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Amino modification enhances reproductive toxicity of nanopolystyrene on gonad development and reproductive capacity in nematode *Caenorhabditis elegans*☆

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ABSTRACT

Although amino modified nanopolystyrene could cause toxicity on environmental organisms, the effect of amino modification on nanopolystyrene toxicity is still largely unclear. We here employed *Caenorhabditis elegans* as an animal model to compare the effects between pristine and amino modified nanopolystyrene particles in inducing reproductive toxicity. Nanopolystyrene (35 nm) could cause the damage on gonad development as indicated by the endpoints of number of total germline cells, length of gonad arm, and relative area of gonad arm. Nanopolystyrene exposure also reduced the reproductive capacity as reflected by the endpoints of brood size and number of fertilized eggs in uterus. Moreover, amino modification enhanced nanopolystyrene toxicity on both the gonad development and the reproductive capacity. Additionally, induction of germline apoptosis and formation of germline DNA damage contributed to the enhancement of nanopolystyrene toxicity in reducing reproductive capacity by amino modification. Our results highlight the potential environmental risk of amino modified nanopolystyrene in inducing reproductive toxicity on gonad development and reproductive capacity of environmental organisms.

1. Introduction

With the increasing production and utilization, a large mount of plastic waste has been generated and released into the environment (Geyer et al., 2017). The microplastics (≤5 mm) can be detected in water body, soil, air, and even our table salt (Yang et al., 2015; Cai et al., 2017; Song et al., 2018; Zhang and Liu, 2018). Some studies have demonstrated that the microplastics could potentially induce the enrichment in a large number of environmental toxicants (e.g., persistent organic pollutants and heavy metals) on their surface (Mato et al., 2001; Katsnelson, 2015). In the environment, the microplastics can be gradually degraded into nanoplastic particles after certain physical, chemical, or biological processes (Mattsson et al., 2015; da Costa et al., 2016).

Polystyrene is one of the most frequently detected plastics in the environment. Nanopolystyrene has been widely used in food containers and stuff package (Abdallah et al., 2018). For example, the nanopolystyrene particles have been frequently used in the production of personal care products. Surface chemical modifications could alter the physicochemical properties and affect the toxicity of nanomaterials (Wang, 2018). Some of the recent studies have suggested that the amino modified nanopolystyrene could alter immune capacity in Mytilus hemocytes, and even induce the development defects in sea urchin embryos (Della Torre et al., 2014; Canesi et al., 2016). However, the effect of amino surface modification on toxicity induction of nanopolystyrene is still largely unclear.

Caenorhabditis elegans is a wonderful animal model for toxicity assessment of various environmental toxicants, such as heavy metals and nanomaterials (Leung et al., 2008; Wang, 2018; Ding et al., 2018; Liu et al., 2019; Moon et al., 2019; Zhao et al., 2019; Lenz et al., 2019). *C. elegans* has also been used for the toxicological evaluation of microplastics, including the nanopolystyrene (Lei et al., 2018a; Lei et al., 2018b; Qu et al., 2018; Hanna et al., 2018;

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Kim et al., 2019; Shao et al., 2019; Qu et al., 2019a). In nematodes, multiple sublethal endpoints, such as brood size and germline apoptosis, can be employed to assess the reproductive toxicity of certain toxicants (Wang, 2019). Our previous study has indicated that exposure to nanopolystyrene (100 nm) at concentrations $\geq$ 10 μg/L could cause several aspects of toxicity on nematodes, including the reduction in brood size (Zhao et al., 2017), which suggests the potential of nanopolystyrene in inducing the reproductive toxicity.

Both pristine and amino modified nanopolystyrene are commonly used plastic particles (Loos et al., 2014). The normally examined pristine nanopolystyrene has the property of negative charge. Surface amino modification can introduce the formation of positive charge on the surface of nanopolystyrene. Gonad is an indispensable reproductive organ (Pazdernik and Schedl, 2013), and germline loss or damage in the gonad will lead to the dysfunctional reproduction in *C. elegans* (Kodoyianni et al., 1992). To determine the effect of amino surface modification on toxicity of nanopolystyrene, we here employed *C. elegans* as an animal model to compare the effects between pristine and amino modified nanopolystyrene particles on induction of reproductive toxicity. Our results suggest that the amino surface modification potentially enhanced the reproductive toxicity of nanopolystyrene (35 nm) on gonad development and reproductive capacity in nematodes. Our data highlights the environmental risk of amino modified nanopolystyrene on environmental organisms. Moreover, our data suggested that the toxicity of nanopolystyrene on environmental organisms may be charge-dependent in the environment.

2. Materials and methods

2.1. Properties of pristine and amino modified nanopolystyrene particles

Both pristine and amino modified nanopolystyrene particles (35 nm) were the gifts from Dr. Yanzheng Yuan's lab (Shandong University, China). Prolonged exposure from L1-larvae to adult day 1 to 100 nm pristine nanopolystyrene ($\geq$10 μg/L) could cause the toxicity in nematodes (Zhao et al., 2018). It was reported that the predicted environmental concentrations of nanoplastics (100 nm) are in the range of $\leq$1 μg/L (Lenz et al., 2016; Al-Sid-Cheikh et al., 2018). The predicted environmental concentrations of nanoplastics will likely further increase as the nanoplastic sizes are decreased, and the environmental concentrations of nanoplastics (50 nm) were predicted to be $\leq$ 15 μg/L (Lenz et al., 2016; Al-Sid-Cheikh et al., 2018). The concentrations of 1, 10, 100, and 1000 μg/L were selected as the working concentrations of nanopolystyrene particles. Different concentrations of nanopolystyrene solutions used were prepared by diluting the stock solutions with liquid K-medium. Physicochemical properties of the used nanopolystyrene particles were examined by transmission electron microscopy (TEM, JEOL Ltd., Japan), Raman spectroscopy (Renishaw Invia Plus laser Raman spectrometer, Renishaw, UK), Fourier transform infrared spectroscopy (FTIR) spectrum (Avatar 370, Thermo Nicolet, USA), dynamic light scattering (DLS), and Zeta potential (Zetasizer Nano-ZS90, Malvern Instruments Ltd., UK).

2.2. Strains maintenance and exposure

The following *C. elegans* strains were used: wild-type N2 and transgenic strain of WS1433/[HUS-1::GFP]. Animals were maintained normally on NGM plates as described (Brenner, 1974). *Escherichia coli* OP50 was used as the food source on NGM plates. L1-larvae were obtained by treating gravid hermaphrodites with bleaching solution (2% HOCl, 0.45 M NaOH) to release the eggs for the aim of synchronous development.

Exposure to pristine or amino modified nanopolystyrene particles was carried out from L1-larvae to adult day 1. During the exposure, the nanopolystyrene solutions were refreshed daily. No obvious aggregation was observed for the nanopolystyrene solutions at the working concentrations for at least two days. Exposure to nanopolystyrene particles was performed in liquid solutions at 20 °C with the addition of food OP50 (~4 x 10^6 colony-forming units (CFUs)) (Shao et al., 2019). After the exposure, both gonad development and reproductive capacity were evaluated.

2.3. Analysis of gonad development

Endpoints of number of total germline cells, length of gonad arm, and relative area of gonad arm were employed to reflect the gonad development (Quevedo et al., 2007; Tijsterman et al., 2002). The 4′,6-diamidino-2-phenylindole (DAPI) is a fluorescent dye that can bind strongly to DNA (Lant and Derry, 2014). Animals were treated with 4% polyformaldehyde for 10-min. After drying, 0.1% polyethylene glycol octyl phenyl ether was added to treat for 5-min, followed with the addition of PBSB (PBS buffer containing 0.5% bovine serum albumin) to treat for 30-min. The animals were further stained with 0.2 μg/mL DAPI dye solution for 30-min without light. After washing with PBSB for three time (each time for 15-min), the gonad development was assessed under a fluorescence microscopy. Fifty nematodes were examined for each treatment.

2.4. Analysis of reproductive capacity

Brood size and number of fertilized eggs in uterus were used to evaluate the reproductive capacity (Qu et al., 2019b; Zhao et al., 2016). After the exposure, the nematodes were rinsed using M9 buffer for three times, and then transferred to empty NGM plates. Totally 30 animals were pick randomly to new NGM plates with a small lawn of fresh OP50. The NGM plates were replaced daily until no eggs were laid. The brood size was counted as the offspring at all stages beyond the egg. The brood size was counted under an optical microscope. To analyze the number of fertilized eggs in the uterus, the differential interference contrast (DIC) microscope was used to capture the images, and the number of fertilized eggs was counted. Thirty nematodes were examined per treatment.

2.5. Germline apoptosis assay

Acridine orange (AO) can penetrate the whole cell membrane and be inserted into nucleus DNA to emit bright green fluorescence (Lant and Derry, 2014). Nematodes were treated with 25 mg/mL AO for 1-h at 20 °C without light. After the AO staining, the nematodes were transferred to NGM plate with OP50 to repel the excessive AO dye in intestine. Germline apoptosis was detected under an epi-fluorescence microscopy (green fluorescence channel with excited light 395 nm and emitted light 509 nm). Fifty nematodes were examined for each treatment.

2.6. DNA damage assay

DNA damage can be detected using transgenic strain WS1433 carrying HUS-1::GFP fusion protein (Hofmann et al., 2002). In nematodes, the HUS-1::GFP signals are localized in the nuclei of proliferating and meiotic germ cells (Hofmann et al., 2002). After exposure, WS1433 worms were rinsed using M9 buffer for three times, and 50 worms were picked randomly to an empty NGM plate for the detection. HUS-1::GFP foci were quantified by counting the

number of bright foci present in the middle/late pachytene germline cells under an epifluorescence microscopy as described (green fluorescence channel with excited light 395 nm and emitted light 509 nm) (Zhao et al., 2016). Fifty nematodes were examined for each treatment.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

After the exposure, the nematodes were washed with M9 buffer at least 3 times in enzymatic-free centrifugal tubes. Total RNA of nematodes in each group after treatment was isolated using Trizol (Sigma-Aldrich, St. Louis, MO, USA). The supernatant was discarded, and 1 mL Trizol (Sigma Aldrich, St. Louis, MO, USA) was added to each EP tube. RNA purity and concentration were guaranteed by the ratio of OD260/280 in the spectrophotometer. Mastercycler gradient PCR system (Eppendorf, USA) was employed to synthesize cDNA with reverse transcriptase reaction. Expression levels of genes required for the control of germline apoptosis and DNA damage were determined by StepOnePlus™ real-time PCR system (Applied Biosystems, Carlsbad, USA) with the SYBR Green qRT-PCR master mix (TOYOBO, Japan). Consistently expressed gene *tba-1* encoding alpha-tubulin protein was selected as reference gene, and comparative cycle threshold methods were used. Three replicates were performed in all reactions. Primers information for qRT-PCR is shown in Table S1.

2.8. RNA interference (RNAi) assay

The prepared L1-larvae were fed with *E. coli* HT115 expressing double-stranded RNA corresponding to certain gene(s) as described (Qu et al., 2019c). After the development into gravid, the adults were transferred onto a fresh plate to obtain the second generation for nanopolystyrene exposure. Before growing onto NGM agar plates, HT115 was transferred into LA broth containing isopropyl 1-thio-β-d-galactopyranoside (IPTG, 5 mM) and ampicillin (100 μg/L). HT115 bacteria harboring empty vector L4440 was used as a negative control. RNAi efficiency was confirmed by qRT-PCR (data not shown).

2.9. Developmental delay assay

The method was performed as described (Page et al., 2012). After the exposure, the exposed nematodes were kept at 20 °C until the control was mostly adults (~2 days). The number of adults and the total number of animals were scored. The percentage adult was calculated from these values (adults/total). Three replicates were performed.

2.10. Statistical analysis

Statistical analysis was analyzed by SPSS 12.0 (SPSS Inc., Chicago, USA). Parameters used were continuous variables, and Agostino *D* test was used to check the normality before the parameters statistics. Differences between groups were tested using one-way analysis of variance (ANOVA), and the differences were checked using a Post Hoc multiple comparisons. For the multiple factor comparison, two-way ANOVA analysis was performed. Probability level of 0.01 was considered statistically significant.

3. Results

3.1. Physicochemical properties of pristine and amino modified nanopolystyrene particles

The TEM images of both pristine and amino modified nanopolystyrene particles were shown in Fig. 1A. Based on the dynamic light scattering (DLS) analysis, the size of pristine nanopolystyrene particles was 34.9 ± 2.7 nm, and the size of amino modified nanopolystyrene particles was 35.5 ± 3.1 nm. The Raman spectroscopy analysis indicated that both pristine and amino modified nanopolystyrene particles showed the peaks at 989.88 cm^{-1} and 1006.03 cm^{-1} (breathing vibration of benzene ring), at 1039.11 cm^{-1} and 1034.00 cm^{-1} (symmetric extension vibration of carbon atoms in benzene ring), at 1191.75 cm^{-1} and 1200.67 cm^{-1} (stretching vibration of carbon atoms between benzene ring and polyethylene group), at 1436.65 cm^{-1} and 1451.24 cm^{-1} (asymmetric bending vibration of carbon atoms and hydrogen atoms), and at 1607.14 cm^{-1}, 1633.33 cm^{-1} and 1601.32 (asymmetric stretching vibration of benzene ring carbon atoms) (Fig. 1B). Additionally, different from the pristine nanopolystyrene, we also detected a stretching vibration between nitrogen atoms at the peak of 1385.21 cm^{-1} and a stretching vibration between carbon and nitrogen atoms at the peak of 1175.03 cm^{-1} in amino modified nanopolystyrene (Fig. 1B). The FTIR analysis demonstrated that the peaks of symmetrical bending vibration (δ_s), symmetric stretching vibration (σ_s), and antisymmetric stretching vibration (σ_{as}) of methylene (CH_2) appeared at 1452.13 cm^{-1}, 2862.37 cm^{-1}, and 2921.14 cm^{-1} for pristine nanopolystyrene, and appeared at 1452.13 cm^{-1}, 2853.16 cm^{-1} and 2922.12 cm^{-1} for amino modified nanopolystyrene (Fig. 1C). The peak (3026.31 cm^{-1}) for pristine nanopolystyrene or peak (3026.21 cm^{-1}) for amino modified nanopolystyrene attributed to the stretching vibration (σ) of unsaturated hydrocarbon group on benzene ring (=CH); the peaks (1492.15 cm^{-1} and 1613.01 cm^{-1}) for pristine nanopolystyrene or the peaks (1492.63 cm^{-1} and 1600.63 cm^{-1}) for amino modified nanopolystyrene were caused by benzene ring skeleton vibration (δ) (C═C); and the peaks (699.55 and 759.33 cm^{-1}) for pristine nanopolystyrene and the peaks (697.14 cm^{-1} and 756.44 cm^{-1}) for amino modified nanopolystyrene were because of the out-of-plane bending vibration (δ) of unsaturated hydrocarbon groups on benzene ring (=C—H) (Fig. 1C). Different from the pristine nanopolystyrene, we also detected the absorption peaks of C—N bonds appeared at 1060.21 cm^{-1} and 1360.21 cm^{-1}, absorption peak of —C═C- bonds appeared at 1693.21 cm^{-1}, and absorption peak of = N—H bonds appeared at 3442.79 cm^{-1} in amino modified nanopolystyrene (Fig. 1C). The zeta potential of pristine nanopolystyrene was −19.8 ± 1.75 mV, and the potential of amino modified nanopolystyrene was +24 ± 0.54 mV.

3.2. Comparison of reproductive toxicity between pristine and amino modified nanopolystyrene particles on gonad development

In this study, the dye of DAPI was used to label the germline in the gonad of nematodes exposed to nanopolystyrene (Fig. 2A). After the exposure, the pristine nanopolystyrene (≥10 μg/L) significantly reduced the number of total germline cells in the gonad, and 1000 μg/L pristine nanopolystyrene could further significantly decrease both the length of gonad arm and the relative area of gonad arm (Fig. 2B and C). Different from these, the amino modified nanopolystyrene (≥1 μg/L) caused the significant reduction in the number of total germline cells in the gonad, and the amino modified nanopolystyrene (≥100 μg/L) resulted in the significant decrease in both the length of gonad arm and the relative area of gonad arm (Fig. 2B and C). Moreover, amino modified nanopolystyrene (10—1000 μg/L) caused more severe reduction in the number of total germline cells in the gonad than pristine nanopolystyrene (10—1000 μg/L), and amino modified nanopolystyrene (1000 μg/L) resulted in more severe decrease in length of gonad arm or relative area of gonad arm than pristine

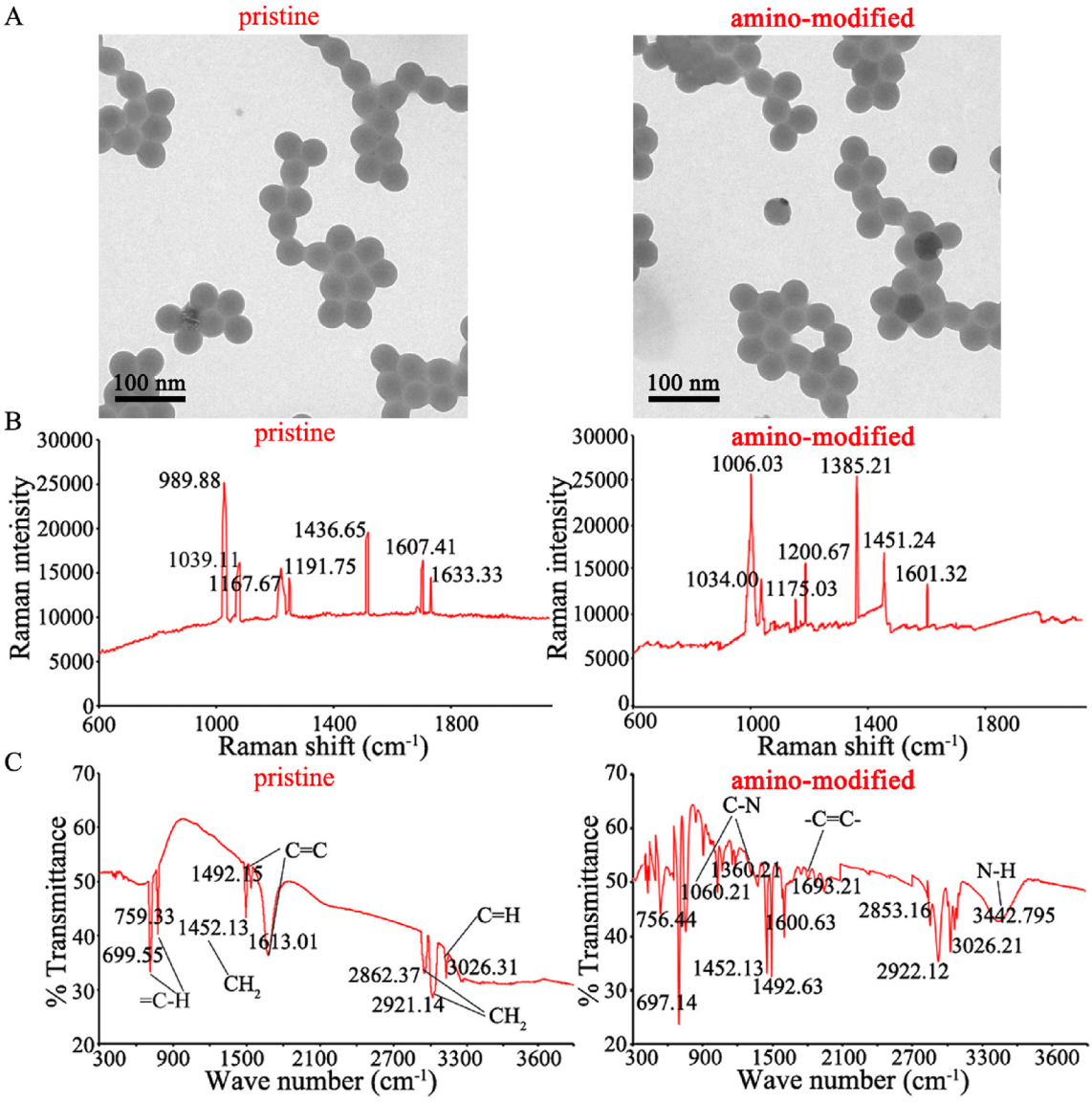

Fig. 1. Properties of pristine and amino modified nanopolystyrene particles. (A) TEM images of pristine and amino modified nanopolystyrene particles (10 mg/L). (B) Raman spectroscopy of pristine and amino modified nanopolystyrene particles. (C) FTIR spectrum of pristine and amino modified nanopolystyrene particles.

nanopolystyrene (1000 μg/L) (Fig. 2B and C).

3.3. Comparison of reproductive toxicity between pristine and amino modified nanopolystyrene particles on reproductive capacity

Besides the gonad development, we also investigated the effects of nanopolystyrene particles on reproductive capacity as reflected by the endpoints of brood size and number of fertilized eggs in uterus. After the exposure, the pristine nanopolystyrene particles (≥10 μg/L) significantly reduced both the brood size and the number of fertilized eggs in uterus (Fig. 3). In contrast, the amino modified particles (≥1 μg/L) could cause the significant reduction in both the brood size and the number of fertilized eggs in uterus (Fig. 3). Moreover, amino modified nanopolystyrene (10–1000 μg/L) induced a more severe decrease in both the brood size and the number of fertilized eggs in uterus than pristine nanopolystyrene (10–1000 μg/L) (Fig. 3).

3.4. Comparison of reproductive toxicity between pristine and amino modified nanopolystyrene particles in inducing germline apoptosis

To further understanding the underlying mechanisms for the observed reduction in reproductive capacity by nanopolystyrene, we next compared the reproductive toxicity between pristine and amino modified nanopolystyrene particles in inducing germline apoptosis. After the exposure, the pristine nanopolystyrene (≥10 μg/L) induced the obvious germline apoptosis (Fig. 4A and B). Different from this, we observed the noticeable germline apoptosis in amino modified nanopolystyrene at concentrations ≥1 μg/L (Fig. 4A and B). Meanwhile, amino modified nanopolystyrene (10–1000 μg/L) induced the more severe induction of germline apoptosis than pristine nanopolystyrene (10–1000 μg/L) (Fig. 4A and B).

CED-3, CED-4, and CED-9 constitute the core molecular basis for apoptosis in nematodes (Lettre and Hengartner, 2006). Exposure to the pristine nanopolystyrene (10 μg/L) only significantly increased

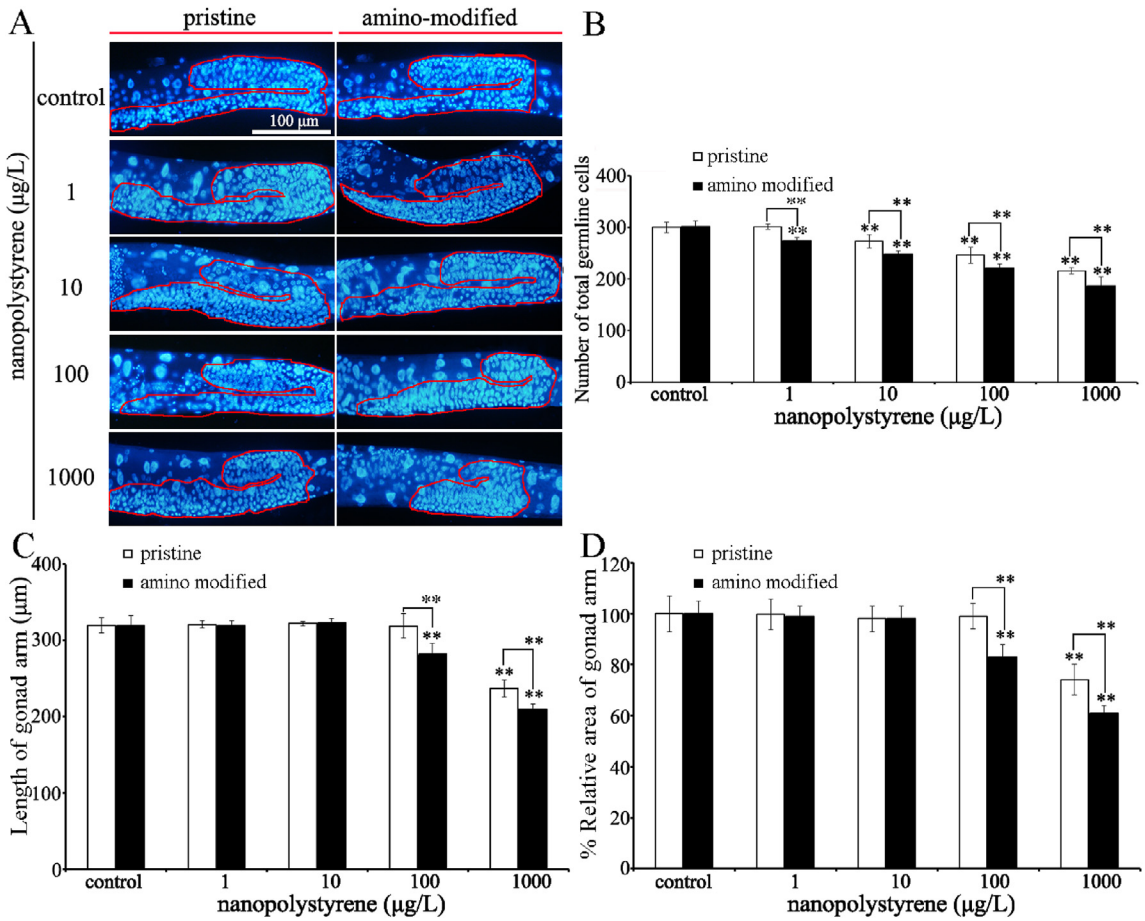

Fig. 2. Amino modification enhanced the reproductive toxicity on gonad development in nematodes. (A) DAPI staining results. N = 50. (B) Comparison of effect of pristine and amino modified nanopolystyrene particles on the number of germline cells. N = 50. (C) Comparison of effect of pristine and amino modified nanopolystyrene particles on the length of gonad arm. N = 50. (D) Comparison of effect of pristine and amino modified nanopolystyrene particles on the relative area of gonad arm. N = 50. Exposure to nanopolystyrenes was performed from L1-larvae to adult day-1. Bars represent means ± SD. $^{**}P < 0.01$ *vs* control (if not specially indicated).

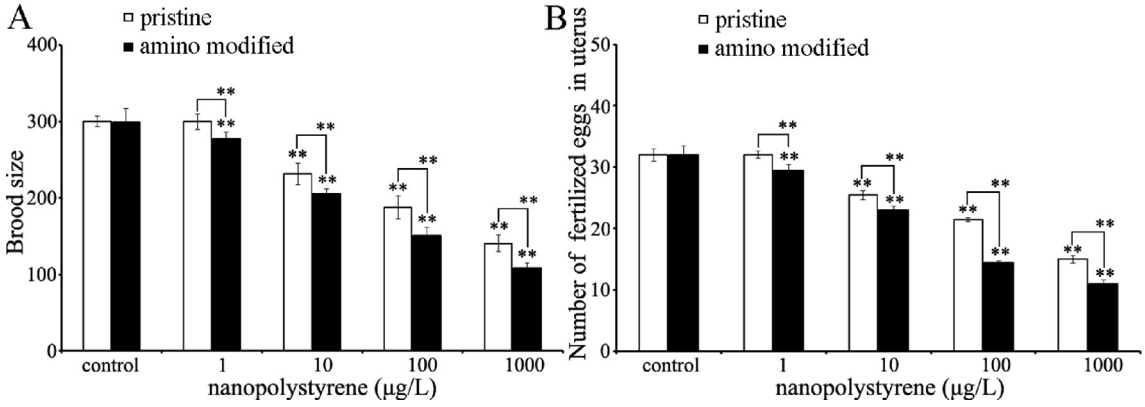

Fig. 3. Amino modification enhanced the reproductive toxicity in reducing reproductive capacity in nematodes. (A) Comparison of effect of pristine and amino modified nanopolystyrene particles on the brood size. N = 30. (B) Comparison of effect of pristine and amino modified nanopolystyrene particles on the number of fertilized eggs in uterus. N = 30. Exposure to nanopolystyrenes was performed from L1-larvae to adult day-1. Bars represent means ± SD. $^{**}P < 0.01$ *vs* control (if not specially indicated).

the *ced-3* expression and decreased the *ced-9* expression (Fig. 4C). In contrast, exposure to amino modified pristine nanopolystyrene (10 μg/L) not only significantly decreased the *ced-9* expression, but also significantly increased expressions of both *ced-3* and *ced-4* (Fig. 4C). Additionally, amino modified nanopolystyrene caused the more severe increase in *ced-3* expression and decrease in *ced-9* expression compared with those in pristine nanopolystyrene exposed nematodes (Fig. 4C). Moreover, we found that RNAi knockdown of *ced-3* or *ced-4* suppressed the germline apoptosis induced by nanopolystyrene, whereas RNAi knockdown of *ced-9* enhanced the germline apoptosis induced by nanopolystyrene (Fig. S1A), which further suggests the involvement of CED-3, CED-4,

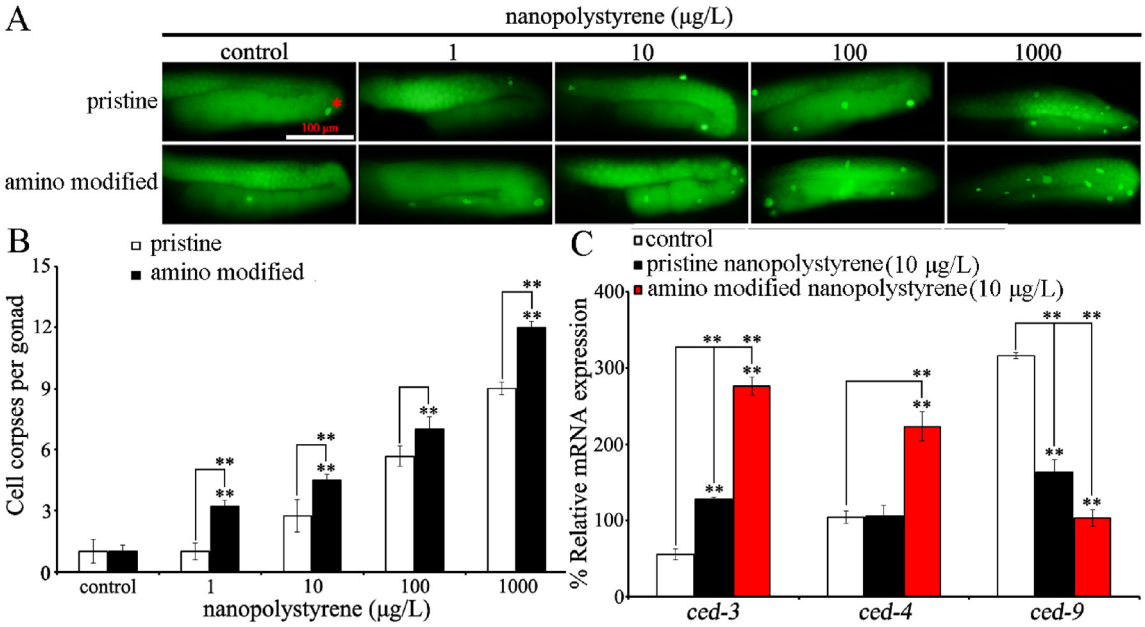

Fig. 4. Amino modification enhanced the reproductive toxicity in inducing germline apoptosis in nematodes. (A) Images showing the induction of germline apoptosis. Asterisk indicates the germline apoptosis signal. Exposure concentrations were 1–1000 μg/L. N = 50. (B) Comparison of effect of pristine and amino modified nanopolystyrene particles in inducing germline apoptosis. Exposure concentrations were 1–1000 μg/L. N = 50. (C) Comparison of effect of pristine and amino modified nanopolystyrene particles in affecting expressions of genes required for the control of germline apoptosis. Exposure concentration was 10 μg/L. Exposure to nanopolystyrenes was performed from L1-larvae to adult day-1. Bars represent means ± SD. $^{**}P < 0.01$ *vs* control (if not specially indicated).

and CED-9 in regulating the induction of germline apoptosis in nanopolystyrene exposed nematodes.

3.5. Comparison of reproductive toxicity between pristine and amino modified nanopolystyrene particles in inducing DNA damage

Induction of germline DNA damage is usually also an important contributor to the reduction in reproductive capacity in nematodes exposed to environmental toxicants (Wang, 2019). HUS-1::GFP was used as a marker to reflect the induction of germline DNA damage (Hofmann et al., 2002). HUS-1, ortholog of Hus1, is a DNA damage checkpoint protein in nematodes (Hofmann et al., 2002). After the exposure, the pristine nanopolystyrene (≥10 μg/L) could induce the obvious germline DNA damage (Fig. 5A and B). Different from this, we detected the noticeable germline DNA damage signals in amino modified nanopolystyrene (≥1 μg/L) exposed nematodes (Fig. 5A and B). Moreover, amino modified nanopolystyrene (10–1000 μg/L) could cause more severe induction of germline DNA damage than pristine nanopolystyrene (10–1000 μg/L) (Fig. 5A and B).

CLK-2, CEP-1, and EGL-1, together with HUS-1, constitute the important molecular basis for DNA damage in nematodes (Kamath et al., 2001; Hofmann et al., 2002; O'Neil and Rose, 2006; Lettre and Hengartner, 2006). In nematodes, CEP-1, an ortholog of human tumor suppressor p53, promotes the DNA damage-induced germline apoptosis by affecting the function of EGL-1, which suggests that the signaling cascade of HUS-1/CLK-2-CEP-1-EGL-1 acts upstream of CED-9 and CED-4-CED-3 to regulate the germline apoptosis (Kamath et al., 2001; Zhao et al., 2016). We further compared the effect of pristine and amino modified nanopolystyrene particles on expressions of *clk-2*, *cep-1*, and *egl-1*. Exposure to the pristine nanopolystyrene (10 μg/L) only significantly increased expressions of *cep-1* and *egl-1* (Fig. 5C). In contrast, exposure to amino modified pristine nanopolystyrene (10 μg/L) could significantly increase the expressions of all the examined three genes (Fig. 5C). Meanwhile, amino modified nanopolystyrene exposure led to the more severe increase in expressions of *clk-2*, *cep-1*, and *egl-1* compared with those in pristine nanopolystyrene exposed nematodes (Fig. 5C). Moreover, RNAi knockdown of *clk-2*, *cep-1*, or *egl-1* inhibited the germline DNA damage induced by nanopolystyrene (Fig. S1B), which also suggests the involvement of CLK-2, CEP-1, and EGL-1 in regulating the induction of reproductive toxicity in nanopolystyrene exposed nematodes.

4. Discussion

In nematodes, our previous study has demonstrated that exposure to nanopolystyrene (100 nm) could reduce the brood size (Zhao et al., 2018), suggesting the potential of nanopolystyrene particles in inducing toxicity on reproductive capacity. In this study, the observed reduction in brood size and number of germline cells in uterus in nanopolystyrene (35 nm) exposed nematodes further conforms the reproductive toxicity in decreasing reproductive capacity (Fig. 3). Besides this, we observed that exposure to nanopolystyrene also caused reproductive toxicity on gonad development, such as decrease in number of total germline cells, reduction in length of gonad arm, and reduction in relative area of gonad arm (Fig. 2). Therefore, nanopolystyrene exposure potentially induces reproductive toxicity on both gonad development and reproductive capacity in nematodes.

In this study, nanopolystyrene (35 nm) at concentrations ≥ 10 μg/L could result in significant decrease in number of total germline cells, reduction in brood size, and decrease in number of fertilized eggs in uterus (Figs. 2B and 3). These observations further suggest that long-term exposure to nanoplastics at predicted environmental concentrations can induce the reproductive toxicity on both gonad development and reproductive capacity in organisms. Meanwhile, we also performed the developmental delay experiment, and found that both pristine and amino modified nanopolystyrene particles at concentrations of 1–100 μg/L did not cause the obvious developmental delay (Fig. S2). The moderate

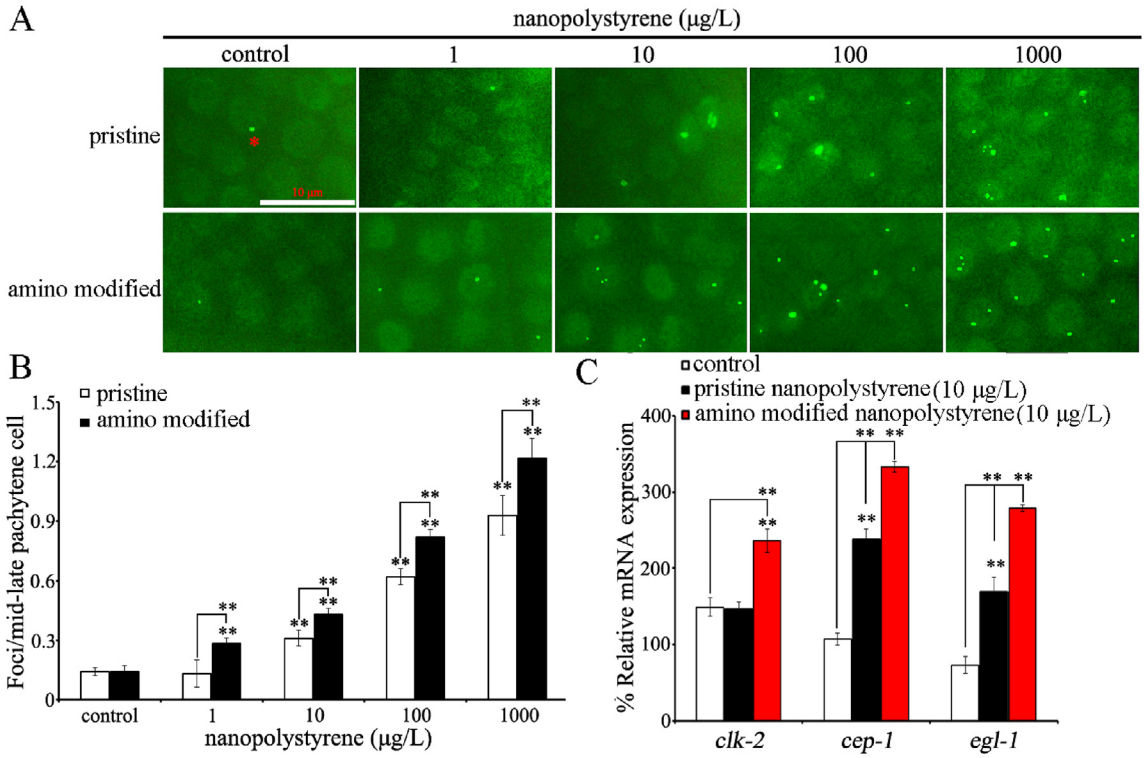

Fig. 5. Amino modification enhanced the reproductive toxicity in inducing DNA damage in nematodes. (A) Images showing the induction of germline DNA damage. Asterisk indicates the germline HUS-1::GFP signal. Exposure concentrations were 1–1000 μg/L. N = 50. (B) Comparison of effect of pristine and amino modified nanopolystyrene particles in inducing germline DNA damage. Exposure concentrations were 1–1000 μg/L. N = 50. (C) Comparison of effect of pristine and amino modified nanopolystyrene particles in affecting expressions of genes required for the control of DNA damage. Exposure concentration was 10 μg/L. Exposure to nanopolystyrenes was performed from L1-larvae to adult day-1. Bars represent means ± SD. $^{**}P < 0.01$ *vs* control (if not specially indicated).

developmental delay could be detected in 1000 μg/L pristine nanopolystyrene exposed nematodes, and amino modification could moderately but significantly strengthen this developmental delay in nematodes exposed to nanopolystyrene (1000 μg/L). These observations imply that exposure to high concentrations (such as 1000 μg/L) of pristine and amino modified nanopolystyrene may induce both the developmental delay and the suppression in reproductive capacity.

Some of previous studies have indicated the potential toxicity of amino modified nanopolystyrene (Della Torre et al., 2014; Canesi et al., 2016). Both the Raman spectroscopy and the FTIR analysis have suggested the amino modification on surface of nanopolystyrene (Fig. 1B and C). The zeta potential analysis further suggested that the formation of positive charge on the surface of nanopolystyrene by amino modification. Using endpoints reflecting the gonad development and the reproductive capacity, we found that amino modification could enhance the reproductive toxicity of nanopolystyrene in nematodes. On the one hand, amino modified nanopolystyrene could induce some aspects of reproductive toxicity at lower concentrations than pristine nanopolystyrene (Figs. 2 and 3). On the other hand, amino modified nanopolystyrene caused more severe reproductive toxicity than pristine nanopolystyrene at the same concentrations (Figs. 2 and 3). Therefore, we provide the evidence to show the potential of amino modification in enhancing reproductive toxicity of nanopolystyrene in nematodes. That is, the toxicity of nanopolystyrene on environmental organisms may be surface charge dependent.

In this study, we further raised two aspects underlying mechanisms for the observed enhancement of nanopolystyrene toxicity in reducing reproductive capacity by amino modification. One of the underlying mechanisms is the induction of gremline apoptosis. Amino modification could induce the more severe induction of germline apoptosis by affecting the expressions of CED-3, CED-4, and CED-9, which are required for the control of apoptosis (Lettre and Hengartner, 2006). CED-9, homolog of cell-death inhibitor Bcl-2, negatively regulates activities of CED-3, a cysteinee aspartate protease, and CED-4, homolog of Apaf1 (Shaham and Horvitz, 1996; Lettre and Hengartner, 2006). Additionally, CED-4 can further stimulate the CED-3 processing during the cell apoptosis (Seshagiri and Miller, 1997). In amino modified nanopolystyrene (10 μg/L) exposed nematodes, the signaling cascade of CED-9-CED-4-CED-3 was affected (Fig. 4C). The pristine nanopolystyrene (10 μg/L) only influenced the signaling cascade of CED-9-CED-3 (Fig. 4C). Another underlying mechanism is the induction of gremline DNA damage. Amino modification also induced the more severe induction of germline DNA damage by affecting the induction of HUS-1, CLK-2, CEP-1, and EGL-1, which are required for the control of DNA damage (Kamath et al., 2001; Hofmann et al., 2002; O'Neil and Rose, 2006; Lettre and Hengartner, 2006). CLK-2, homolog of telomere length-regulating protein Tel2p, acts together with HUS-1 to activate the activity of CEP-1, homolog of human tumor suppressor p53 (Kamath et al., 2001; Stergiou et al., 2007). EGL-1, homolog to the protein containing BH3 domain, is DNA damage checkpoint and cell death activator by acting upstream of core apoptosis signaling pathway (Lettre and Hengartner, 2006; O'Neil and Rose, 2006). In amino modified nanopolystyrene (10 μg/L) exposed nematodes, the signaling cascade of HUS-1/CLK-2-CEP-1-EGL-1 was affected (Fig. 5C). The pristine nanopolystyrene (10 μg/L) only affected the signaling cascade of HUS-1-CEP-1-EGL-1 (Fig. 5C).

Intestine is the primary targeted organ for environmental toxicants in nematodes (Wang, 2019). In contrast, the possible effects of amino modification on the behaviors of nanopolystyrene in

intestinal lumen of nematodes are still largely unknown. Recently, it was reported that the positively charged nanomaterials tended to heteroagglomerate with *E. coli*, implying that the amino modification may affect the effects of nanomaterials on feeding in nematodes (Hanna et al., 2018). Besides this, the correlation of amino modification on zeolitic imidazolate framework-90 (ZIF-90) nanocrystals with the enhancement in perturbation of cell membrane and cell uptake has also been detected (Yen et al., 2016).

Together, we employed the animal model of *C. elegans* to determine the effect of amino modification on reproductive toxicity of nanopolystyrene (35 nm). In nematodes, nanopolystyrene could induce the reproductive toxicity on both gonad development and reproductive capacity. Moreover, we found that the amino modification could effectively enhance these reproductive toxicities in nanopolystyrene exposed nematodes. Induction of germline apoptosis and formation of germline DNA damage may contribute greatly to the observed enhancement in nanopolystyrene toxicity in reducing reproductive capacity by amino modification. Our results imply the potential of amino modified nanopolystyrene in inducing at least two aspects of reproductive toxicity on environmental organisms.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.112978.

References

Abdallah, M.A., Sharkey, M., Berresheim, H., Harrad, S., 2018. Hexabromocyclododecane in polystyrene packaging: a downside of recycling? Chemosphere 199, 612–616.

Al-Sid-Cheikh, M., Rowland, S., Stevenson, K., Rouleau, C., Henry, T.B., Thompson, R.C., 2018. Uptake, whole-body distribution, and depuration of nanoplastics by the scallop pecten maximus at environmentally realistic concentrations. Environ. Sci. Technol. 52, 14480–14486.

Brenner, S., 1974. Genetics of *Caenorhabditis elegans*. Genetics 77, 71–94.

Cai, L., Wang, J., Peng, J., Tan, Z., Zhan, Z., Tan, X., Chen, Q., 2017. Characteristic of microplastics in the atmospheric fallout from Dongguan city, China: preliminary research and first evidence. Environ. Sci. Pollut. Res. Int. 24, 24928–24935.

Canesi, L., Ciacci, C., Fabbri, R., Balbi, T., Salis, A., Damonte, G., Cortese, K., Caratto, V., Monopoli, M.P., Dawson, K., Bergami, E., Corsi, I., 2016. Interactions of cationic polystyrene nanoparticles with marine bivalve hemocytes in a physiological environment: role of soluble hemolymph proteins. Environ. Res. 150, 73–81.

da Costa, J.P., Santos, P.S.M., Duarte, A.C., Rocha-Santos, T., 2016. Nanoplastics in the environment - sources, fates and effects. Sci. Total Environ. 566–567, 15–26.

Della Torre, C., Bergami, E., Salvati, A., Faleri, C., Cirino, P., Dawson, K.A., Corsi, I., 2014. Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of development of sea urchin embryos *Paracentrotus lividus*. Environ. Sci. Technol. 48, 12302–12311.

Ding, X.-C., Rui, Q., Zhao, Y.-L., Shao, H.-M., Yin, Y.-P., Wu, Q.-L., Wang, D.-Y., 2018. Toxicity of graphene oxide in nematodes with deficit in epidermal barrier caused by RNA interference knockdown of *unc-52*. Environ. Sci. Technol. Lett. 5, 622–628.

Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. Sci Adv. 3, e1700782.

Hanna, S.K., Montoro Bustos, A.R., Peterson, A.W., Reipa, V., Scanlan, L.D., Hosbas Coskun, S., Cho, T.J., Johnson, M.E., Hackley, V.A., Nelson, B.C., Winchester, M.R., Elliott, J.T., Petersen, E.J., 2018. Agglomeration of Escherichia coli with positively charged nanoparticles can lead to artifacts in a standard Caenorhabditis elegans toxicity assay. Environ. Sci. Technol. 52, 5968–5978.

Hofmann, E.R., Milstein, S., Boulton, S.J., Ye, M., Hofmann, J.J., Stergiou, L., Gartner, A., Vidal, M., Hengartner, M.O., 2002. *Caenorhabditis elegans* HUS-1 is a DNA damage checkpoint protein required for genome stability and EGL-1-mediated apoptosis. Curr. Biol. 12, 1908–1918.

Kamath, R.K., Martinez-Campos, M., Zipperlen, P., Fraser, A.G., Ahringer, J., 2001. Effectiveness of specific RNA-mediated interference through ingested double stranded RNA in *C. elegans*. Genome Biol. 2, 1–10.

Katsnelson, A., 2015. News feature: microplastics present pollution puzzle. Proc. Natl. Acad. Sci. U.S.A. 112, 5547–5549.

Kim, H.M., Lee, D.K., Long, N.P., Kwon, S.W., Park, J.H., 2019. Uptake of nanopolystyrene particles induces distinct metabolic profiles and toxic effects in *Caenorhabditis elegans*. Environ. Pollut. 246, 578–586.

Kodoyianni, V., Maine, E.M., Kimble, J., 1992. Molecular basis of loss-of-function mutations in the *glp-1* gene of *Caenorhabditis elegans*. Mol. Biol. Cell 11, 1199–1213.

Lant, B., Derry, W.B., 2014. Immunostaining for markers of apoptosis in the *Caenorhabditis elegans* germline. Cold Spring Harb. Protoc. https://doi.org/10.1101/pdb.prot080242.

Lei, L., Liu, M., Song, Y., Lu, S., Hu, J., Cao, C., Xie, B., Shi, H., He, D., 2018a. Polystyrene (nano)microplastics cause size dependent neurotoxicity, oxidative damage and other adverse effects in *Caenorhabditis elegans*. Environ. Sci.: Nano 5, 2009–2020.

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K.M., He, D., 2018b. Microplastic particles causes intestinal damage and other adverse effects in zebrafish *Danio Retio* and nematode *Caenorhabditis elegans*. Sci. Total Environ. 619–620, 1–8.

Lenz, R., Enders, K., Nielsen, T.G., 2016. Microplastic exposure studies should be environmentally realistic. Proc. Natl. Acad. Sci. U.S.A. 113, E4121–E4122.

Lenz, K.A., Miller, T.R., Ma, H., 2019. Anabaenopeptins and cyanopeptolins induce systemic toxicity effects in a model organism the nematode Caenorhabditis elegans. Chemosphere 214, 60–69.

Lettre, G., Hengartner, M.O., 2006. Developmental apoptosis in *C. elegans*: a complex CEDnario. Ann. Rev. Mol. Cell Biol. 7, 97–108.

Leung, M.C., Williams, P.L., Benedetto, A., Au, C., Helmcke, K.J., Aschner, M., Meyer, J.N., 2008. *Caenorhabditis elegans*: an emerging model in biomedical and environmental toxicology. Toxicol. Sci. 106, 5–28.

Liu, P.-D., Shao, H.-M., Ding, X.-C., Yang, R.-L., Rui, Q., Wang, D.-Y., 2019. Dysregulation of neuronal Gαo signaling by graphene oxide in nematode *Caenorhabditis elegans*. Sci. Rep. 9, 6026.

Loos, C., Syrovets, T., Musyanovych, A., Mailänder, V., Landfester, K., Nienhaus, G.U., Simmet, T., 2014. Functionalized polystyrene nanoparticles as a platform for studying bio-nano interactions. Beilstein J. Nanotechnol. 5, 2403–2412.

Mato, Y., Isobe, T., Takada, H., Kanehiro, H., Ohtake, C., Kaminuma, T., 2001. Plastic resin pellets as a transport medium for toxic chemicals in the marine environment. Environ. Sci. Technol. 35, 318–324.

Mattsson, K., Hansson, L.A., Cedervall, T., 2015. Nano-plastics in the aquatic environment. Environ. Sci. Process Impacts 17, 1712–1721.

Moon, J., Kwak, J.I., An, Y.J., 2019. The effects of silver nanomaterial shape and size on toxicity to *Caenorhabditis elegans* in soil media. Chemosphere 215, 50–56.

O'Neil, N., Rose, A., 2006. DNA repair. WormBook. https://doi.org/10.1895/wormbook.1.54.1.

Page, K.E., White, K.N., McCrohan, C.R., Killilea, D.W., Lithgow, G.J., 2012. Aluminium exposure disrupts elemental homeostasis in *Caenorhabditis elegans*. Metallomics 4, 512–522.

Pazdernik, N., Schedl, T., 2013. Introduction to germ cell development in *Caenorhabditis elegans*. Adv. Exp. Med. Biol. 757, 1–16.

Qu, M., Xu, K.-N., Li, Y.-H., Wong, G., Wang, D.-Y., 2018. Using *acs-22* mutant *Caenorhabditis elegans* to detect the toxicity of nanopolystyrene particles. Sci. Total Environ. 643, 119–126.

Qu, M., Liu, Y.-Q., Xu, K.-N., Wang, D.-Y., 2019a. Activation of p38 MAPK signaling-mediated endoplasmic reticulum unfolded protein response by nanopolystyrene particles. Adv. Biosyst. 3, 1800325.

Qu, M., Qiu, Y.-X., Lv, R.-R., Yue, Y., Liu, R., Yang, F., Wang, D.-Y., Li, Y.-H., 2019b. Exposure to MPA-capped CdTe quantum dots causes reproductive toxicity effects by affecting oogenesis in nematode *Caenorhabditis elegans*. Ecotoxicol. Environ. Saf. 173, 54–62.

Qu, M., Kong, Y., Yuan, Y.-J., Wang, D.-Y., 2019c. Neuronal damage induced by nanopolystyrene particles in nematode *Caenorhabditis elegans*. Environ. Sci.: Nano. https://doi.org/10.1039/C9EN00473D.

Quevedo, C., Kaplan, D.R., Derry, W.B., 2007. AKT-1 regulates DNA-damage-induced germline apoptosis in *C. elegans*. Curr. Biol. 17, 286–292.

Seshagiri, S., Miller, L.K., 1997. Caenorhabditis elegans CED-4 stimulates CED-3 processing and CED-3-induced apoptosis. Curr. Biol. 7, 455–460.

Shaham, S., Horvitz, H.R., 1996. An alternatively spliced *C. elegans ced-4* RNA encodes a novel cell death inhibitor. Cell 86, 201–208.

Shao, H.-M., Han, Z.-Y., Krasteva, N., Wang, D.-Y., 2019. Identification of signaling cascade in the insulin signaling pathway in response to nanopolystyrene particles. Nanotoxicology 13, 174–188.

Song, Y.K., Hong, S.H., Eo, S., Jang, M., Han, G.M., Isobe, A., Shim, W.J., 2018. Horizontal and vertical distribution of microplastics in Korean coastal waters. Environ. Sci. Technol. 52, 12188–12197.

Stergiou, L., Doukoumetzidis, K., Sendoel, A., Hengartner, M.O., 2007. The nucleotide excision repair pathway is required for UV-C-induced apoptosis in

Caenorhabditis elegans. Cell Death Differ. 14, 1129–1138.
Tijsterman, M., Okihara, K.L., Thijssen, K., Plasterk, R.H., 2002. PPW-1, a PAZ/PIWI protein required for efficient germline RNAi, is defective in a natural isolate of *C. elegans*. Curr. Biol. 17, 1535–1540.
Wang, D.-Y., 2018. Nanotoxicology in *Caenorhabditis elegans*. Springer Nature Singapore Pte Ltd.
Wang, D.-Y., 2019. Target Organ Toxicology in *Caenorhabditis elegans*. Springer Nature Singapore Pte Ltd.
Yang, D., Shi, H., Li, L., Li, J., Jabeen, K., Kolandhasamy, P., 2015. Microplastic pollution in table salts from China. Environ. Sci. Technol. 49, 13622–13627.
Yen, C.I., Liu, S.M., Lo, W.S., Wu, J.W., Liu, Y.H., Chein, R.J., Yang, R., Wu, K.C., Hwu, J.R., Ma, N., Shieh, F.K., 2016. Cytotoxicity of postmodified zeolitic imidazolate framework-90 (ZIF-90) nanocrystals: correlation between functionality and toxicity. Chemistry 22, 2925–2929.
Zhang, G.-S., Liu, Y.-F., 2018. The distribution of microplastics in soil aggregate fractions in southwestern China. Sci. Total Environ. 642, 12–20.
Zhao, L., Qu, M., Wong, G., Wang, D.-Y., 2017. Transgenerational toxicity of nanopolystyrene particles in the range of μg L^{-1} in the nematode *Caenorhabditis elegans*. Environ. Sci.: Nano 4, 2356–2366.
Zhao, L., Dong, S.-S., Zhao, Y.-L., Shao, H.-M., Krasteva, N., Wu, Q.-L., Wang, D.-Y., 2019. Dysregulation of *let-7* by PEG modified graphene oxide in nematodes with deficit in epidermal barrier. Ecotoxicol. Environ. Saf. 169, 1–7.
Zhao, Y.-L., Wu, Q.-L., Wang, D.-Y., 2016. An epigenetic signal encoded protection mechanism is activated by graphene oxide to inhibit its induced reproductive toxicity in *Caenorhabditis elegans*. Biomaterials 79, 15–24.