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Galleria mellonella as a Simple Yet Reliable In Vivo Model for Nanotoxicology: Techniques and Applications

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ABSTRACT

Nanomaterials are a rapidly advancing tool with applications across various scientific fields. However, their interactions with living organisms have raised numerous safety concerns, making it crucial to develop reliable models to predict and evaluate associated toxicity effects. Traditional in vitro assays fail to mimic the true physiological responses of living organisms to nanomaterials, whereas murine and other in vivo models are time-consuming, costly, and ethically controversial. The greater wax moth, *Galleria mellonella*, has emerged as a promising in vivo model for nanotoxicology, serving as an effective bridge between in vitro and in vivo mammalian testing. This model combines simplicity and ethical viability with a human-conserved innate immune system, making it ideal for immunotoxicity testing. While it cannot fully replace more complex animal models, *G. mellonella* represents a valuable alternative for early-stage nanotoxicology screening and deserves greater recognition and integration into toxicological research. In this review, we examine all the methodologies and applications of *G. mellonella* in nanotoxicological studies, highlighting its potential as a reliable and ethical model for assessing nanomaterial safety.

This article is categorized under:

Toxicology and Regulatory Issues in Nanomedicine > Toxicology of Nanomaterials

1 | Introduction

Nanotechnology is a multidisciplinary science that mixes principles and techniques from physics, chemistry, biology, materials science, and engineering to design and develop materials at the nanoscale (Campagnolo et al. 2024). Nanomaterials such as nanoparticles (NPs), nanowires, nanotubes, or thin films exhibit unique physicochemical properties, primarily due to their small size and large surface-area-to-volume ratio. These characteristics have enabled significant advancements, especially in the therapeutic field. Nanomaterials help overcome the limitations of conventional drug delivery methods by improving stability, solubility, and transport across biological membranes, thereby extending the duration of circulation and enhancing the

effectiveness of the drug. Additionally, nanomaterials allow targeted drug delivery, enhancing therapeutic efficacy and precision (Havelikar et al. 2024). However, the same properties that offer these benefits also raise safety concerns about potential toxicity at the cellular and organismal levels (Ahmad et al. 2022).

Evaluating nanomaterial safety requires models that accurately mimic physiological conditions. Traditional in vitro models are often too simple, making in vivo models the most reliable approach for evaluating the safety of nanomaterials (Domingues et al. 2022). In this context, *Galleria mellonella* stands out as a simple and alternative invertebrate model for initial nanotoxicology assessments due to its practical advantages and the variety of assays and techniques it offers (Villani et al. 2025). Despite the increasing interest in *G. mellonella* as a model organism, its application in nanotoxicology remains limited and fragmented. Most toxicology studies rely only on survival assays, without addressing other important health assessments such as cellular assays to check immunomodulation or behavioral studies. Other assays like molecular assessment of gene expression, metabolomics, or oxidative stress are rarely used; so too are the biodistribution or imaging assays that can be widely useful to better determine the possible toxicity of nanomaterials and smooth the path to the mammalian assays. Moreover, there is a lack of standardized protocols for these assays that results in variability in toxicity levels for the same material (Opris et al. 2025), which complicates cross-study comparisons and reproducibility.

This review aims to provide a comprehensive overview of the different techniques and applications used with *G. mellonella* in nanotoxicology, as well as its current limitations.

2 | Toxicity Concerns and Safety Frameworks of Nanomaterials

While the advantages of nanomaterials for new therapies are clear, their potential risks to human health and the environment cannot be overlooked. Due to their unique physicochemical properties, nanomaterials are often more reactive than their bulk counterparts, potentially damaging cells and impacting the health of the organism by causing some undesired effects (Ahmad et al. 2022). These include immune dysregulation (e.g., inflammation, immunosuppression or autoimmunity) (Dobrovolskaia and Mcneil 2013; Engin and Hayes 2018), oxidative stress, and genetic damage by direct or indirect DNA interactions (Cheng et al. 2022). Some nanomaterials can penetrate into the CNS, raising concerns about neurological effects (Koedrith et al. 2021; Leudjo Taka et al. 2021; Tiple et al. 2020). Moreover, exposure is not limited to therapeutic applications. Humans are increasingly in contact with nanomaterials from environmental sources, particularly nanoplastics, which are widespread in air, soil, and oceans. Beyond being a significant environmental concern, nanoplastics may enter the body through inhalation and ingestion, interacting with various biological systems (Zhu et al. 2024) and potentially causing metabolic disruption and other cellular stress responses (Yee et al. 2021).

Once inside the body, a major determinant of nanomaterial behavior is the protein or biomolecular corona, formed through interactions between nanomaterials and different biomolecules including proteins, lipids, polysaccharides, metabolites, and nucleic acids (Blanco-Cabra et al. 2024). This corona is a critical factor in the biodistribution and toxicity of nanomaterials inside the body, as it is known to influence the interaction with the immune system and the clearance process (Karmali and Simberg 2011). Consequently, the destiny of nanomaterials inside the body is highly dependent on immune system recognition, and studying nanomaterials interactions with the immune system is therefore essential for predicting potential undesired effects (Fadeel 2019; Hussain et al. 2012).

Despite these concerns, it is well established that many of these adverse effects often occur in a dose-dependent manner (Koedrith et al. 2021; Vilas-Boas and Vinken 2021). This reinforces the importance of comprehensive in vitro and in vivo studies to determine accurate threshold exposure levels, elucidate dose–response relationships, and distinguish between safe and potentially harmful concentrations. In this context, the field of nanotoxicology has emerged as a specialized branch of toxicology focused on investigating the health and environmental risks associated with nanomaterials and supporting the development of nanospecific safety assessment strategies (Fadeel and Keller 2024).

However, regulatory frameworks have not yet fully adapted to the unique nanomaterial properties. Regulatory bodies, such as the FDA, still often rely on bulk material data to make regulations, which may not totally represent nanomaterials (Havelikar et al. 2024; Krug and Nau 2022). This regulatory gap underscores the urgent need for specific guidelines for nanotoxicology testing, including validated in vitro and in vivo models, which are pivotal in evaluating interactions with biological systems and assessing the safety of nanomaterials.

2.1 | In Vitro and In Vivo Nanotoxicology Testing

The interactions of nanomaterials with the body that lead to nanotoxicology highlight the essential need for trustworthy models to evaluate their safety. In vitro models are considered a fast and inexpensive first approach to assess these effects, without using animal experimentation (Huang et al. 2021). However, despite their practicality, they present important limitations. Typically, most assays rely on traditional 2D cell cultures, which poorly represent the reality of nanomaterial interactions within cells in the body (Cao et al. 2021).

Several methodological issues further constrain in vitro assessments. The experimental media used to dissolve nanomaterials often differ significantly from physiological fluids, resulting in variations in agglomeration and adsorption. Additionally, these cultures are generally performed on immortalized cell lines rather than on representative primary cells, which pose a challenge when trying to correlate and transfer dosages between in vitro assays and in vivo models (Hussain et al. 2015). Furthermore, cell toxicity is usually assessed by classical colorimetric viability assays (e.g., MTT), in which false-negative results have been reported due to the interaction of some nanomaterials with fluorescent dyes (Arora et al. 2012; Clift et al. 2011; Vukomanovic and Torrents 2019). Similar interferences have also been described with enzymatic immunoassays

(Kroll et al. 2009). Adding to these limitations, in vitro testing is generally restricted to acute exposure scenarios, being unsuitable for studying long-term or chronic effects (Huang et al. 2021).

To overcome these drawbacks, more advanced in vitro models have been established over the last few decades, including 3D cell culture and microfluidic systems such as organ-on-a-chip technologies. These models can employ a wide range of cell sources to improve biological relevance, including primary cells, stem cells, and reprogrammed cells like induced pluripotent stem cells (iPSCs) (Xie et al. 2024). Features like tight junctions and cell-matrix interactions, which play a key role in tissue integrity and homeostasis, offer a promising alternative for drug testing due to their higher physiological resemblance to native tissues (Masri et al. 2024). Moreover, co-culture systems involving epithelial cells with macrophages, endothelial cells, or other cell types aim to integrate the tissue microenvironment and are also currently being used for a more reliable assessment of nanotoxicology (Braun et al. 2021). Despite their potential, significant challenges in standardization, reproducibility, and modeling still exist for the widespread implementation of these advanced cell models (Ma et al. 2021). On the other hand, in silico models, such as machine learning approaches, have the potential to become complementary tools for screening and supporting traditional toxicological studies (Verma et al. 2023; von Ranke et al. 2022).

Nevertheless, it should be noted that although the use of several models and methodologies is often necessary and provides a higher predictive value, differences in correlation and translation between in vitro and in vivo assays still exist (Maurer-Jones and Haynes 2012). Consequently, in vivo testing remains the gold standard for determining the potential risks of nanomaterials. Mammalian models, such as mice and rats, are therefore extensively used in pharmacological and toxicological studies. However, these models are not entirely analogous to the human body, and certain differences (anatomical, immunological, biochemical, and physiological) impact the reliability of assays, especially regarding pharmacokinetics (Valic and Zheng 2019). For instance, the mucosal barrier in mice and rats is thinner than in humans, and there are notable variations in the gastric pH and intestinal transit time, which can lead to significant differences in the nanomaterial uptake (Domingues et al. 2022). Despite these differences, their high physiological and genetic similarity to humans makes mammalian models valuable for studying organ-specific toxicities (He 2016). Nonetheless, mammalian models demand specialized facilities and entail high operational costs and maintenance requirements. These challenges, together with the ethical considerations that mammalian models require due to their consciousness and capacity to experience pain (particularly the mandatory 3Rs principle: Replacement, Reduction, Refinement), have encouraged the adoption of alternative models (Erkekoğlu et al. 2011). These include "lower vertebrates" like the embryos of the zebrafish *Danio rerio* and invertebrates such as the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, or the greater wax moth *G. mellonella*. The characteristics of these in vivo models, including maintenance, handling, ethics, model characterization, immune system, temperature, and nanotoxicology testing, are compared in Table 1.

Among the non-mammalian models, the vertebrate zebrafish *D. rerio* has been widely utilized in toxicology due to its genetic and physiological similarities to mammals, particularly in systems and pathways involved in toxic responses (Ávila et al. 2018). Zebrafish embryos offer additional advantages, such as transparency and external development, making them ideal for developmental toxicity studies. However, maintaining these embryos requires complex infrastructure, and a significant drawback is their optimal growth temperature, which is much lower than the human body temperature, potentially affecting the relevance of certain toxicological studies (de Souza Anselmo et al. 2018). The nematode *C. elegans* is another well-established model for nanotoxicology. It is exceptionally easy to maintain and provides valuable insights into molecular mechanisms of toxicity. Yet, one key limitation is the lack of a circulatory system, which precludes intravenous administration of nanomaterials for systemic toxicity studies (Li et al. 2021) (Table 1).

Another commonly used in vivo model is *D. melanogaster* (fruit fly), one of the best-characterized eukaryotic organisms worldwide and a widely used in vivo model. While it does not fully replicate the vertebrate response to toxicants, it shares several molecular pathways with mammals. Remarkably, it surpasses zebrafish in some respects, particularly in its ability to facilitate the identification of specific genes involved in toxicity, often at significantly lower costs (Demir 2020). However, this model still has some of the zebrafish limitations, including its optimal growth temperature of 25°C, which is significantly lower than the human body temperature (Ng et al. 2019). In this regard, the greater wax moth *G. mellonella* is a better model, since its favorable growth temperature matches that of the human body (Table 1). This, along with other benefits, has contributed to the increasing popularity of *G. mellonella* as an in vivo model in recent years. The advantages and features of this insect's larval stage will be discussed in detail in the following sections, underscoring its potential as a robust alternative model for nanotoxicology studies.

3 | *Galleria mellonella* as an In Vivo Model

G. mellonella is an insect from the Lepidoptera order that is naturally found in beehives across most continents. Over the last decades, its popularity as an in vivo alternative infection model has grown significantly, and it is now widely used to assess pathogen virulence and to study the efficacy of antimicrobial agents (Ménard et al. 2021; Pereira et al. 2020; Serrano et al. 2023). This wide use is mainly due to a low maintenance cost, easy manipulation, short life cycle, lack of ethical constraints, and structural and functional similarities to the human innate immune system. Interestingly, larvae can also be maintained at 37°C, facilitating the mimicking of the human body (Pereira et al. 2020). In addition, precise doses can be inoculated through intra-haemocoel injection or by force-feeding in larvae, enabling the screening of drugs and other compounds (Fuchs et al. 2010).

These features have also led to the use of *G. mellonella* as a model for studying the toxicity of a wide range of substances, including antimicrobials (Ignasiak and Maxwell 2017), food preservatives (Maguire et al. 2016) and industrial toxic substances such as ionic

TABLE 1 | Comparison characteristics of in vitro and in vivo models. Created with Biorender.com.

Models	Maintenance	Handling	Ethics	Model characterisation	Immune System	Temperature	Nanotoxicology testing
In vitro 	Fast, easy and cheap	Easy	Lack of constraints	Depends on cell line	Lack of immune system	Favorable growth at 37°C	Poor physiological representation Difficulty in dose translation Limited long-term studies
C. elegans 	Easy and cheap	Difficult to inject	Lack of constraints	Very good	Only innate immune system	Optimal growth at ≈20°C	Lack of circulatory system
D. melanogaster 	Easy and cheap	Requires loupe to inject	Lack of constraints	Very good	Only innate immune system	Optimal growth at ≈25 °C	Circulatory system (open) Well-described genes involved in toxicity
G. mellonella 	Easy and cheap	Easy	Lack of constraints	Limited characterization	Only innate immune system	Favorable growth at 37°C	Circulatory system (open) Broad applications in nanotoxicology testing
D. rerio 	Complex infrastructure	Requires loupe to inject	Ethical regulation	Good	Innate and adaptative immunity	Optimal growth at ≈28 °C	Circulatory system (closed) Transparent embryos for live biodistribution studies
Mammalian 	High cost and maintenance	Difficult	Ethical regulation	Very good	Innate and adaptative immunity	Favorable growth at 37°C	High genetic and physiological similarity to humans Most reliable for toxicity assessments

Note: Shading represents whether the characteristics of the different models are considered advantageous (green), disadvantageous (red), or neutral (gray) for nanotoxicology studies.

liquids (Piatek et al. 2021), among others. Importantly, multiple studies have demonstrated that toxicity data obtained from *G. mellonella* show strong statistical correlations with results from both mammalian models and in vitro cell lines. Although survival values may not be identical across different models, they have comparable ranges and exhibit strong statistical correlation, reflecting consistent toxicity trends across systems (Do Carmo Neto et al. 2024; Ignasiak and Maxwell 2017; Maguire et al. 2016; Moya-Andérico, Vukomanovic, et al. 2021; Piatek et al. 2021). Therefore, *G. mellonella* has significant potential for becoming a nanotoxicology model, allowing the evaluation of nanomaterial effects within a range of methodologies and serving as a bridge between in vitro assays and more complex in vivo models, reducing the use of mammals in nanotoxicology testing (Demirtürk et al. 2024; Villani et al. 2025).

3.1 | *Galleria mellonella* Immunological and Anatomical Features for Nanotoxicology

Considering the importance of the immune system in nanotoxicology studies, it is essential to understand the immune response of the in vivo models employed. *G. mellonella* possesses only an innate immune system, which is divided into two main branches: cellular immunity and humoral immunity. On one hand, the cellular response is mediated by their main immune cells, hemocytes, which are mostly found freely circulating in the hemolymph or attached to some organs. There are different types of hemocytes, including prohemocytes, plasmatocytes, granulocytes, spherulocytes, oenocytoids, and coagulocytes (Pereira et al. 2020). Upon the entry of a foreign body, hemocytes carry out functions such as phagocytosis, nodulation, or encapsulation (Figure 1). Thus, the number of hemocytes can differ along the larval cycle and can be influenced by specific triggers such as nanomaterials. On the other hand, the humoral response includes the secretion of antimicrobial peptides (AMPs), opsonins, and melanin (Figure 1). Melanin synthesis and deposition, triggered by the enzyme phenoloxidase (PO) help eliminate the exogenous material and prevent its dissemination. Herewith, melanization can vary widely, beginning sooner or later depending on the foreign agent, and with larvae turning completely dark as a sign of death. Hence, both cellular and humoral responses are interconnected and confer the larvae with a primary line of defense (Serrano et al. 2023; Wojda et al. 2020).

Additionally, *G. mellonella* possesses several well-characterized immune pathways (Toll, Imd and JAK–STAT) that are essential for cytokine-like molecules regulation and production of AMPs in the fat body (Wrońska et al. 2024) (Figure 1). These pathways are well-conserved in insects, although they have been particularly more studied and characterized in *D. melanogaster* (Geng et al. 2016; Wang et al. 2024). Notably, these pathways share functional similarities with components of the human immune system. The insect Toll signaling pathway resembles the human Toll-like receptor (TLR) pathway (Tanji and Ip 2005), and also shows partial conservation with the human interleukin 1 (IL-1) pathway (Wojda et al. 2020). In fact, IL-1-like molecules, which play a significant role in human immunotoxicity against nanomaterials, have been identified in *G. mellonella* (Wittwer et al. 1999; Wrońska et al. 2024; Zhang et al. 2022). Similarly, the Imd pathway can be compared to the human TNF-α pathway. Both Toll and Imd pathways lead to the activation of Dif, Dorsal, and Relish, respectively, which are analogous to NF-kB

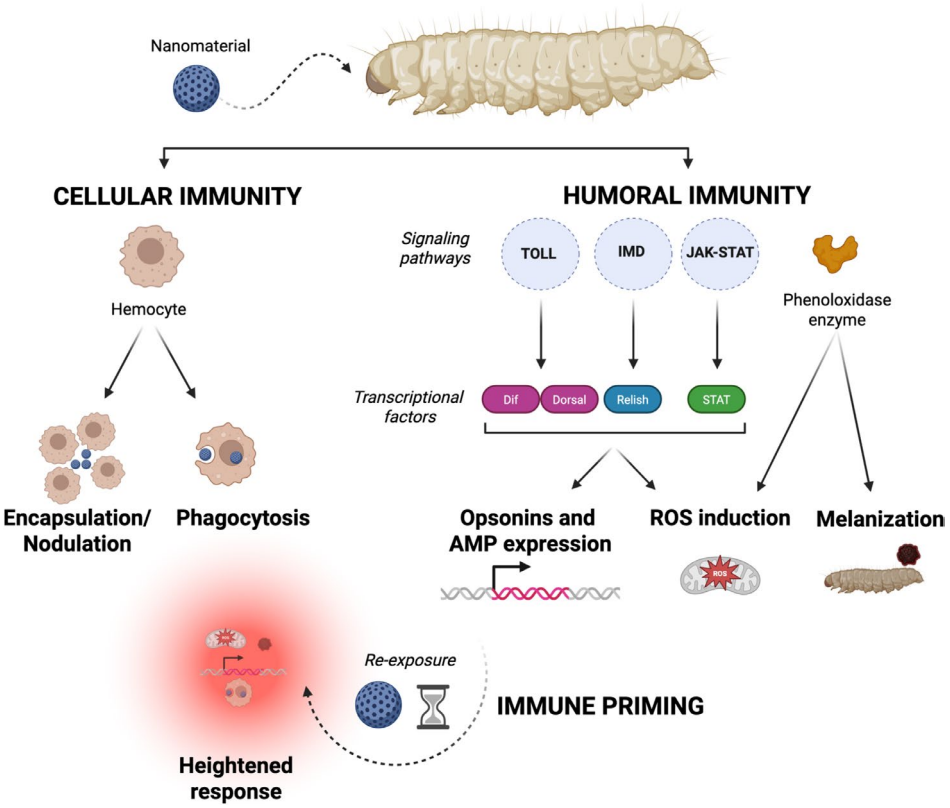

FIGURE 1 | Simplified representation of *G. mellonella* immunity in response to nanomaterials. Created with Biorender.com.

factors in mammals. These transcriptional factors translocate into the nucleus to induce the expression of AMPs (Tanji and Ip 2005). Likewise, the function of the JAK/STAT pathway has been associated with the interferon-gamma signaling in humans. This molecular cascade is involved not only in the expression of AMPs, but also in the expression of other immune-related molecules and defense mechanisms, such as the production of ROS (Kaczmarek et al. 2025) (Figure 1).

These well-conserved immune pathways, along with their relevance to human immunity, support the use of *G. mellonella* in toxicology studies. It has already been employed to assess immune responses to food preservatives and other chemicals, demonstrating its utility in immunotoxicology research (Erbaş et al. 2022; Gwokyalya and Altuntaş 2019; Yucel and Kayis 2018).

Given the critical role of the immune system in nanotoxicology, an emerging concept of interest is the "trained immunity." Despite being traditionally considered a nonspecific first-line mechanism, the innate immune system is now understood to exhibit a protective immune response resulting from epigenetic and metabolic cell reprogramming, following re-exposure to an immunostimulant trigger. While trained immunity can offer promising advances, especially in the field of infections and cancer, a dysregulated response can induce damaging effects, such as hyperinflammation (Netea et al. 2020). The implications of this trained immunity on the nanomaterials toxicity are still quite unknown. It is thought that these heightened inflammatory responses can amplify the toxic effects of nanomaterials, but most studies related to a priming response have only been performed in vitro (Lebre et al. 2022). One of these in vitro studies reported epigenetic modifications in murine bone marrow-derived macrophages following initial exposure to pristine graphene. Later, these macrophages showed an enhanced immune response upon a different stimulus (Lebre et al. 2020). Another study observed an immune tolerance response: AuNPs were found to interfere with and downregulate the immune response triggered by live *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) in human primary monocytes. However, the exact mechanism underlying this effect was not detailed (Swartzwelter et al. 2020). Similarly, a shift in the immune response was also described in hemocytes from mussels of the *Mytilus* genus following re-exposure to nanoplastics (Auguste et al. 2020).

In invertebrates, this phenomenon is referred to as "immune priming", a term distinct from "trained immunity," which is typically reserved for vertebrates, although these types of responses are conserved through evolution among pluricellular organisms (Mulder et al. 2019; Muñoz-Wolf and Lavelle 2021; Netea et al. 2020; Sheehan et al. 2020) (Figure 1). *G. mellonella*, lacking an adaptive immune system, provides an excellent simplified model to explore nanomaterials' immunotoxicity and immune priming effects (Boraschi et al. 2020; Italiani et al. 2020; Moya-Andérico, Vukomanovic, et al. 2021). For instance, low doses of ZnO NPs in *G. mellonella* have been shown to alter hemocyte behavior towards a *Candida albicans* infection, suggesting a priming response (Xu et al. 2021). A lot of unawareness remains on the subject, but although limited research has been performed about it in this invertebrate model, evidence suggests that nanomaterials can induce epigenetics and metabolic modifications, potentially modulating innate immune memory and being able to cause undesired effects (Italiani et al. 2020; Sierra et al. 2016).

Beyond immunity, *G. mellonella* also shows anatomical similarities to mammals that are relevant for nanotoxicology. For instance, the fat body functions analogously to the human liver, being one of the primary tissues exposed to xenobiotics accumulation and absorption, along with the gut (Li et al. 2019; Mese et al. 2022; Tuncsoy et al. 2019) (Figure 2). In the same way, Malpighian tubules can relate to the human renal system, while hemolymph acts as a circulatory fluid akin to blood (Chen and Keddie 2021; Singkum et al. 2019) (Figure 2).

Thus, the anatomical and functional similarities between *G. mellonella* and mammals, particularly regarding their immune systems, provide a valuable framework for studying immunotoxicity at the molecular level and highlight the potential of this model for assessing nanotoxicity of nanomaterials.

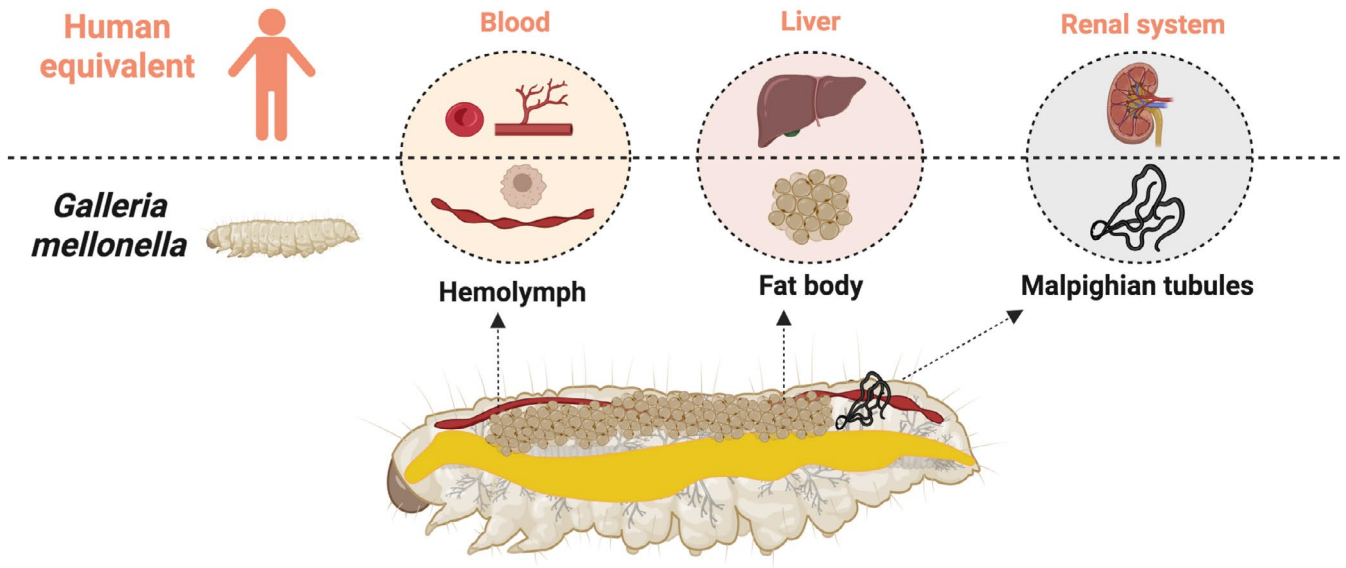

FIGURE 2 | *G. mellonella* key anatomical structures relevant for nanotoxicology evaluation correlated with their human equivalent. Created with Biorender.com.

4 | Methodologies to Test Nanotoxicology in *Galleria mellonella*

This section compiles a range of applications and techniques that *G. mellonella* offers for nanotoxicology studies. *G. mellonella* larvae allow the testing of very precise doses of nanomaterials by injecting them through the hemocoel or by force-feeding. Additionally, nanomaterials can also be fed to the larvae as part of the diet (Figure 3A). Once the nanomaterial is inside the larval body, nanotoxicology can be assessed by exploring the whole larvae or specific tissues, either hemolymph or solid tissues (Figure 3B, Moya-Andérico, Vukomanovic, et al. 2021). To recover the hemolymph tissue, larvae can be anesthetized on ice before tail excision, allowing them to bleed (Moya-Andérico et al. 2020). For the rest of the solid tissues (e.g., fat body or the gut), freezing the larvae prior to dissection allows for better manipulation and tissue isolation under the stereoscopic microscope (Admella and Torrents 2023) (Figure 3B). Although some of the techniques and applications can be applied to both the entire larvae and the specific tissues, distinct sections have been made to simplify and organize the different procedures, represented in Figure 4. Additionally, Table 2 summarizes various parameters and assays performed in *G. mellonella* to evaluate nanotoxicology, which are further reviewed in the following sections.

4.1 | Whole Larvae Methodologies

One of the most commonly used methodologies to evaluate nanotoxicology is the study of larvae survival with Kaplan–Meier survival analysis (Villani et al. 2025). This approach allows the determination of the LD_{50} value (Figure 4A-1), a parameter that indicates the dose of a compound required to kill 50% of the tested population (Ignasiak and Maxwell 2017). This value allows for toxicity comparisons among different animal models (Moya-Andérico, Vukomanovic, et al. 2021) and it is frequently employed in *Galleria* studies. For this reason, studies relying solely on this method are not included in the summary presented in Table 2. Beyond survival, larval health status can also be evaluated using a health evaluation (Figure 4A-2), which considers different factors (activity, cocoon formation, melanization, or development time). For instance, Zorlu et al. applied this type of evaluation to test the effect of dietary TiO_2 NPs on the biological parameters of the larvae (Zorlu et al. 2018) (Table 2). Furthermore, these individual parameters can be integrated into a "health index scoring system," where different health degrees are corelated with a punctuation, and the sum of all categories and points can reflect the general state of the larvae (Tsai et al. 2016).

About the nanotoxicology effects on the larvae comportment, behavioral tracking studies can be performed in the whole larva, including movement or chemotaxis tests to analyze larva activity (Figure 4A-3). Behavioral patterns and traveled distance are parameters that can reveal the impact of nanomaterials on locomotion. Moya-Andérico et al. injected toxic doses of Au NPs, Ag NPs, and Se NPs, observing defective movement in the larva with Ag NPs, and a total lack of movement in the latter (Moya-Andérico, Vukomanovic, et al. 2021) (Table 2). These types of studies have also been performed in other Lepidoptera models, like the *Bombyx mori*. In this case, larvae were exposed to polystyrene NPs for 10 days, and inconsistent hyperactive movements were recorded after. Since chemoreception is important for food location in insects, they showed that polystyrene NPs were mainly altering the start of the feeding process (Parenti et al. 2020).

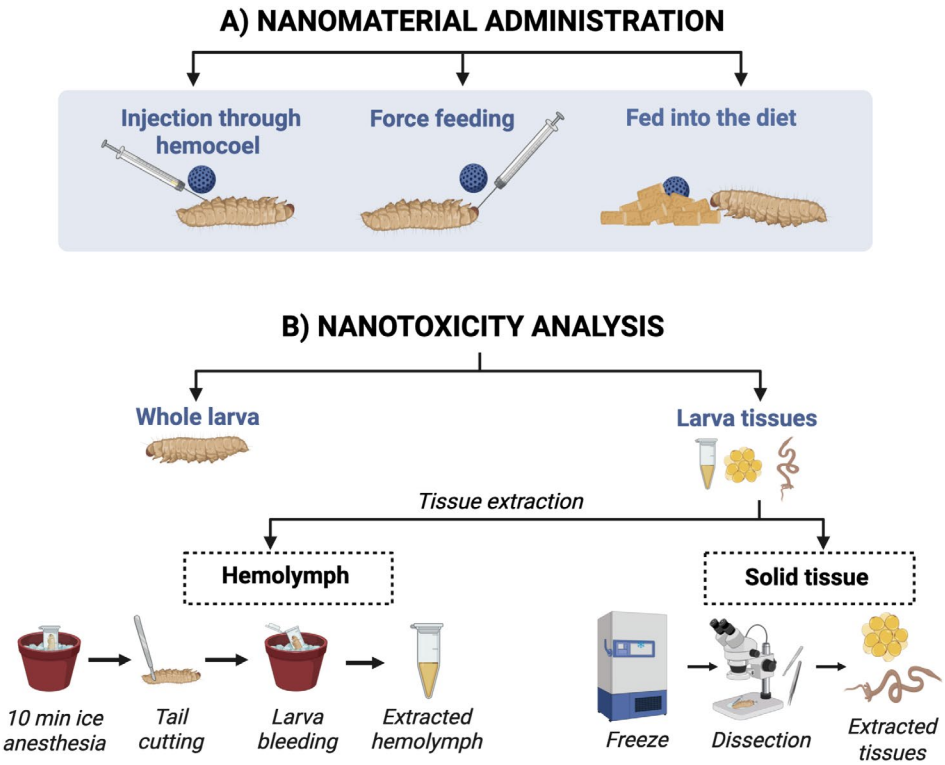

FIGURE 3 | (A) Methodologies to administrate nanomaterials into *G. mellonella*. (B) Methodologies to analyze nanotoxicity in the whole larva or by extracting the larva tissues. Created with Biorender.com.

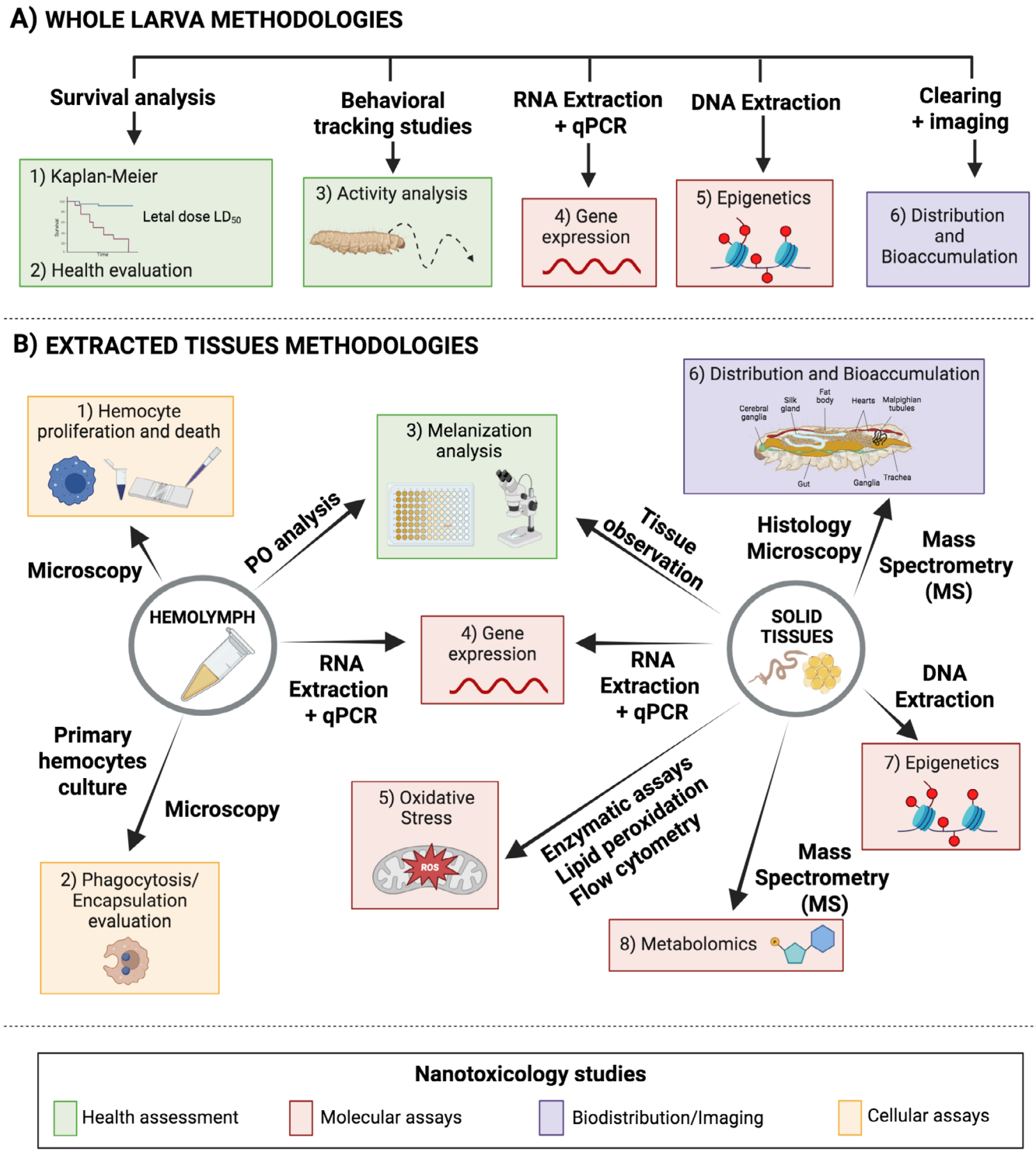

FIGURE 4 | Methodologies in *G. mellonella* to test nanotoxicology. (A) Methodologies from whole larvae. (B) Methodologies from larva hemolymph and solid tissues extracted. Created with Biorender.com.

In addition, larval RNA can be extracted by homogenizing the entire larva. Downstream applications such as quantitative real-time polymerase chain reaction (qRT-PCR) (Campo-Pérez et al. 2025) can be used to analyze the expression of relevant genes for nanotoxicology assessment (Figure 4A-4). A similar procedure can be applied for extracting DNA (Kong et al. 2019), enabling subsequent epigenetic studies (Figure 4A-5).

Finally, imaging the whole larvae of *G. mellonella* is highly difficult due to the opacity effects caused by light scattering. Moya-Andérico et al. developed a clearing protocol that allowed making the larvae cuticle transparent, so fluorescent NPs could be visualized under the confocal microscope (Moya-Andérico, Admella, and Torrents 2021) (Table 2). This strategy can be useful to study nanomaterials distribution and bioaccumulation in the whole larvae (Figure 4A-6).

TABLE 2 | Summary of nanotoxicology assays in *G. mellonella* larvae.

Nanomaterial	Nanomaterial characteristics	Route of administration	Nanomaterial dose	Assay type	Assay time	References
Ag NPs	Size: 12 nm ζ: −17 mV	Injection	2.5–2500 mg/kg larva	Kaplan–Meier survival	72 h	Moya-Andérico, Vukomanovic, et al. (2021)
			50–2500 mg/kg larva	Hemocyte proliferation		
			2841 mg/kg larva	Activity analysis	73 s	
			250 mg/kg larva	Phagocytosis evaluation	20 h	
			250 & 2841 mg/kg larva	Distribution and Bioaccumulation	37 h	
Au NPs	Size: 15 nm ζ: −14 mV	Injection	2.5–2500 mg/kg larva	Kaplan–Meier survival	72 h	
			50–2500 mg/kg larva	Hemocyte proliferation		
			1250 and 2841 mg/kg larva	Distribution and Bioaccumulation		
			1250 mg/kg larva	Phagocytosis evaluation	20 h	
			2841 mg/kg larva	Activity analysis	73 s	
Se NPs	Size: 9 nm ζ: −16 mV	Injection	2.5–2500 mg/kg larva	Kaplan–Meier survival	37 h	
			50–2500 mg/kg larva	Hemocyte proliferation		
			114 mg/kg larva	Distribution and Bioaccumulation		
				Activity analysis	73 s	
CuO NPs	Size: 33.0 ± 8.3 nm ζ: −17.1 ± 0.4 mV	Fed into the diet	0.1–10,000 μg/mL	Kaplan–Meier survival	15 days	Tunçsoy et al. (2021)
			10–1000 mg/L	Oxidative stress enzymatic assays		
				Hemocyte proliferation and death		
			10 μg/L	Bioaccumulation	15 days	Tuncsoy et al. (2019)
				Oxidative stress enzymatic assays		
	Size: 38 nm	Force-fed	1–1000 μg/larva	Kaplan–Meier survival	24 & 72 h	Eskin and Bozdoğan (2022)
			10–150 μg/larva	Hemocyte proliferation and death		
$Cu_3(PO_4)_2$ Nanoflowers	Size: 12–18 μm	Force-fed	100–20,000 ppm/larva	Kaplan–Meier survival	24 h	Eskin et al. (2021)
			100–6000 ppm/larva	Hemocyte proliferation		
				oxidative stress enzymatic assays		

(Continues)

TABLE 2 | (Continued)

Nanomaterial	Nanomaterial characteristics	Route of administration	Nanomaterial dose	Assay type	Assay time	References
TiO_2 NPs	Size: 25 nm ζ: 26.1 mV	Fed into the diet	100–5000 ppm	Health evaluation (larval and pupal developmental time)	> 45 days	Zorlu et al. (2018)
				Oxidative stress enzymatic assays	Unspecified	
	Size: 28.82 ± 11.07 nm	Fed into the diet	5–1250 μg/mL	Bioaccumulation	15 days	Tuncsoy and Mese (2021)
				Oxidative stress enzymatic assays		
				Hemocyte proliferation		
ZnO NPs	Size: 70 nm ζ: 23 mV	Force-fed	0.1–100 μg/larva	Kaplan–Meier survival	24 h	Eskin et al. (2019)
			0.5–5 μg/larva	Hemocyte proliferation		
		Fed into the diet	100–5000 ppm	Kaplan–Meier survival	Unspecified	Eskin and Nurullahoğlu (2022)
				Hemocyte proliferation		
				Encapsulation evaluation	4 & 24 h	
				Melanization analysis		
Al_2O_3 NPs	Size: 43.56 ± 6.44 nm	Injection	10–100 μg/mL	Bioaccumulation	48 h	Tuncsoy and Tuncsoy (2023)
				Oxidative stress enzymatic assays		
Al NPs	Size: < 50 nm PDI: 0.1 ζ: 20.3 mV	Fed into the diet	10–3000 ppm	Kaplan–Meier survival	25 days	Demirtürk et al. (2024)
			50–1000 ppm	Melanization analysis	4 and 24 h	
				Encapsulation evaluation		
Polystyrene NPs	Size: 275.6–351.7 nm PDI: 0.1–0.2 ζ: −19.3 mV	Fed into the diet	10–3000 ppm	Kaplan–Meier survival	25 days	
			50–1000 ppm	Melanization analysis	4 and 24 h	
				Encapsulation evaluation		
Polypropylene nanoplastics	Unspecified	Fed into the diet	Unspecified	Kaplan–Meier survival	4–5 days	Rost-Roszkowska et al. (2024)
				Distribution and Bioaccumulation	24 and 48 h	
				Oxidative stress analysis by ROS staining and flow cytometry		
				Hemocyte proliferation and death		
Polymeric NPs with rhodamine	Size: 200–300 nm PDI: 0.085–0.122 ζ: −13 mV	Injection	10 μg/larva	Clearing + distribution and bioaccumulation	10 days	Moya-Andérico, Admella, and Torrents (2021)
	Size: 30 nm ζ: −21 mV	Injection	1 μg/larva	Phagocytosis evaluation in primary hemocytes culture	24 h	Admella and Torrents (2022)

Note: Shading in the assay type column follows the same nanotoxicology study categories shown in Figure 4: health assessment (green), molecular assays (red), biodistribution/imaging (purple), and cellular assays (yellow).

4.2 | Methodologies From Larva Hemolymph

The hemolymph is a vital tissue in *G. mellonella*, offering a wide range of possibilities for nanotoxicology studies.

The cellular immune response is a crucial aspect to evaluate. The number of circulating hemocytes changes after activation of the immune system. Therefore, hemocyte levels are an important and used parameter when evaluating nanotoxicology (Eskin and Nurullahoğlu 2022; Eskin et al. 2019; Moya-Andérico, Vukomanovic, et al. 2021). Counts can be performed for the different hemocyte types to assess cell proliferation or cell death (Figure 4B–1), since a lower number of hemocytes may indicate damage or toxicity to the larvae's hematopoietic organs (Eskin et al. 2019). In a study performed by Eskin and Bozdoğan, hemocytes were examined under the microscope following eosin and hematoxylin staining to assess viability. Apoptotic, necrotic, mitotic, and micro-nucleated cells were counted and classified according to the aspect and morphology of chromatin and cytoplasm in larvae injected with CuO NPs to report changes in hemocyte percentages due to toxicity (Eskin and Bozdoğan 2022) (Table 2).

Encapsulation or phagocytosis by hemocytes should also be studied (Figure 4B-2), as these cells play a significant role in engulfing foreign or invading bodies (Serrano et al. 2023). For these studies, hemocytes can be visualized under the microscope directly from extracted hemolymph. Another tool for testing nanomaterial internalization could involve the use of primary cell cultures, which provide a more suitable platform than cell lines, although such cultures remain limited in invertebrate models (Weng et al. 2022). Accordingly, a protocol for culturing *G. mellonella* hemocytes in vitro was developed by Admella and Torrents, as a system for testing NPs toxicity. The phagocytic response was analyzed by confocal microscopy one day after the addition of polymeric NPs in the cell culture (Admella and Torrents 2022) (Figure 4B-2) (Table 2).

Melanization typically occurs around foreign particles or nanomaterials, and it can be assessed from the hemolymph by quantifying the activity of PO, the main enzyme involved in this response (Figure 4B-3), as done with some studies reviewed in Table 2 (Demirtürk et al. 2024; Eskin and Nurullahoğlu 2022).

At the molecular level, the signaling pathways Toll and Imd are activated in response to pathogens, but it seems that pattern recognition receptors can also detect foreign bodies like nanomaterials (Figure 1). By extracting RNA just from the hemolymph, the expression of immune-relevant genes, such as antimicrobial peptides and opsonins, along with antioxidant genes can be easily analyzed (Weng et al. 2022) (Figure 4B-4). Interestingly, genes involved in epigenetics and metabolism reprogramming can also be assessed by qRT-PCR to study the immune priming response (Mukherjee et al. 2019).

4.3 | Methodologies From Solid Tissues

Specific solid tissues, such as the gut and fat body, can provide deeper and more specific insights into nanotoxicology. Regarding larval melanization, it can be evaluated with the dissected larvae under a stereoscopic microscope (Admella and Torrents 2023) (Figure 4B-3).

An additional significant effect of nanotoxicology is the generation of oxidative stress. Reactive oxygen species (ROS) produce damage and can be measured by ROS positive staining (Rost-Roszkowska et al. 2024) (Figure 4B-5). By visualizing the tissue histological samples under the microscope, this approach allows the localization and monitoring of cells expressing ROS in different specific larvae tissues. Interestingly, the use of oxidative stress kits in combination with flow cytometry facilitates the quantification of ROS+ and ROS− cells (Rost-Roszkowska et al. 2024) (Table 2). Oxidative stress can also be studied biochemically by measuring the activity of different antioxidant enzymes in the selected tissues (Figure 4B-5). This technique is widely employed in *G. mellonella* as changes in enzyme activity are used as biomarkers for evaluating nanotoxicology. The following enzymes have been the most assessed in several studies: acetylcholinesterase (AchE), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and Glutathione-S-Transferase (GST) (Eskin et al. 2021). Tuncsoy et al. described that CuO NPs produced toxicity in the larvae, and antioxidant enzymes were induced in response (Tuncsoy et al. 2019). In a later study, it was demonstrated that increased SOD and CAT activities occurred in the larvae fat body after exposure to Al_2O_3 NPs (Tuncsoy and Tuncsoy 2023) (Table 2). Interestingly, another effect of ROS is lipid peroxidation, which can be determined with the content of malondialdehyde (MDA) (Zorlu et al. 2018) (Figure 4B-5) (Table 2).

Larvae dissection, which has been previously used as a tool for studying the dissemination of bacterial infections (Admella and Torrents 2023), is also suitable for analyzing the biodistribution and bioaccumulation of nanomaterials (Figure 4B-6). Heavy metals like Zn, Cu, and Ti accumulate in the midgut and fat body of the larvae (Mese et al. 2022). Tuncsoy and Mese described high levels of Ti bioaccumulation in the Malpighian tubules of *G. mellonella* in larvae reared with TiO_2 NPs (Tuncsoy and Mese 2021) (Table 2). In addition, histological analysis can be performed on tissue sections to assess nanomaterial distribution. With this technique, employing an optical microscope, Moya-Andérico et al. showed the accumulation of Se NPs in the most caudal areas of the larvae, surrounded by lymphoid tissue, while some NPs were also distributed near muscle or Malpighian tubules (Moya-Andérico, Vukomanovic, et al. 2021) (Table 2). Histological studies using electronic microscopy, including transmission electron microscopy (TEM) or scanning electron microscopy (SEM), would even be more suitable techniques, allowing the study of subcellular localization of nanomaterials and cell morphology changes (Rost-Roszkowska et al. 2024). In the case of metallic nanomaterials, metal levels from different tissues have also been evaluated in a more sensitive way with inductively coupled plasma mass spectrometry (ICP-MS) (Tuncsoy and Tuncsoy 2023) (Figure 4B-6) (Table 2).

Finally, histone acetylation and DNA methylation can be measured with specific kits for epigenetic studies (Figure 4B-7), as reported by Mukherjee et al. while metabolomic techniques (Figure 4B-8) such as liquid chromatography–mass spectrometry (LC–MS) could be employed for a better understanding of the metabolic and energetic changes occurring during immune

priming (Méndez-López et al. 2024; Mukherjee et al. 2019; Wu et al. 2022).

5 | *Galleria mellonella*: Model Limitations and Future Perspectives

Despite the growing popularity of *G. mellonella* over the recent years as an infection model, only a limited number of studies have in fact evaluated nanomaterials toxicity in this model (summarized in Table 2). A major limitation remains the lack of standardization among different laboratories, leading to disparity in larvae rearing conditions, including temperature, humidity, and diet, that can significantly influence larval development and behavior. Additional variables such as larvae starvation, which reduces the immune response, and the developmental stage at which experiments are performed hinder the comparison and consistency of results, further complicating reproducibility. Moreover, unlike *D. melanogaster*, few companies offer standardized larvae, while most *G. mellonella* suppliers are not intended for research purposes and vary in their rearing conditions, contributing to inter-laboratory variability (Pereira et al. 2020; Tsai et al. 2016; Villani et al. 2025).

It is worth noting that the lack of an adaptive immune system in *G. mellonella* larvae enables the study of specific innate immune responses (Ménard et al. 2021). However, it is well established that nanomaterials also interact and modulate the adaptive immune response (Pondman et al. 2023), which limits the model's capacity to fully reproduce the immunological complexity found in humans.

Regarding pharmacokinetics, evidence suggests similarities between silkworms and mammals (Hamamoto et al. 2019). In line with this, comparable drug distribution patterns and dosages have been described in *G. mellonella*, particularly when evaluating antifungal compounds (Astvad et al. 2017; Jemel et al. 2020). Additionally, *G. mellonella* possesses cytochrome P450 enzymes and glutathione S-transferases, which are involved in xenobiotic detoxification and further support its relevance as a pharmacokinetic model. However, despite the evolutionary conservation of these enzymes and some metabolic pathways across insects and humans, the clearance of nanomaterials can differ (as it does within even different species of mammals), limiting direct translation and comparability to humans (Vogel et al. 2011). Moreover, the lack of standardized protocols and the limited number of studies hinders data correlation with mammalian models and the validation of *G. mellonella* as a robust nanotoxicology model.

Considering these challenges, and as the field of nanotechnology continues to grow, we encourage the development of clear guidelines for implementing *G. mellonella* as an alternative model for nanotoxicology testing. We hope that the wide range of techniques and methodologies *G. mellonella* offers will promote further and more comprehensive nanotoxicology studies in this promising model.

6 | Conclusion

Nanomaterials encompass a wide range of materials and compounds at the nanoscale. Beyond their advantages and promising applications, exposure to them is inevitable, and their safety must be carefully evaluated. The insect *G. mellonella* presents significant potential for the initial evaluation of various aspects and effects of nanomaterial toxicity, using a variety of well-established methodologies. This invertebrate model could contribute to reducing the number of mammals used in this field and minimize the need for in vitro testing, as many preliminary parameters can already be effectively assessed within this system. However, we aim to raise awareness of the current lack of standardization and other limitations that hinder its broader implementation as an alternative model for nanotoxicology testing. While we acknowledge that it cannot fully replace superior and more complex animal models, *G. mellonella* can definitely serve as a valuable substitute for other early-stage screening systems.

Author Contributions

Núria Blanco-Cabra: conceptualization (equal), data curation (equal), formal analysis (equal), investigation (equal), methodology (equal), validation (equal), visualization (equal), writing – original draft (lead), writing – review and editing (equal). **Joana Admella:** conceptualization (equal), data curation (equal), formal analysis (equal), investigation (equal), methodology (equal), resources (equal), software (equal), validation (equal), visualization (equal), writing – original draft (lead), writing – review and editing (lead). **Eduard Torrents:** conceptualization (lead), formal analysis (supporting), funding acquisition (lead), investigation (equal), methodology (equal), project administration (lead), resources (equal), supervision (lead), validation (lead), visualization (equal), writing – original draft (supporting), writing – review and editing (supporting).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Related WIREs Articles

Predicting nanomaterials pulmonary toxicity in animals by cell culture models: Achievements and perspectives

Toxicokinetics, dose-response, and risk assessment of nanomaterials: Methodology, challenges, and future perspectives

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