Toxic Effects of Hydroxyl- and Amine-functionalized Silica Nanoparticles (SiO₂ and NH₂-SiO₂ NPs) on *Caenorhabditis elegans*

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ABSTRACT

Silica nanoparticles (SiO₂ NPs) are engineered nanomaterials (ENMs) that have a wide range of application. Increased use in manufacturing has led to concerns about their environmental impact and possible adverse health effects. We conducted a comparative toxicity assessment of bare SiO₂-NPs and amine-functionalized SiO₂ NPs (NH₂-SiO₂ NPs) utilizing the *Caenorhabditis elegans* (*C. elegans*) *in vivo* model. L1 nematodes were exposed to exposure concentrations of 0.25, 0.5, 2.5, and 5 mg mL⁻¹ until the worms reached the L4 stage. The chronic lethality and lifespan assays revealed a significant decrease in survival rate and lifespan at 2.5 and 5 mg mL⁻¹ for nematodes exposed to bare SiO₂ NPs (89% and 88%; 22 days, $p < 0.05$ and 14 days, $p < 0.05$) and at 5 mg mL⁻¹ for the NH₂-SiO₂-NPs-exposed group (86%; 20 days, $p < 0.001$). Exposure to all SiO₂ NP concentrations reduced progeny production to 79–60% while exposure to 2.5 and 5 mg mL⁻¹ of NH₂-SiO₂ NPs significantly reduced the brood size to 64–63%. Neurobehavioral toxicity was also observed in the SiO₂ NP-exposed worms, which displayed significantly decreased head thrashing for up to 92–71% and in the NH₂-SiO₂ NPs-exposed worms which showed significantly reduced head thrashing movement for up to 91–85% at concentrations of 0.5–5 mg mL⁻¹. Body bending movements were also significantly reduced at 0.5–5 mg mL⁻¹ SiO₂ NPs (71–34%) and 2.5–5 mg mL⁻¹ NH₂-SiO₂ NPs (94–66%). Significant shortening of body size was also observed in nematodes exposed to 0.5–5 mg mL⁻¹ for both SiO₂ NPs (93–81%) and NH₂-SiO₂ NPs (94–88%). Overall, bare SiO₂ NPs were observed to be more toxic due to the negatively charged surface OH groups, which may have disrupted protein homeostasis, resulting in the observed toxicities. We suggest that functionality is an important indicator in nanosafety evaluations.

Keywords: *Caenorhabditis elegans*; Silica nanoparticles; Nanotoxicity; Amine-functionalized silica nanoparticles; Hydroxyl-functionalized silica nanoparticles.

INTRODUCTION

Silicon dioxide nanoparticles (SiO₂ NPs) are engineered nanomaterials that can be categorized as solid, porous, or mesoporous (Esim *et al.*, 2019). The ease of modification of the surface chemistry of SiO₂ NPs has been widely exploited to control their interactions with biological systems used for drug delivery and to serve their purpose related to the remediation of environmental contaminants (Farrukh *et al.*, 2014; Gomes *et al.*, 2016; Mao *et al.*, 2020). It has been reported that surface modification of SiO₂ NPs enhances colloidal stability, biocompatibility, and target specificity as

well as improving cellular uptake, all of which are necessary components of drug delivery and bioimaging applications (Besic Gyenge *et al.*, 2011; Kralj *et al.*, 2012; Liberman *et al.*, 2014). The alteration of the surface chemistry of SiO₂ NPs has also been found to be beneficial in water treatment applications such as in nanofiltration processes (Ang *et al.*, 2019) as well as other types of environmental contaminant remediation methods (Huang *et al.*, 2003; Kim *et al.*, 2005; Walcarius and Delacôte, 2005; Chen *et al.*, 2019; Lin *et al.*, 2019). SiO₂ NPs are also utilized as food additives, to improve building and construction materials, in microelectronics manufacturing, and as an ingredient in agricultural mixtures (Kang *et al.*, 2011; Go *et al.*, 2017; Palla *et al.*, 2017; Shang *et al.*, 2019; Park *et al.*, 2019).

Synthesized SiO₂ NPs commonly have hydroxyl groups (OH) on their surfaces, which essentially only require basic silane chemistry for further required functionalization (Acharya *et al.*, 2017). The OH groups provide colloidal stability due

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to the negatively charged surface in the physiological environment as well as enhance linking interactions between the SiO₂ NP surfaces and target biomolecules (Osseo-Asare and Arriagada, 1999; Haensch *et al.*, 2010). In addition, the contaminant adsorption of SiO₂ NPs can be improved through the control of their surface chemistry (Li *et al.*, 2019). Aside from the OH group-containing SiO₂ NPs, amine-functionalized SiO₂ NPs (NH₂-SiO₂ NPs) are also popular for surface modification purposes, such as in processes that involve the attachment of a variety of organic groups (Ghosh *et al.*, 2013). Crosslinkers are easily coupled with the amino group and the positively charged surface of the particles enables gene therapy applications due to strong binding with DNA (Hsiao *et al.*, 2019). Aside from the surface chemistry, the end use of SiO₂ NPs is also dependent on the synthesis parameters that control their other features, including structure, stability, particle size, and porosity (Wilczewska *et al.*, 2012).

In general, the wide-range of applications of SiO₂ NPs has increased their rate of manufacture, where in 2014, the global production volume was reported to be 185–1400 kilotons (Keller and Lazareva, 2013; Pulit-Prociak and Banach, 2016). The dispersion of SiO₂ NPs into the environment can be attributed to their end use (Piechulek *et al.*, 2019), and their surface functionalization plays an important role in their environmental fate and transport (Jarvie *et al.*, 2009). Most engineered oxide nanoparticles such as SiO₂ NPs have been observed to be introduced into the environment through industrial wastewater and sewage discharges (Boxall *et al.*, 2007). Surface-functionalized SiO₂ NPs as compared to uncoated SiO₂ NPs have been shown to be removed more easily during primary wastewater treatment through the sedimentation process (Jarvie *et al.*, 2009). Other probable routes for SiO₂ NPs released into the air include direct introduction of the particles or through vehicle exhaust, while aerial deposition, leakage, and spills release SiO₂ NPs into surface waters (Bahadar Zeb *et al.*, 2018; Piechulek *et al.*, 2019). Overall, soils and sediments have been observed to be the major environmental sinks of these releases (Piechulek *et al.*, 2019). The median SiO₂ NPs levels predicted in central European surface waters had values of 3.5 µg L⁻¹ while natural and urban soils, sewage sludge-treated soil, and landfill waste had values of 160, 390, and 490 µg kg⁻¹, respectively (Wang *et al.*, 2016; Wang and Nowack, 2018).

Agricultural formulations containing SiO₂ NPs that are extensively used, are considered as major routes to which they are introduced into the food chain (Iavicoli *et al.*, 2017; Shang *et al.*, 2019). SiO₂ NPs can also be introduced into the terrestrial and aquatic food chains through the uptake process of plants and crops as well as through via trophic transfer (Unrine *et al.*, 2012; Skjolding *et al.*, 2014; Gupta *et al.*, 2016). Furthermore, the usage of SiO₂ NPs as food additives is also a straightforward route for exposure. The United States Food and Drug Administration (FDA) and the European Unions have imposed standards on the allowable concentrations of silica additives in food (2% and 1% per weight), respectively (EU, 2011; FDA, 2015). Around 80% of silica food additives were found to be in the nano-size range during the digestion process in the intestines where the gut epithelium is most likely to be affected (Peters *et al.*, 2012).

The workplace has been reported to provide a major risk of exposure in humans (Kim *et al.*, 2014; Oh *et al.*, 2014).

Although SiO₂ NPs are nontoxic, previous studies have reported adverse effects in *in vitro* and *in vivo* models, including inflammatory effects, cytotoxicity, and hemolysis (Park and Park, 2009; Slowing *et al.*, 2009; Al-Rawi *et al.*, 2011; Panas *et al.*, 2013; Panas *et al.*, 2014). The toxicity of unmodified SiO₂ NPs stems from the available OH groups on their surfaces and their strong interactions with the components of biological membranes such as lipids and proteins (Slowing *et al.*, 2009). Other findings have suggested that SiO₂ NP exposure is associated with respiratory and cardiovascular illnesses, multiple organ damage, oxidative stress, and cellular- and molecular-level damage (Chen *et al.*, 2004; Chen and von Mikecz, 2005; Lin *et al.*, 2006; Chen *et al.*, 2008; Shang *et al.*, 2009; Choi *et al.*, 2010; Lu *et al.*, 2010; Yang *et al.*, 2010; van der Zande *et al.*, 2014; Niu *et al.*, 2016). Amine functionalized amorphous SiO₂ NPs have demonstrated less toxicity as compared to bare amorphous SiO₂ NPs in both previous *in vitro* and *in vivo* studies (Marzaioli *et al.*, 2014; Nagano *et al.*, 2017), although other studies have reported otherwise (Yu *et al.*, 2011; Kurtz-Chalot *et al.*, 2014). The varying toxicities of NH₂-SiO₂ NPs may be attributed to the exposure conditions (Boussif *et al.*, 1995; Xia *et al.*, 2008; Hsiao *et al.*, 2019) as well as the extent of amino group surface coverage (Yu *et al.*, 2011; Morris *et al.*, 2016) and steric hindrance brought about by the linker structure which binds the amino groups to the SiO₂ NP surface (Hsiao *et al.*, 2019). Amino groups can be protonated at low pH conditions such as in the endolysosomal compartment of cells which triggers the “proton sponge effect” (rupture of lysosomes due to water influx) as demonstrated in amine-modified polystyrene (PS-NH₂)-exposed human and murine macrophages (Boussif *et al.*, 1995; Xia *et al.*, 2008). The interaction between the reactive OH groups and the cell membrane may be reduced depending on the degree of amino group surface substitution (Yu *et al.*, 2011; Morris *et al.*, 2016) and linker-related steric hindrance (Hsiao *et al.*, 2019). Hsiao *et al.* (2019) demonstrated that exposure conditions reduced the toxicity and improved the biocompatibility of amine functionalized amorphous SiO₂ NPs rather than the degree of amino group surface substitution.

Caenorhabditis elegans (*C. elegans*), a nematode living in soil, has been established as an *in vivo* model for various applications such as in drug, nanomaterial, and chemical assessments in the biomedical field as well as for studying the toxicity of environmental contaminants such as metals, chemicals, fine particulate matter, and nanomaterials (Chung *et al.*, 2019; Queiros *et al.*, 2019). Its advantages over other *in vivo* models include a short life cycle and lifespan, easy maintenance, completely sequenced genome, large brood size, and a small, transparent body (Brenner, 1974; Shen *et al.*, 2018). In addition, the *C. elegans* features can act as an intermediate between *in vitro* and mammalian testing (Hunt, 2017). *C. elegans* are potential targets for NP deposition since their habitat primarily overlaps with the known major sinks of NPs in the environment such as sludge treated soils and sediments found along riverbanks (Mikecz, 2018; Sinis *et al.*, 2019). Only a few studies have investigated the toxicity

of SiO₂ NPs utilizing *C. elegans in vivo* model (Pluskota et al., 2009; Scharf et al., 2013; Jung et al., 2015; Acosta et al., 2018; Eom and Choi, 2019; Piechulek et al., 2019; Li et al., 2020). Our study is aimed towards comparing the toxicity of dense SiO₂ NPs and NH₂-SiO₂ NPs through an evaluation of various *C. elegans* toxicological endpoints such as chronic lethal toxicity, lifespan, brood size, body length, and locomotion, as well as to contribute to the existing global data on possible indicators for nanosafety. To the best of our knowledge, this is the first comparative study on the toxicity of OH- and NH₂-functionalized silica-NPs using the *in vivo* model, *C. elegans*.

METHODS

Sample Preparation and Exposure Concentrations

Dense SiO₂ and NH₂-SiO₂ NPs were purchased from Merck KGaA, Darmstadt, Germany. These particles were dispersed in ddH₂O (pH = 7) to prepare stock solutions with a concentration of 25 mg mL⁻¹. Each NP type was diluted using ddH₂O to exposure concentrations of 0.25, 0.5, 2.5, and 5 mg mL⁻¹ (pH = 7). The exposure method was carried out by adding 200 µL of each of the exposure concentrations on bacterial lawns with a mean area of 5.05 cm² resulting in particle per loading area concentrations of 9.9 (0.25 mg mL⁻¹), 19.8 (0.5 mg mL⁻¹), 99 (2.5 mg mL⁻¹), and 198 (5 mg mL⁻¹) µg cm⁻², respectively (Pluskota et al., 2009), while the control group fed on a bacterial lawn that was solely supplemented with water. To achieve a homogeneous dispersion, the samples were sonicated for 1 hr before conducting the exposure experiments.

Characterization

The chemical structure of the NP samples was verified using attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy (Perkin Elmer Spectrum 100 FTIR Spectrometer, Waltham, MA, USA). The NPs were then pelletized at a fixed weight of 100 mg. The samples were then placed on an ATR crystal stage with the samples being scanned 16 times at a resolution of 4 cm⁻¹. The morphology and size distribution of the NPs were verified using field emission scanning electron microscopy (FESEM, S-4800 Hitachi Ltd. Tokyo, Japan). The samples were then transferred onto the sample holder using double-sided carbon tape and then coated with platinum (Pt) under vacuum conditions of 15 mA for 150 seconds. The FESEM acceleration voltage was maintained at 3 kV. The charges of the NP samples were verified using the dynamic light scattering (DLS) instrument (Zeta Nano ZS, Malvern, UK). The dispersion of the particles was performed using ultrasonication in deionized water with a stock solution concentration of 20 mg mL⁻¹. The samples were then placed in the sample cells to determine the charge. Using a thermal gravimetric analysis (TGA, Q500, TA Instrument, USA), the decomposition temperature of the samples was determined under a nitrogen atmosphere. Samples of 2–5 mg each were transferred onto a Pt pan and then placed on the TGA furnace.

C. elegans Cultivation and Exposure Conditions

The nematodes were maintained in nematode growth

medium (NGM) plates seeded with OP50 *E. coli*. At a temperature of 20°C. Age synchronization was achieved using the alkaline bleaching method (Porta-de-la-Riva et al., 2012). Eggs collected using the age synchronization method were maintained in M9 medium and incubated until they hatched into L1 worms for experimentation. The NGM plates contained bacteriological agar and bactopectone, which were obtained from Laboratories Conda (S.A., Spain) and NaCl obtained from Honeywell Fluka™ (New Jersey, USA). Additional ingredients including CaCl₂, K₂HPO₄, and cholesterol were obtained from Sigma-Aldrich (St. Louis, MO, USA) while MgSO₄ was acquired from Avantor Performance Materials, Ltd. (Gyeonggi-do, South Korea). OP50 *E. coli* cultures were acquired from the Bioresources Collection and Research Center (Hsinchu, Taiwan) and the Luria-Bertani broth was obtained from Sigma-Aldrich (St. Louis, MO, USA). For the bleaching solution, NaOCl was obtained from J.T. Baker (Central Valley, PA), and KOH was obtained from Duksan Pure Chemicals (Gyeonggi-do, South Korea). The KH₂PO₄ used for the phosphate buffer was acquired from Avantor Performance Materials, LLC (Radnor, PA, USA), and the Na₂HPO₄ used for the M9 buffer was obtained from Honeywell Fluka™ (New Jersey, USA). All physiological observations were done under a dissecting microscope (Olympus, SZX10, Waltham MA, USA).

Chronic Lethality Assay

The age-synchronized nematodes were exposed to the different concentrations of SiO₂ NPs and NH₂-SiO₂ NPs at the L1 stage at a constant temperature of 20°C until they reached their gravid adult stage. The lethal chronic toxicity of the samples was assessed immediately after the exposed worms reached the L4 stage. The nematodes were scored with regard to whether they were dead or alive by gently prodding them using a worm picker. Any worms that responded after prodding were scored as alive while worms that did not respond to touch were scored dead. Three biological replicates were performed, and a total of 150 worms were assayed.

Lifespan Assay

Using the different exposure concentrations of the SiO₂ NPs and NH₂-SiO₂ NPs, age-synchronized worms were exposed at the L1 stage until they reached their gravid adult stage. After exposure, the nematodes were transferred to fresh plates for the first 4–5 days of egg-laying and were transferred every other day to fresh plates after the egg-laying period. The exposed worms were assessed for their lifespan and were scored as either dead or alive every day. Three biological replicates and a total of 150 worms were evaluated. The temperature was maintained at 20°C.

Reproductive Assay

After chronic exposure, the age-synchronized worms were assessed for 4–5 days of egg-laying at 20°C. The nematodes were exposed to the various exposure concentrations of SiO₂ NPs and NH₂-SiO₂ NPs at the L1 stage, and the L4 worms were later assayed for their progeny production. One worm was transferred to each plate, and each worm was again

transferred to fresh plates during the egg-laying period. Each of the old plates containing the laid eggs were incubated, and the progeny worms were counted. Three biological replicates and a total of 60 worms were examined in this assay.

Locomotion Assay

The neurobehavior of the nematodes was assessed using two toxicological endpoint assays: head thrashing and body bending. The nematodes were exposed using the same exposure concentrations of SiO₂ NPs and NH₂-SiO₂ NPs starting at the L1 stage at 20°C until the worms reached the L4 stage. For the head thrashing assessment, the worms were individually transferred on a droplet of M9 placed on a glass slide, and the movements were recorded using the dissecting microscope camera and then measured for a duration of 1 minute. Three biological replicates were performed, and a total of 30 worms were evaluated. For the body bending assay, individual worms were placed on a fresh NGM plate without any bacterial lawn and then left to adjust in the incubator at the same exposure temperature. The worms were then carefully assessed under a dissecting microscope with a mounted camera recording the movement for 20 seconds. The number of body bends was then determined for all of the non-exposed and exposed groups. Three biological replicates were performed, and a total of 60 worms were evaluated.

Body Size Measurement

For the body size measurement, the L1 worms were exposed to the different exposure concentrations of the target samples and then allowed to reach adulthood. L4 worm images were captured using a dissecting microscope (Olympus, SZX10,

Waltham MA, USA) at a 1.5X magnification, and the sizes were measured using the Java-based open source imaging software, ImageJ (<http://imagej.nih.gov/ij/>) (Wisconsin, USA). Three biological replicates were performed, and a total of 150 worms for each experimental group were measured.

Statistical Analysis

The test for normality was performed on all the data. Normal and non-normal distribution were determined using the Shapiro-Wilk test. Normally distributed data were examined for significance using a one-way ANOVA, and non-normal distributed data were examined using the non-parametric test, the Kruskal-Wallis H test. The Mann Whitney U test was used to determine the significant differences between two independent samples. A survival plot or the Kaplan-Meier plot was constructed to evaluate the effects of the different concentrations of SiO₂ NPs and NH₂-SiO₂ NPs on the lifespan or ageing of the nematodes. All figures for the toxicological endpoint data were made using GraphPad Prism 6 (San Diego, California, USA). All statistical analyses were carried out using SPSS version 23 (International Business Machines Corp., New York, USA).

RESULTS AND DISCUSSION

Characterization of SiO₂ NPs and NH₂-SiO₂ NPs

Figs. 1(A) to 1(D) show the morphology and size distribution of two commercial NPs, SiO₂ and NH₂-SiO₂. The SiO₂ NPs were determined to have an average size distribution of 31 ± 4 nm, and the NH-SiO₂ NPs had an average size distribution of 37 ± 15 nm. As compared to the

Fig. 1. Morphology and size distribution of (A and B) SiO₂ and (C and D) NH₂-SiO₂ NPs.

NH₂-SiO₂ NPs, a narrower size distribution was observed in the SiO₂ NPs. Although a frequency of 35 nm particles was observed in NH₂-SiO₂ NPs, both types showed a similar average size, which is important when comparing the toxicity of both NPs in terms of their functionalization.

Fig. 2(A) shows the ATR-FTIR spectra of the NPs. The peak at 805 cm⁻¹ in the case of both NPs corresponds to the Si-O-Si stretching while peaks at 1030 cm⁻¹ for the SiO₂ NPs and at 1080 cm⁻¹ for the NH₂-SiO₂ NPs represent the siloxane vibrations of the (SiO)_n groups. The O-H stretching band for the SiO₂ NPs was observed at 3360 cm⁻¹, and the peak at 1640 cm⁻¹ can be attributed to the adsorbed water molecules. The peak at 1622 cm⁻¹ for the NH₂-SiO₂ NPs represents the N-H bending of the amine groups. The CH band of the propyl group of NH₂-SiO₂ NPs can be located at 2919 cm⁻¹ (Bois *et al.*, 2003; Azarshin *et al.*, 2017). Thus, this confirms that the two NP samples have different functionalization. Due to their differences in functionalization, their particle charge was also different (Fig. 2(B)). At pH = 7, the charges of the two NPs are as follows: SiO₂ = -31.57 ± 1.06 mV, and NH₂-SiO₂ = 16.73 ± 0.83. The OH group of the SiO₂ NPs could deprotonate, which resulted in a negative total net charge, whereas the amine groups on the NH₂-SiO₂ NPs could protonate, which provided a positive charge. The degradation behavior of the nanoparticles was determined in a temperature range of 50°C–850°C (Fig. 2(C)). The NH₂-SiO₂ and SiO₂ NPs showed a total loss of 2.05 and 2.32 wt.%, respectively.

The SiO₂ NPs had a higher total loss value due to the higher content of organic compounds such as OH groups and adsorbed water, which relatively decomposed within the temperature range. The water content in both types of NPs was removed starting at 50°C–120°C, wherein the SiO₂ and NH₂-SiO₂ NPs lost about 0.35 and 0.06 wt.%, respectively. The OH groups on the SiO₂ NP surfaces and the NH₂ groups on the NH₂-SiO₂ NP surfaces degraded at temperatures starting at 120°C–550°C.

Effects of Exposure Conditions and Chronic Lethality of SiO₂ NPs and NH₂-SiO₂ NPs

In our study, the nematodes' exposure at the L1 stage until adulthood to the different concentrations of the target samples is considered to be chronic exposure due to the relatively short life cycle of the nematodes (Comber *et al.*, 2008). Although environmental dispersion concentrations of SiO₂ NPs do not fall within the indicated exposure concentrations in this study, other scenarios that present clusters of local particle accumulation, such as surface coating local leaching, leakage and accidental spills, as well as occupational environments, permit the possibility of such concentration levels being present specifically (Scharf *et al.*, 2013). Additionally, *C. elegans* thrive in the soil, which is a major sink for released nanomaterials in the environment (Sinis *et al.*, 2019); therefore, supplementation of the target NPs on the bacterial lawn shows relevance to actual conditions where

Fig. 2. (A) ATR-FTIR spectra, (B) particle charge, and (C) TGA of SiO₂ and NH₂-SiO₂ NPs.

they are exposed to NPs. Before interpreting any results, Sinis *et al.* (2019) emphasized the importance of taking into account the exposure conditions used. Factors such as homogeneity, monodispersity, and colloidal stability can be altered when using conventional liquid media (e.g., M9, K-medium) due to the high ionic strength coupled with high chloride content, which may promote aggregation and precipitation of NPs and thereby compromise the size integrity of the NPs. Salt exposure may also cause some toxicity in nematodes (Sinis *et al.*, 2019). Chronic exposure with starvation of the nematodes is not suggested due to outcomes being affected by the additional biological surface and unprecedented NP interactions with the supplied bacterial food in liquid medium. In addition, NPs mixed in solid NGM may present a homogeneous distribution of NPs (Kim *et al.*, 2012); however, nematodes are only known to be in close contact with the NGM surface. We chose to allocate our exposure concentrations on a bacterial lawn to improve the distribution of NPs as well as to reduce stress and provide adequate food for the nematodes (Pluskota *et al.*, 2009; Contreras *et al.*, 2013; Contreras *et al.*, 2014).

As shown in Fig. 3, nematodes exposed to the 2.5 and 5 mg mL⁻¹ of SiO₂ NPs showed a significant decrease in survival rate (89% and 88%) immediately after exposure ($p < 0.05$) while only the highest concentration showed significant lethality in the nematodes exposed to NH₂-SiO₂ NPs (86%, $p < 0.01$).

In a study conducted by Eom and Choi (2019), L4 nematodes exposed to an SiO₂ NP (60–100 nm size distribution) concentration of 500 mg L⁻¹ in K-medium for 24 hours did not show any significant mortality while exposure to a lower concentration of 50 mg L⁻¹ in the same medium for 48 hours induced significant mortality with a calculated LC₅₀ of 123 mg L⁻¹. Li *et al.* (2020) investigated the lethal effects of SiO₂ NPs at 30 nm in worms exposed from the L1 stage to adulthood in K-medium and observed no lethality at any of the exposure concentrations, which ranged from 0.1–100 µg L⁻¹. The supplementation of SiO₂ NPs on a bacterial lawn may affect the lethality in *C. elegans* in comparison to liquid exposure methods; thus our study cannot be directly compared

to the studies mentioned above. Overall, our results exhibited slightly higher lethality in worms exposed to SiO₂ NPs as compared to NH₂-SiO₂ NPs.

Lifespan Effects

The toxic effects of exposure to the SiO₂ NPs and NH₂-SiO₂ NPs on the aging of *C. elegans* were also evaluated. Day 1 of the life span was designated as the day after the exposure was conducted and the worms already reached adulthood. The exposed worms were then transferred to fresh NGM plates within the egg-laying period and were scored every day. In the case of bare SiO₂ NPs, a significant decreasing trend in the lifespan and the average lifespan of the nematodes were observed at exposure concentrations of 2.5 ($p < 0.05$) and 5 ($p < 0.05$) mg mL⁻¹ (Fig. 4(A)), with the average lifespans significantly shortening to 22 and 14 days (Fig. 4(C) and Table 1), respectively. In addition, the total population of the *C. elegans* was observed to decrease below 50% at days 12 and 6. Nematodes exposed to NH₂-SiO₂ NP concentrations of 5 mg mL⁻¹ exhibited a significantly reduced lifespan (Fig. 4(B)), with an average lifespan of 20 days (Fig. 4(D) and Table 1) and a below 50% decrease in the total population at day 6.

Although in the present study, the same exposure technique and similar exposure concentrations were used as in Pluskota *et al.* (2009), whose results showed that SiO₂ NPs did not significantly reduce the worm lifespan. The dissimilar outcome may be attributed to their exposure being performed on L4 worms, which excluded any developmental-related function degeneration. However, in this study, L1 worms were exposed until adulthood, thus suggesting the possibility of overlapping effects of developmental- and age-related organ degeneration on ageing in *C. elegans*. The younger stage of the nematodes in their study led to greater sensitivity and resulted in more obvious outcomes during chronic exposure (Tyne *et al.*, 2013; Luo *et al.*, 2017). Additionally, in comparison with our study, the size distribution of the SiO₂ NPs was relatively smaller (30 nm), which may have contributed to the higher toxicity in the lifespan of the worms. Acosta *et al.* (2018) demonstrated that the nano-sized SiO₂

Fig. 3. Chronic lethality effects of exposure to bare SiO₂ NPs and NH₄-SiO₂ NPs. The bars in the figures represent means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ with respect to the control group unless otherwise specified.

Fig. 4. Lifespan assay on *C. elegans* exposed to SiO_2 NPs and $\text{NH}_4\text{-SiO}_2$ NPs shows the (A and B) survival rate of the exposed worms for the duration of their lifespan and their calculated (C and D) average lifespan.

Table 1. Summary of the average lifespans of nematodes exposed to SiO_2 and $\text{NH}_2\text{-SiO}_2$ NPs.

SiO_2 NP Concentration (mg mL^{-1})	Average Lifespan	S.D.	n	p-value	$\text{NH}_2\text{-SiO}_2$ NP Concentration (mg mL^{-1})	Average Lifespan	S.D.	n	p-value
Control	29	0.5774	150	-	Control	29	0.5774	150	-
0.25	28	1.0000	150	3.46E-01	0.25	29	0.577	150	1.00
0.5	26	0.5774	150	6.10E-02	0.5	28	1.0000	150	8.86E-01
2.5	22	2.5166	150	2.33E-02 ^a	2.5	28	1.0000	150	8.86E-01
5	14	1.0000	150	1.16E-02 ^a	5	20	0.5774	150	3.00E-06 ^b

Note: *p* values were calculated using a one-way ANOVA statistical test to determine the significance of lifespan reduction in comparison to the control group.

^a *p* < 0.05.

^b *p* < 0.001.

NPs (338 nm) significantly decreased the lifespan of the worms at concentrations of 0.5, 5, and 50 $\mu\text{g L}^{-1}$ as compared to their harmless micro-sized counterparts (1930 μm and 1114 μm). Although a study by Acosta *et al.* (2018) utilized a larger NP size distribution and sublethal or environmentally-relevant exposure concentrations as compared to those used in the present study, which thus showed a more pronounced toxicity toward the longevity of the nematodes, the NP exposure done in liquid media may have contributed to higher toxicity as compared to when the target NPs were supplemented on a bacterial lawn in the present study. Age-

related degeneration of nematode functions such as neurobehavior and egg-laying processes can be associated with long term exposure to NPs (Mikecz, 2018; Piechulek *et al.*, 2019), particularly in worms exposed during adulthood (Pluskota *et al.*, 2009). Sublethal concentrations of SiO_2 NPs have been reported to have no significant effects on the nematode's longevity via a high-throughput analysis but rather were found to accelerate age-related degeneration of reproductive and behavioral fitness (Jung *et al.*, 2015; Mikecz, 2018). Our study presented the higher toxicity of bare SiO_2 NPs as compared to $\text{NH}_2\text{-SiO}_2$ NPs at high concentrations.

Reproductive Toxicity

Oral uptake of nanomaterials may lead to their distribution to secondary organs such as the reproductive tract due to their ability to penetrate and translocate from primary organs such as the intestinal epithelial cells, which results in defects in the reproduction of *C. elegans* (Pluskota et al., 2009; Acosta et al., 2018; Sinis et al., 2019). Furthermore, nanomaterials may find entrance through the vulvar slit and consequently interact with reproductive components such as the plasma membrane of the vulvar cells and spermathecae (Scharf et al., 2013). Therefore, evaluation of reproductive-related endpoints such as the brood size is necessary to discern a possible translocation process of the nanomaterials in the nematode. After exposing the worms from the L1 stage into the L4 stage, the exposed adult worms were individually transferred to an NGM plate, and each worm, allowed to lay eggs for a duration of 4–5 days. Each worm was transferred to a new NGM plate every day, keeping the old plates in the incubator for the eggs to hatch and then counted to determine the total brood size (Fig. 5(A)). The nematodes exposed to all the bare SiO₂ NP exposure concentrations experienced significantly reduced progeny production in a range of 79–60% as compared to the unexposed worms ($p < 0.001$). A significant decrease in the progeny number was only observed in the NH₂-SiO₂ NPs-exposed nematodes at concentrations of 2.5 and 5 mg mL⁻¹ ($p < 0.001$) at 64–63%. As compared to NH₂-SiO₂ NPs, SiO₂ NPs showed higher reproductive toxicity even at lower concentrations (Fig. 5(B)). Eventually, both types exhibited the same toxic intensity at higher exposure concentrations of 2.5 and 5 mg mL⁻¹.

Pluskota et al. (2009) observed that L4 nematodes exposed to all SiO₂ NP concentrations with a size distribution of 50 nm showed a significant decrease in progeny production. In addition, an increase in the bag-of-worms (BOW) phenotype, an egg-laying deficiency characterized by eggs hatching inside the body, was observed in 39–55% of worms exposed to 2.5 mg mL⁻¹ of the SiO₂ NPs. This was attributed to movement deficiencies in the vulvar muscles rather than vulvar developmental abnormalities since L4 worms already exhibited developed vulvas. Therefore, the results reported by Pluskota et al. (2009) represented an age-related degeneration of the reproductive capability of the nematodes exposed to the bare SiO₂ NPs rather than a developmental defect. In a study conducted by Scharf et al. (2013), L1 worms exposed to 2.5 mg mL⁻¹ of SiO₂ NPs supplemented on a bacterial lawn until adulthood were shown to have developmentally defective egg-laying, which was determined to be the result of SiO₂ NP-induced protein aggregation, causing serotonin presynaptic accumulation, which also leads to reduced neurotransmission capacity. To that end, such conclusions might explain the similar observations that were found in our study with regards to the observed developmentally related defects in brood production. As compared to the bare SiO₂ NPs, the NH₂-SiO₂ NPs showed less toxicity toward the egg-laying capacity of *C. elegans*. The disturbance in protein homeostasis described by Scharf et al. (2013) can be explained by the fact that the presence of the negatively charged OH groups on the surface can lead to strong interactions with cellular membrane components such as positively charged lipids and proteins (Slowing et al., 2009) while the presence

Fig. 5. Measurement of the (A) brood size of NH₂-SiO₂ and SiO₂ NPs-exposed *C. elegans* (B) comparison between the two samples at different concentrations using a statistical analysis to determine any significant differences. The bars in the figures represent means $\pm$ SD. *** $p < 0.001$ with respect to the control group unless otherwise specified.

of positively charged amino groups are more likely to interact with negatively charged genetic materials such as the DNA (Hsiao *et al.*, 2019). Although the degree of amino group surface substitution and steric hindrance due to the presence of linker have been associated with the reduction of toxicity and improvement of biocompatibility of amine-functionalized silica-NPs in previous studies (Yu *et al.*, 2011; Morris *et al.*, 2016; Hsiao *et al.*, 2019), the exposure condition is more likely the probable cause as demonstrated by Hsiao *et al.* (2019). In their study, they observed a significant reduction in toxicity and increased biocompatibility of amine functionalized amorphous SiO₂ NPs in the presence of serum proteins as compared to when the serum proteins were absent, regardless of the degree of amino group surface substitution.

At a molecular level, genes such as the *msp* (Major Sperm Protein), which is related to reproduction in *C. elegans*, has been reported to be downregulated after exposure to SiO₂ NPs, an outcome that was attributed to the clathrin-mediated endocytosis pathway uptake mechanism (Eom and Choi, 2019). Although a detailed study of the mechanism of NH₂-SiO₂ NPs-induced reproductive toxicity in *C. elegans* is yet to be conducted, the possibility of toxic effects at a molecular level, particularly on the reproductive genes, is possible, but is likely only at higher concentrations as compared to bare SiO₂ NPs. Acosta *et al.* (2018) reported lower toxicity of mesoporous silica (MSP) at concentrations of 0.5, 5.0, and 50 µg mL⁻¹ in an M9 buffer with size distributions of > 50 nm and in MSPs functionalized with a starch derivative, emphasizing the effects of NP properties such as the size and functionality. Li *et al.* (2020) observed that SiO₂ NPs with size distributions of 30 nm significantly reduced brood size and induced germline apoptosis in nematodes exposed to SiO₂ NP concentrations of 100 µg L⁻¹. Overall, the functionality, size, and dosage of silica-NPs are found to be important indicators of the extent of their toxicity on nematodes, particularly in terms of their reproductive ability.

Neurobehavioral Toxicity

Two of the commonly used locomotion tests for the neurobehavior of *C. elegans* were performed in this study, the head thrashing and body bending assays. Head thrashing movements are defined as changes in the bending of the mid-body while body bends are defined as a directional change in the part of the worm corresponding to the posterior bulb of the pharynx with respect to the y axis while the nematode is traveling along the x axis (Nouara *et al.*, 2013). Head thrashes were counted within the duration of 1 minute, and body bends were counted within intervals of 20 seconds. Nematodes exposed to the bare SiO₂ NPs concentrations of 0.25 ($p < 0.01$), 0.5 ($p < 0.001$), 2.5 ($p < 0.001$), and 5 ($p < 0.001$) mg mL⁻¹ were found to exhibit significantly decreased head thrashing movements of between 92 and 71% (Fig. 6(A)). The NH₂-SiO₂ NP-exposed nematodes were found to have significantly reduced head thrashing movements at exposure concentrations of 0.5 ($p < 0.05$), 2.5 ($p < 0.001$), and 5 mg mL⁻¹ ($p < 0.001$), with reduction percentages of 91%, 88%, and 85%, respectively. As compared to the NH₂-SiO₂ NPs, bare SiO₂ NPs appeared to affect the head thrashing movement

more, with an observed significant difference at the highest concentration ($p < 0.01$) (Fig. 6(B)). The body bending of the exposed nematodes was assessed by letting them crawl on an NGM plate with a freshly spread thin OP50 lawn and counting the number of times the nematode part corresponding to the posterior bulb of the pharynx changed in direction with respect to the y axis while travelling along the x axis in an interval of 20 seconds (Nouara *et al.*, 2013). A significant reduction in the body bending movement was observed at SiO₂ NP concentrations of 0.5–5 mg mL⁻¹, with the body bending number reduced to a range of 71–34%. NH₂-SiO₂ NPs concentrations of 2.5 and 5 mg mL⁻¹ significantly decreased the nematode body bending movement by 94–66% (Fig. 6(C)). Significant differences in both sample types were observed at concentrations of 0.5, 2.5, and 5 mg mL⁻¹ (all with p values < 0.001), with SiO₂ NPs showing higher toxicity (Fig. 6(D)).

As compared to the lifespan assay, the reproductive and the locomotion tests appeared to be more sensitive towards SiO₂ NP exposure, which indicates evident neurotoxic properties. Scharf *et al.* (2013) attributed such neurodegenerative effects to accumulated ubiquitinated insoluble proteins and nuclear amyloid, which damage the neural circuits responsible for the neurobehavior of worms. This was supported by their investigation on the proteomic characteristics of exposed nematodes, which showed that the aggregation of SiO₂ NPs mainly affects the protein homeostasis pathway-related proteins. In addition, both the negative effects on locomotion and reproductive behavior were also evident in behavioral phenotypes treated with anti-amyloid compounds. Aside from presynaptic serotonin accumulation and egg-laying defects, the sensitivity of serotonergic HSN motor neurons towards SiO₂ NPs can also result in neurotransmission problems. Acosta *et al.* (2018) demonstrated the toxicity of nano-sized mesoporous silica-based particles (338 nm) at concentrations of 5 and 50 µg mL⁻¹ on 2-day and 9-day adult nematodes and reported a significant dose-dependent reduction in their body bending rates (p values of 0.08 and 0.01). In contrast, starch functionalization enhanced biocompatibility and reduced the SiO₂ NPs toxicity in the worms. At a concentration of 10–100 µg L⁻¹, L1 to adult day-1 nematodes exposed to SiO₂ NPs (30 nm) showed significant slowing in head thrashing and body bending movements (Li *et al.*, 2020). Overall, previous studies have demonstrated SiO₂ toxicity based on factors such as functionality, size, and dosage. Although this is the first comparative study to compare the toxicity of NH₂-SiO₂ NPs with SiO₂ NPs and to study the toxicity mechanism, the former had less toxic effects as compared to the latter, which was attributed to the negatively charged nature of the OH groups, which are more likely to interact with proteins (Hsiao *et al.*, 2019). However, increasing the dosage of NH₂-SiO₂ NPs has been found to lead to significant toxic effects comparable to its counterpart.

Growth Effects

Developmental toxicity in *C. elegans* can be represented by its capacity to grow (Li *et al.*, 2020). After exposure, the exposed nematodes were measured for body size to evaluate the effects of the target samples on their growth. *C. elegans*

Fig. 6. Neurobehavioral testing comprising the (A) measurement of head thrashes and (B) body bends were performed to evaluate neurotoxicity. In addition, a comparison between the two silica-NPs was also performed by determining their significant differences at different concentrations for the (C) head thrashing and (D) body bending tests. The bars in the figures represent means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to the control group unless otherwise specified.

exposed to bare SiO₂ NP concentrations of 0.5, 2.5, and 5 mg mL⁻¹ were found to have body sizes significantly reduced to 93% ($p < 0.001$), 86% ($p < 0.001$), and 81% ($p < 0.001$), respectively, while NH₂-SiO₂ NPs induced significant decreases in growth, which was evident in the reduced body lengths of 94% ($p < 0.001$), 89% ($p < 0.001$), and 88% ($p < 0.001$) at similar exposure concentrations (Fig. 7(A)). Although both target samples showed reduced growth at similar concentrations, bare SiO₂ NPs showed higher toxicity at concentrations of 2.5 ($p < 0.01$) and 5 mg mL⁻¹ ($p < 0.001$) (Fig. 7(B)). The mean body size measurements of the worms exposed to 0.25, 0.5, 2.5, and 5 mg mL⁻¹ of SiO₂ NPs were 1020, 969, 896, and 842 μ m while the NH₄-SiO₂ NP-exposed nematodes showed mean body sizes of 1027, 976, 927, and 909 μ m, respectively.

Piechulek *et al.* (2019) observed a reduction in the body length of L4 nematodes exposed to 200 μ g mL⁻¹ of silica-NPs synthesized using different methods (12nm HTFH, 25 nm Hartlen and 50nm Stoeber silica-NPs) and BULK silica (500 nm) for 72 h. Nematodes exposed to BULK silica had a normal body size (1233 μ m) in comparison to the untreated group (1194 μ m). In contrast, regardless of the synthesis method, nematodes exposed to the different silica-NPs showed a mean body length of 736 μ m, which is relatively similar to that of the *pep-2* deletion mutants, which lack the OPT-2/PEP-2 peptide transporter (838 μ m). This result indicated that although the OPT-2/PEP-2 peptide transporter is active in nematodes exposed to silica-NPs,

nutrient peptides reportedly trapped in vesicles formed when intestinal cells absorbed the reporter β -Ala-Lys-AMCA. This prevented essential proteins such as di- and tri-peptides from undergoing further hydrolysis and amino acid metabolism (Smith *et al.*, 2013). Our study demonstrated that nematodes exposed to SiO₂ NPs exhibited the same reduced body size phenotypes resulting from a disturbance in protein homeostasis. We concluded that the OH groups on the bare SiO₂ NP surfaces contributed more to the disruption of protein homeostasis effects as compared to the amine groups on the surface of the NH₂-SiO₂ NPs, which resulted in the observed toxicity. The interaction of silica-NPs with biomolecules can be credited to their differences in terms of charge, with the negatively charged OH groups having higher affinity to lipids and proteins and positively charged amine groups having higher affinity to genetic materials.

CONCLUSION

The present study is the first comparative analysis of the toxic effects of two commonly utilized silica-NPs (SiO₂ and NH₂-SiO₂ NPs) in terms of functionality and dosage in the *in vivo* model, *C. elegans*. Our target concentrations were relatively higher as compared to the reported predicted concentrations of silica-NPs in the environment from previous studies. However, we cannot ignore the possibility that local particle accumulation resulting from untoward accidents such as local leaching of surface coating, occupational

Fig. 7. Body size measurement of the unexposed and exposed nematodes. Significant differences in body size measurements were determined between the (A) control group and the exposed group and between (B) the two different samples at different concentrations. The bars in the figures represent means $\pm$ SD. *** $p < 0.001$ with respect to the control group unless otherwise specified.

environments, and leakage and accidental spills may occur. Although significant chronic toxicity was only observed at higher exposure concentrations of both silica-NP types, developmental toxicities were observed. Nematodes exposed to both silica NP types were shown to have significantly reduced average lifespans, brood size, locomotion, and body size. However, the functionality assessment differentiated their toxic intensities, with SiO₂ NPs exhibiting negatively charged OH groups on the surface being more toxic due to their higher interaction with biomolecules, particularly proteins resulting in a disturbance in protein homeostasis as compared to the positively charged amino groups on the NH₂-SiO₂ NP surface. Protein homeostasis disruption has been associated with egg-laying and neurotransmission defects as well as to reductions in body size, all of which may contribute to a reduced lifespan in nematodes. To that end, our study suggests functionality as an important indicator for assessing the toxicity of nanoparticles.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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