

Article

Toxicological Effects and Potential Therapeutics of Chronic Exposure to Polyurethane Nanoplastics in *Caenorhabditis elegans*

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Abstract

Despite growing concerns about the ecological and health risks of nanoplastics at environmentally relevant concentrations (ERCs), the effects of polyurethane nanoplastics (PU NPs) on environmental organisms remain unclear. This study assessed the toxicity of PU NPs in the µg/L range in *Caenorhabditis elegans* (*C. elegans*) through chronic exposure. Our results showed that 10 µg/L PU NP exposure significantly reduced brood size, head thrashes, and body bends, while 100 µg/L PU NP exposure decreased lifespan, and 1000 µg/L PU NP exposure increased mortality in wild-type *C. elegans*. Analysis of oxidative stress showed that both 10 and 1000 µg/L PU NP exposures elevated reactive oxygen species (ROS), SKN-1::GFP, and GST-4::GFP levels. Notably, while ROS production rose at 1000 µg/L, SKN-1::GFP and GST-4::GFP expression decreased compared to the 10 µg/L group, suggesting a compensatory response in *C. elegans* at lower exposure levels. The expression of oxidative stress-related genes and phenotype of differentially expressed genes indicated that *C. elegans* was in a compensatory phase when exposed to 10 µg/L of PU NPs, participating in the protective response of *C. elegans* to PU NPs. However, when exposed to 1000 µg/L of PU NPs, *C. elegans* was in a decompensatory phase, participating in the toxic regulation of PU NPs. In addition, under 10 µg/L PU NP exposure, cinnamon essential oil (CIEO) can enhance the expression of more antioxidant enzymes, thereby increasing the protective effect. Under 1000 µg/L PU NP exposure, CIEO could alleviate the toxic response of *C. elegans* to PU NPs exposure by promoting the expression of *skn-1*. Molecular docking analysis showed that the main active component of CIEO, cinnamaldehyde (CID), has a strong affinity with SKN-1/Nrf2. Our study is the first to emphasize the toxic effects of PU NPs on environmental organisms at ERCs and that CIEO might serve as a potential antidote for nanoplastic poisoning.

Keywords: polyurethane nanoplastics; *Caenorhabditis elegans*; chronic exposure; antioxidant mechanisms; cinnamon essential oil



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1. Introduction

Since the large-scale production of plastics commenced in the 1950s, annual output has steadily increased [1]. By 2016, global plastic production had reached 3.35×10^8 metric tons, with a mean annual growth rate of approximately 4% [2,3]. Owing to high emission rates and low recycling efficiency, considerable amounts of plastic waste have been detected in the environment [4–6]. Plastic waste entering the environment disintegrates, through physical, chemical, and/or biological degradation, into particles whose diameter is ≤ 5 mm in at least one dimension, giving rise to “microplastics”—a term first coined in 2004 [6–9]. However, subsequent research indicates that microplastics do not constitute the final form of plastic waste in the environment, as they are further degraded into nanoplastics, measuring 1 nm to 1 μ m in at least one dimension [10,11]. It is predicted that as nanoplastic particle size diminishes, their environmentally relevant concentrations (ERCs) increase significantly due to surface area and volume considerations, with all sizes typically in the μ g/L range [11–13]. Recently, the potential risks of nanoplastics, especially types frequently detected in the environment such as PU NPs, to environmental organisms and humans have garnered substantial attention [14–16].

According to existing research, the environmental detection abundances of different micro(nano)plastics follow this order: expanded polystyrene foam (EPS) > polyurethane (PU) > polypropylene (PP) > polyamide (nylon, PA) > polyvinyl chloride (PVC) [17,18]. Currently, most studies in the micro(nano)plastic field utilize polystyrene (PS) as a model to explore environmental risks, and little is known about the toxicity of PU NPs at ERCs [11]. PU is an organic polymer material synthesized through the polyaddition reaction of polyisocyanates and polyols, exhibiting unique properties such as high tensile strength, abrasion and fatigue resistance, and low-temperature flexibility [19,20]. These properties have enabled its widespread use in industries such as apparel, household appliances, construction, and automotive industries [20,21]. Additionally, PU nanoparticles have been applied in drug delivery systems, biomedical engineering, advanced coatings and adhesives, environmental remediation, and smart textiles/electronics [21,22]. Global polyurethane production is estimated to reach approximately 9 million tonnes annually, with a steady year-to-year increase [23]. Thus, the potential environmental risks posed by PU nanoplastics (PU NPs) warrant urgent attention.

As a critical spice and traditional medicinal herb, cinnamon plays a pivotal role worldwide [24,25]. Cinnamon essential oil (CIEO), extracted from cinnamon, contains volatile constituents—predominantly cinnamaldehyde (CID)—and non-volatile components such as polysaccharides, polyphenols, flavonoids, and diverse bioactive compounds [25,26]. Studies demonstrate that CIEO exhibits superior antioxidant activity to that of aqueous and ethanol extracts, while its major component CID also demonstrates significant antioxidant effects [27,28]. Through free radical scavenging and the inhibition of oxidative stress responses, CIEO mitigates oxidative damage, suggesting therapeutic potential for various oxidative stress-related disorders [28]. However, limited studies have investigated the potential of CIEO and/or CID to mitigate biotoxicity induced by environmental toxicants (especially PU NPs), and how their antioxidant properties modulate organismal antioxidant systems to mediate detoxification processes.

Caenorhabditis elegans (*C. elegans*), a transparent invertebrate model organism, provides multiple advantages as a research tool [29]. Owing to its low maintenance cost, compact size, short lifespan, and highly conserved genome with vertebrates, *C. elegans* is extensively employed in biology, genetics, drug screening, environmental toxicological evaluation, and investigations of underlying molecular mechanisms [30,31]. Sublethal endpoints such as lifespan (reflecting overall health), body length (developmental status), locomotor behavior (motor neuron functionality), and intestinal permeability (toxicity to the primary

target organ) serve as indicators for assessing the toxicity of environmental toxicants to nematodes [32]. With the rapid development of nanotechnology, the biosafety assessment of nanomaterials has become increasingly important. Consequently, *C. elegans* has been applied to explore the distribution of environmental nanomaterials (including nanoplastics), transgenerational toxicity, and screening of preventive drugs [30,33,34].

In the present study, we systematically evaluated the toxic effects of PU NPs on *C. elegans* at ERCs (in the $\mu\text{g}/\text{L}$ range) using multiple endpoints, including lethality, brood size, locomotor behavior, and lifespan. By employing transgenic *C. elegans* strains and measuring in vivo reactive oxygen species (ROS) production, we investigated the roles of key oxidative stress-related enzymes or molecules in PU NP-induced toxicity. Furthermore, through transcriptional expression analysis of oxidative stress-related coding genes and RNA interference (RNAi) knockdown technology, we identified specific molecules involved in responses to varying concentrations of PU NPs and characterized their phenotypic alterations. Additionally, we elucidated how CIEO differentially modulates the molecular mechanisms underlying organismal responses to different PU NP exposure concentrations by mobilizing diverse antioxidant enzymes or molecules. Our results demonstrated that exposure to PU NPs at ERCs elicits toxic effects in environmental organisms, while CIEO exhibits therapeutic potential against PU NP intoxication.

2. Materials and Methods

2.1. Physicochemical Properties of PU NPs

In this study, the stock solution of PU NPs (50 mg/mL) was first gradient-diluted to a storage concentration of 1000 $\mu\text{g}/\text{mL}$ using M9 buffer (3 g KH_2PO_4 , 6 g Na_2HPO_4 , 5 g NaCl, and 1 mL 1 M MgSO_4 per 1 L), followed by further dilution to working concentrations of 1, 10, 100, and 1000 $\mu\text{g}/\text{L}$. The physicochemical properties of PU NPs were characterized by scanning electron microscopy (SEM, GeminiSEM 360, ZEISS, Oberkochen, Germany) and dynamic light scattering (DLS) to describe particle size and morphology, identified by Raman spectroscopy (LabRAM HR evolution, HORIBA, Kyoto, Japan) and Fourier transform infrared spectroscopy (FTIR, Nicolet iS10, Thermo Fisher, Waltham, MA, USA) for chemical composition, and analyzed by a Malvern particle size analyzer (Zetasizer Nano ZS90, Malvern Instruments Ltd., Malvern, UK) for surface charge and hydrated particle size. The detection methods and procedures described above have been detailed in previous research [35].

2.2. Nematode Maintenance

In this study, the following *C. elegans* strains were utilized: wild-type N2 and transgenic strains, LD1/*ldIs7[skn-1b/c::GFP+rol-6(su1006)]* and CL2166/*dvIs19[(pAF15)gst-4p::GFP::NLS]*, with detailed strain information provided in Table S1. All strains were obtained from the *Caenorhabditis* Genetics Center (CGC). Nematodes were cultured at 20 °C on Nematode Growth Medium (NGM) agar plates seeded with *Escherichia coli* (*E. coli*) OP50 lawns. The NGM composition per liter was as follows: 17 g agar, 3 g NaCl, 2.5 g bactopectone, 25 mL 1 M KH_2PO_4 , 1 mL 1 M MgSO_4 , 1 mL 1 M CaCl_2 , and 1 mL 5 mg/L cholesterol.

2.3. PU NPs Exposure

C. elegans were exposed to 1–1000 $\mu\text{g}/\text{L}$ of PU NPs in M9 buffer containing UV-inactivated *E. coli* OP50, as previously described [36,37]. To synchronize L1 larvae, gravid hermaphrodites were lysed using a bleaching solution (0.45 M NaOH and 2% HClO); eggs were collected and cultured until hatching. Long-term exposure to PU NPs commenced at the L1 larval stage and continued until the first day of adulthood (approximately 108 h).

For each experimental group, ~500 nematodes were introduced into 35 mm culture dishes (Corning, New York, NY, USA) containing 2.5 mL of exposure solution: M9 buffer for the control group or 1–1000 µg/L PU NP suspensions for experimental groups. The medium, OP50, and PU NPs solutions were replaced daily, and each experiment included at least 3 biological replicates for the control group.

2.4. Toxicity Evaluation Endpoints

Lethality, brood size, locomotion behavior, and lifespan were used as evaluation endpoints to assess the general toxicity of PU NPs, as previously described [38]. All experiments were repeated at least 3 times.

Lethality, a commonly used toxicity endpoint for evaluating environmental toxins in *C. elegans*, was defined as the percentage of survival after exposure [35]. Nematodes were classified as dead when they exhibited no movement following repeated stimulation with a worm picker. At least 100 nematodes per group were used for lethality testing.

Brood size, a classic indicator of reproductive capacity in *C. elegans*—particularly in studies of reproductive toxicity induced by environmental toxins, including nanoplastics—was used to evaluate the effect of PU NPs exposure on reproductive function [39]. After exposure, nematodes were washed with M9 buffer and transferred to NGM plates devoid of *E. coli* OP50. Brood size was determined by counting the number of eggs laid by each nematode, with 30 nematodes analyzed per group.

Locomotion behavior, including head thrash and body bend—indicators of motor neuron function—are commonly used evaluation endpoints in *C. elegans* [36]. Head thrash is defined as the rapid change in bending direction at the midbody, while body bend refers to the wavelength of nematode movement. Nematodes were randomly placed on NGM plates within 2 h post-exposure, and locomotor behaviors were counted using an optical microscope. At least 30 nematodes per group were assessed for locomotor behavior.

Lifespan, a classic indicator of nematode overall health, is defined as the period from egg hatching (day 0) to the termination of survival [36]. Lifespan tests were conducted at 20 °C, with nematodes randomly placed on agar plates containing *E. coli* OP50 within 2 h post-exposure. During the lifespan assay, hermaphrodite nematodes were transferred daily for the first 7 days of adulthood. Nematodes were classified as dead when they showed no response to repeated stimulation with a worm picker, with at least 30 nematodes per group analyzed for lifespan.

2.5. ROS Analysis

Intracellular free radicals were manifested as ROS, which were routinely quantified using a reactive oxygen species assay kit (Beyotime Biotechnology Co., Ltd., Shanghai, China) [37]. After exposure, nematodes were harvested and washed at least 3 times with M9 buffer, with the final supernatant decanted. A 10 mM 2',7'-dichlorofluorescein diacetate (DCFH-DA) ROS solution was added to each centrifuge tube, and the nematodes were incubated at 20 °C for 3 h. The base of each centrifuge tube was gently tapped every hour to ensure homogeneous mixing. Thereafter, nematodes were rinsed three to five times with M9 buffer to remove unbound probe, the supernatant discarded, and the prepared nematodes deposited dropwise onto 2% agar pads. Fluorescence microscopy (excitation wavelength 488 nm, emission wavelength 525 nm) was used to capture images, which were processed with ImageJ software (Version 1.8.0, NIH, Bethesda, MD, USA). ROS levels were quantitatively analyzed by assessing the fluorescence intensity of reactive oxygen species in nematodes. Each experimental group comprised a minimum of 30 nematodes, with three parallel samples set for both experimental and control groups.

2.6. Experimental Methods for Detecting Transgenic Nematodes

After exposure, fluorescent images of transgenic nematode strains *ldIs7* and *dvIs19* were captured using a fluorescence microscope. The acquired images were processed and analyzed with Image-Pro Plus (Media Cybernetics Image Techno. Co., Rockville, MD, USA). A semi-quantitative analytical approach was utilized to characterize fluorescent disparities between PU NP-exposed groups and the control group (unless otherwise stated). A minimum of 30 nematodes per group was employed for fluorescent analysis.

2.7. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Target gene-specific primers were designed and synthesized by Sangon Biotech Co., Ltd., Shanghai, China. Total RNA was extracted post-exposure using the TRIzol® method (Thermo Fisher Scientific Inc., Waltham, MA, USA), followed by purification with the RNeasy kit (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was conducted using the PrimeScript™ RT kit (Takara, Kusatsu, Japan), and PCR products were amplified with SYBR Green qRT-PCR Master Mix (Toyobo, Osaka, Japan) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The *tba-1* gene encoding α -tubulin was selected as the reference gene in this study. Relative expression levels of target genes in exposed groups versus controls were calculated using the $2^{-\Delta\Delta CT}$ method. For qRT-PCR analysis, 500 nematodes were used per group with three parallel reactions set for each group. All qRT-PCR primers are listed in Table S2. Information for genes involved in oxidative stress in *C. elegans* is shown in Table S3.

2.8. RNAi

RNAi induction in *C. elegans* was accomplished by feeding engineered bacteria expressing double-stranded RNA (dsRNA), a widely established methodology [40]. For gene silencing, RNAi technology was applied following the protocol described previously [36]. Gravid hermaphrodite nematodes were transferred to NGM plates overlaid with specific RNAi bacteria, and their third-generation progeny were utilized for toxicity assessment. HT115 (DE3) bacteria served as the control. To validate the interference efficacy of dsRNA in RNAi nematodes, RNAi efficiency was evaluated via qRT-PCR by determining the mRNA expression levels of target genes (Figure S1). RNAi primers are listed in Table S4.

2.9. CIEO Component Analysis and Treatment

The CIEO was dissolved in a solution containing 1% dimethyl sulfoxide (DMSO), with the final concentration in the liquid culture system fixed at 2.5 mg/mL, as per our prior study [25]. To confirm the safety and efficacy of CIEO at 2.5 mg/mL on *C. elegans*, we analyzed the effects of CIEO treatments at concentrations of 0, 1, 2.5, 5, and 10 mg/mL on mortality rate and locomotion behavior, and found that exposure to 2.5 mg/mL CIEO had no impact on the mortality rate or locomotion behavior (Figure S3). Sufficient L1-stage nematodes were treated with 1% DMSO, 10 or 1000 μ g/L of PU NPs, and 10 or 1000 μ g/L of PU NPs combined with 2.5 mg/mL CIEO, respectively. After exposure, the expression levels of genes within the antioxidative system were assayed using the methodologies described above.

2.10. Molecular Docking

To evaluate the binding energy and interaction modes between the main component of CIEO, CID, and its target SKN-1/Nrf2, we employed AutodockVina 1.2.2, a computational protein–ligand docking software [41]. The molecular structure of CID was obtained from the PubChem Compound Database (<https://pubchem.ncbi.nlm.nih.gov/> accessed on 10 June 2025) [42]. The 3D coordinates of the protein SKN-1/Nrf2 (PDB code: 7k2a;

resolution: 1.90 Å) were downloaded from the PDB (<http://www.rcsb.org/> accessed on 10 June 2025). Preprocessing of CID and SKN-1/Nrf2 involved converting files to PDBQT format, removing all water molecules, and adding polar hydrogen atoms. The grid box was centered to encompass the functional domain of each protein, enabling free molecular movement, with the docking pocket defined as a 30 Å × 30 Å × 30 Å cubic volume and a grid spacing of 0.05 nm. Molecular docking analyses were performed using AutodockVina 1.2.2 (<https://autodock.scripps.edu/> accessed on 10 June 2025), with model visualization facilitated by the same software.

2.11. Statistics and Data Analysis

Statistical analyses were conducted using SPSS 25.0 (SPSS Inc., Chicago, IL, USA). All parameters employed in this study were continuous variables. The *Agostino D* test was utilized to assess variance homogeneity. Intergroup differences were analyzed via one-way analysis of variance (ANOVA), with multiple comparisons performed using the SNK-q test. Results were deemed statistically significant at a confidence level of $p < 0.05$.

3. Results

3.1. Physicochemical Properties of PU NPs and CIEO

The physicochemical properties of PU NPs were characterized by SEM, Raman shift, FTIR spectrum, DLS, and zeta potential. A representative SEM image of PU NPs is illustrated in Figure 1A. Raman spectroscopy analysis revealed that the peak at 610.17 cm^{-1} was due to the bending vibration of $\text{C}=\text{O}$; the peak at 1000.03 cm^{-1} was due to the breathing/deformation vibration of C-H in the benzene ring; the peak at 1280.08 cm^{-1} was due to the stretching vibration of C-N and the bending vibration of N-H; the peak at 1452.36 cm^{-1} was due to the bending/swinging vibration of CH_2 ; the peak at 1610.24 cm^{-1} was due to the in-plane vibration of NH; the peak at 2857.89 cm^{-1} was due to the stretching vibration of CH_2 ; the peak at 3069.57 cm^{-1} was due to the stretching vibration of aromatic C-H; and the peak at 3398.76 cm^{-1} was due to the stretching vibration of N-H (Figure 1B). FTIR analysis showed that the peak at 3328.19 cm^{-1} was due to the secondary amine group ($-\text{NH}-$) in the urethane bond; the peak at 3080.46 cm^{-1} was due to the $=\text{C}-\text{H}$ in the aromatic ring; the peak at 1600.57 cm^{-1} was due to the $\text{C}=\text{C}$ in the benzene ring; the peak at 1450.03 cm^{-1} was due to all $-\text{CH}_2$ groups in the PU molecule; and the peaks at 1267.53 cm^{-1} and 1150.15 cm^{-1} were due to the C-O in the urethane (Figure 1C). The zeta potential of PU NPs was -8.7 ± 0.7 mV, and DLS analysis indicated the hydrodynamic diameter of PU NPs was 205.9 ± 2.7 nm, with their particle size remaining stable over time (Figure S3). Collectively, these data confirm that the nanoplastic particles used in this study were spherical PU NPs with a diameter of approximately 200 nm and a negative surface charge.

3.2. General Toxicity Evaluation of *C. elegans* Exposed to PU NPs in the Range of $\mu\text{g/L}$

Lethality, brood size, head thrashes, body bends, and lifespan serve as established universal endpoints for assessing the toxicity of environmental toxicants, including nanoplastics [38,43]. Our study demonstrated that exposure to 1000 $\mu\text{g/L}$ of PU NPs induced a significant increase in mortality ($p < 0.01$) in wild-type *C. elegans* (Figure 2A). Conversely, exposure to 10 $\mu\text{g/L}$ of PU NPs was sufficient to elicit a decline in reproductive capacity ($p < 0.01$), with a clear dose-effect relationship as the concentration increased (Figure 2B). Locomotion behavior analysis showed that 10 $\mu\text{g/L}$ of PU NPs significantly impaired motility in wild-type *C. elegans*, as reflected by reduced head thrashes ($p < 0.01$) and body bends ($p < 0.01$), both exhibiting a distinct dose-effect relationship with increasing concentration (Figure 2C,D). Lifespan analysis further showed that exposure to 100 $\mu\text{g/L}$ of PU NPs significantly shortened lifespan ($p < 0.05$) in a dose-dependent manner (Figure 2E).

Collectively, these results indicate that PU NPs at $\mu\text{g/L}$ concentrations exert notable toxic effects on wild-type *C. elegans*.

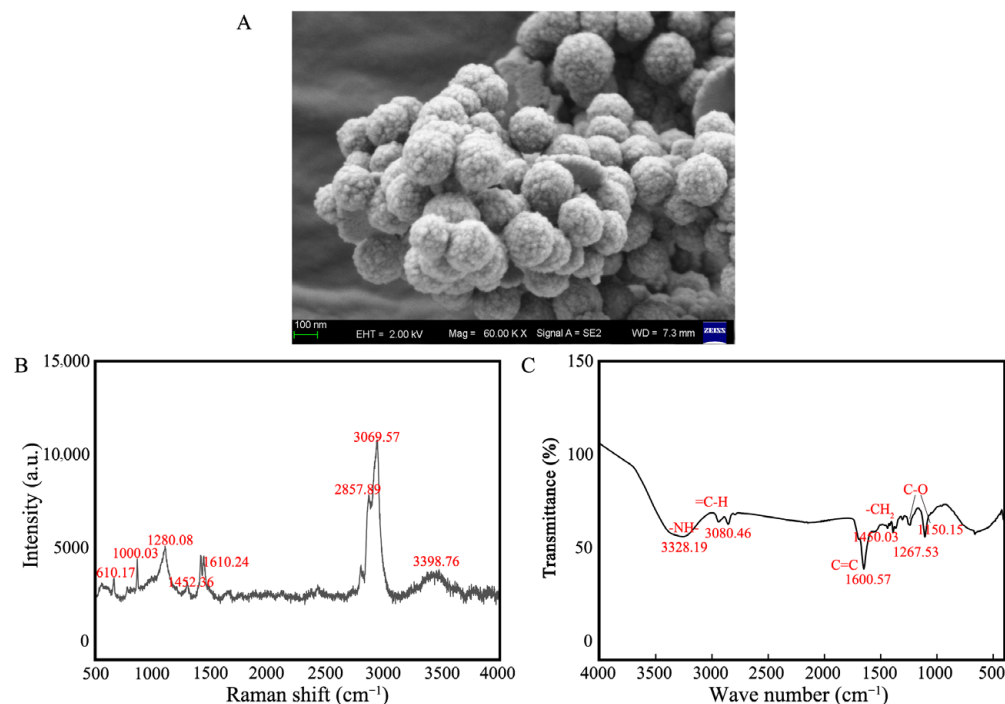


Figure 1. Physical and chemical properties of PU NPs and CIEO used in the present study. (A) SEM image of PU NPs. (B) Raman spectrum image of PU NPs. (C) FTIR spectroscopy of PU NPs.

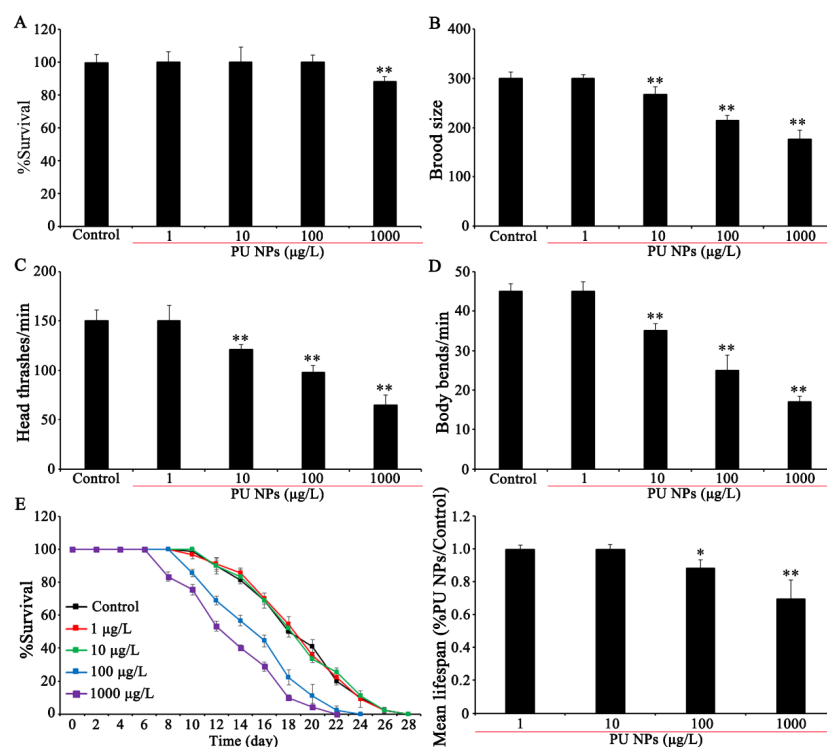


Figure 2. General toxicity evaluation of *C. elegans* exposed to PU NPs in the $\mu\text{g/L}$ range. (A) General toxicity evaluation on lethality. (B) General toxicity evaluation on brood size. (C) General toxicity evaluation on head thrashes/min. (D) General toxicity evaluation on body bends/min. (E) General toxicity evaluation on lifespan. Prolonged exposure to PU NPs was performed from L1-stage larvae to adult day 1. Bars indicate means $\pm$ SD. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control (if not specially indicated).

3.3. The Anti-Oxidative System Exhibited Differential Regulatory States in Nematodes Exposed to 10 and 1000 µg/L of PU NPs

To further elucidate the regulatory role of oxidative stress in *C. elegans* response to µg/L levels of PU NP exposure, we chose doses of 10 and 1000 µg/L to dissect underlying regulatory mechanisms. Exposure of wild-type *C. elegans* to both 10 and 1000 µg/L of PU NPs significantly elevated intracellular ROS production ($p < 0.01$), with a more pronounced ROS accumulation at the higher concentration (Figure 3A). *skn-1* serves as the only evolutionary homolog of Nrf2, and the signaling pathways it mediates constitute central mechanisms underpinning the biological response to oxidative insult in *C. elegans* [33]. Upon exposure to oxidative stress, SKN-1 is activated and undergoes nuclear translocation [44]. In *C. elegans*, *gst-4* encodes glutathione transferase 4, a key enzyme in the antioxidant stress response system [45]. We employed transgenic strains *ldls7* and *dols19* to investigate the functions of SKN-1/Nrf2 and the antioxidant enzyme GST-4 in mediating toxicity responses to µg/L levels of PU NP exposure. After exposure to 10 or 1000 µg/L of PU NPs, *C. elegans* displayed enhanced nuclear localization of SKN-1::GFP ($p < 0.01$) (Figure 3B) and increased expression of GST-4::GFP ($p < 0.01$) (Figure 3C) compared with the control group. Notably, the 1000 µg/L PU NP exposure group exhibited attenuated SKN-1 nuclear translocation and reduced GST-4::GFP expression relative to the 10 µg/L group ($p < 0.01$) (Figure 3B,C). These findings suggest that dose-dependent PU NP exposure triggers differential antioxidant responses to mitigate toxicity.

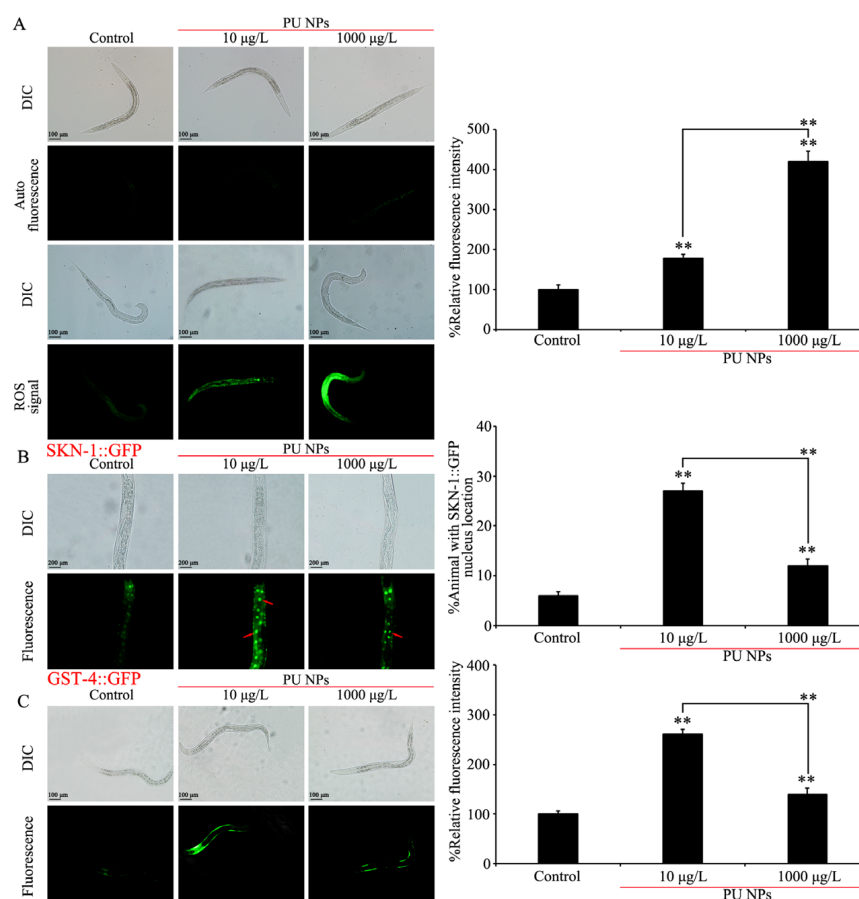


Figure 3. Effects of the anti-oxidative system regulating the toxicity of PU NP exposure in the range of µg/L in *C. elegans*. (A) Effect of exposure to PU NPs in inducing intestinal ROS production. (B) Effect of exposure to PU NPs on the expression of SKN-1::GFP. Arrows indicate the nuclear translocation signal. (C) Effect of exposure to PU NPs on the expression of GST-4::GFP. Prolonged exposure to PU NPs was performed from L1-stage larvae to adult day 1. Bars indicate means $\pm$ SD. ** $p < 0.01$ vs. control (if not specially indicated).

3.4. Identification of Anti-Oxidative Enzymes in the Role of Regulating PU NP-Induced Toxicity at 10 and 1000 µg/L of PU NPs

In *C. elegans*, the antioxidative system comprises superoxide dismutase (SOD-1-5), catalases (CTL-1-3), electron transport chain (ISP-1 and CLK-1), mitochondrial complex components (MEV-1 and GAS-1), Nrf2 (SKN-1), and glutathione S-transferase (GST-4) [11,46,47]. Our research indicated that exposure of wild-type *C. elegans* to either 10 or 1000 µg/L of PU NPs did not alter the expression patterns of *sod-1*, *sod-2*, *sod-4*, *sod-5*, *clk-1*, *isp-1*, *mev-1*, *gas-1*, *ctl-2*, and *ctl-3* at the transcriptional level (Figure 4A). In contrast, 10 µg/L PU NPs exposure significantly increased the transcriptional expression levels of *sod-3*, *ctl-1*, *skn-1*, and *gst-4* (Figure 4A). Notably, 1000 µg/L PU NP exposure also induced upregulation of these genes, though their transcriptional levels were markedly lower than those in the 10 µg/L group while remaining significantly higher than the control (Figure 4A).

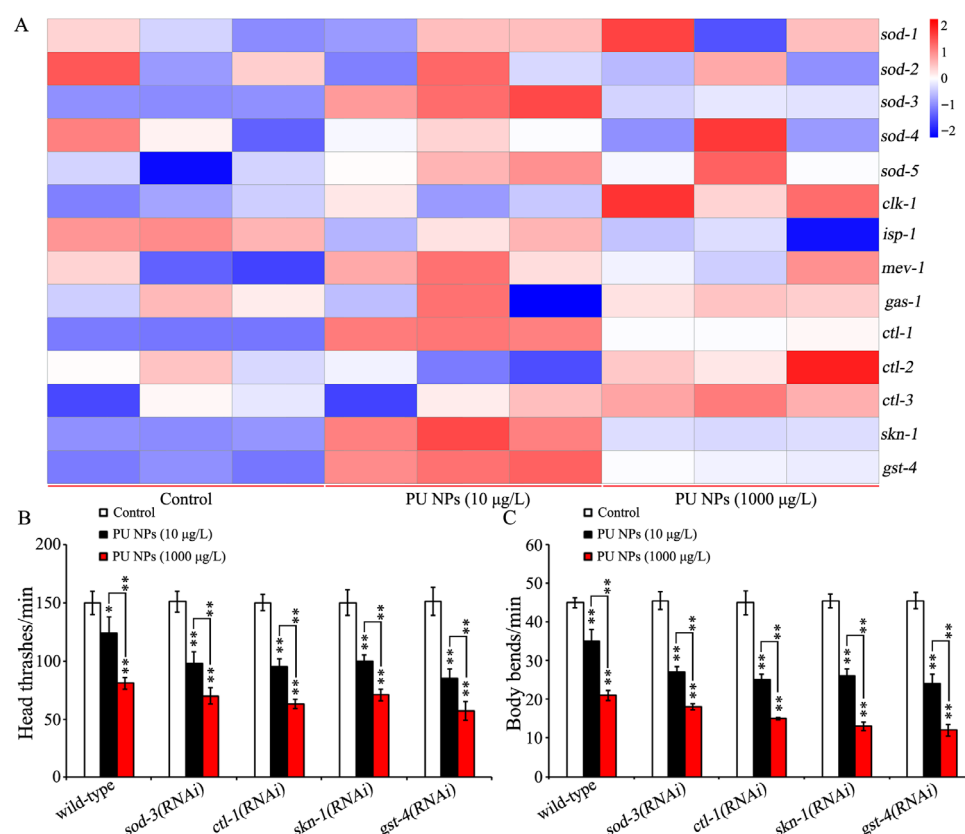


Figure 4. Expressional alterations of genes involved in the anti-oxidative system in PU NP-exposed *C. elegans*. (A) Expression patterns of genes required for the control of oxidative stress in PU NP-exposed (10 and 1000 µg/L) wild-type *C. elegans*. (B) Effect of RNAi knockdown of *sod-3*, *ctl-1*, *skn-1*, or *gst-4* on head thrashes in PU NP-exposed (10 and 1000 µg/L) *C. elegans*. (C) Effect of RNAi knockdown of *sod-3*, *ctl-1*, *skn-1*, or *gst-4* on body bends in PU NP-exposed (10 and 1000 µg/L) *C. elegans*. Prolonged exposure to PU NPs was performed from L1-stage larvae to adult day 1. Bars indicate means $\pm$ SD. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control (if not specially indicated).

Using RNAi technology and locomotor behavior as endpoints, we further investigated the phenotypes of *sod-3*, *ctl-1*, *skn-1*, and *gst-4* knockdown in *C. elegans* exposed to 10 or 1000 µg/L of PU NPs. Compared with wild-type nematodes, RNAi-mediated knockdown of these genes did not elicit detectable alterations in head thrashes ($p < 0.01$) or body bends ($p < 0.01$) (Figure 4B,C). However, compared with wild-type *C. elegans* exposed to PU NPs, the knockdown of *sod-3*, *ctl-1*, *skn-1*, or *gst-4* rendered *C. elegans* more susceptible to PU NP exposure, evidenced by pronounced reductions in head thrashes ($p < 0.01$) and body bends ($p < 0.01$)—a decline more pronounced in the 1000 µg/L group than in the 10 µg/L group

(Figure 4B,C). These results indicate that at the lower dose of 10 $\mu\text{g/L}$, *C. elegans* activates a protective mechanism via the upregulation of antioxidative enzymes (SOD-3, CTL-1, and GST-4). Conversely, 1000 $\mu\text{g/L}$ PU NP exposure inhibits expression of these enzymes with increasing dosage, thereby mediating toxic effects.

3.5. CIEO Treatment Enhanced the Protective Response of *C. elegans* to 10 $\mu\text{g/L}$ PU NP Exposure by Promoting the Expression of More Antioxidant Enzyme Systems

To investigate the modulation of CIEO treatment on the expression of antioxidant enzyme systems in *C. elegans* under 10 $\mu\text{g/L}$ PU NP exposure, we assessed the transcriptional profiles of genes encoding superoxide dismutases, catalases, electron transport chain components, mitochondrial complex subunits, SKN-1/Nrf2, and glutathione S-transferases. Our results indicated that, compared with the 10 $\mu\text{g/L}$ PU NP exposure group, the CIEO treatment group not only sustained higher expression levels of *sod-3*, *ctl-1*, and *gst-4* but also upregulated the transcription of *sod-2*, *sod-5*, *clk-1*, *ctl-2*, and *skn-1*, thereby enhancing the protective response of *C. elegans* to 10 $\mu\text{g/L}$ PU NP exposure (Figure 5A).

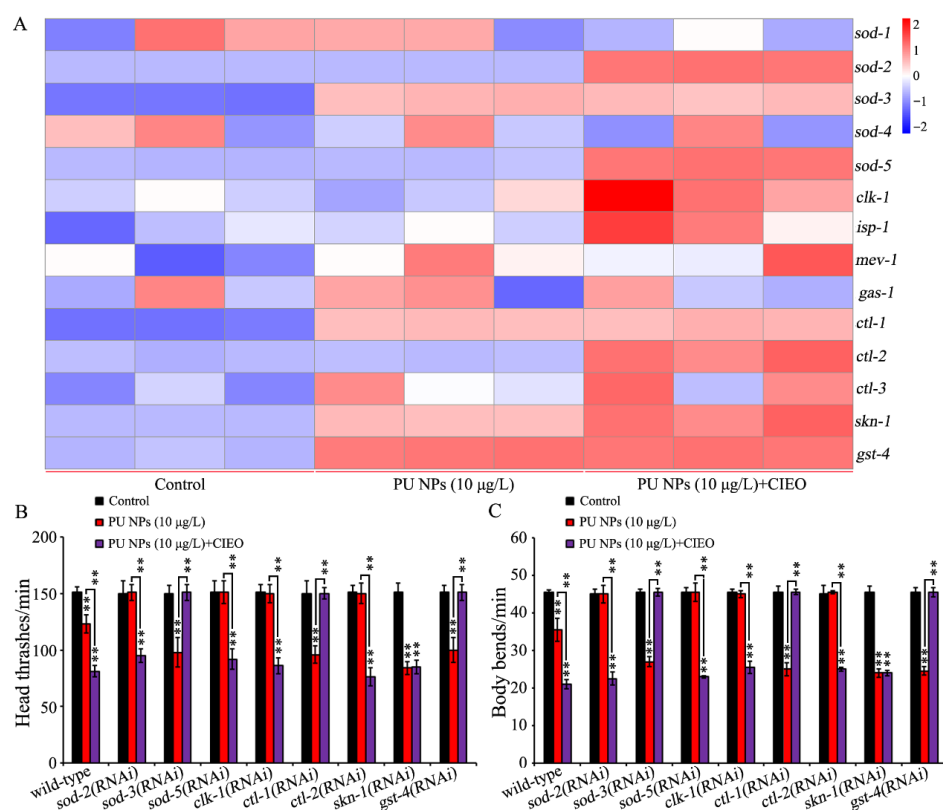


Figure 5. Effects of CIEO treatment on the expressional alterations of genes involved in the anti-oxidative system in PU NP-exposed (10 $\mu\text{g/L}$) *C. elegans*. (A) Expression patterns of genes required for the control of oxidative stress in 10 $\mu\text{g/L}$ PU NP-exposed group and CIEO-treated after 10 $\mu\text{g/L}$ PU NP-exposed groups of wild-type *C. elegans*. (B) Effect of RNAi knockdown of *sod-3*, *ctl-1*, *skn-1*, or *gst-4* on head thrashes in 10 $\mu\text{g/L}$ PU NP-exposed group and CIEO-treated after 10 $\mu\text{g/L}$ PU NP-exposed group of wild-type *C. elegans*. (C) Effect of RNAi knockdown of *sod-3*, *ctl-1*, *skn-1*, or *gst-4* on body bends in 10 $\mu\text{g/L}$ PU NP-exposed group and CIEO-treated after 10 $\mu\text{g/L}$ PU NP-exposed group of wild-type *C. elegans*. Prolonged exposure to PU NPs was performed from L1-stage larvae to adult day 1. Bars indicate means $\pm$ SD. ** $p < 0.01$ vs. control (if not specially indicated).

Using RNAi technology and locomotion assays as endpoints, we analyzed phenotypic responses following RNAi-mediated knockdown of antioxidant-related genes (*sod-2*, *sod-3*, *sod-5*, *clk-1*, *ctl-1*, *ctl-2*, *skn-1*, and *gst-4*) in *C. elegans* exposed to 10 $\mu\text{g/L}$ of PU NPs. Compared with the wild-type exposure group, RNAi knockdown of *sod-2*, *sod-5*, *clk-1*,

or *ctl-2* failed to elicit significant susceptibility or resistance in *C. elegans* under 10 µg/L PU NP exposure, whereas knockdown of *sod-3*, *ctl-1*, *skn-1*, or *gst-4* induced significant susceptibility (Figure 5B,C). Notably, in the context of CIEO treatment, RNAi knockdown of *sod-2*, *sod-5*, *clk-1*, *ctl-2*, or *skn-1* resulted in significant susceptibility ($p < 0.01$) to 10 µg/L PU NP exposure (Figure 5B,C). These results indicate that the expression of enzyme systems associated with *sod-2*, *sod-5*, *clk-1*, *ctl-2*, and SKN-1/Nrf2 is essential for CIEO treatment to augment the protective response of *C. elegans* to 10 µg/L PU NP exposure.

3.6. CIEO Treatment Alleviated the Toxic Response of *C. elegans* to 1000 µg/L PU NP Exposure by Promoting the Expression of *Skn-1*

To investigate how CIEO treatment regulated the expression of antioxidant enzyme systems in *C. elegans* upon exposure to 1000 µg/L of PU NPs, we characterized the transcriptional profiles of genes encoding superoxide dismutases, catalases, electron transport chain components, mitochondrial complex subunits, SKN-1/Nrf2, and glutathione S-transferases. Our results showed that, compared with the 1000 µg/L PU NP exposure group, the CIEO treatment group attenuated the excessive upregulation of *sod-3*, *ctl-1*, and *gst-4*, restoring their expression to control-level baselines to mitigate the toxic response of *C. elegans* to 1000 µg/L of PU NPs (Figure 6A). Conversely, the CIEO treatment group further enhanced *skn-1* expression to alleviate toxicity (Figure 6A).

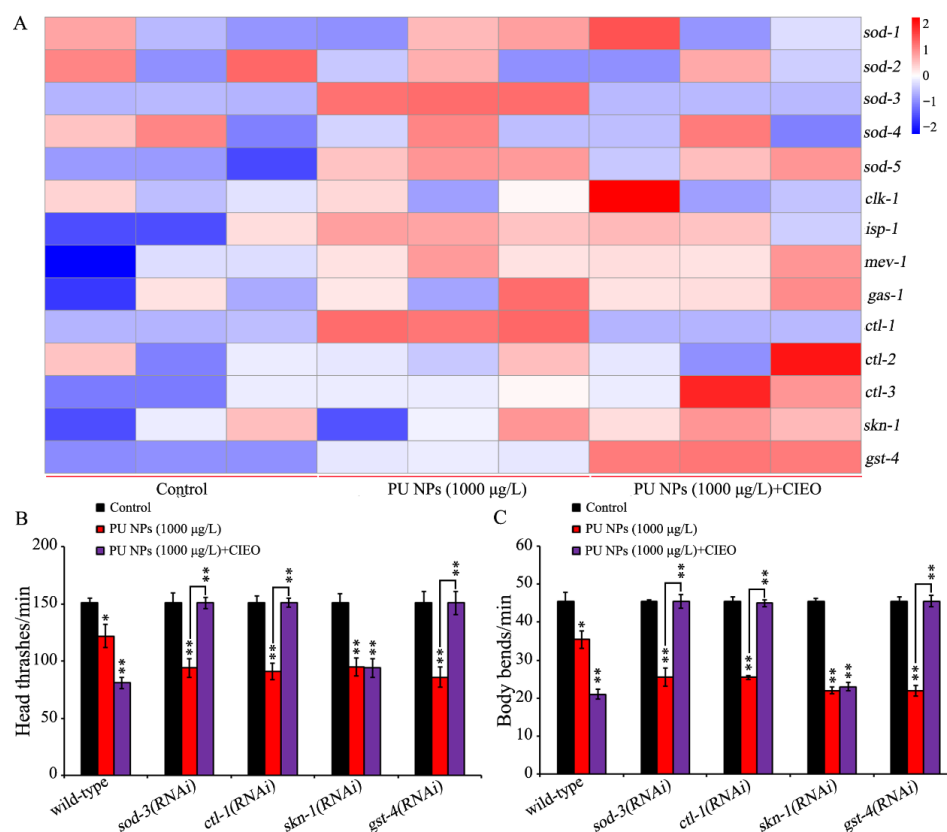


Figure 6. Effects of CIEO treatment on the expressional alterations of genes involved in the anti-oxidative system in PU NP-exposed (1000 µg/L) *C. elegans*. (A) Expression patterns of genes required for the control of oxidative stress in 1000 µg/L PU NP-exposed and CIEO-treated after 1000 µg/L PU NP-exposed groups of wild-type *C. elegans*. (B) Effect of RNAi knockdown of *sod-3*, *ctl-1*, *skn-1*, or *gst-4* on head thrashes in 1000 µg/L PU NP-exposed group and CIEO-treated after 1000 µg/L PU NP-exposed group of wild-type *C. elegans*. (C) Effect of RNAi knockdown of *sod-3*, *ctl-1*, *skn-1*, or *gst-4* on body bends in 1000 µg/L PU NP-exposed group and CIEO-treated after 10 µg/L PU NP-exposed group of wild-type *C. elegans*. Prolonged exposure to PU NPs was performed from L1-stage larvae to adult day 1. Bars indicate means $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$ vs. control (if not specially indicated).

Using RNAi technology and locomotion behavior as endpoints, we analyzed phenotypes after the RNAi knockdown of *sod-3*, *ctl-1*, *skn-1*, or *gst-4*. Compared with the wild-type exposure group, RNAi knockdown of *sod-3*, *ctl-1*, *skn-1*, or *gst-4* induced significant susceptibility to 1000 µg/L of PU NPs (Figure 6B,C). Notably, in the context of CIEO treatment, RNAi knockdown of *sod-3*, *ctl-1*, or *gst-4* did not elicit significant susceptibility ($p < 0.01$), whereas knockdown of *skn-1* still resulted in pronounced susceptibility ($p < 0.01$) (Figure 6B,C). These findings indicate that CIEO treatment mitigates toxic responses to 1000 µg/L of PU NPs in *C. elegans* via transcriptional upregulation of *skn-1*.

3.7. Effects of CIEO Treatment on the Expression of SKN-1::GFP After PU NP Exposure and Molecular Docking of CID with SKN-1/Nrf2

Using the transgenic *C. elegans* strain LD1, we subsequently investigated the impact of CIEO treatment on the expression of SKN-1::GFP following exposure to PU NPs. Our results revealed that in the 10 µg/L PU NP exposure group, CIEO treatment further enhanced the translational expression of SKN-1/Nrf2 (Figures 7A and S2). In the 1000 µg/L PU NP exposure group, CIEO treatment ameliorated the reduction in SKN-1/Nrf2 expression observed compared with the 10 µg/L exposure group and promoted the translational expression of SKN-1/Nrf2 (Figures 7A and S2). These findings are in accordance with prior observations of *skn-1* transcriptional expression levels, indicating that SKN-1/Nrf2 plays a critical role in the regulatory mechanism by which CIEO treatment alleviates toxicity induced by PU NP exposure in *C. elegans*.

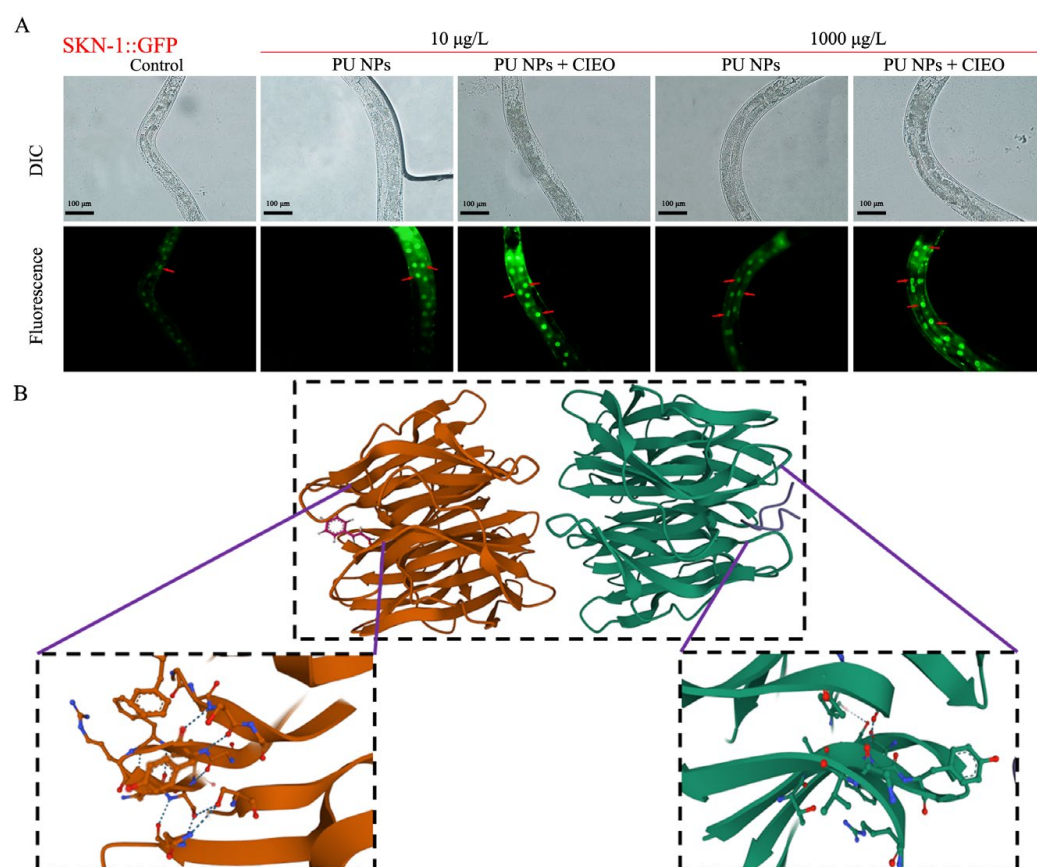


Figure 7. Effects of CIEO treatment on the expression of SKN-1::GFP after PU NP exposure and molecular docking of CID with SKN-1/Nrf2. (A) Effects of CIEO treatment on the expression of SKN-1::GFP. Arrows indicate the nuclear translocation signal. (B) Molecular docking visualization of CID with SKN-1/Nrf2.

To assess the binding affinity of CID—the primary component of CIEO—for SKN-1/Nrf2, molecular docking analysis was performed using AutoDock Vina v.1.2.2. This analysis yielded binding poses, interaction profiles, and corresponding binding energies for CID-SKN-1/Nrf2 complexes (Figure 7B). Our results showed that CID engages SKN-1/Nrf2 via observable hydrogen bonds and robust electrostatic interactions. Furthermore, CID effectively occupies the hydrophobic pockets of each target, with the top ten binding energies ranging from -4.899 to -4.801 kcal/mol, indicative of highly stable binding interactions.

4. Discussion

The concept of microplastics has emerged as a focal point of scientific inquiry since its introduction in 2004 [8]. These entities do not represent the terminal form of plastic waste in ecosystems, as environmental processes facilitate their degradation into nanoplastics [6]. In recent years, the scientific community has extensively deployed sentinel organisms—*Daphnia magna*, zebrafish, and *C. elegans*—to probe the environmental and health hazards, together with their underlying toxicological mechanisms, elicited by micro(nano)plastics [16,48,49]. Integrating environmental survey data with computational simulations, it is reported that ERCs of micro(nano)plastics generally fall within the $\mu\text{g/L}$ range [11,12]. However, most current studies employ exposure concentrations in the mg/L range, substantially higher than ambient exposure levels experienced by environmental organisms [12,13]. Furthermore, while research has predominantly focused on PS as the model micro(nano)plastic, toxicological assessments of other plastic types, such as PU, remain critically underrepresented.

In this study, we employed a prolonged exposure protocol in *C. elegans*, from the L1 larval stage to adulthood, to evaluate the toxicity of PU NPs. Exposure to $1000 \mu\text{g/L}$ of PU NPs significantly increased lethality (Figure 2A). At $10 \mu\text{g/L}$, brood size, head thrashes, and body bends were reduced (Figure 2B–D), while $100 \mu\text{g/L}$ shortened lifespan (Figure 2E). Locomotion and brood size were more sensitive than lethality or lifespan, consistent with prior findings [50]. Oxidative stress plays a dual role in toxicant response: a moderate ROS level can be protective, but ROS in excess causes damage [51]. PU NP concentrations of $10 \mu\text{g/L}$ and $1000 \mu\text{g/L}$ increased intracellular ROS by 1.78-fold and 4.21-fold, respectively (Figure 3A), linking ROS overproduction to the lethality observed at high concentrations. Our investigation into the role of oxidative stress at 10 and $1000 \mu\text{g/L}$ of PU NPs was motivated by two observations: First, $10 \mu\text{g/L}$ —the lowest dose inducing detectable toxicity—caused reduced brood size and locomotion in *C. elegans* (Figure 2B–D). Second, $1000 \mu\text{g/L}$ significantly increased mortality, suggesting the exceedance of tolerance limits (Figure 2A). Exposure to $10 \mu\text{g/L}$ of PU NPs upregulated SKN-1/Nrf2 and GST-4 expression by 4.5- and 2.6-fold, respectively, with nuclear translocation of SKN-1 (Figure 3B,C). In contrast, the $1000 \mu\text{g/L}$ group showed only 2-fold and 1.4-fold upregulation—a reduction of 55.6% and 46.2% compared to the low-dose group—indicating overwhelmed antioxidant capacity. Transcriptional analysis further revealed the upregulation of *sod-3*, *ctl-1*, *skn-1*, and *gst-4* in both groups, but marked downregulation at $1000 \mu\text{g/L}$ relative to $10 \mu\text{g/L}$ (Figure 4A). RNAi knockdown of these genes increased susceptibility to PU NPs (Figure 4B,C). The integrated data demonstrate concentration-dependent dual roles: these antioxidants mediate protection at $10 \mu\text{g/L}$ but contribute to toxicity at $1000 \mu\text{g/L}$. ROS levels increased moderately at $10 \mu\text{g/L}$, inducing compensatory upregulation of oxidase systems. At $1000 \mu\text{g/L}$, excessive ROS led to decompensation, characterized by reduced antioxidant gene expression despite remaining above controls. Thus, moderate SKN-1/Nrf2 and GST-4 upregulation reflects adaptive protection, while excessive ROS triggers decompensatory toxicity [52,53].

CID, the primary active component of CIEO, mediates its antioxidant effects [25,54–56]. In this study, wild-type *C. elegans* were exposed to PU NPs (at 10 or $1000 \mu\text{g/L}$) with

simultaneous CIEO treatment. Through gene expression and RNAi phenotypic analysis, we elucidated CIEO's antioxidant mechanism. At 10 µg/L of PU NPs, CIEO co-treatment upregulated *sod-2*, *sod-5*, *ctl-2*, and *skn-1*, enhancing protective responses. Conversely, at 1000 µg/L, it attenuated *sod-3*, *ctl-1*, and *gst-4* expression while promoting *skn-1*, thereby alleviating toxicity—a finding confirmed by RNAi (Figures 5 and 6). SKN-1/Nrf2, a key regulator of oxidative stress homeostasis in *C. elegans*, serves as a central mediator in this context [33]. The transcription factor SKN-1/Nrf2, a central oxidative stress regulator, was upregulated by CIEO at both concentrations but mediated distinct protective mechanisms: bolstering defenses at low concentration and mitigating toxicity at high concentration (Figures 5A,B, 6 and 7A). This study is the first to investigate PU NP toxicity under environmental conditions in *C. elegans*. CIEO alleviates toxicity by enhancing protective pathways and suppressing toxic cascades, demonstrating its therapeutic potential against environmental toxicants.

The current study has limitations in the following areas: (I) Although *C. elegans* has many advantages as a model organism in toxicology research, its lack of complex organ systems in practical applications limits the study of toxicity mechanisms involving organ-specific metabolism, immunity, or neural behavior. Extrapolating the results to mammals (including humans) carries risks. While its short life cycle facilitates research, it also makes it difficult to simulate the long-term cumulative effects of chronic low-dose exposure over the human lifespan. (II) Although a detailed characterization of PU NPs was performed in this study, once PU NPs enter biological or environmental media, they undergo dynamic and complex transformations. For example, they may form a protein corona, altering their surface properties and biological recognition [6]; they may also dissolve, aggregate, or transform, causing the actual exposure dose to differ from the initially characterized state [6]. (III) Molecular docking is a valuable starting point and auxiliary tool in toxicology research. It can provide hypotheses on molecular interactions, guide experimental design, and accelerate screening. However, it is essentially a computational prediction based on simplified physicochemical models. It cannot simulate complete biological systems, and its predictions carry uncertainties.

5. Conclusions

In this study, we leveraged the in vivo evaluation system of *C. elegans*, with a prolonged exposure paradigm, to systematically characterize the toxicity of PU NPs at µg/L concentrations. Exposure to ≥10 µg/L of PU NPs elicited significant toxicity in *C. elegans*, while ≥1000 µg/L exposure induced lethality. Analysis of ROS and spatiotemporal dynamics of key antioxidant enzymes/molecules revealed that 10 µg/L PU NP exposure upregulated protective intracellular antioxidant enzymes. In contrast, 1000 µg/L exposure overwhelmed defense mechanisms, leading to toxic downregulation. CIEO treatment was found to potentiate protective responses at 10 µg/L of PU NPs by promoting expression of diverse antioxidant enzymes and SKN-1/Nrf2, while mitigating toxic responses to 1000 µg/L exposure via SKN-1/Nrf2 activation. Molecular docking confirmed strong binding affinity between CID, the primary bioactive component of CIEO, and SKN-1/Nrf2. Our study demonstrated that PU NPs at microgram-per-liter concentrations pose ecological risks. We identified CIEO as a potential antidote against PU NP toxicity, highlighting its therapeutic potential for environmental remediation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nano16040220/s1>, Figure S1. RNAi knockdown efficiency of (A) *sod-2*, (B) *sod-3*, (C) *sod-5*, (D) *clk-1*, (E) *ctl-1*, (F) *ctl-2*, (G) *skn-1*, and (H) *gst-4*. Bars represent means ± SD. ** $p < 0.01$ vs wild-type; Figure S2. Effects of CIEO treatment on the expression of SKN-1::GFP after PU NP exposure. Prolonged exposure to PU NPs was performed from L1-stage

larvae to adult day 1. Bars indicate means $\pm$ SD. ** $p < 0.01$ vs control; Figure S3. Effects of CIEO treatment on the alteration of mortality rate and locomotion behavior. Effects of CIEO treatment on the alteration of (A) mortality rate, (B) head thrashes, and (C) body bends. Prolonged exposure to CIEO was performed from L1-stage larvae to adult day 1. Bars indicate means $\pm$ SD. * $p < 0.05$ vs control; Figure S4. Distribution of hydrous particle diameter PU NPs (1 mg/L) in M9 buffer; Table S1. Information for *C. elegans* strains used in present study; Table S2. Primer information for qRT-PCR; Table S3. Information for genes involved in oxidative stress in *C. elegans*; Table S4. Primer information for RNA interference.

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Conflicts of Interest: Author Xingmin Liu was employed by the company Centre Testing International of Qingdao Co., Ltd. The remaining 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.

Abbreviations

The following abbreviations are used in this manuscript:

ERCs	environmentally relevant concentrations
PU NPs	polyurethane nanoplastics
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
ROS	reactive oxygen species
CIEO	cinnamon essential oil
CID	cinnamaldehyde
EPS	expanded polystyrene foam
PU	polyurethane
PP	polypropylene
PA	polyamide
PVC	polyvinyl chloride
PS	polystyrene
RNAi	RNA interference
DLS	dynamic light scattering
FTIR	Fourier transform infrared spectroscopy
CGC	Caenorhabditis Genetics Center
NGM	Nematode Growth Medium
<i>E. coli</i>	<i>Escherichia coli</i>
DCFH-DA	2',7'-dichlorofluorescein diacetate
qRT-PCR	quantitative reverse transcription polymerase chain reaction
dsRNA	double-stranded RNA
DMSO	dimethyl sulfoxide

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