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EDITED BY
Gurumurthy Hegde,
Christ University, India

REVIEWED BY
Ekaterina Obraztsova,
Institute of Bioorganic Chemistry (RAS), Russia
Luiz K. C. De Souza,
Federal University of Amazonas, Brazil

*CORRESPONDENCE
Antonio G. Souza Filho,
agsf@fisica.ufc.br
Diego Stéfani Teodoro Martinez,
diego.martinez@lnnano.cnpem.br

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Toxicology of carbon nanomaterials in the *Caenorhabditis elegans* model: current status, characterization, and perspectives for testing harmonization

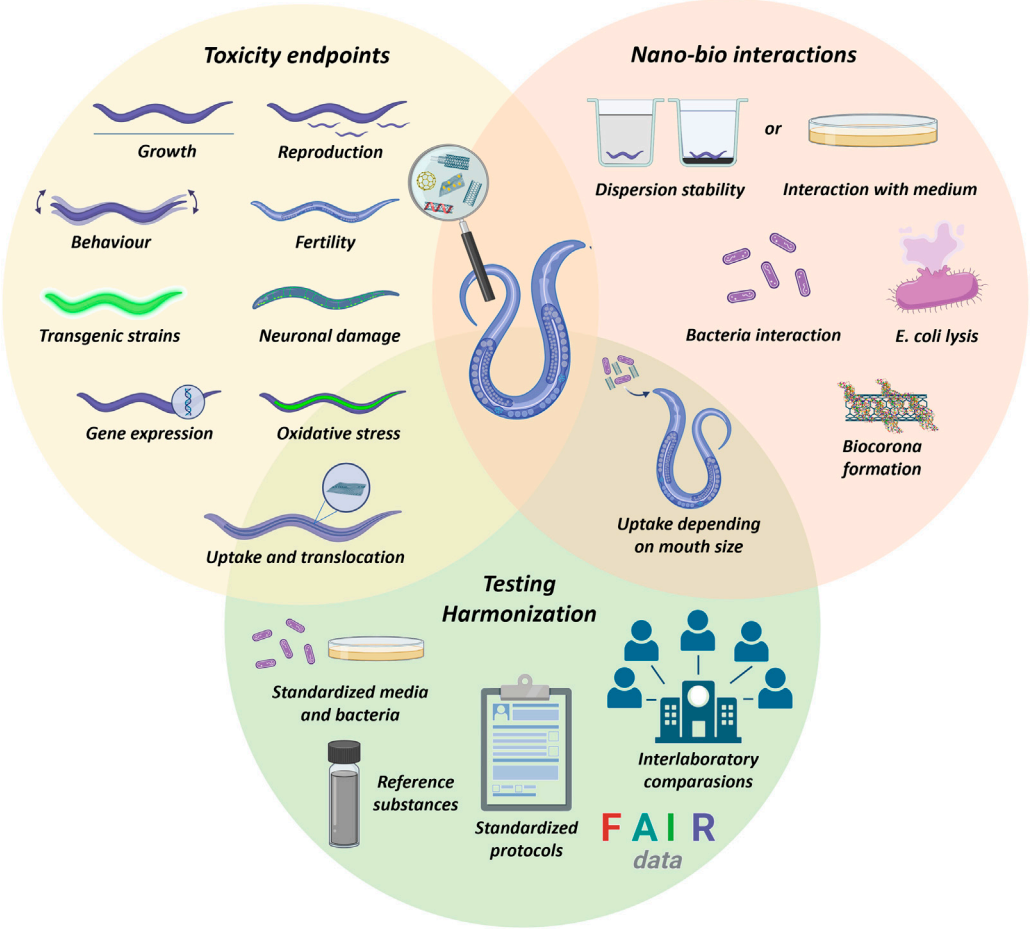
Francine Côa[1,2], Leandro de Sá Bortolozzo[1,3], Daiana Silva Ávila[4], Antonio G. Souza Filho[5]* and Diego Stéfani Teodoro Martinez[1,2,3]*

[1]Brazilian Nanotechnology National Laboratory (LNNano), Brazilian Center for Research in Energy and Materials (CNPEM), São Paulo, Brazil, [2]Center for Nuclear Energy in Agriculture (CENA), University of São Paulo (USP), São Paulo, Brazil, [3]School of Technology, University of Campinas (UNICAMP), Sao Paulo, Brazil, [4]Grupo de Pesquisa em Bioquímica e Toxicologia em Caenorhabditis elegans (GBToxCe), Universidade Federal do Pampa (UNIPAMPA), Uruguaiana, Rio Grande do Sul, Brazil, [5]Departamento de Física, Universidade Federal do Ceará (UFC), Ceará, Brazil

Carbon nanomaterials are promising advanced materials for novel technologies. Therefore, biosafety studies are mandatory to support their safe development, uses, and disposal in sustainable innovation. Traditional toxicological assays are typically expensive, time-consuming, and have low throughput; they have been replaced by new approach methodologies (NAMs) focused on *in vitro*, *in chemico* and *in silico* approaches, along with alternative models. *Caenorhabditis elegans* has emerged as a complete model organism for predictive toxicology due to its transparent body, short reproductive and life cycles, and fully sequenced genome with high homology with the human genome. In this review, we discuss the current status, state-of-the-art characterization techniques, and scientific gaps in nanotoxicity studies involving the carbon nanomaterials and the *C. elegans* model considering the last two decades of research. Moreover, we show the existing supportive tools to evaluate the internalization and biodistribution of carbon nanomaterials in *C. elegans* and discuss their advantages and limitations. Methodological and experimental gaps must still be discussed with the scientific community; hence, we bring this discussion to light and point out future orientations and perspectives. This review will contribute for guiding the research with *C. elegans* and harmonization of assays/protocols linked to computational tools and nanoinformatics approaches during the development of carbon nanomaterials.

KEYWORDS

new approach methodologies, alternative models, ecotoxicology, nanosafety, nanobiotechnology, nanoinformatics



GRAPHICAL ABSTRACT

1 Introduction

Carbon nanomaterials (CNMs) are allotropes of carbon that have received significant attention because their unique properties enable novel products and technologies. They have been exploited in the biomedical field for drug delivery, imaging, tissue engineering, and target photothermal therapy (Maiti et al., 2019; Madima et al., 2020). CNMs have also been applied in composites, catalysis, electronic devices, food industries, environmental remediation, and agricultural technologies (Jangid and Prabhu Inbaraj, 2021; Singh et al., 2021; Cui et al., 2022; Hashim et al., 2022). However, this growing interest is approaching a relevant impasse as they can be released into the environment, thus reaching organisms and humans (Ding et al., 2022). Therefore, nanotechnology risks must be well investigated toward the safe, sustainable, and responsible application of CNMs (Caldeira et al., 2022).

Over the last decades, *in vitro* and *in vivo* models have been applied to provide pivotal insights into our understanding of nanomaterial-mediated toxicity mechanisms. The ethical ambition to conduct experiments without using animals has resulted in intense efforts over the past few decades from industry, academia, and regulatory bodies to develop and implement new approach methodologies (NAMs). The expansion of *in silico* (structure-based/toxicokinetic models) and *in vitro* methods (e.g., 3D cells culture, organs-on-chips, and organoids), together with the establishment of alternative model organisms, compose substantial new approach methodologies for analyzing the harmful effects of nanomaterials, supporting the next-generation risk assessment strategy (Pimtong et al., 2021; Magurany et al., 2023).

Caenorhabditis elegans is one of the biological models that has been exploited to study the biological effects of nanomaterials (NMs), considering the One-Health perspective for protecting animal, human, and environmental health (Gao, 2021). It was the first organism sequenced at the multicellular level, and it has complete and well-described digestive, reproductive, endocrine, sensory, and neuromuscular systems with signaling pathways quite similar to humans (Cook et al., 2019; Hastings et al., 2019).

The adoption of *C. elegans* in biological assays is considered to be advantageous because its culture in laboratory is inexpensive, and it has a high reproductive capacity (~300 progeny per hermaphrodite adult) and a short life cycle, which are valuable relative to other *in vivo* models that have longer life cycles and complex manipulation. Due to its abundance in ecosystems and its key role in soil nutrient decomposition and cycling, *C. elegans* is a valuable marker of environmental changes. Unlike cell and tissues

cultures, *C. elegans* responds to a stimulus by metabolically activating its neuronal, motor, digestive, and reproductive systems, besides endocrine signaling and behavioral responses. It is also highly sensitive to both environmental pollutants and drugs, which permits rapid toxicological screening by studying several complex and functional endpoints, such as lifespan and survival, growth rate, locomotory ability, reproduction, fertility, oxidative stress, intestinal damage, and alterations in the metabolic profiles (Tejeda-Benitez and Olivero-Verbel, 2016). Due to its transparent cuticle, optical, and fluorescence microscopy techniques can be used to visualize the uptake, translocation, and excretion of pollutants *in vivo* as well as structure-specific gene expression in transgenic strains (e.g., ROS induction marked by fluorescent proteins as GFP) (Batasheva et al., 2019).

Several regions of *C. elegans* genes are similar to human genes, and it exhibits highly conserved levels compared to other vertebrates in terms of gene functions and metabolic pathways (e.g., insulin/IGF-1 signaling (IIS) pathway) (Hastings et al., 2019). Most human neurotransmitter systems are present in this nematode, such as acetylcholine (ACh), glutamate, γ-aminobutyric acid (GABA), serotonin, and dopamine, making *C. elegans* an interesting animal to study neurotoxicology. A complete diagram of the chemical and electrical connections present in *C. elegans* neurons is available (Hall et al., 2005). Connectomes that encompass the *C. elegans* body were recently entirely characterized (Cook et al., 2019). Its alimentary system presents many characteristics that are similar to that of mammals, such as an acidified lumen, secretion of digestive enzymes, and peristalsis (Chauhan et al., 2013). Due to these features, *C. elegans* can be applied as a platform for understanding CNM toxicity at the organism, tissue, and molecular levels and extrapolating these effects to other complex organisms. However, nematodes have no blood–brain barrier; thus, pollutants directly interact with their nervous system (Hunt, 2017). Furthermore, the lack of a respiratory and circulatory system, as well as specific organs (e.g., brain, heart, lungs, eyes, and kidney) is a challenge to full elucidation of the potential toxicity of pollutants to humans (Gonzalez-Moragas et al., 2015).

Over the last 20 years, several relevant toxicity studies have been performed with *C. elegans* and CNMs. However, this knowledge has not been summarized yet. In addition, from our perspective, some scientific challenges and methodological particularities are mentioned but not thoroughly discussed in several studies. In this context, this review intends to sum up the current knowledge about the effects of carbon nanomaterials against the *C. elegans* model, as well as address the main techniques to support this evaluation while pointing out their advantages and limitations. Finally, we also raised the scientific and experimental gaps in nanotoxicity studies by offering some recommendations to advance this research field.

2 Toxicity of carbon nanomaterials on *Caenorhabditis elegans*: the state of the art

Current literature demonstrates that biological effects of carbon nanomaterials are strongly dependent on the physicochemical properties of the NM in question (e.g., morphology, size, surface charge, and chemistry) as well as its dispersion state, concentration, and conditions of exposure (e.g., period of duration, life stage of nematode, strain, and presence/absence of bacteria). Supplementary Table S1 considers these aspects, summarizes the published studies with *C. elegans* and these materials, and details the experimental conditions used.

Studies have reported accumulation of graphene oxide (GO) in the nematode gut and its translocation to reproductive organs, neurons, and even to eggs. A significant reduction in *C. elegans* reproductive capacity along with induction of germline apoptosis has been observed, besides the interruption of its cell cycle (Zhao et al., 2016; Chatterjee et al., 2017). GO exposure also reduces the nematode lifespan (Wu et al., 2013b), impairs its locomotion (Wu et al., 2014a), defecation behavior, and immune response (Wu et al., 2013a; Wu et al., 2014b). A potential neurotoxic effect of GO was also noticed because it accumulates in *C. elegans* head and decreases the dopaminergic and glutamatergic neurotransmitter contents (Kim et al., 2020).

Several biological mechanisms related to GO toxicity have been studied with *C. elegans*, such as apoptosis, RNA interference, and miRNA function. Overexpression of reproductive-related genes, such as *egl-1* and *ced-13*, and even apoptosis-correspondent genes, such as *ced-3*, *ced-4*, and *ced-9*, due to multigenerational GO exposure was found (Jin et al., 2022). The expression of oxidative stress-related genes (*sod-1*, *sod-3*, and *clt-2*) was also reported (Tsai et al., 2021), as well as genes important for the function of AFD neurons (*ceh-14* and *ttx-1*). GO also acted by dysregulating the expression of some proteins, such as NLP30, CNC-2, ISP-1, SOD-3, LYS-1, LYS-8, SSP-1, DOD-6, and F55G11.4 (Ding et al., 2018; Jin et al., 2022). Some strategies have been reported to reduce the toxicity of GO to *C. elegans*, such as chemically degrading it by sodium hypochlorite (Bortolozzo et al., 2021) or modifying its surface with bovine serum albumin (Sivaselvam et al., 2020; Côa et al., 2022).

The induction of oxidative stress and the reduction of nematode lifespan, growth, fertility, and reproduction have been observed in nematodes exposed to multi-walled carbon nanotubes (MWCNTs) (Sinha et al., 2016; Côa et al., 2022). The translocation of carbon nanotubes (CNTs) from pharynx to the intestine and gonads has been also reported (Wu et al., 2013a), although the pharynx has been pointed out as a key barrier in preventing this translocation to secondary organs (Zhuang et al., 2016). MWCNTs also affected the nematode behavior and brood size in a transgenerational study with activation of germline long non-coding RNA *linc-7* (Zhao et al., 2022). The expression patterns of some genes required for intestinal development and the defecation cycle have been affected by exposure to MWNCTs (Shu et al., 2015). CNTs also dysregulated the expression of *tbh-1* associated with the octopamine signal, or the expression of IncRNAs, such as *linc-2*, *-7*, *-9*, *-32*, and *-50* (Zhao et al., 2022). miRNAs can be also dysregulated in the nematodes after prolonged exposure to MWCNT (0.1–10 mg L^{-1}); it was observed that the expression levels of some miRNAs increased (e.g., *mir-77*, *mir-52*, *mir-36*, and *mir-40*) and others decreased (e.g., *mir-57*, *mir-355*, *mir-249*, and *mir-1*) in response to MWCNTs (Zhao et al., 2014).

Carbon quantum dots (C-QDs) can also be taken up by *C. elegans* via oral ingestion; they accumulate in gonad and germ cells without representing potential toxicity to *C. elegans* (Singh et al.,

2018; Atchudan et al., 2019; Han et al., 2019). Hence, these carbon nanomaterials could be safely applied in bioimaging due to their fluorescent characteristics and apparent biocompatibility (Li et al., 2020).

Up to now, key studies have provided valuable insights into CNM toxicity *to C. elegans*. The surface chemistry of carbon nanomaterials has been discussed as a precursor to alterations in the toxicological profile and translocation of these materials in the *C. elegans* body (Nouara et al., 2013; Chatterjee et al., 2014; Yang et al., 2015; Chatterjee et al., 2017; Rive et al., 2019). Nevertheless, some aspects remain to be clarified, such as: 1) how the shape of carbon nanomaterials impacts biological effects; 2) whether the CNM colloidal behavior influences the obtained results in terms of toxicity; 3) how specifically CNMs are translocated from the primary to secondary target organs of *C. elegans* (Yao et al., 2022); 4) whether CNMs can be transferred to the next generations and what are the impacts of that; 5) whether the characteristics of CNMs can change over time in a toxicity assay with *C. elegans*, mainly in the presence of food (bacteria). In addition, toxicokinetic studies could be performed with *C. elegans* to predict the fate and behavior of CNMs in an entire animal with metabolically active systems toward obtaining valuable data for human health protection and explaining the fate and dynamics of NMs in the ecosystem.

The implementation of *C. elegans* in microcosm systems could be an interesting method of evaluating the behavior of CNMs in environmentally relevant scenarios and assessing the transport of CNMs in the food chain. This nematode could also be advantageous to further study of the toxicity of mixtures of CNMs with pre-existing environmental pollutants, as well as the impacts of physical, chemical, and biological transformations of CNMs in the environment to the toxicological response (e.g., photodegradation, homo or heteroaggregation, or coating with biomolecules).

3 Tools for evaluating nanomaterial internalization and biodistribution in *Caenorhabditis elegans*

Assessing the uptake and biodistribution of nanomaterials in organisms is a basic aspect of understanding and interpreting their potential effects, besides to identifying their persistence and behavior in the environment (Petersen et al., 2023). There are several methods to evaluate the biodistribution of organic and inorganic substances from biological matrices. However, identifying and quantifying nanomaterials is challenging, especially those that are carbon-based, which need to be distinguished from the carbon background from the organisms.

The uptake of nanomaterials by *C. elegans* occurs mainly through the mouth, which size is approximately 1 µm at the adult stage (Leung et al., 2008; Hunt, 2017). When internalized, CNMs can be translocated from the gastrointestinal region to proximities of the nervous system (Wang et al., 2017). Reproductive organs such as gonads and spermatheca are other targeted tissues. Some robust techniques are presented in Table 1, and their advantages and limitations are examined. Although imaging techniques are strategic instruments, it is notable that other tools are better exploited to understand the biodistribution of carbon nanomaterials in *C. elegans*. Adopting a multi-analytical approach from the materials and biological sciences seems to be the most promising alternative because no one technique is sufficient to fully access and understand NM fate and behavior.

Moreover, some instruments do not detect carbon nanomaterials with high precision, and the signal-to-noise ratio of these materials in the tissues is a challenge, especially when the purpose is to evaluate environmentally relevant concentrations (range at ng L^{-1}). Labeling carbon nanomaterials with metals, fluorescent dyes (e.g., rhodamine B), or ^{14}C could be a robust method of improving the ability to quantify these materials, especially in *in situ* conditions (Goodwin et al., 2018). However, these labels must be toxically tested and must remain attached to the carbon nanostrcuture during their internalization and analysis (Petersen et al., 2023).

The influence of organic molecules adsorbed on the CNM surfaces during the experiments (e.g., biocorona formation) is also an open and core question because it may impact detection and quantification methods. This impact has not been discussed in the literature; thus, the available techniques must be revisited.

Applying orthogonal methods would improve the discussions and reliability of results, thus reducing interferences and misinterpretations because the results would be based on measuring the same parameter using different physical principles (Simon et al., 2023). Radio-labeling CNMs is an option to help the development of new methods (Petersen et al., 2023). As important as choosing the right technique, the accuracy of sample preparation is a key element of avoiding bias and artifacts of analysis that can compromise the biological outcomes (Johnson et al., 2017a). In this direction, there is a field to be exploited because there are no standardized procedures to separate or extract CNMs from the biological matrices, especially from the nematode cuticle. However, one protocol was developed to remove non-ingested gold nanoparticles from the *C. elegans* cuticle and medium surrounding before bioaccumulation studies (Johnson et al., 2017b), this approach could be the basis for this additional research.

4 Scientific and methodological challenges in assays

4.1 Factors influencing the nano–bio interactions of *Caenorhabditis elegans* and carbon nanomaterials

Relevant efforts have been made to study carbon nanomaterial toxicity with *C. elegans*. However, difficulties inherent to nanotoxicity assays, the lack of details of the adopted methodologies, and the absence of standardized protocols may lead to inconsistent, hardly comparable, and irreproducible results (Selck et al., 2016). Moreover, specific factors may affect the interface between nematodes and nanomaterials in biological assays with *C. elegans*. All these key factors are summarized in Figure 1 and discussed in this topic.

Different from conventional chemicals (i.e., organic and inorganic molecules or bulk materials), the intrinsic physicochemical properties of nanomaterials (e.g., size, morphology, surface chemical

composition, and charge) play a substantial role in their toxicity as they govern their contact and interface with organisms (Johnston et al., 2010; Ou et al., 2016). The NM surface charge (i.e., positive or negative), for instance, may interfere in the attractive force between the NMs and the *C. elegans* cuticle (Gonzalez-Moragas et al., 2017a). Moreover, differences in the synthesis method for the same nanomaterial, variations between batches and purification protocols, and impurities from synthesis are also critical issues for toxicity assessment (Liu et al., 2022). In view of this, it is key to characterize the physico-chemical properties of nanomaterials to understand their biological effects (ISO, 2012). A multi-technique approach and the use of orthogonal techniques are of fundamental importance and has been extensively documented in nanosafety guidelines and described in previous reviews (Gao and Lowry, 2018; Simon et al., 2023).

Another key element is the proper preparation of stock dispersions for biological experiments. They must present good colloidal stability to guarantee accurate dilutions as the low stability of NMs stock dispersions leads to low reproducibility. Ultrasounds or surfactant strategies have been used for this purpose but each one has drawbacks. The use of surfactants (e.g., tetrahydrofuran—THF) should be avoided because they may be toxic to the organisms (Zhu et al., 2006; Petersen and Henry, 2012). Probe-type ultrasounds are not suitable to prepare dispersions of carbon nanomaterials because high energies may modify the CNM properties, such as decreasing their size and/or increasing their structural defects (Mullick Chowdhury et al., 2014; Le et al., 2019). Ultrasonic baths are more appropriate for dispersing carbon nanomaterials because water temperature does not exceed 20 ± 2°C; high temperatures lead to strong CNM agglomeration that cannot be reversed by applying more energy as we empirically observed in the routine of our research group. Moreover, it is recommended that the period of sonication be studied toward protecting the CNM structure (OECD, 2017).

The carbon nanomaterial properties and exposure conditions (i.e., media composition and food availability) are key for toxicity because they can modify the NM behavior and their interactions with organisms. In addition, the life stage of nematodes and the exposure period are essential criteria to be considered (Liu et al., 2020).

The assessment of NM toxicity is typically carried out in either solid (nematode growth media (NGM)) or liquid (K-medium, S basal, M9) media with different chemical compositions. However, it is worth mentioning that they were developed to assess the toxicity of conventional chemicals. Therefore, they cannot be used for nanotoxicity studies without modification. In solid media, nematodes may be exposed to different concentrations of NMs due to the non-homogeneous mixture of these materials over the agar. Nanomaterials are also subject to interact with solid media components (e.g., phosphate) or bacteria, which can change the NM properties affecting the biological outcomes (Gonzalez-Moragas et al., 2017b). *C. elegans* may respond with an aversive behavior in the solid medium by avoiding exposure to carbon nanomaterials (Xiao et al., 2018).

In liquid media, multiple factors are associated with the biological effects of nanomaterials, such as aggregation/agglomeration, sedimentation, and transformations (i.e., dissolution or interaction with the food source and bacteria). These phenomena are strongly influenced by the medium's characteristics, such as pH, ionic strength, and temperature. High ionic strength, for example, can reduce the electrostatic or steric stabilization of CNMs, thereby promoting the formation of aggregates/agglomerates and, subsequently, sedimentation (Yang et al., 2016).

Aggregation and sedimentation are phenomena that affect the conditions in which the nematodes are exposed to CNMs. The formation of aggregates/agglomerates and the settling of CNMs to the bottom of the exposure wells, where nematodes are located, may primarily influence the amount of material internalized by nematodes because the *C. elegans* buccal cavity has a size-selective filtering mechanism that captures the food and excludes large particles (>1 μm at l stage and >3 μm when adults) (Fang-Yen et al., 2009). As the toxic effect is associated with the amount of internalized substances, understanding the aggregation and agglomeration behavior is decisive for making assumptions about the toxicity of the tested material.

Furthermore, the settling of CNMs may also lead to differences between the nominal dose (i.e., what was administered in the test) *versus* "the delivered dose" that reaches the nematode at the bottom of well in the assays, resulting in levels of exposure higher than was calculated by initial dispersion measurements (Wang et al., 2018).

It is expected that the toxicological effect and the dose that affects 50% of organisms (LC_{50} or EC_{50}) may vary for the same studied material in different exposure conditions (i.e., different medium or in presence/absence of bacteria), which makes the risk assessment process a massive challenge as it is based on LC_{50} values. This challenge should be solved by developing standardized dosimetry methodologies that would allow discerning the administered dose of the delivered/bioavailable dose. Even though no standardized protocol has been designed yet to study the sedimentation behavior of CNMs, significant advances have been made with cells that could guide further studies with *C. elegans* (DeLoid et al., 2017).

Moreover, the aggregation/agglomeration behavior assessment of carbon nanomaterials could be supported by the OECD guidelines nº 318 (OECD, 2017) and nº 317 (OECD, 2022), which are not specific to the CNM case but can be useful to initiating these discussions. In addition, dynamic light scattering could be a useful technique to assess the CNM aggregation, similar to what was adopted in our recent publication to study the stability of GO and MWCNT (Côa et al., 2022).

Another variable is related to the fact that NMs can undergo surface modifications when they encounter biomolecules in biological media. At this point, adding a food source (bacteria, commonly *Escherichia coli*) is an overlooked step that may result in misinterpretations because bacteria may interact with nanomaterials, leading to unknown and different effects.

The importance of assessing the influence of bacteria in studies with *C. elegans* was pointed out in several studies for conventional chemicals (Donkin and Williams, 1995; Ke and Aschner, 2019) and different NMs (Starnes et al., 2015; Luo et al., 2016; Hanna et al., 2018), but it is still far from being understood for carbon nanomaterials. A report has described, for example, that positively charged NMs interacted with bacteria, making the food unavailable to the nematodes (Hanna et al., 2018). As its developmental physiology is sensitive to nutrient depletion

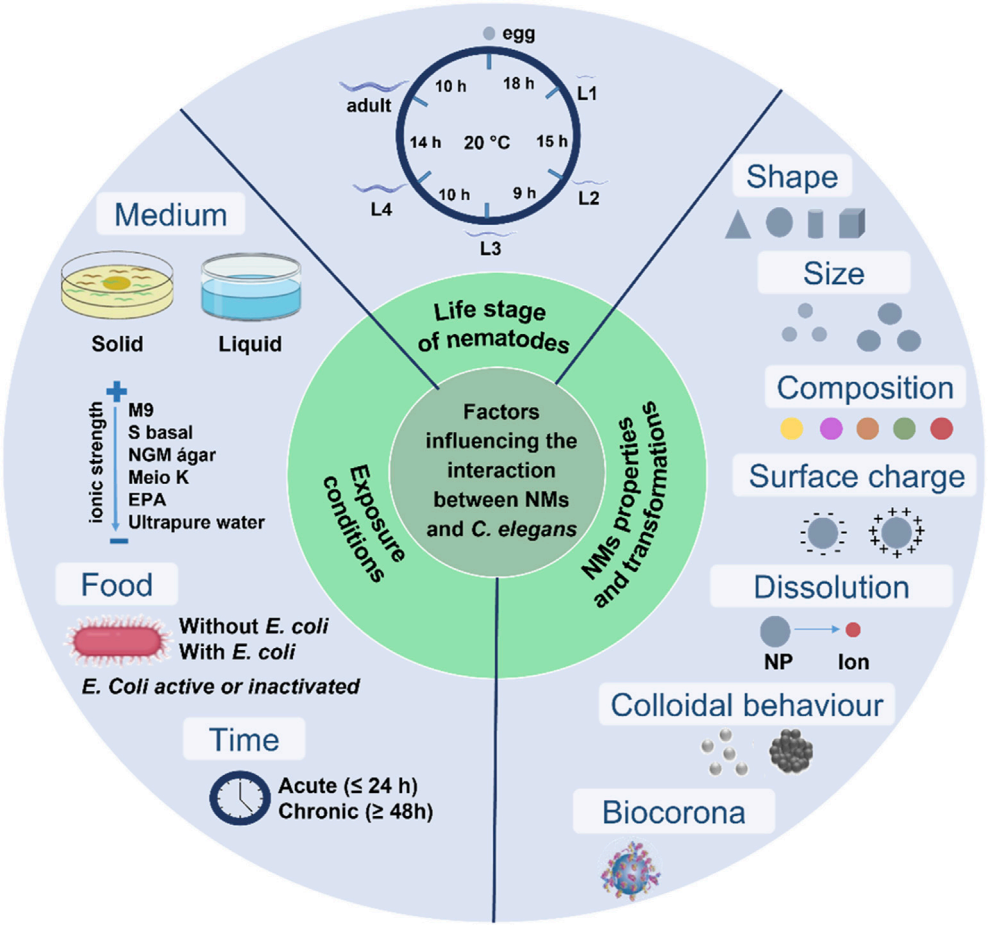


FIGURE 1
Factors that may influence the nano–bio interactions in assays with a *Caenorhabditis elegans* biological model.

(Kaplan et al., 2018), a decrease in nematode growth, reproduction, and fertility was observed. Other researchers noticed a possible dietary transfer of NMs to the food chain when *C. elegans* was fed with *E. coli* after its interaction with the tested NMs (Priyam et al., 2021; How and Huang, 2023).

Indeed, the interaction of NMs with bacteria can affect the bacteria's integrity, causing its lysis and release of its biomolecules, such as proteins, nucleic acids, lipids, and carbohydrates, in biological medium (Schaechter and Neidhardt, 1987). Consequently, these molecules can be adsorbed onto the NM surface, forming a coating known as biocorona, which will strongly affect the NM biological response and toxicity because the entity that cells "see" is not the naked nanomaterial but a hybrid nanomaterial–biomolecule instead (Markiewicz et al., 2018). The same effect can occur when NMs attach to the bacteria's surface and are taken up by the nematodes. Inside the nematode pharynx, the biomolecules that composed the bacteria will be released by the physical degradation induced by the pharyngeal grinder; thus, these biomolecules will interface with the reactive surface of NMs. These nano–bio interactions may result in the biocorona formation as demonstrated in Figure 2, which will change particle surface properties and their agglomeration and settling behavior, altering the way by which the nematodes will be exposed to CNMs (Lynch et al., 2014).

Although the biocorona concept has been extensively discussed in the nanotoxicology field over the last 20 years (Mahmoudi et al., 2023), the occurrence of the biocorona formation at the interface of bacteria–NM interactions during the toxicological assays remains unknown for the *C. elegans* scientific community. Therefore, it must be incorporated into the scope of the studies because many were performed in the presence of bacteria, as was pointed out in Supplementary Table S1.

Moreover, these discussions indicate that exposure conditions must be standardized to allow the comparability of the data that are fundamental to predicting the potential risks of nanomaterials. One possible alternative is to review and adapt one guideline developed by the International Standard Organization (ISO) for studying the toxicity of environmental samples on the growth, reproduction, and fertility of *C. elegans*. ISO 10872:2020 is not focused on nanomaterials, but it establishes qualitative and quantitative parameters that must be reached to confirm the viability of *C. elegans* in the assays. The exact quantity of bacteria to be added to the tests was prescribed in this guideline, and a reference substance was indicated to verify the sensitivity of the *C. elegans* culture. Both elements are a starting point for the discussions because the current methodologies found in the literature do not incorporate a reference substance or even report the bacteria quantity applied. Obviously,

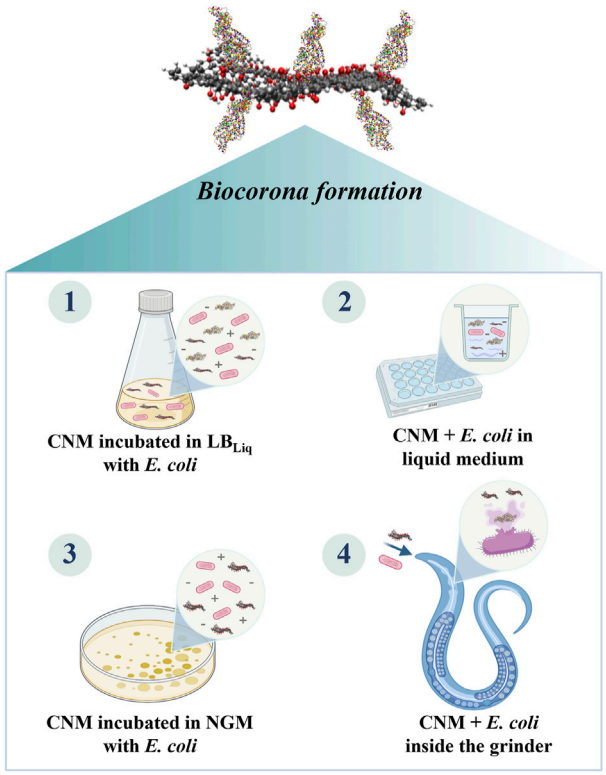


FIGURE 2
Scenarios in which the surface of CNMs can be coated by biomolecules during the biological assays with *Caenorhabditis elegans* leading to the biocorona formation.

adapting this protocol to different types of nanomaterials will require much work; hence, it will be discussed in the next topic.

In addition to the harmonization, one of the challenges to understanding CNM toxicity is interpreting and comparing the large volume of published data collected under a wide range of exposure conditions. The generation of data and no compilation of that with the physicochemical characterization of carbon nanomaterials do not contribute to generating mathematical models that can predict the toxicological effects (Lynch et al., 2021). Scientific publications in this field need to improve this aspect. The publication of raw data and data sharing needs to be encouraged to translate the collected data into knowledge (Harper et al., 2013).

4.2 Experimental gaps in toxicity tests with *Caenorhabditis elegans* and carbon nanomaterials

Although *C. elegans* has been successfully used as a model in nanotoxicology, some methodological barriers must be debated, including the lack of a standardized protocol to perform the toxicity assays and the investigation of the mechanisms of toxicity.

First, the laboratory practices and conditions must be carefully controlled. Different types of agars, the quality of reagents and water, and the variability in nutrient and vitamin content of media are factors that can alter the nematode gene expression, fertility, growth rate, resistance to pathogens, and lifespan. Furthermore, laboratory conditions, such as temperature and humidity, are critical to the *C. elegans* culture, influencing, for example, the prevalence of males in the culture. Finally, mutations in the bacterial strains need to be avoided, as well as contaminations with other bacteria or pathogens that can reduce the viability of *C. elegans* (Höss et al., 2012; Pho and MacNeil, 2019).

Developing a standardized medium for NM testing must be a priority in the *C. elegans* community as it is a central point to achieving comparable data. During the NANoREG project (Kleiven and Oughton, 2015), the guideline ISO 10872 was revised toward harmonizing the medium for nanotoxicological studies because most media have high chloride content and high ion strength. Two media were proposed: i) the EPA moderately hard reconstituted water, which has a low ionic strength (4 mM), and ii) simulated soil pore water (SSPW), which is an ecologically relevant medium for soil matrices, named ISO, 2010. However, until this date, few studies have been carried out with those media, thus making difficult to attest whether they are more suitable for nanotoxicity studies. It is important to emphasize that each NM has a distinct colloidal behavior; therefore, characterizing the NMs in the medium should be a key step to be incorporated in a standardized protocol.

E. coli concentration and viability are criteria that must be fully described and harmonized. By using a reference substance (BAC-C16) and applying the ISO 10872:2017, Hanna et al. (2016) observed that a minimum bacteria density (i.e., 500 FAU) is required for nematode development in chronic assays. Furthermore, they also noticed that a variation in bacterial density in 50 FAU occasioned inhibition of nematode growth by 19%, demonstrating that applying a standardized bacterial density is fundamental to robust experiments. Hanna et al. (2016) also tested the feasibility of this protocol for testing positive polystyrene nanoparticles. They reported that feeding the nematodes with UV-killed *E. coli* decreased the polystyrene NPs toxicity, suggesting that bacterial viability is a critical parameter. Moreover, they also observed that positive polystyrene NPs agglomerated with *E. coli*, making the food unavailable to *C. elegans* and causing inhibition of nematode growth. These findings confirm that the viability and density of *E. coli* are major parameters to be considered in the adoption of ISO 10872: 2020 for nanotoxicity studies. At this point, it is important to highlight that some NMs can affect bacterial integrity or be taken up together with bacteria, leading to the biocorona formation, as previously mentioned. All these aspects depend on the NM type and biological medium; therefore, they need to be investigated.

Special attention should be given to techniques that allow characterizing CNMs in experimental media (*in situ*), such as the cryo-TEM technique. However, because these techniques are time-consuming, expensive, difficult to implement, and require specialized equipment that sometimes is not available for complex matrices, an alternative is applying strategic experimental controls. The National Institute of Standards and Technology (NIST) has published a list of experimental controls to be included in assays to improve their reliability (Petersen, 2015). Other experimental controls could be added to a standardized protocol toward distinguishing the artifacts from the biological outcomes in the CNM case.

Another difficulty is related to accurately counting organisms during the experiments to produce the dose–response curves.

TABLE 1 Summary of promising tools to study carbon nanomaterial fate, uptake, and biodistribution in *Caenorhabditis elegans*.

Detection method or technique	Obtained information	Advantage	Limitation	Previous studies performed with these tools
Optical microscopy	Internalization and biodistribution	Easy sample preparation and fast and simple analysis	Low spatial resolution (>300 nm), only qualitative, limited to carbon nanomaterials marked with dyes or that significantly accumulate inside the nematode	Yang et al. (2015); Li et al. (2017); Han et al. (2019)
Fluorescence microscopy	Internalization and biodistribution	Easy sample preparation, low cost, high contrast, and straightforward operation	Low spatial resolution (>300 nm), non-quantitative, and organisms can be affected by exposure to the light from the fluorescence microscopy. In addition, it is restricted to fluorescent CNMs or CNMs labeled with fluorophores	Mohan et al. (2010); Sonkar et al. (2012); Wu et al., 2013b; Wu et al., (2016); Goodwin et al. (2014); Li et al., 2014; 2017 (2020); Singh et al. (2018); Xu et al. (2018); Atchudan et al. (2019); Cong et al. (2019); Hendler-Neumark et al. (2021)
Laser scanning confocal microscopy (LSCM)	Uptake and biodistribution	Easy sample preparation, tomographic capacity (reconstruction of 3D images), ability to track dynamic events	Limited depth (200 μm), expensive in relation to conventional microscopy, complex and slow image processing. Limited to fluorescent carbon nanomaterials or samples marked with fluorophores. This technique can lead to the photodamage effect and requires a high number of images for robust statistical analysis	Qu et al. (2011); Sonkar et al. (2012); Zhang et al. (2012); Goodwin et al. (2014); Yang et al. (2015); Pramanik et al. (2016); Singh et al. (2018); Xu et al. (2018); Zhao et al. (2018); Guo et al. (2020); Sivaselvam et al. (2020); Lu et al. (2022)
Enhanced dark-field hyperspectral microscopy (EDFM-HSI)	Biodistribution (in the nematode cuticle and, in some cases, in tissues)	Minimal sample preparation, non-destructive, simple personnel training, and ability to combine imaging with spectroscopy	Lack of spatial resolution, time-consuming, the equipment's computer must be fast, image acquisition is not automated, and the analysis requires ultraclean glass slides. The spectrum of analyzed material needs to be distinct from the *C. elegans* spectrum. There is no standard spectrum library. There are many potential artifacts of analysis	Fakhrullina et al. (2015); Bortolozzo et al. (2021); Stavitskaya et al. (2021)
Scanning electron microscopy (SEM)	Interaction of CNMs with nematode cuticle	This technique can be successfully applied when CNMs are visible on exterior surfaces, and it can be combined with energy-dispersive X-ray spectroscopy (EDS) to analyze the elemental composition	Low spatial resolution, only qualitative information, and it is not accurate to identify carbon nanomaterials	—
Transmission electronic microscopy (TEM)	Biodistribution (cellular level)	High resolution (>1 nm), intracellular location, and translocation routes	Non-quantitative, low contrast for carbon nanomaterials, requires complex sample preparation as ultrathin sections need to be prepared (50–100 nm), and cellular structures need to be contrasted	Wu et al. (2013b); Yang et al. (2015)
Confocal Raman spectroscopy	Internalization and biodistribution (in organs and tissues)	Simple sample preparation and analysis, does not require labels, and enables CNM characterization	Time-consuming, semi-quantitative information, calibration required for quantitative analysis, and limited ability to detect trace quantities	Chatterjee et al. (2017); Kim et al., 2018;, Singh et al. (2018); Côa et al. (2022)
Inductively coupled plasma-mass-spectroscopy (ICP-MS)	CNM internalization (whole body)	Allows the detection and quantification of CNMs coordinated or incorporated with a metal ion, with a high level of accuracy (ppt levels)	Requires coordination or incorporation of a metal ion into the CNM structure, which involves choosing metals that are not natural constituents of *C. elegans*. Necessary to apply acid digestion before the analysis in which contaminations could lead to interfering signals. Coordinated metal can be released from CNM structure during the exposure, or inside the nematode gut when in contact with the acid environment, leading to misinterpretations regarding the CNM concentration	—

(Continued on following page)

TABLE 1 (*Continued*) Summary of promising tools to study carbon nanomaterial fate, uptake, and biodistribution in *Caenorhabditis elegans*.

Detection method or technique	Obtained information	Advantage	Limitation	Previous studies performed with these tools
Autoradiograph	Biodistribution of ^{14}C labeling after tissue biological oxidation	Allows analyzing and quantifying the spatial distribution of CNMs by the radioactivity signal at low detection limits (ppb to ppt levels). It is also valuable to identify degradation products.	Requires a special synthesis, expensive, and dangerous. Most CNMs are not radiolabeled in the real world; therefore, sometimes this analysis is largely theoretical rather than practical. This technique can lead to misinterpretation because a radioactive isotope can become separated from the CNMs through the nematode body	—
Liquid scintillation counting (LSC)	Biodistribution of ^{14}C labeling after tissue biological oxidation	Quantitative (ppt to ppb levels) and permits the detection of biotransformed CNMs in the tissues	Expensive and dangerous CNM synthesis. Requires calibrating the instrument with radioactive carbon-14 chemicals, and a minimal amount of sample is required to release enough gas for the analysis	—
Microwave-induced heating system	Carbon nanotube detection and quantification in the organisms	Low cost. It is most applicable for MWCNT quantification as it absorbs the microwave radiation, and the CNT concentration is proportional to the temperature increase observed	Not commercially available, not very suitable for the graphene material family, and requires calibrating the instrument	—
Near-infrared fluorescence (NIRF) microscopy	SWCNT and MWCNT quantification	High spatial resolution, high sensitivity, and relatively low cost. It permits good tissue penetration and can quantify and detect the CNMs at very low limits of detection using the unique electronic bandgap properties of carbon allotropes	Not applicable for functionalized SWCNT or MWCNT	—

Although it appears to be simple, it is a time-consuming and error-prone process when researchers have many samples. The adopted methodologies are usually approximations that are not properly reported in publications (Scanlan et al., 2018). At the beginning of the research, high doses of carbon nanomaterials (>10 mg L^{-1}) were tested with *C. elegans*. In addition to not being environmentally relevant, they lead to artifacts of analysis because carbon nanomaterials can aggregate in liquid media and settle at the bottom of the well, not allowing the nematodes to be accurately counted as we have been observed in our assays. Adopting automatic measurements, such as those performed by the Copas Biosort equipment, can be problematic because it is difficult to identify and separate the nematodes from aggregates. On the other hand, these automatic measurements are advantageous alternatives when environmentally relevant concentrations are studied.

There is a barrier in the studies conducted to investigate the trophic transfer of nanomaterials using *E. coli* and *C. elegans*. In these methodologies, authors interacted the NMs and *E. coli* in a Luria–Bertani (LB) broth. Afterward, nematodes were exposed to this mixture. However, this procedure may not be suitable because LB is a highly nutrient-rich microbial medium composed of peptone, yeast extract, and NaCl that will interact with the NM surface. In this way, a biocorona could be formed on NMs (as illustrated in Figure 2), and the toxicity outcomes will be related to this biocorona, not only to the effects of NMs. Therefore, it is important that we thoroughly examine the current exposure protocols to ensure they align with the main purpose.

The use of fluorescent probes (e.g., CM-H_2DCFDA, Nile red, or acridine orange) for labeling damage on nematode tissues is also a topic of concern. As carbon nanomaterials are highly adsorbent and, typically, are taken up and accumulate in the nematode intestine, they can hinder the permeability of these reagents to the analyzed tissue. This aspect may generate uncertain results, mainly if high concentrations of CNMs are used. Thus, it may be more suitable to replace the fluorescent probes for transgenic strains marked to reduce or increase the damaged tissue's fluorescence.

Developing harmonized protocols is a major issue toward producing comparable experimental data and minimizing the generation of artifacts. A rigorous posture is expected from nanotoxicology scientists to investigate both toxicity and the possibility of interfering/bias during the tests. Implementing good laboratory practices (GLP) to maintain the *C. elegans* culture and carry out assays is imperative to ensure the generated data's consistency, reliability, reproducibility, quality, and integrity (Pho and MacNeil, 2019). Finally, interlaboratory comparisons need to be encouraged in the *C. elegans* community because they offer valuable insights about factors that need to be controlled in the protocols to enhance the accuracy and reliability of results (Höss et al., 2012).

5 Conclusion and future perspectives

Significant advances have been made toward understanding carbon nanomaterial toxicity. Overall, graphene-based materials,

carbon nanotubes, and fullerene may potentially be toxic to *C. elegans* as they may affect its survival, reproduction, growth, fertility, germline cells, the functionality of the intestinal barrier and neurons, as well as deregulate the gene expression and important signaling pathways. However, most studies have focused on the biological effect. They overlooked the importance of exploring materials science, especially to clarify what are the properties of carbon nanomaterials that influence their toxicological profile to *C. elegans*. There is a knowledge gap in relation to the colloidal behavior of CNMs in the performed assays. Therefore, future studies should focus on correlating the biological results with the physicochemical properties and colloidal stability of CNMs. There is also a lack of multigenerational and transgenerational studies explaining how the observed effects can be transferred to the next generations, especially focused on epigenetic modifications.

Some experimental issues require investigation to guarantee the reliability of existing results, such as i) the influence of *E. coli* on CNM behavior, fate, and toxicity during the exposure, ii) transformations that these materials may suffer inside the nematode with emphasis on chemical and physical analyses and toxicological response, and iii) the products excreted after CNM ingestion. Some tools (e.g., microwave-induced heating system or autoradiograph) could be better exploited to measure the amount of CNM ingested and accumulated by *C. elegans* and to understand how CNMs are translocated to secondary target organs of this nematode.

As several factors influence the nano–bio interactions in the *C. elegans* experiments, many methodological gaps need to be revisited by considering the new facets described herein. These issues need to be standardized in protocols that include strategic experimental controls, reference substances, and guidance on the best practices. Cause-and-effects diagrams should be employed to gather information about possible interferences in the tests. The application of the FAIR (Findability, Accessibility, Interoperability, and Reuse of digital assets) data principle is also imperative within the *C. elegans* scientific community.

We hope these outstanding questions can encourage scientists to take advantage of the possibilities offered by *C. elegans* to continue elevating the studies to a higher level of knowledge in the coming decades. Furthermore, the perspectives raised herein open the door for new studies that would help toxicologists to validate *C. elegans* as a model organism to be implemented worldwide within the perspective of new and reliable approach methodologies for regulatory purposes.

Author contributions

FC contributed to the conception, structure of the paper, analysis, writing, and interpretation of available literature. LB contributed to the development of the initial draft and interpretation of available literature. DSA and AGSF reviewed and improved the output for important intellectual content. DSTM supervised, reviewed, and was responsible for project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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