

Surface chemistry, charge and ligand type impact the toxicity of gold nanoparticles to *Daphnia magna*[†]

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Nanoparticles (NPs) are the basis of a range of emerging technologies used for a variety of industrial, biomedical, and environmental applications. As manufactured NP production increases, so too does the concern about their release into the environment and potentially harmful effects. Creating nanomaterials that have minimal negative environmental impact will heavily influence the sustainability of nanomaterials as a technology. In order to create such NPs, the mechanisms that govern NP toxicity need to be better elucidated. One aspect of NP structure that may influence toxicity is the identity and charge of ligand molecules used to functionalize the NP surface. These surface chemistries have the potential to increase or decrease negative biological impacts, yet their impacts are poorly understood. In this study, the toxicity of three types of functionalized ~4–5 nm gold NPs (AuNPs), polyallylamine hydrochloride (PAH–AuNPs), citrate (Cit–AuNPs) and mercaptopropionic acid (MPA–AuNPs) as well as cetyltrimethylammonium bromide-functionalized gold nanorods (CTAB–AuNRs) were evaluated in the toxicological model species, *Daphnia magna*. In order to get the most detailed information on NP toxicity in *D. magna*, both acute and chronic toxicity assays were performed. Acute exposure toxicity assays show that overall the negatively-charged AuNPs tested are orders of magnitude less toxic than the positively-charged AuNPs. However, chronic exposure assays show that both positively and negatively-charged particles impact reproduction but potentially through different mechanisms and dependent upon functional group. In addition, while select ligands used in NP functionalization (such as CTAB) that are toxic on their own can contribute to observed NP toxicity, our acute toxicity assays indicate that minimally toxic ligands (such as PAH) can also cause significant toxicity when conjugated to NPs. This research demonstrates that surface chemistry plays a pivotal role in NP toxicity and that surface chemistry has the potential to affect the sustainability of these materials.

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Nano impact

Understanding the impacts of engineered NP exposure on aquatic organisms is essential to predict the environmental implications of nanotechnology, however issues with nanomaterial synthesis and characterization often plague these studies. In this study the acute and chronic toxicity of well characterized gold nanoparticles functionalized with ligands of differing charges were investigated in *Daphnia magna*. We found that AuNP toxicity was highly dependent on both NP charge and ligand identity where positively charged nanomaterials were more toxic and certain ligands were only toxic when associated with the nanomaterial. Select negatively charged nanomaterials also impacted reproduction. This experiment illustrates the need for well-characterized nanomaterials to determine the impacts of nanomaterial properties on toxicity to inform the safe and sustainable development of nanomaterials.

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Introduction

In the U.S. alone, the engineered nanoparticle (NP) industry is a multibillion-dollar industry and is predicted to increase to a one-trillion-dollar industry by 2015.¹ One study estimates that 63–91% of over 260 000–309 000 metric tons of the world NP production in 2010 entered our environment through landfills.² As production increases, there is a concern about



cellular imaging,⁴² bio-chemical sensing,⁴³ drug and gene delivery,^{44,45} to medical therapeutics.⁴⁶ The successful implementation of AuNPs in these applications depend strongly upon appropriate particle functionalization.⁴⁷

Although many studies on toxicity of NPs have been conducted to date, the mechanisms that govern NP toxicity are still in question as experimental artifacts and the design of experiments can impede understanding of the fine scale interactions of NPs with biological entities.⁶ For example, the charge of the NP surface has been implicated as a major factor in toxicity.^{7–12} Yet the initial charge of a particle may be altered during an experiment due to interactions with media.^{13–16} Particle size and surface chemistry have also been suggested to impact NP toxicity^{17–23} as both particle size and surface chemistry can influence particle uptake and partitioning in organelles and tissues.^{24–29} The density of ligands on the NP surface is often poorly characterized, however it may also have an impact on toxicity.^{30,31} Aggregation over time in experimental media or in the environment, can either increase or decrease NP toxicity and may have a significant impact on toxicity.^{32–34} Byproducts from particle synthesis or the ligands alone may cause toxicity.³⁵ Few studies attempt to include controls for several of these factors, which can complicate conclusions of properties associated with toxicity. Finally, many studies that have been conducted to date have been carried out using *in vitro* systems and only over short time periods, which may not accurately reflect real world whole organismal interactions with NPs.^{8,36,37}

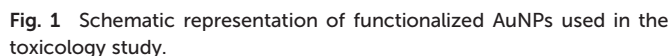
Materials and methods

Nanoparticle synthesis and functionalization

All materials were used as received, unless otherwise noted. Gold tetrachloroaurate trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), 3-mercaptopropionic acid (MPA), sodium borohydride (NaBH_4), polyallylamine hydrochloride (PAH; M_w 15 000 g mol^{-1}), and silver nitrate (AgNO_3) were obtained from Sigma Aldrich. Hexadecyltrimethyl ammonium bromide (CTAB), L-ascorbic acid, and trisodium citrate were obtained from Sigma. Deionized water was prepared using a Barnstead NANOPURE water filtration system. PALL Minimate tangential flow filtration capsules for AuNP purification, with 50 kD pore size was obtained from VWR. TEM grids, SiO on copper mesh (PELCO) were used for transmission electron microscopy studies. Functionalized AuNPs were synthesized using previously reported methods in a millifluidic reactor, which enabled high-throughput NP synthesis. The millifluidic reactor for NP synthesis was assembled (as previously described) from commercially available components: a peristaltic pump (Cole-Palmer Masterflex L/S), Tygon polyvinyl tubing (ID = 2.79 mm), polyethylene Y-mixers (ID = 1.79 mm), and polyethylene joints.⁴⁸ AuNPs were synthesized in the reactor at an overall flow rate of 50.0 mL min^{-1} , and experienced a residence time of 3.0 min for the spherical AuNP syntheses, and 20.0 min for the gold nanorod synthesis.

NPs stabilized with four different surface chemistries were synthesized: (1) citrate-functionalized NPs (“Cit–AuNPs”), (2) poly(allylamine) hydrochloride (“PAH–AuNPs”), (3) mercaptopropionic acid-functionalized NPs (“MPA–NPs”) and (4) cetyl trimethylammonium bromide-functionalized gold nanorods (“CTAB–AuNRs”) (Fig. 1).

Cit–AuNPs (5.0 nm). 5.0 nm citrate AuNPs were synthesized using previously reported procedures.⁴⁹ In a typical synthesis, 4.0 L of a growth solution, consisting of aqueous gold tetrachloroaurate (HAuCl₄, 10.0 mM) and 10.0 mM sodium citrate_(aq), as well as a 4.0 L of an aqueous solution of sodium borohydride (5.0 mM) were prepared. The two solutions were flowed together in a millifluidic synthesis reactor at a flow rate of 50.0 mL min^{−1}, and experienced a residence time of



MPA–AuNPs (4.0 nm). Thiol-stabilized AuNPs were prepared by direct synthesis with sodium borohydride according to previously reported methods.^{50,52} Briefly, two 4.0 L aqueous solutions (a growth solution and a reducing agent solution) were prepared. The growth solution contained HAuCl₄ (3.0 mM) and MPA (6.0 mM) dissolved in nanopure deionized water. The pH of the growth solution was adjusted to approximately 8.5 by the addition of sodium hydroxide. The other 4.0 L solution consisted of 5.0 mM-aqueous NaBH₄. The two

CTAB-stabilized gold nanorods (AuNRs). CTAB-stabilized gold nanorods with aspect ratio (length/width) 4.0 were synthesized using our previously reported seeded growth procedures.⁵³ Two solutions were prepared: a growth solution and a “Seed” solution. For the growth solution, 10.0 mL HAuCl₄ (0.1 M), 16.0 mL AgNO₃ (0.01 M), and 11.0 mL of ascorbic acid (0.1 M) were added to 1.0 L of a 0.1 M aqueous CTAB solution. For the seed solution, 2.4 mL of a previously prepared gold NP seed dispersion (aged 2 hours) was added to 998.0 mL of a 0.1 M CTAB solution. The solutions were mixed within the flow reactor (flow rate = 50.0 mL min⁻¹), and the AuNR growth solution experienced a residence time of approximately 15.0 min in the reactor before being deposited into an *aqua regia*-cleaned 4.0 L bottle. AuNRs were purified and concentrated by centrifugation and washing (two times, 20 min at 11 000 rcf) with nanopure deionized water.

Ligand densities for particles at the beginning of the experiment were determined using XPS. Detailed procedures for XPS sample preparation and analysis are presented in the ESI.[†] Briefly, NP solutions were dripped onto Si wafers, and XPS features characteristic for each molecule were measured and normalized against gold as an internal standard. A quantitative numerical modeling procedure was validated and used to correct for NP curvature and scattering effects. These measurements are presented in Table 3.

Table 1 AuNP characterization as synthesized

AuNP sample	SPR λ_{max} (nm)	d_{core} (nm)	D_{h} (nm)	ζ -potential (mV)
CTAB-AuNRs	512 778	50×14	20.7 ± 0.5	16.7 ± 1.5
PAH-AuNPs	524	4.7 ± 1.2	17.9 ± 0.9	17.9 ± 0.9
Cit-AuNPs	518	4.9 ± 1.4	12.8 ± 1.2	-15.3 ± 1.5
MPA-AuNPs	512	3.8 ± 1.1	8.0 ± 1.2	-18.5 ± 1.3

Table 3 AuNP ligand density determined by XPS

AuNP sample	Measured density
CTAB-AuNR	7.6×10^{14} and 1.39×10^{15} molecules cm^{-2}
PAH-AuNP	1.12×10^{15} formula units cm^{-2}
Cit-AuNP	4.7×10^{14} molecules cm^{-2}
MPA-AuNP	5.6×10^{14} molecules cm^{-2}

sample is given as a single standard deviation from the mean core diameter. The surface charge of the AuNPs was determined using ζ -potential analysis (Table 1).

The ligand density of the AuNP library was determined using XPS and with the exception of CTAB-AuNR Table 3 shows that the ligand densities for Cit-AuNP and MPA-AuNP are nearly the same and are also nearly identical to values previously reported for densely-packed self-assembled monolayers on planar gold surfaces.⁶¹ Because CTAB-AuNR is a rod shaped particle, a max/min range based on the assumption of a planar and spherical particle has been provided. As the rods still possess a significant amount of curvature the ligand density should be closer to the lower density estimate. Though there are physically more CTAB molecules, CTAB coats the AuNR in a bilayer.^{62,63} Thus, the increased number of CTAB ligands is not due to a higher packing density. The number of ligands on the outer leaflet of CTAB-AuNR is comparable to the other functionalized particles in this study. For PAH-AuNP, the polymeric nature of the PAH ligand makes it difficult to determine an equivalent molecular density.

Stability of AuNPs in daphnid media

In order to better connect the physiochemical properties of the AuNPs tested with their acute mortality in *Daphnia magna*, we monitored the changes in the AuNP physiochemical properties the AuNPs undergo following dispersion in daphnid media using UV-vis absorption spectroscopy, DLS, and ζ -potential analysis. The positively-charged AuNPs (the CTAB-AuNRs and the PAH-AuNPs) showed no evidence of changes in size, shape, or aggregation state (based on their UV-vis spectra, Fig. S3† and DLS analysis, Table 2). In contrast, the negatively-charged AuNPs show evidence of aggregation following dispersion in daphnid media. This aggregation is evidenced by broadening of the Surface Plasmon Resonance (SPR) absorbance (~ 520 nm) in the UV-vis spectra, and a significant increase in their hydrodynamic diameter over the course of the 48 h exposure time (D_{h} , see Table 2). Interestingly, the

structure of the aggregated Cit-AuNPs appears to be different from the MPA-AuNP aggregates. TEM analysis of the AuNP aggregates shows significant fusion of the Cit-AuNP cores to form larger AuNPs and wire-like structures after 48 h, while the MPA-AuNP aggregates appear to consist primarily of large, loosely bound networks of AuNPs with an ~ 4 nm primary particle size (Fig. 2). The MPA-AuNPs could be re-suspended from their aggregated state by gentle agitation, while the Cit-AuNPs could not, which would be consistent with the structure of the aggregates observed in the TEM images (Fig. S5†).

Effects of AuNPs on daphnid acute mortality

Particle surface chemistry and stability played an important role in daphnid survival, with positively-charged particles, CTAB-AuNRs and PAH-AuNPs, being orders of magnitude more toxic than the negatively-charged particles, Cit-AuNPs and MPA-AuNPs. CTAB and PAH-AuNPs significantly affected daphnid mortality (93% mortality, $U = 0$, $p < 0.05$; and 40% mortality, $U = 0$, $p < 0.05$, respectively) at concentrations as low as $10 \mu\text{g L}^{-1}$ (Fig. 3). However, PAH and CTAB-AuNPs demonstrated similar toxicity at $5 \mu\text{g L}^{-1}$, showing 13% mortality. Cit and MPA-AuNPs did not significantly affect *Daphnia magna* mortality at all concentrations tested ($p > 0.05$), which reached an order of magnitude higher than the positively charged particle. Positively-charged particles were also more stable in the MHRW media (Table 2).

Of the four ligands tested, CTAB was the only ligand that by itself significantly affected *D. magna* mortality compared to control daphnids (Fig. 3). CTAB ligand caused the same daphnid mortality as CTAB-AuNR acute exposure, causing 13% mortality at $5 \mu\text{g L}^{-1}$, 93% mortality at $10 \mu\text{g L}^{-1}$ and 100% mortality at $50 \mu\text{g L}^{-1}$ (Fig. 3). The PAH ligand, unlike the PAH-AuNP, did not affect daphnid mortality at the concentrations that were significantly impacting daphnid mortality in the PAH-AuNP acute assay (Fig. 3). Citrate and MPA free ligands, like Cit and MPA-AuNPs, had no impact on daphnid mortality.

When comparing toxicity to that of supernatants and filtrates (collected from the particle purification process), only the supernatants significantly impacted daphnid survival with PAH-AuNP supernatant being the more toxic of the two positively-charged supernatants (Fig. 2). PAH-AuNP supernatant significantly affected daphnid mortality up to $5 \mu\text{g L}^{-1}$ causing 73% mortality ($U = 0$, $p < 0.05$) and 100% mortality at 10 and $50 \mu\text{g L}^{-1}$ ($U = 0$, $p < 0.05$). UV-vis and TEM analysis of the PAH-AuNP supernatant indicated that, unlike the other supernatants tested in this study, the PAH-AuNP

Table 2 AuNP characterization in daphnid media

AuNP sample	Time (h)	D_{h} (nm)	Z-potential (mV)
CTAB-AuNRs	0	16.6 ± 2.0	28.1 ± 1.6
CTAB-AuNRs	48	17.5 ± 0.2	27.7 ± 1.8
PAH-AuNPs	0	52.8 ± 1.7	11.8 ± 5.2
PAH-AuNPs	48	55.9 ± 4.1	20.4 ± 0.4
Cit-AuNPs	0	21.9 ± 0.9	-15.3 ± 1.5
Cit-AuNPs	48	90.8 ± 5.9	-5.6 ± 0.2
MPA-AuNPs	0	50.7 ± 1.1	-9.1 ± 5.3
MPA-AuNPs	48	750.6 ± 8.2	-11.0 ± 1.0



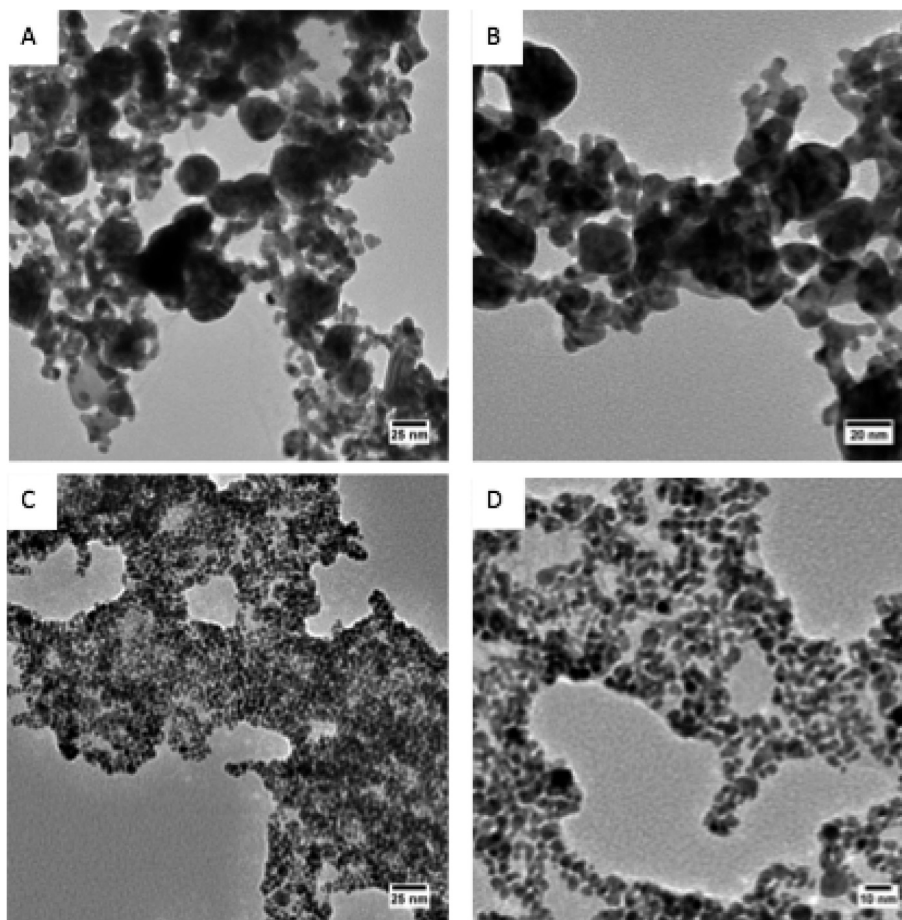


Fig. 2 Agglomerated vs. aggregated AuNPs. TEM images of 4.0 nm Cit-AuNPs (A, B) and MPA-AuNPs (C, D) immersed in daphnid media for 48 h. Close examination of the aggregated Cit-AuNPs reveal that the AuNPs have formed extended networks of irregular nanowires and large particles. In contrast, the MPA-AuNPs remain dispersed, but closely associated individual AuNPs. Scale bar in (A) and (C) is 25 nm. Scale bar in (B) is 20 nm. Scale bar in (D) is 10 nm.

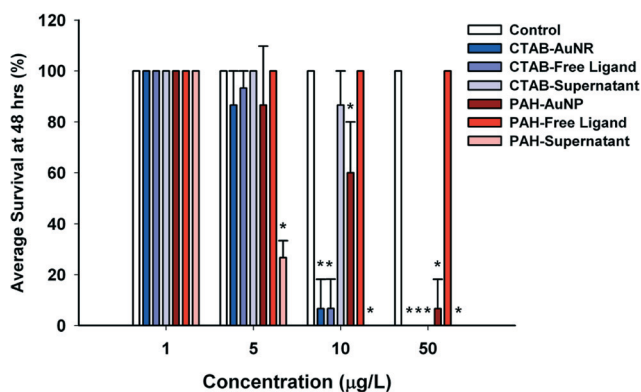


Fig. 3 Effects of AuNPs, free ligands and impurities on daphnid acute mortality. Effects of AuNP exposure on daphnid mortality after 48 hour exposure to 1, 5, 10 and 50 $\mu\text{g L}^{-1}$ CTAB or PAH-AuNPs, free ligands and supernatants. Acute mortality evaluated by Mann-Whitney *U* test for two independent samples. Asterix indicate significant difference from control ($p < 0.05$).

supernatant contained a low concentration of very small ($d_{\text{core}} < 4.0$ nm) AuNPs. The UV-vis spectrum of the PAH-AuNP supernatant and a representative TEM image are provided in

the ESI† as Fig. S7. CTAB–AuNR supernatant significantly caused mortality at 50 $\mu\text{g L}^{-1}$, eliciting 100% mortality in *D. magna* exposures ($U = 0$, $p < 0.05$).

Effects of chronic AuNP exposures on daphnid survival, reproduction, and body size

PAH (positively charged) and Cit-AuNPs (negatively charged) were the only functionalized AuNPs that significantly decreased daphnid reproduction over the 21 day chronic exposure (Fig. 4a and b). PAH-AuNPs significantly decreased reproduction at $5 \mu\text{g L}^{-1}$ (12% decrease, $U = 10$, $p < 0.05$) (Fig. 4a). In contrast, the free ligand PAH, significantly increased reproduction in daphnids (10% increase, $U = 7$, $p < 0.05$; and 13% increase, $U = 8$, $p < 0.05$) at both $1 \mu\text{g L}^{-1}$ and $5 \mu\text{g L}^{-1}$ concentrations. Daphnid exposed to the CTAB free ligand also experienced increased reproduction by 20% in $5 \mu\text{g L}^{-1}$ exposures ($U = 3$, $p < 0.05$) but the CTAB supernatant decreased reproduction by 18% at $1 \mu\text{g L}^{-1}$ ($U = 9.5$, $p < 0.05$). For negatively charged particles, Cit-AuNPs significantly decreased daphnid reproduction (97% decrease, $U = 0$, $p < 0.05$) at 25 mg L^{-1} averaging only 3 *D. magna* neonates per individual compared

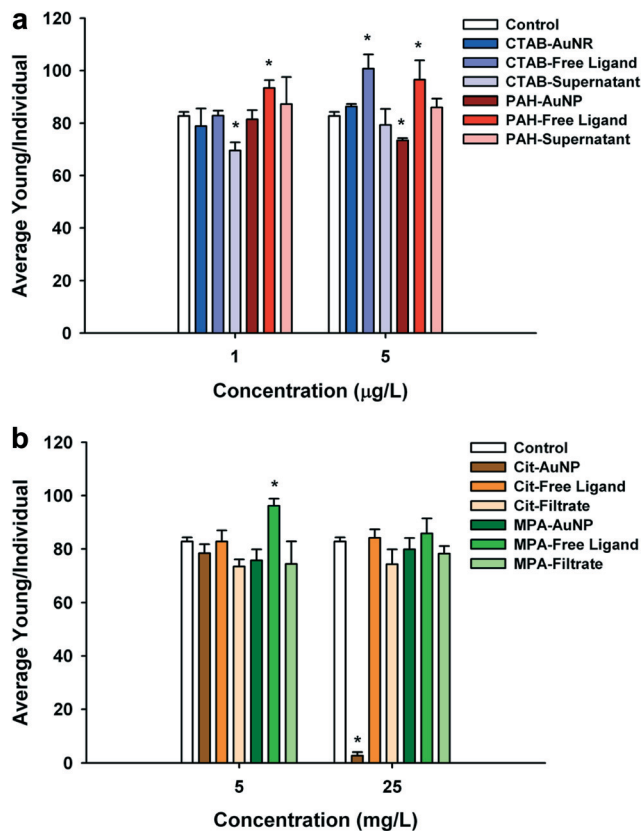


Fig. 4 Effects of AuNPs, free ligands and impurities on daphnid reproduction in chronic assays. Effects of AuNP exposure on (a) daphnid reproduction after 21 day exposure to 1 and 5 $\mu\text{g L}^{-1}$ CTAB or PAH-AuNPs, free ligands and supernatants and (b) daphnid reproduction after 21 day exposure to 5 and 25 mg L^{-1} Cit or MPA-AuNPs, free ligands and filtrates. Reproduction evaluated by Mann-Whitney *U* test for two independent samples. Asterisk indicate significant difference from control ($p < 0.05$).

to 83 neonates per individual control daphnids (Fig. 4b). MPA-AuNPs had no impact yet at 5 mg L⁻¹ the MPA free ligand increased reproduction by 14% ($U = 4$, $p < 0.05$).

Daphnia magna exposed to 25 mg L⁻¹ Cit-AuNPs and MPA-AuNPs exhibited a significant reduction in adult body size as compared to control daphnids ($U = 0, p < 0.05$; and $U = 0, p < 0.05$) (Fig. 5), while daphnids exposed to CTAB and PAH showed no significant reduction in daphnid body size. At 25 mg L⁻¹, Cit-AuNPs reduced daphnid growth by 15% compared to control daphnids, having an average length of 3.36 mm compared to 3.85 mm, respectively and exposure to MPA-AuNPs elicited a 7% reduction in daphnid body size, averaging 3.57 mm body length. Since chronic exposures were conducted at sub lethal levels as determined by acute exposures we did not observe any significant mortality for any of the chronic NP exposures.

Effects of surface chemistry on AuNP uptake in *Daphnia magna*

The surface chemistry of the AuNPs was found to have minimal influence on NP uptake by daphnids in this study (Fig. S6†), as ICP-MS analysis indicated that all the AuNPs tested were found to be taken up by daphnids in similar amounts (on a

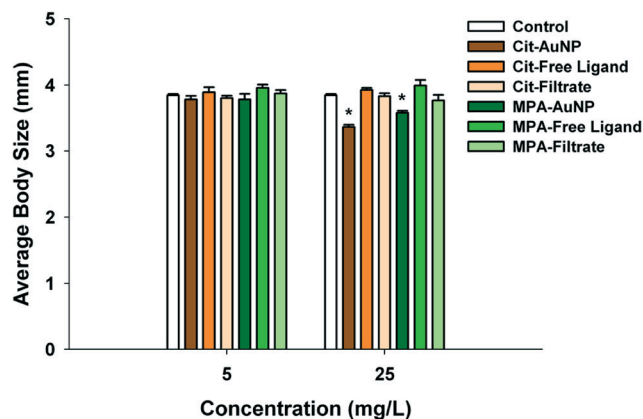


Fig. 5 Effects of AuNPs on daphnid body size after 21 day chronic exposure. Effects of AuNP exposure on daphnid body size after 21 day exposure to 5 and 25 mg L⁻¹ of Cit or MPA AuNPs. Body size evaluated by Mann-Whitney *U* test for two independent samples. Asterix indicate significant difference from control ($p < 0.05$).

per mass basis). However, MPA-AuNPs were significantly taken up ($p < 0.05$) to a greater extent when compared to PAH-AuNPs and Cit-AuNPs even though it is still relatively comparable throughout treatments. Additionally, there was no significant difference in the quantity of NPs on the daphnid carapace and minimal accumulation across all treatments when iodide treated samples were compared to samples without the iodide treatment.

Discussion

Initial particle charge significantly impacted overall toxicity observed in this study, with positively-charged particles being more toxic than their negatively-charged counterparts. Positively-charged particles, PAH and CTAB-AuNPs, were orders of magnitude more toxic than negatively-charged citrate and MPA-AuNPs, significantly eliciting mortality in *Daphnia magna* down to a concentration of $10 \mu\text{g L}^{-1}$. In addition, PAH-AuNPs significantly affected daphnid reproduction at $5 \mu\text{g L}^{-1}$ whereas Cit-AuNPs affected *Daphnia magna* reproduction at 25 mg L^{-1} (Fig. 4a and b). Toxicity caused by imparting positive charge to NPs has been shown with other cells, mammals and aquatic organisms such as algae, bivalves and fish.^{7,10,64-67} One study in particular, demonstrated a charge dependent response when exposing zebrafish to AuNPs. When 0.8 nm and 1.5 nm AuNPs functionalized with a cationic surface group, trimethylammoniummethanethiol (TMAT), were more toxic than anionic 2-mercaptoethanesulfonate (MES) or neutral charged 2-(2-mercaptoethoxy)ethanol (MEE) surface groups, affecting mortality, morphology, behavior and developmental endpoints to a greater extent.⁶⁵

One possible reason that PAH and CTAB-AuNPs were more toxic than their negatively-charged counterparts is potentially due to these particles having a high affinity towards the negative charged surfaces of cellular membranes.⁶⁸ The high level of attraction that positive charged particles have with cellular membranes increases cellular uptake of these particles through

mechanisms such as phagocytosis, pinocytosis and membrane disruption, which creates holes in the membrane due to the densely populated charge on the NP surface.^{69,70} This allows the positive charged particles to enter the intracellular matrix and potentially damage cell organelles integral to cellular functions. Lovern *et al.* (2008)⁷¹ and García-Camero *et al.* (2013)⁷² observed the uptake of gold NPs *in vivo* with minimal evidence of AuNPs ability to cross the *Daphnia magna* digestive tract. However, these studies were not focused on positively-charged particles, which have a greater potential to be further incorporated into the organism. The lack of tissue accumulation of negatively-charged NPs could be one of the potential mechanisms that caused the positively charged NPs to be more toxic and could explain the low toxicity observed with the negatively charged particles.

Another potential explanation for the difference seen in toxicity between the differentially charged particles in our experiment may be due to particle stability and aggregation in our media (MHRW). Positively charged particles, PAH and CTAB-AuNPs, did not aggregate significantly in the media (as can be seen in the UV-vis spectra of these AuNPs, provided in the ESI,† Fig. S3), while negatively charged particles, MPA and Cit-AuNPs, underwent greater aggregation during our experiments (Table 2). Stable particles, PAH-AuNP and CTAB-AuNP, remained less than 100 nm in size (55.9 ± 4.1 and 17.5 ± 0.2 , respectively) and the unstable particles, MPA-AuNP and Cit-AuNP, aggregated or agglomerated to a large extent, drastically increasing their size over the period between media exchanges (750.6 ± 8.2 and 90.8 ± 5.9 , respectively) (see Fig. 6 and S3–S5†). Aggregation-dependent toxicity has been seen in other studies using zebrafish embryos, leading us to believe that aggregation can affect NP toxicity.^{73,74} Although daphnids clearly ingested all functionalized particles (Fig. S6†) the aggregation size may cause a difference in their interactions with daphnid, potentially affecting the surface area of the particle available for interaction with the cells within the organism, as well as the potential uptake into cells.

The smaller size of the positively-charged particles in our study may have increased their toxicity by enabling them to cross the gut lumen of the daphnids, potentially further interacting with *D. magna* cells and organelles vital to daphnid homeostasis and other functions. This could account for the high mortality seen at low concentrations, especially because the PAH-AuNP supernatant exposure produced high mortality and TEM images revealed a small amount of <4 nm PAH-AuNPs present in the supernatant suspension (Fig. S7†). Small, 10 nm positively-charged amine coated AuNPs have been shown to enter a freshwater bivalve's branchial and digestive epithelial cells, entering the cytoplasm where the particles were then able to penetrate the cells nucleus and lysosomal vesicles.⁶⁴ AuNPs with core diameters less than 50 nm (15 and 50 nm AuNPs) have also been shown to affect inner organs of rats to a greater extent than larger particles (160 nm).^{24,75} Similar size dependent toxicity has been shown in aquatic organisms with various NP types.¹⁷ Negatively-charged particles aggregating to a large size have

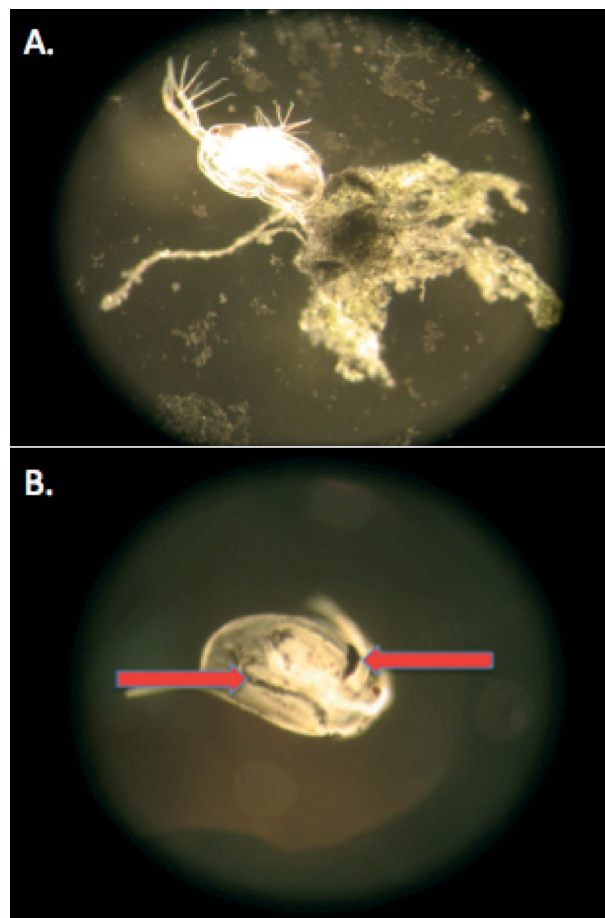


Fig. 6 Nanoparticles adhered to daphnid exoskeleton. Cit-AuNPs adhered to a daphnid carapace. (a) Cit-AuNP algal-agglomerate adhering to daphnid carapace. (b) Cit-AuNPs are ingested by daphnids but may also be found on the outside of the daphnids.

fewer mechanisms to enter the cell, reducing the ability to cross the cellular membrane and damage cell organelles. Main routes of entry of larger particles are through macropinocytosis, phagocytosis and clathrin mediated endocytosis.⁶⁹ Other studies have shown that larger particles and aggregates are less toxic than their smaller, monodispersed counterparts.^{21,32,34} For example, increasing the extent of aggregation therefore increasing the particle size of MPA-AuNPs, reduced their toxicity to zebrafish embryos.^{73,74}

MPA and Cit-AuNPs did impact reproduction (at high concentrations of Cit-AuNP, Fig. 4b) and body size (Fig. 5), however we observed unusual aggregation in the chronic exposures for these two particles. Cit-AuNPs, the more toxic of the two negatively-charged particles, formed agglomerates as appose to aggregates (Fig. 2). These agglomerates could not be re-suspended and formed a film on the bottom of the beaker. TEM imaging of the AuNP aggregates confirms that the Cit-AuNPs form extended wires and larger spherical AuNPs as a result of their aggregation process as appose to the MPA-AuNPs, which remain ~4.0 nm particles, but form large networks of closely associated AuNPs. We hypothesize that these agglomerates could cause an impact on the energy



budgets of these organisms either through blocked nutrient absorption or a decrease in food consumption⁷⁶ or due to an impact on swimming and molting as these particles were found to adhere to the exoskeleton, adding mass to the swimming organism (Fig. 6). Lee and Ranville (2012)⁷⁷ while conducting a 48 hour acute assay using *D. magna*, found that organisms exposed to aggregated Cit-AuNPs shed their exoskeletons while the controls did not. The increased molting observed in the Cit-AuNP exposed daphnids demonstrates that *Daphnia magna* may use this tactic to avoid any unwanted effects caused by the adhered Cit-AuNPs, however, the increased molting requires energy, leaving less for daphnid growth and reproduction.

Certain ligands used to alter the surface chemistry of NPs are more toxic than others, which could also explain the increased toxicity of CTAB-AuNRs in particular in our study. For the CTAB ligands, the acute ligand toxicity exactly matched the acute toxicity of the CTAB-AuNR, while the PAH ligand was only toxic when paired with the gold NP (Fig. 3). For CTAB-AuNRs, it is widely accepted that improper purification or ligand desorption can result in free-floating CTAB ligands in NP suspensions, which leads to this functionalized particle being highly toxic.¹³ This potentially downplays the effect of charge on toxicity for this functionalized AuNP. However, both ligand concentrations in the particle suspensions were much lower than what we tested in the free ligand control experiments implying that there is another mechanism that causes the ligands as well as the particles to become more toxic. We hypothesize that the increase in PAH and CTAB toxicity when attached to the gold NPs may be due to an increase in the concentration of PAH and CTAB inside the daphnids gut due to their ingestion with the NP and localization of ligand on or in daphnid cells. There were some indications of an increase in the reproduction of daphnids in the presence of the free ligand of CTAB, PAH and MPA. This may be due to some physiological use for the daphnids of these functional groups but may also be due to a hormesis effect of the ligands where a small amount of these chemicals is stimulatory to the organism's biochemical mechanisms that deal with toxins and therefore an up-regulation of other pathways dealing with reproduction. This may be a life history strategy for the organism to increase reproduction in a time of stress, as seen by others.^{78,79}

Lastly, in this experiment, the effects of ligand density and gold dissolution on NP toxicity are thought to be minimal. The similarity in ligand densities determined by XPS results (Table 3) for these ligands demonstrates that the differences in subsequent biological interaction are not likely to be associated with differences in ligand density. Additionally, it should be noted that while functionalized AuNPs may be susceptible to aggregation under specific environmental conditions, AuNPs are not readily susceptible to dissolution or oxidation under typical environmental conditions (which is in contrast to many functionalized silver or metal oxide NPs).^{38–41} As a consequence, when we considered how AuNP stability influences toxicity in this discussion, we focused primarily on

the stability of AuNPs against aggregation, rather than dissolution or oxidation.

The results of this study are important, because it identifies mechanisms for AuNP toxicity by examining NP toxicity with different charges using an environmentally relevant organism. The present study and our previous work²³ demonstrate the need for functionalized NPs to be evaluated in chronic exposures, as acute exposures do not explain the full adverse affects of NPs on aquatic organisms, as seen with Cit-AuNPs. This study also demonstrates that characterization of NP size after exposure to media as well as accounting for free ligand and synthesis impurity toxicity are important in determining the mechanism for NP toxicity.

Conclusion

NPs have the potential to be highly beneficial to society, however in order to create NPs that are beneficial, while minimizing the environmental implications of these materials, the mechanisms that govern the toxicity of NPs need to be better elucidated. We found that surface chemistry plays a significant role in NP toxicity; not only does the charge of the ligand on the AuNP surface influence both acute and chronic toxicity, but the identity of the ligand itself can influence toxicity. Interestingly, ~4.0 nm AuNPs functionalized with a non-toxic ligand (PAH) showed significant acute and chronic toxicity. In addition, the smallest particles (<4 nm) as seen in the PAH-AuNP supernatant exposures, could potentially be much more harmful to *Daphnia magna* than comparatively larger sized PAH-AuNPs (>4 nm). Furthermore, the chronic toxicity assays performed in this study indicate that AuNPs that show minimal acute toxicity can induce significant long-term effects, impacting daphnid reproduction. Therefore, chronic toxicity studies are essential for elucidating potential long-term exposures of manufactured NPs. Testing libraries of NPs with a variety of surface chemistries with different effective charges and functionalities in a given study will help discover trends in NP toxicity and translate these experiments to predictions for other types of NPs.

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