transmission can be traced and found in *C. elegans*. For example, the neurotoxic effect of particular nanoparticles can be monitored utilizing eight dopaminergic (DAergic) neurons available in worms that express GFP driven by the dopamine transporter (*dat-1* gene) promoter. Thus, it is easy to understand neurological disorders such as amyotrophic lateral sclerosis (ALS) and Parkinson's disease with worm locomotion behavior, and GFP assays can be a direct link to motor neuronal functions (Hu et al. 2018b) (Fig. 17.4).

Neurons are known for their sensitivity and are under constant exposure for the ill effects of nanoparticles and ENMs such as quantum dots (QDs). In the biomedical field, TiO_2 NPs are regularly used in various applications including cancer treatment and as therapies for antiparasitic and antimicrobial drugs (Nadeem et al. 2018). However, recent findings have shown that exposure of TiO_2 nanoparticles to *C. elegans* can have adverse impact on the worms, such as the decrease in their population, size, movement, offspring generation, fecundity, and pharyngeal pumping rate (Li et al. 2012; Zhao et al. 2013). Engineered nanoparticles such as TiO_2 NPs cause a reduction in locomotion behavior and decrease in premature pharyngeal pumping in Bristol-type N2 (Yu et al. 2015). In neurons, TiO_2 nanoparticles are reported to have reduced axon length, which is likely to obstruct the worm locomotion behavior. According to DNA microarray analysis, expression levels of metal-binding or detoxification gene change significantly. These changes elucidate that TiO_2 NPs are toxic to *C. elegans* nervous system (Hu et al. 2018a).

Piechulek and von Mikecz (2018) employed fluorescence reporter strains, with expressions of tryptophan hydroxylase-1::DsRed to find whether Ag-nanoparticles can mimic behavioral defects in worms. They correlated the fluorescence intensity levels with the rate of aggregations of axonal proteins and neurodegeneration of serotonergic and sensory neurons. Notably, they also found that serotonergic ADF neurons are more sensitive as targets for Ag-nanoparticle toxicity, whereas GABAergic neurons can withstand degeneration under the same condition (Piechulek and von Mikecz 2018). Gold NPs are also important components of the biomedical field

which is commonly used for bioimaging, biosensing, facial creams, and targeted therapeutic purposes. In *C. elegans*, the AuNPs with 11-mercaptoundecanoic acid (MUA) and without MUA are reported to have a toxic effect on worm body length, locomotion behavior, along with a change in axonal neuronal growth. Similarly, AuNP exposure reduces axons generation in cultured neurons of worms. In another gene expression study, it was observed that there is change in expression level, cellular defense gene (clec-174), body morphogenesis gene (cut-3), gene which is expressed in neurons of embryonic tissues (*dpy-14*), and gene (*mtl-1*) which is involved in metal detoxification and regulations (Hu et al. 2018b).

Some other studies have shown that exposure of Al_2O_3 NPs has significantly reduced the locomotion behavior in worms. Recently, Yu et al. (2015) have reported that exposure of Al_2O_3 nanoparticles enhanced neural disorders related to phenotype in worms, such as neural disorder in D-type GABAergic neuron manifested with an adverse effect on the thermotaxis behavior and thermotaxis perception. These changes suggested that Al_2O_3 nanoparticles are neurotoxic in nature (Yu et al. 2015). Nitric oxide plays a key role in neurotransmission, water and salt balance, growth, and immune function. Rogers et al. have shown that CeO_2 nanoparticle exposure can downregulate nitric oxide synthase (NOS) activity while diminishing the production of NOS and NO observed and impaired nervous system functioning (Rogers et al. 2015).

17.6.2 Effect of Nanoparticles on *C. elegans* Immune System (Immunotoxicity)

Immune toxicity is the new and emerging field which deals with interactions of nanoparticles with the immune cells. Nanoparticles can directly cause damage to immune cells either by apoptosis or necrosis or indirectly by deregulations of immune-specific signaling pathways. Nanoparticle interactions cause a change in an inflammatory response which either generates ROS or releases proinflammatory cytokines. These changes are measured by the expression level of surface markers, cytokine production, cell differentiation, and activation of immune cells and antimicrobial peptides (AMPs) (Hartung et al. 2013).

Antimicrobial peptides are important components of the innate immune response of *C. elegans*, and on nanoparticles, exposure alternations in expression levels of gene engaged in antimicrobial peptide formation can lead to immune toxicity. *C. elegans* requires the activation of the p38 MAPK pathway for generation of the innate immune response (Shakoor et al. 2016). In *C. elegans*, *lys-1* and *lys-8* genes encode lysozymes, *dod-6* gene encodes a protein downstream of DAF-16, F55G11.4 gene encodes a protein containing a CUB-like domain, and spp-1 gene encodes a caenopore. Similarly, in nematodes, it has been observed that mutation of *lys-1*, *lys-8*, or *spp-1* RNAi knockdown of the antimicrobial gene enhanced the susceptibility of *C. elegans* to GO-induced toxicity that is manifested with intestinal ROS production and decreasing locomotion behavior (Mallo et al. 2002; Ren et al. 2017).

17.6.3 Effect of Nanoparticles on Development and Reproductive System of *C. elegans*

The reproductive system represents an important system for nanotoxicity studies with adverse effects of nanoparticles observed on the development of offspring, along with sexual activity and fertility rate of organisms. Although the reproductive system is considered as a secondary target organ of NPs, it was suggested that nanoparticles can access reproductive system via two routes: through its movement from the pharynx to the intestinal system and through the vulva. For experimental purposes, nanoparticles can also be delivered to the reproductive system by microinjection to the gonads (Pluskota et al. 2009). Recently, a high-throughput complex object parametric analyzer and sorter (COPAS) assay optimization and methods for assessing the developmental and reproductive toxicity of ENMs using *C. elegans* model have been reported. This can aid in accelerating reproductive and developmental toxicity studies of different ENMs and in the assessment of dose- and size-specific response on the development of nanoparticles in the near future (Kim et al. 2019) (Fig. 17.4).

In some cases, the properties of nanoparticles such as their surface charge, size, and stability, for example, quantum dots, can also reach the reproductive system, and this is dependent on their surface charge (Qu et al. 2011; Choi et al. 2010). This is manifested in the form of defecting egg-laying capacity and shortened life span in comparison with the control (Hsu et al. 2012). Similarly, SiO_2 nanoparticles are reported to be toxic to *C. elegans* with the declined rate of progeny production, internal hatch (i.e., "bag of worms"), abnormalities of reproductive organs, and BOW phenotype observed.

On the contrary, it was observed that surface coating and modifications of nanoparticles can ameliorate the toxicity of some nanoparticles such as citrate Ag-nanoparticles. The observed reproductive defects that were obvious within 24-h exposure of young worms were not observed when worms are exposed to PVP-coated Ag NPs (Roh et al. 2009). Even among the nanoparticles derived from the same metal source, their reproductive toxicity levels might differ significantly. ZnO nanoparticles are reported to have more toxic effects with enhanced germ cell apoptosis in *C. elegans*, in comparison with $ZnCl_2$, and this has been linked to cep-1/p53-dependent pathway (O'Donnell et al. 2017). Multigenerational effects of gold nanoparticles (AuNPs) and induction of multigenerational *C. elegans* germ cell death on worms were also reported based on the exposure mode (Luo et al. 2016; Moon et al. 2016).

17.7 Conclusion

Nanoparticles have various biological applications in the field of biomedical research such as therapeutics, biosensors, and bio-imaging; hence, it is essential for researchers to learn more about the toxic effects mediated through regular exposure to different nanomaterials. In practical terms, the ease of use of *C. elegans* has

attracted itself as a model for high-throughput nanotoxicity studies. Various lethal and sublethal endpoints which are used for safety evaluations of nanoparticles in *C. elegans* have shown the accuracy of *C. elegans* in predicting toxicity levels as required for translational correlations to higher mammalian systems. *C. elegans* also enhances systemic approaches in measurements of toxicities via its well-defined nervous, immune, and reproductive systems. With the availability of genetic tools such as RNAi and the ease of generating transgenic and mutant strains, toxicogenomic approach and nano-biological interactions can also be easily carried out using this model. At the cellular and molecular level, fundamental insights from research on *C. elegans* have led to a better understanding of the roles of oxidative stress in nanoparticle-mediated toxicity and how ER stress and other significant pathways are involved. With the recent technical advances in *C. elegans* handling, culture, and phenotyping, it is now increasingly possible to conduct mass screens in whole, intact organisms for developmental nanotoxicity risk assessment assays in the near future.

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