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Redesign of hydrophobic quantum dots mitigates ligand-dependent toxicity in the nematode *C. elegans*

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Abstract

Surface properties of engineered nanomaterials (ENMs) have been shown to influence their interaction with biological systems. However, studies to date have largely focused on hydrophilic materials, likely due to biocompatibility concerns and aqueous exposure conditions necessary for many model systems. Therefore, a knowledge gap exists in nanotoxicity literature for impacts of hydrophobic ENMs, with studies of hydrophobic materials largely limited to carbon ENMs. Here we demonstrate testing of hydrophobic quantum dots (QDs) using the nematode *C. elegans*, a model soil organism cultured on solid media and amenable to hydrophobic exposures. To evaluate the influence of hydrophobicity, we compared CdSe/ZnS QDs functionalized with hydrophobic trioctylphosphine oxide (TOPO) to identical QDs functionalized with hydrophilic dihydrolipoic acid-polyethylene glycol (DHLA-PEG) and alternative hydrophobic CdSe/ZnS QDs functionalized with oleic acid (OA). Results show that hydrophobic TOPO QDs are significantly more toxic than hydrophilic DHLA-PEG QDs, and substitution of TOPO with OA yields relatively non-toxic hydrophobic QDs. Fluorescence microscopy shows TOPO QDs loosely associated with the organism's cuticle, but atomic force microscopy shows no difference in cuticle structure from exposure. Importantly, TOPO ligand alone is as toxic as TOPO QDs, and our data suggests that TOPO may impact neuromuscular function, perhaps upon displacement from the QD surface. This study demonstrates the importance of examining ligand-

specific impacts of hydrophobic ENMs and indicates OA-functionalized QDs as a potential alternative to TOPO QDs for reduced toxicity.

Keywords

quantum dot, hydrophobic, nanotoxicity, *C. elegans*, trioctylphosphine oxide, oleic acid

Abbreviations

engineered nanomaterial	(ENM)
quantum dot	(QD)
trioctylphosphine oxide	(TOPO)
oleic acid	(OA)
dihydrolipoic acid-polyethylene glycol	(DHLA-PEG)

1. Introduction

The surface chemistry of engineered nanomaterials (ENMs) has been established as an important property that influences the interaction of ENMs with biological systems and thus toxicity (Albanese *et al.* 2012, Sun *et al.* 2019, Johnston *et al.* 2020, Waris *et al.* 2021). ENM surface chemistry can differ between core materials based on their inherent properties and can also be altered by addition of surface ligands to impart desired properties to materials. Much work has gone into functionalizing ENMs with hydrophilic ligands to improve biocompatibility and stability of ENMs for potential medical applications and for use in systems for toxicity testing, which require aqueous-stable materials for reliable results (Dhawan and Sharma 2010, Schubert and Chanana 2018). As a result, a significant knowledge gap exists in the literature for toxicity of hydrophobic ENMs, although many commercially relevant materials—such as semiconductor quantum dots (QDs) used in displays—are hydrophobic as-used (Batley *et al.* 2013). Hydrophobicity is an important property of ENMs demonstrated and modeled to impact ENM toxicity (Kim *et al.* 2013, Manshian *et al.* 2014, Allen *et al.* 2017, Farnoud and Nazemidashtarjandi 2019, Fontana *et al.* 2021), but, to date, studies of hydrophobic ENM toxicity have focused largely on carbon ENMs (e.g. nanotubes, nanosheets, fullerenes)(Lin *et al.* 2017, Chen 2019).

More broadly, hydrophobicity is recognized as an important determinant of chemical uptake and distribution within cells and organisms (D. Cronin 2006). Hydrophobic compounds are able to access and traverse the cell membrane, concentrating in lipids of cells and tissues, which leads to bioaccumulation (Puckowski *et al.* 2016). By localizing to lipophilic cellular compartments such as the membrane, hydrophobic compounds can cause biological impacts by altering membrane structure and organization (Barnoud *et al.* 2014) or by increasing their relative concentration in the membrane and thus impact function of membrane proteins (Chisari *et al.* 2009). In contrast, hydrophilic compounds often require active or passive transport via membrane proteins to enter the cell, which can pose a barrier to non-specific uptake of hydrophilic compounds (Klaper and Niemuth 2016, Padhye *et al.* 2020). Thus, although hydrophobicity has been well studied and shown to be important in governing the impact and partitioning of pharmaceuticals and environmental contaminants, it has not been well explored for ENMs (Steinhäuser and Sayre 2017).

For hydrophilic ENMs, previous research has demonstrated that the choice of ligand has an impact on the interaction with organisms and ultimately the toxicity (Bozich *et al.* 2014, Dominguez *et al.* 2015, Sun *et al.* 2019). This may be due to the ligand itself (Feng *et al.* 2015), but may be a nano-specific impact (Qu *et al.* 2015). In this way, changing the surface ligands of an ENM is one strategy for material redesign that can improve safety or sustainability, potentially without impacting underlying function. However, this has not been as well-studied for hydrophobic surface modifications.

Due to their excellent fluorescence properties, colloidal QDs are being extensively investigated for uses as varied as imaging agents, catalysts, and electronic display components (Panfil *et al.* 2018). Due largely to use in displays, the world market for QDs is expected to reach $3.4 billion by 2021 (Pramanik *et al.* 2018). QDs are being used for backlighting in displays by LG, Samsung, Sony and other display manufacturers (Chen *et al.* 2017). At present, Cd-based QDs outperform alternatives in areas such as narrowness of emission and quantum yield, but the inherent toxicity of the Cd ion has raised concerns, and research into heavy metal-free alternatives is extensive (Yang *et al.* 2018). Despite these concerns, Cd-based QDs are being

used in commercial displays, where they are still superior to alternative materials, such as InP QDs (Sadasivan *et al.* 2016).

QDs are typically synthesized with hydrophobic trioctylphosphine oxide (TOPO) ligands, although alternative hydrophobic ligands such as oleic acid (OA) and hexadecylamine are sometimes used (Zheng *et al.* 2019). Frequently, hydrophobic ligands are cap-exchanged with hydrophilic alternatives, such as dihydrolipoic acid-polyethylene glycol (DHLA-PEG), in order to disperse QDs in aqueous media for fluorescence imaging (Zheng *et al.* 2019).

The preponderance of QD nanotoxicology research to date has been on hydrophilic ligand-functionalized QDs (Rocha *et al.* 2017, Wang and Tang 2018, Hu *et al.* 2021). As in industry, much in this literature is made of the potential toxicity of Cd. However, little has been done to evaluate the toxicity of as-synthesized hydrophobic QDs, likely because they aggregate and sediment in the aqueous media used in most common toxicology models (e.g. cell culture, *Daphnia magna*, *Danio rerio*). This behavior also means they would most likely end up in organic matter or sediments in the environment (Navarro *et al.* 2010), or alternatively in soils upon waste disposal. The potential for toxicity from hydrophobic materials (Kim *et al.* 2013, Manshian *et al.* 2014, Allen *et al.* 2017, Farnoud and Nazemidashtarjandi 2019) means that testing for toxicity of hydrophobic QDs specifically in sediments and soil should be included.

To overcome complications of testing hydrophobic materials in aqueous models, we used the nematode *C. elegans*, which can be grown and exposed on solid media. By mixing hydrophobic particles directly with the *C. elegans* OP50 *E. coli* food on solid agar plates, we were able to create QD-bacteria lawns on which worms could be exposed to hydrophobic ENMs. In addition, because of the transparency of *C. elegans*, we were able to take advantage of the fluorescence of QDs to determine their *in vivo* localization upon *C. elegans* exposure. Studies of hydrophilic QDs in *C. elegans* have found them to be relatively non-toxic compared to equivalent concentrations of Cd (Hsu *et al.* 2012, Contreras *et al.* 2013, Wang *et al.* 2016). However, no studies have been done looking at toxicity of hydrophobic QDs in *C. elegans*.

For this study, we compared impacts of hydrophobic versus hydrophilic CdSe/ZnS QDs on *C. elegans* lifespan and molecular impacts. In addition, we compared the impacts of the conjugation with two different hydrophobic ligands to determine if toxicity is due to the hydrophobicity in general or due to the nanoparticle-ligand combination. Finally, we examined if toxicity due to hydrophobic ligand conjugation was nano-specific or due to the ligands themselves. The initial hydrophobic versus hydrophilic nanomaterial study included TOPO-functionalized CdSe/ZnS QDs compared to cap-exchanged hydrophilic DHLA-PEG CdSeCdSe/ZnS QDs, and each respective free ligand. In the second study, hydrophobic TOPO-functionalized CdSe/ZnS QDs were compared to an alternative hydrophobic OA-functionalized CdSe/ZnS QDs and their respective ligands (Figure 1a). In addition, localization was investigated using fluorescence microscopy, and impacts on *C. elegans* cuticle were investigated with atomic force microscopy (AFM). Finally, we compared the molecular impacts of QD and ligand exposure using fluorescent probes for internal *C. elegans* structure and gene expression.

2. Materials and Methods

2.1. Particle synthesis and characterization

2.1.1. Materials

Zinc formate (98%), zinc acetate and 1-dodecylphosphonic acid (DPA, 95%) were purchased from Alfa Aesar. Diisooctylphosphonic acid (90%), 1-Octadecene (ODE), Trioctylphosphine oxide (TOPO), trioctylphosphine (TOP, 97%), oleic acid, tetramethylammonium hydroxide solution (TMAH, 25 wt % in methanol), cadmium acetylacetonate (CdAcAc, 99.9%), sodium chloride, LUDOX TMA colloidal silica (34 wt % suspension in H_2O), hexamethyldisilathiane ($(TMS)_2S$, synthesis grade), Paraffin Oil (puriss.), and zinc oxide were purchased from Sigma-Aldrich. Selenium powder (Se, 99.5%), 1-hexadecylamine (HDA, 90%), oleylamine (C18 content 80–90%), and sodium hydroxide were purchased from Acros Organic.

2.1.2. TOPO-functionalized CdSe/ZnS QDs (A20a, A18d)

Two batches of TOPO-functionalized CdSe/ZnS QDs were synthesized for this study (A20a and A18d)(Fig 1a). Core synthesis for TOPO-functionalized QDs followed a previously published procedure (Lyons *et al.* 2017). In a 50 ml three –neck round bottom flask, 2g of TOPO, 8g of HDA, 10ml TOP, 0.76mL Diisooctylphosphonic acid, and 0.311g of cadmium acetylacetonate

were heated to 100 °C under nitrogen gas. The vessel was placed under vacuum for 20 min to remove water and oxygen, and then backfilled with nitrogen gas. Next, the vessel was raised to 320 °C. At this temperature, a TOPSe precursor (0.351g Selenium powder in 4 mL TOP) was swiftly injected for CdSe nucleation. CdSe cores were allowed to grow at 270 °C for 5 min, and then slowly cooled to room temperature.

For ZnS shell growth, the Successive Ionic Layer and Adsorption (SILAR) Technique was used (Xie *et al.* 2005, Lyons *et al.* 2017, Williams *et al.* 2018). A 1:1 mixture of CdSe cores in their reaction mixture with acetone was purified via centrifugation at 2000*g* for 5 min. The pellet was suspended in chloroform, and then analyzed via UV/Vis spectroscopy for size and concentration following calculations provided by the literature (Jasieniak *et al.* 2009). These calculations were then used to add 0.15μmol CdSe cores, 6 mL ODE, 6 mL Oleylamine, 4 mL TOP, and 10 mg dodecylphosphonic acid to a 50 mL three-neck round-bottom flask. The mixture was heated to 100 °C under nitrogen gas, placed under vacuum for 30 min to removed oxygen and water, and then put back in a nitrogen environment. Concurrently, a 0.05M zinc formate in oleylamine precursor and a 0.25M $(TMS)_2S$ in TOP precursor were made under nitrogen environments to add the zinc and sulfur elements for the shell, respectively. At 100 °C the first amount of the zinc precursor was injected over 15 min using a syringe pump, calculated to add one shell layer (Xie *et al.* 2005). The vessel's temperature was raised to 160 °C, and then the first amount of the sulfur precursor was injected over 15 min. This first layer was allowed to anneal 20 min. The temperature was raised to 180 °C, where calculated amounts of zinc and sulfur precursors were added sequentially over 15 min, followed by 20 min of annealing. The temperature was raised to 190 °C for the addition of the third layer and annealing. Finally at 200 °C, 0.5 mL oleic acid was added dropwise for a 1 h annealing of the TOPO-functionalized CdSe/ZnS core/shell QD. These QDs were precipitated with acetone and centrifugation prior to use.

2.1.3. DHLA-cap exchange of TOPO-functionalized QDs (A20b)

DHLA-PEG QDs (batch A20b) were cap-exchanged from TOPO QD batch A20a (above)(Fig 1a). Dihydro Lipoic acid-polyethylene glycol (DHLA-PEG-OCH_3) was prepared and purified with slight modifications to a previously reported protocol (Mei *et al.* 2008, 2009). DHLA ligand, sodium hydroxide, zinc acetate, and methanol were sonicated together in a septum-closed

vial filled with nitrogen gas. Purified QDs (A20a) were stirred with a minimal amount of chloroform, dried, and put under a flow of nitrogen. The DHLA ligand solution was added to the QDs and left to stir overnight at 50 °C under nitrogen gas. The next day, ethyl acetate and hexane were added to QDs, and then stirred and allowed to separate. The hexane layer was removed. The QDs were dried under vacuum, and then re-dispersed in Millipore water. This QD solution was passed through a 0.45 SFCA syringe filter into a 30,000 MWCO spin filtration device for washing through centrifugation 3 times at 2000*g* (Liu and Snee 2011). The ligand exchange reaction with DHLA-PEG enables the transfer of the QD to aqueous media. It significantly decreases the impact of TOPO-coated QDs on *C. elegans* by removing TOPO molecules from the QD surface and by reducing the impact of residual, bound TOPO through steric hindrances provided by the DHLA-PEG ligands (Wenger *et al.* 2017).

2.1.4. Oleic Acid-functionalized CdSe/ZnS QDs (D3a)

OA-functionalized hydrophobic QDs were synthesized as batch D3a (Fig 1a). In a 25 mL 3-neck round-bottom flask with a stir bar, 5 mmol of CdO, 10 mL paraffin oil, and 2.4 mL oleic acid were heated under nitrogen gas to dissolve the metal. In a 100 ml 3 neck round-bottom flask with stir bar, 50 ml of paraffin oil, and 1 mmol of Se powder were heated to 100 °C under nitrogen gas. The vessel was placed under vacuum for 30 min, and then put back under nitrogen gas to heat it to 240 °C. At 240 °C, 3.6 mL of the CdO mixture was quickly injected and allowed to react for 15 min. Finally, the vessel was allowed to cool to room temperature using an oil bath.

These OA-functionalized core QDs were purified via the following methods: A 1:1 mixture of the CdSe core QDs in their reaction mixture and methanol was purified via centrifugation at 2000*g* for 5 min. The top layer was discarded. The bottom QD-layer was retained and diluted to a 1:1 mixture in methanol for centrifugation two more times. An aliquot of the final bottom layer was diluted in chloroform for optical characterization, size calculation, and concentration calculation as directed by the literature (Jasieniak *et al.* 2009).

The SILAR method was used to shell these TOPO-free QDs as well, following the same temperatures and times, but the zinc precursor was 0.1628 g (0.1 M) zinc oxide in 13.7 mL

paraffin oil and 5 mL oleic acid and the sulfur precursor was 0.1 M sulfur powder in ODE (Deng *et al.* 2005). The shelled QDs were precipitated with methanol and centrifugation prior to use.

2.1.5. Absorbance and Fluorescence Instrumentation for Particle Characterization

UV−Vis absorption spectra were obtained using a Thermo Scientific Evolution 201 UV−Vis spectrophotometer. Fluorescence spectroscopy measurements were performed using a PTI-Horiba QuantaMaster 400 fluorometer, equipped with an integration sphere for emission quantum yield measurements, and with a PicoMaster TCSPC detector for fluorescence lifetime measurements.

2.1.6. High-Resolution TEM

QDs were further characterized using a high-resolution Titan 80−300 analytical transmission electron microscope (TEM) between 43-490kV. Prior to imaging, samples were prepared by purifying a small amount of the QD solution and drop-casting 20 µL of the solution onto 400 mesh, copper grids with an ultrathin carbon film on a holey carbon support film (Ted Pella, Inc.). The grids were then placed in a vacuum oven overnight before being inserted into the transmission electron microscope.

2.2. C. elegans *exposure and imaging*

2.2.1. C. elegans *culture, synchronization, exposure*

C. elegans N2 worms and OP50 *E. coli* were obtained from the Caenorhabditis Genetics Center at the University of Minnesota. Worms were cultured on large (150 mm) solid nematode growth medium (NGM) plates seeded with OP50 at 20 °C according to maintenance protocols outlined on WormBook (Stiernagle 2006). Synchronized cultures of L1 larvae were prepared for exposures using bleaching Protocol 6 from Wormbook (Stiernagle 2006) and synchronized overnight on a large NGM plate without food. For lifespan assays, worms were grown to young adults by growing the synchronized population on an OP50-seeded large NGM plate for 3 d.

For lifespan assays, exposure plates were supplemented with floxuridine (FUDR) at 12 mg/L to prevent overgrowth by hatched larvae and minimize transferring of adult worms. Small (60 mm) FUDR-supplemented NGM exposure plates were made up in triplicate by combining 200 µL of

5x treatment solutions or solvent with 800 µL of concentrated OP50, vortexing to mix, and adding 300 µL of the resulting suspension to each of the 3 plates. Exposure plates were allowed to dry overnight in a fume hood to remove any solvent. For hydrophilic exposures (DHLA-PEG QDs and ligand, Cd control, and water control) autoclave-sterilized Milli-Q® ultrapure water was used as the solvent. For hydrophobic exposures (TOPO QDs and ligand, OA QDs and ligand, and hexane control) hexane ≥ 95% (Fluka) was used as the solvent. Cadmium control concentration was calculated based on equivalence to Cd contained in A20a QDs at 1 µM: 236 µg/mL as 438 µg/mL $CdSO_4$. Concentrations for ligands were selected based on total amounts added during QD synthesis equivalent to 1 µM QDs: 6.4 mg/mL TOPO, 7.3 mg/mL DHLA-PEG, 3.7 mg/mL OA. Ten synchronized young adult worms were picked to each exposure plate and scored live/dead each day, determined by tapping on the plate and poking worms with a sterilized Pt worm pick to evaluate for movement, until all worms were counted as dead (no movement in response to stimulus).

For AFM and fluorescence imaging of *C. elegans*, NGM exposure plates (100 mm) were made with 1 mL of exposure solution (200 µL 5x treatment solution and 800 µL concentrated OP50, vortexed to mix) added to each plate, and plates dried overnight in a fume hood. Concentration for Cd control was 47 µg/mL, equivalent to 0.2 µM QDs. Concentration for TOPO ligand controls was 740 µg/mL, based on amount added for synthesis of 0.2 µM QDs. Synchronized L1 larvae were washed from their plate using M9 media after a period of 24 h of starvation, concentrated by centrifuging at 2500*g* 20 °C for 1 min, and resuspended in M9 at 10 worms per µL. From this stock, 150 µL (1500 larvae) were added to each exposure plate.

2.2.2. Larval fixation for smFISH and AFM

At 2 h and 6 h time points, *C. elegans* L1 larvae were washed from exposure plates with M9 and fixed in 4% paraformaldehyde for 45 min following the protocol for smFISH outlined by Ji and van Oudenaarden (Ji and van Oudenaarden 2012). Fixed worms for AFM imaging (2 h exposure) were stored in PBS, while worms for smFISH (6 h exposure) were stored and permeabilized in 70% RNAse-free ethanol at 4 °C.

2.2.3. Fluorescence imaging of C. elegans *for particle localization and smFISH*

C. elegans samples were imaged with a Zeiss LSM710 confocal microscope. Fluorescence images were taken with a 40× (NA 1.1) water immersion objective lens using a 1-5 mW laser power. For initial assessment of QD localization, worms were picked from exposure plates onto agar pads with no rinse steps and anesthetized with levamisole using methods described in WormBook (Shaham 2006).

Single-molecule fluorescence *in situ* hybridization (smFISH) was used to probe the expression of *cdr-1* and *gpdh-1* genes. The *cdr-1* gene is expressed in the *C. elegans* gut in response to Cd exposure, as might be expected from CdSe/ZnS QDs (Liao *et al.* 2002). The *gpdh-1* gene is expressed in the gut in response to osmotic stress, as might be expected from cuticle damage (Lamitina *et al.* 2006, Dodd *et al.* 2018). FISH probes were labeled with Alexa647 at the 5' end and purchased from Integrated DNA Technologies. Probe sequences are included in Supplementary Table S1. Hybridization was performed by referring to previously established protocol, using probes at a concentration of 100 nM (Ji and van Oudenaarden 2012). Worms were also stained with DAPI (Sigma-Aldrich) at 5 ng/mL and Alexa Fluor 488® phalloidin (Invitrogen) at 156 nM to label nuclei and actin, respectively. SmFISH images were taken with the wide-field fluorescence microscope mode (60× oil immersion objective lens, NA 1.4; laser power 30 mW, exposure time 0.5-1s, EM gain 30-50). 20-30 z-axis sections were imaged to cover most of the worm body volume. In post-processing, RNA molecules were identified at each scanning plane with a Gaussian mask fitting algorithm and projected to the final reconstructed images (Cui *et al.* 2017). The processed images were subjected to counting with home-built MATLAB programs (the script is available upon request). The gene expression level was quantified in the gut (see Fig S3) and normalized per 1000 μm^3 (approximately the size of a single cell).

2.2.4. Imaging of C. elegans *cuticle using AFM*

C. elegans were adhered to glass bottom dishes (50 mm, #14027, Ted Pella) coated with poly-L-lysine solution (PLL, average mol. Wt. 70,000-150,000, 0.1%). The bottoms of the dishes were covered with 2 mL of PLL solution and heated to evaporate off the water and leave a coating of PLL. 20 µL of previously fixed *C. elegans* were deposited into the center of the dish. An inverted optical microscope (Axiovert 200, Zeiss) with a 100x oil immersion objective was used to locate

the *C. elegans* within the field of view. An Asylum Research MFP-3D-Bio AFM (Oxford Instruments) was mounted on top of the optical microscope. Prior to tip engagement on the surface, the eyepiece was used to center the AFM probe (NPG-D, Bruker, nominal spring constant of 0.06 N/m) above a *C. elegans*. Imaging was conducted in contact mode at a scan rate of 0.2 Hz. For each image, the setpoint was optimized so that the minimal amount of force needed to adequately track the surface was applied, which allowed for minimal sample damage and/or movement. The worms remained hydrated in PBS buffer throughout the entirety of imaging. At least three worms of each condition were imaged. Imaging analysis was conducted using Gwyddion (Nečas and Klapetek 2012).

2.2.5. Statistics

Statistical analysis was carried out using SPSS version 22 for Mac. All data were analyzed for normality using the Shairo-Wilk test and equality of variance using Levene's test. Survival data were analyzed using the non-parametric Kruskal-Wallis test with Bonferroni-adjusted post-hoc comparisons. For smFISH experiments, *gpd-1* data were analyzed using a one-way ANOVA with Tukey post-hoc tests, and *cdr-1* data were analyzed using a one-way Welch ANOVA with Dunnett T3 post-hoc tests to account for inequality of variance. Level of significance for statistical analyses was set as $p < 0.05$.

3. Results and discussion

3.1. QD characterization

The optical properties and sizes of the QDs were characterized following their synthesis. The initially synthesized TOPO-functionalized QDs (batch A20a) had an excitation peak at 619 nm and emission peak at 653 nm (Fig 1b and 1c). DHLA-PEG-functionalized QDs (batch A20b) were cap-exchanged from A20a TOPO-functionalized QDs. The second round of TOPO-functionalized QDs synthesized (batch A18d) had an excitation peak at 596 nm and emission peak at 608 nm (Fig 1b and 1c). The OA-functionalized QDs (batch D3a) had a first excitation peak at 515 nm and emission peak at 548nm (Fig 1b and 1c). The emission of each initially synthesized QD was also analyzed for lifetime and quantum yield as a check for QD quality according to previous studies (Fig S1)(Lyons *et al.* 2017). The results of these investigations concluded a lifetime of 27.81 ± 0.11 ns for A20a, 31.96 ± 0.64 ns for A18d, and 20.49 ± 0.03 ns

for D3a. Further, TEM analysis of the QDs was used to confirm the size of the QD calculated from absorbance via equations from the literature.(Jasieniak *et al.* 2009) TEM analysis revealed A20a are 9.26 ± 1.26 nm, A18d are 5.54 ± 0.49 nm, and D3a are 3.67 ± 0.41 nm in diameter (Fig S2). These parameters are similar to those reported for CdSe/ZnS QDs elsewhere (Williams *et al.* 2018).

3.2. Hydrophobic vs hydrophilic QD survival comparison

TOPO QDs caused significant toxicity, reducing *C. elegans* adult lifespan from 2 weeks to only a few days. Importantly, a similar impact was seen for TOPO ligand exposure alone. As shown in Fig 2, lifespan for TOPO ligand and TOPO QD-exposed (A20a) animals were both significantly shorter than water or hexane controls, 1.8 ± 0.2 d and 2.8 ± 0.3 d versus 14.2 ± 0.4 d and 12.8 ± 0.4 d respectively. Lifespan did not differ significantly between TOPO ligand and TOPO QD-exposed worms, and the level of toxicity associated with TOPO QD and ligand exposure exceeds even that of all Cd contained in dosed QDs. While Cd control treatment did cause a significant decline in lifespan versus water and hexane controls, decreasing lifespan to 8.7 ± 0.2 d, this was of a lesser degree than the impact of TOPO ligand or TOPO QDs.

Replacing TOPO with the hydrophilic DHLA-PEG ligand by cap-exchange (A20b) dramatically reduces QD toxicity at 1 μM exposure, for a lifespan of 10.4 ± 0.5 d. DHLA-PEG QDs had a small but significant impact on lifespan vs the water control, but DHLA-PEG ligand alone had no impact (lifespan 12 ± 1 d). Toxicity observed for DHLA-PEG QDs is similar to that seen with other hydrophilic QDs in *C. elegans* (Hsu *et al.* 2012, Contreras *et al.* 2013, Wang *et al.* 2016). Water and hexane controls did not differ significantly from each other.

Similar to the results observed here, TOPO-functionalized CdSe QDs were shown to cause toxicity in hepatocytes, with this toxicity being mitigated by cap-exchange with a hydrophilic mercaptoacetic acid ligand (Derfus *et al.* 2004), similar to mitigation seen here with DHLA-PEG functionalization. In contrast to these results, water solubilization of TOPO-functionalized CdSe/ZnS QDs by coating with gum arabic created considerable toxicity in *Daphnia magna* versus cap-exchanging with mercaptopropionic acid (Lee *et al.* 2010), demonstrating that the

method of creating a hydrophilic particle from TOPO-functionalized QDs can greatly influence particle toxicity, with cap exchange providing toxicity mitigation superior to surface coating.

The importance of surface coating in determining ENM toxicity is well-established, including for QDs (Hu *et al.* 2016) and other ENMs, such as Au (Bozich *et al.* 2014, Dominguez *et al.* 2015, Feng *et al.* 2015, Qiu *et al.* 2015). Given the drastic difference in toxicity we observed between DHLA-PEG and TOPO QDs, and with the impact of TOPO QDs and ligands exceeding even those of Cd, we chose to explore the interaction of TOPO QDs and ligand with *C. elegans* further.

3.3. Investigation of TOPO QD and ligand impacts by fluorescence imaging, AFM, and smFISH

Other studies have shown accumulation of hydrophilic QDs in the *C. elegans'* intestine (Qu *et al.* 2011, Contreras *et al.* 2013, Wang *et al.* 2016). However, we only observed hydrophobic TOPO QDs associated with the exterior of *C. elegans*. Initial fluorescence imaging of picked, non-fixed animals revealed TOPO QD agglomerates (Å 18d) on the exterior of worms (Fig 3). The aggregation of TOPO QDs and their adherence to the surface of *C. elegans* is similar to the aggregation and adherence of TOPO-functionalized CdS QDs to root surfaces observed in soybean (Majumdar, Ma, *et al.* 2019). Our observation of TOPO QDs only on the exterior of *C. elegans* led to the hypothesis that TOPO QDs and ligand may cause toxicity by damaging the cuticle, the collagenous outer layer responsible for protecting *C. elegans* from their environment (Chisholm and Xu 2012). However, AFM imaging of fixed, exposed animals demonstrated no observable impact on cuticle structure in larvae from TOPO QDs or ligand, even at an extremely lethal 5 μM dose (Fig 4). In addition, no QDs were visible in fixed samples, likely due to washing steps removing QDs associated with the worm surface for fixed sample imaging.
The absence of cuticle impact is underscored by the lack of any change in expression of *gpdh-1* from exposure to TOPO QDs or ligands (Fig 5a). The *gpdh-1* gene would be expected to be upregulated in response to osmotic stress if the cuticle had in fact been damaged by TOPO QDs or ligand (Lamitina *et al.* 2006, Chisholm and Xu 2012, Dodd *et al.* 2018), but smFISH analysis of *gpdh-1* in the gut determined its expression did not differ significantly among treatment groups. Expression of Cd-responsive *cdr-1* in the gut also did not differ significantly between

TOPO QDs and controls, although Cd control did show significant upregulation of *cdr-1* compared to hexane control (Fig 5b). Lack of a significant response of *cdr-1* reinforces the observation in lifespan assays (Fig 2) that Cd toxicity alone cannot account for the toxicity of TOPO QDs. Also undermining QD dissolution and Cd release as a likely source of toxicity, QDs and QD agglomerates were still visible on exposure plates (not shown) and visibly associated with the worm exterior (Fig 3), suggesting most QDs remained intact during exposure. Thus, as is the case for hydrophilic QDs reported in the literature (Hsu *et al.* 2012, Contreras *et al.* 2013, Wang *et al.* 2016), released Cd is unlikely to be the source of observed toxicity for TOPO QDs, and in fact cannot account for observed toxicity from TOPO QDs in this study. Instead, TOPO QD toxicity is likely attributable to the TOPO ligand itself.

3.4. Potential for TOPO displacement from the QD surface

The lack of observable uptake of particles by worms, the lack of damage to the cuticle by associated QDs, and the equivalent toxicity observed for TOPO QD and ligand exposures, suggest that free TOPO may be the source of observed toxicity in TOPO QD exposures. The TOPO ligand is easily displaced by other molecules according to the literature (Bullen and Mulvaney 2006), and is considered to be a weaker binding ligand than organic acids such as stearic acid or OA (Knittel *et al.* 2013). TOPO has been shown to be displaced by amines (Koole *et al.* 2008), thiols (Breus *et al.* 2007), and humic and fulvic acids (Navarro *et al.* 2009), all of which are likely to be found in complex biological matrices. Thus, upon mixture with concentrated OP50 *E. coli*, TOPO molecules may be displaced from the QD surface in an *in situ* cap-exchange with biomolecules excreted by bacteria or present in the bacterial media. Free TOPO displaced from the QD surface would then be able to come into contact with *C. elegans* and induce toxicity. It should be noted that direct transfer to TOPO-bound ligands to *C. elegans* membranes upon association of TOPO-coated QD with *C. elegans* could provide an additional toxicity mechanism. Regardless of whether the adverse interactions of TOPO-coated QD with *C. elegans* are driven by free or bound TOPO molecules, our study clearly shows the importance of the capping ligand, in this case TOPO in the toxicity of the QD. The toxicity of free TOPO has been previously demonstrated in cells (Hoshino et al. 2004), supporting that TOPO in a free or bound form is a likely source of the toxicity observed here in *C. elegans*.

3.5. Potential neuromuscular impacts of TOPO

Fluorescence imaging of phalloidin-stained worms revealed marked straightening of the pharynx in TOPO ligand and QD exposed larvae (Fig 6). This straightening, along with observation of spasming in animals upon exposure to TOPO QDs and ligand (personal observation), suggests a potential neuro-muscular mechanism of TOPO toxicity. TOPO is amphiphilic, containing both polar and non-polar portions due to its P=O bond dipole and alkyl chains, respectively. The P=O bond creates a strong dipole (Gilheany 1994), on the order of 5 D for TOPO (whereas OA has a much weaker dipole: 0.7 D for the cis conformer and -0.1 D for the trans)(Wawrzynczyk *et al.* 2013). Amphiphilic dyes such as DiI preferentially stain the plasma membrane of *C. elegans* exposed sensory neurons (Tong and Bürglin 2010), and amphiphiles such as the hydrophobic anion TPB- have been shown to incorporate into the plasma membrane of cells where they can impact membrane charge (Zimmermann *et al.* 2008) and even act as antagonists of GABA and NMDA neural receptors (Chisari *et al.* 2011, Linsenbardt *et al.* 2013).

Hydrophobicity has been demonstrated to be an important determinant of the biological potency of compounds, with increased hydrophobicity increasing pharmaceutical impact on GABA receptor activity *in vitro*, likely due to increased concentration of hydrophobic compounds in the membrane (Chisari *et al.* 2009). The observed accumulation of ion channel proteins in TOPO-QD exposed soybean roots supports the impact of TOPO QDs on membrane proteins and ion transport (Majumdar, Pagano, *et al.* 2019). The localization of Cd to membranes in soybeans exposed to TOPO QDs also supports membrane impacts of these ENMs (Majumdar, Ma, *et al.* 2019). Specifically supporting neurotoxicity as a mechanism, axonal degeneration has been observed in PC12 cells exposed to CTAB/TOPO QDs (Mahto *et al.* 2010). Thus, observations in our study and others support membrane impacts of TOPO QDs, with neurotoxicity as one potential effect of these membrane impacts.

The GABA receptor is the target of the anti-parasitic drug ivermectin, which also impacts the pharynx and causes death in *C. elegans* (Lespine *et al.* 2009). Organophosphorus (OP) compounds are also known to act as nerve agents (e.g. sarin, VX) and used as pesticides (e.g. dichlorvos, tetrachlorvinphos), with the P=O bond facilitating inhibition of acetylcholinesterase (AChE), which results in neurotoxicity (Jokanović 2018). OP pesticides have been shown to

inhibit AChE and cause mortality in *C. elegans* at concentrations as low as 3 μM (Rajini *et al.* 2008). *C. elegans* have NMDA, GABA, and acetylcholine receptors (Bargmann 1998). Thus, due either to its amphiphilic nature and likely infiltration of membranes, its P=O bond, or both, TOPO may potentially disrupt normal neuron function, which could lead to the changes in the pharynx, spasming, and mortality observed in our study.

3.6. Hydrophobic TOPO vs OA QD survival comparison

The toxicity of TOPO raises the question if hydrophobicity alone is sufficient to confer enhanced toxicity to QDs, or if alternative hydrophobic ligands can be used that are relatively non-toxic. As shown in Fig 7, synthesizing QDs with alternative OA ligands completely negates QD toxicity even at 1 μM exposure. While TOPO ligand and 0.2 and 1 μM TOPO QD (A18d) exposures all significantly shortened lifespan compared to water and hexane controls (1.3 ± 0.1 d, 6.8 ± 0.3 d, and 3.1 ± 0.4 d versus 14.4 ± 0.3 d and 13.4 ± 0.4 d, respectively), OA ligand and OA QD-exposed (D3a) animals at 0.2 or 1 μM (lifespans 14.5 ± 0.3 d, 13.3 ± 0.2 d, and 12.4 ± 0.3 d, respectively) showed no difference in lifespan from water or hexane controls. Thus, not all hydrophobic ligands induce toxicity, and it is possible to redesign QDs to produce less-toxic hydrophobic QDs, in this case by substituting TOPO with OA. This is a valuable result, as previous studies of TOPO QD toxicity have focused on comparisons to hydrophilic QDs without including alternative hydrophobic QDs—necessary to determine if TOPO toxicity is due to hydrophobicity in general or, as is demonstrated in this study, is specific to the TOPO ligand itself (Derfus *et al.* 2004, Hoshino *et al.* 2004, Lee *et al.* 2010).

To our knowledge, the comparative toxicity of OA-functionalized QDs has not been explored in the literature. A comparison has been made between hydrophilic polyvinylpyrrolidone and OA-functionalized Ag ENMs in earthworms (Shoults-Wilson *et al.* 2011), with no significant difference in toxicity observed between Ag ENMs functionalized with these two ligands. Given this and the relative lack of toxicity of OA QDs compared to the more common TOPO QDs observed in our study, further exploration of OA as an alternative hydrophobic ligand for QDs and other ENMs is warranted.

3.7. Implications

We have shown toxicity of hydrophobic QDs to be due specifically to TOPO ligand. Although QDs for displays in current technology will be embedded in polymers (Gallagher *et al.* 2018), TOPO would be expected to contribute to the hazardous waste from QD manufacturing (Engül and Theis 2011). The relatively non-toxic nature of OA-functionalized hydrophobic QDs suggests that this method of synthesis for QDs may greatly reduce the potential toxicity of QD manufacturing waste. Some literature also suggests that performance of OA QDs may be superior to TOPO QDs (Chen *et al.* 2009, Lee *et al.* 2013). Life-cycle assessment and thorough performance comparisons between TOPO and OA-functionalized QDs should be carried out to determine if hydrophobic OA QDs represent a viable 'greener' alternative to standard TOPO QDs.

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Credit Author Statement

Nicholas Niemuth: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Resources, Writing-Original Draft, Writing - Review & Editing; **Denise Williams**: Resources, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization; **Arielle C. Mensch**: Methodology, Investigation, Writing - Original Draft, Visualization; **Yi Cui***:* Methodology, Formal analysis, Investigation, Data curation, Writing - Original Draft, Visualization; **Galya Orr:** Conceptualization, Methodology, Validation, Writing- Reviewing and Editing, Funding acquisition, Supervision; **Ze'ev Rosenzweig**: Conceptualization, Methodology, Validation, Writing- Reviewing and Editing, Funding acquisition, Supervision; **Rebecca Klaper:** Conceptualization, Methodology, Validation, Writing- Reviewing and Editing, Funding acquisition, Supervision.

Declaration of interests

The authors declare no competing interests.

Supplementary data

Supplementary material

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Figure 1. CdSe/ZnS quantum dots and spectra. Quantum dots (QDs) used for this study displayed (a) in a cartoon format illustrating the ligands and types of comparisons between QDs of different functionalizations in this study. Normalized (b) absorbance and (c) emission intensity of QDs.

Figure 2. Impact of hydrophobic and hydrophilic quantum dots on *C. elegans* lifespan. Percent survival and adult lifespan of *C. elegans* exposed to water control, hexane control, Cd at 1 μM equivalent, trioctylphosphine oxide (TOPO) ligand at 1 μM equivalent, TOPO-functionalized CdSe/ZnS quantum dots (QDs) at 1 μM, dihydrolipoic acid-polyethylene glycol (DHLA-PEG) ligand at 1 μM equivalent, and DHLA-PEG CdSe/ZnS QDs at 1 μM. For bar graphs, columns with different letters indicate significant difference ($p < 0.05$) by Kruskal-Wallis non-parametric analysis with Bonferroni-adjusted pairwise comparisons, and error bars represent standard error of the mean.

Figure 3. Association of TOPO-functionalized QDs with *C. elegans* cuticle. Bright field (a) and red fluorescence images (b) of *C. elegans* exposed to trioctylphosphine oxide (TOPO)-functionalized CdSe/ZnS quantum dots at 5 μM for 24 h. Worms were picked from exposure plate using Pt wire. White arrows indicate TOPO quantum dot agglomerates associated with the cuticle of exposed *C. elegans*.

Fig 4. Atomic force microscopy of *C. elegans* Atomic force microscopy of *C. elegans* L1 larvae exposed for 2 h on an OP50 lawn spiked with: (a,b) water, (c,d) TOPO ligand at 0.2 μM equivalent, (e,f) TOPO QDs at 0.2 μM, and (g,h) TOPO QDs at 5 μM. No difference in cuticle structure was evident after exposure. All lateral scale bars are 2 μm.

Figure 5. Expression of *gpdh-1* and *cdr-1* by smFISH. Expression of (a) *gdph-1* and (b) *cdr-1*

in gut tissue of synchronized L1 larvae exposed to water control, hexane control, Cd control at 0.2 μM equivalent, trioctylphosphine oxide (TOPO) ligand at 0.2 μM equivalent, and TOPO-functionalized quantum dots at 0.2 μM for 6 h. No significant differences between treatments were detected in *gpdh-1* expression (a) by one-way ANOVA. Columns with different letters in (b) indicate significant difference ($p < 0.05$) in *cdr-1* expression by one-way Welch ANOVA with Dunnett T3 pairwise comparisons. Error bars represent standard error of the mean.

Fig 6. Staining of *C. elegans* actin. Phalloidin staining of actin in *C. elegans* L1 larvae exposed for 6 h on a lawn of OP50 containing: (a) water, (b) hexane solvent control, (c) Cd equivalent to 0.2 μM QDs, d) TOPO ligand at 0.2 μM equivalent, and e) TOPO QDs at 0.2 μM. Note abnormal straightening of the pharynx in TOPO ligand and QD-exposed animals (yellow arrows). Scale bar represents 10 μm.

Fig 7. Comparison of impacts of hydrophobic QDs with different ligands on *C. elegans* lifespan. Percent survival and adult lifespan of *C. elegans* exposed to water control, hexane control, Cd at 1 μM equivalent, oleic acid (OA) ligand at 1 μM equivalent, OA-functionalized CdSe/ZnS QDs at 0.2 and 1 μM, TOPO ligand at 1 μM equivalent, and TOPO-functionalized CdSe/ZnS QDs at 0.2 and 1 μM. For bar graphs, columns with different letters indicate significant difference ($p < 0.05$) by Kruskal-Wallis non-parametric analysis with Bonferroni-adjusted pairwise comparisons, and error bars represent standard error of the mean.

Graphical abstract

Highlights

- Hydrophobic TOPO QDs are significantly more toxic than hydrophilic DHLA-PEG QDs.
- TOPO ligand alone is as toxic as TOPO QDs.
- TOPO QDs loosely associate with but do not damage the cuticle.
- Straightening of the pharynx and spasming suggest neuro-muscular TOPO toxicity.
- Substitution of TOPO with OA yields relatively non-toxic hydrophobic QDs.