Title: Effects of Charge and Surface Ligand Properties of Nanoparticles on Oxidative Stress and Gene Expression within the Gut of *Daphnia magna*

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Abstract

Concern has been raised regarding the current and future release of engineered nanomaterials into aquatic environments from industry and other sources. However, not all nanomaterials may cause an environmental impact and identifying which nanomaterials may be of greatest concern has been difficult. It is thought that the surface groups of a functionalized nanoparticles (NPs) may play a significant role in determining their interactions with aquatic organisms, but the way in which surface properties of NPs impact their toxicity in whole organisms has been minimally explored. A major point of interaction of NPs with aquatic organisms is in the gastrointestinal tract as they ingest particulates from the water column or from the sediment. The main goal of this study was to use model gold NP (AuNPs) to evaluate the potential effects of the different surfaces groups on NPs on the gut of an aquatic model organism, *Daphnia magna*. In this study, we exposed daphnids to a range of AuNPs concentrations and assessed the impact of AuNP exposure in the daphnid gut by measuring reactive oxygen species (ROS) production and expression of genes associated with oxidative stress and general cellular stress: *glutathione S-transferase (gst)*, *catalase (cat)*, *heat shock protein 70 (hsp70)*, and *metallothionein1 (mt1)*. We found ROS formation and gene expression were impacted by both charge and the specific surface ligand used. We detected some degree of ROS production in all NP exposures, but positively charged AuNPs induced a greater ROS response. Similarly, we observed that, compared to controls, both positively charged AuNPs and only one negatively AuNP impacted expression of genes associated with cellular stress. Finally, ligand-AuNP

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4 exposures showed a different toxicity and gene expression profile than the ligand alone,
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7 indicating a NP specific effect.
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30 **Key Words:** ROS, *gst*, gene expression, nanotoxicity, oxidative stress
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1. Introduction

The toxicity of manufactured nanoparticles (NPs) to organisms in the environment has been a subject of interest due to their increasing use in commercial products and their potential environmental release. NPs can enter aquatic environments via waste generated from the use of consumer products, biomedical chemicals, agriculture, and industrial uses (Benn and Westerhoff, 2008; Klaine et al., 2008). For example, metal silver NPs used as antimicrobials in consumer products such as socks and titanium oxide used in sunscreens and paints have already been detected in effluent and sludge from wastewater treatment plants (Farkas et al., 2011; Kaegi et al., 2008; Kim et al., 2010a; Westerhoff et al., 2011). Research on the toxic effects of these NPs on aquatic organisms reported in the literature has largely focused on metals and metal oxides such as silver (Newton et al., 2013; Ribeiro et al., 2014; Scown et al., 2010), copper oxide (Fan et al., 2012; Griffitt et al., 2009; Heinlaan et al., 2008), titanium dioxide (Dalai et al., 2014; Kim et al., 2010b; Wiench et al., 2009), and zinc oxide (Poynton et al., 2011; Santo et al., 2014). However, many other NPs are in development that are more complex and include ligands that can change the surface properties of NPs and thus their interactions in the environment. The impacts of these surface properties on toxicity have been minimally explored.

The chemical properties imparted by the ligands attached to NP surfaces greatly affect charge, surface area, particle size, and aggregation potential (Elsaesser and Howard, 2012; Lapresta-Fernández et al., 2012; Rana and Kalaichelvan, 2013), and may play an important role in toxicity. Comparative studies have reported that the charge of the surface group attached to NPs can promote or prevent aggregation and

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4 increase of decrease uptake of NPs and therefore impact their toxicity. However, even
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6 across similar organisms results have not been consistent as to which type of charge
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8 causes the greatest impact. For example, negatively charged COOH-quantum dots
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10 have been shown to be taken up to a greater extent by daphnids after 24h exposure
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12 than neutral or positively charged quantum dots (Feswick et al., 2013). In contrast
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14 Bozich et al (2014) found that negatively charged AuNPs are taken up to an equal
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16 extent as positively charged particles. Aggregation may play a role in toxicity, as
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18 acetylated or carboxylated multi-walled carbon nanotubes functionalized with
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20 polyethyleneimine (PEI-MWCNT) increase aggregation and subsequent toxicity in the
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22 gut of daphnids (Petersen et al., 2010). Depuration rate may also play a role in toxicity,
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24 and stabilizing ligands attached to NPs have been shown to impact rate of depuration
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26 (Skjolding et al., 2014). The impacts may also be ligand specific. For example,
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28 negatively charged gamma-cyclodextrin fullerenes cause greater mortality in daphnids
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30 compared to functionalized carbon NPs with similar charge (Arndt et al., 2013), and
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32 positively charged polyallylamine hydrochloride AuNPs (PAH-AuNP) are more toxic
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34 than cetyltrimethylammonium bromide AuNPs (CTAB-AuNP) also positively charged
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36 (Bozich et al., 2014).
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45 In order to understand the mechanisms responsible for the toxicity of particular
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47 functionalized NPs, it will be necessary to determine the molecular changes that occur
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49 as the NPs contact tissues within an organism. Oxidative stress is a commonly
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51 measured cellular molecular response to NP exposure and can be an indication of
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53 damage and cellular repair but is also a natural response to a foreign object (Meng et
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55 al., 2009; Nel et al., 2006). Gene expression may provide a molecular level of
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4 understanding of how nanomaterials interact with the organism and how NPs with
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6 different properties may differentially impact an organism (Klaper et al., 2014). For
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8 example, exposing zebrafish to negatively charged AuNPs induces genes associated
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10 with mitochondrial dysfunction in brain and muscle tissues (Geffroy et al., 2012).
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12 Exposing golden clams to AuNPs caused not only oxidative stress but an induction of
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14 metallothionein (Renault et al., 2008). Overall, molecular indicators may provide an
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16 indication of the specific mechanisms for NP toxicity.
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21 The aim of this study was to evaluate the effects of surface functionalization of
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23 NPs on the molecular impact of NPs in *Daphnia magna*, an aquatic model species.
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25 Molecular responses were measured as generation of reactive oxygen production
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27 (ROS) and expression of genes associated with the antioxidant and stress systems
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29 within the gut of *D. magna*. The water flea *D. magna* is a common model for
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31 ecotoxicological testing of nanomaterials due to its sensitivity to environmental changes,
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33 its clonal reproduction, and simplicity to culture (Baun et al., 2008). Daphnids naturally
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35 ingest particulates from the water column or from the sediment (Horton et al., 1979) and
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37 have been shown to readily take up and accumulate NPs in the gut within 6-12 h after
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39 exposure (Feswick et al., 2013; Lee and Ranville, 2012; Lovern et al., 2008). We chose
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41 to examine the impacts of NPs with different ligands on the cells of the gut and
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43 associate these findings with our previous study examining apical endpoints of mortality
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45 and reproduction (Bozich et al., 2014). In our study, daphnids were exposed to a range
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47 of concentrations of four AuNPs carrying different ligands: the positively charged
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49 cetyltrimethylammonium bromide (CTAB) and polyallylamine hydrochloride (PAH), and
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51 the negatively charged mercaptopropionic (MPA) acid and citrate. AuNPs were used as
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4 they are easily functionalized, chemically inert and due to their increased use in
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6 biomedical research (Liu and Ye, 2013), food industry (Shah and Belozerova, 2009),
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8 environmental remediation (Wong et al., 2009), and electronics (Prime et al., 2009;
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10 Tsoukalas, 2009). A growing concern has been raised due to the likely release of
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12 AuNPs in the environment (Yah et al., 2012). AuNPs are also a model particle that is
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14 easily functionalized and characterized with respect to the surface charge and density of
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16 the ligands on the NP surface. The goal of this study was to use these particles as a
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18 model for investigating the impact of different surface properties on NP toxicity so that
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20 this information may then be used to inform design for future commercial NPs. In our
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22 previous work we showed that positively charged particles, and specifically PAH-AuNPs
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24 are more toxic than negatively charged particles, but also that the ligand composition
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26 plays a key role in toxicity, such that not all charged particles are equally toxic. This
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28 study further explores the specific molecular changes that occur in gut tissue in
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30 response to each ligand-NP combination.
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41 **2. Materials and Methods**

42 *2.1 Nanoparticles preparation and characterization*

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44 For this study we used a library of four AuNPs (Figure 1) created using
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46 previously reported methodologies (Bozich et al., 2014; Lohse et al., 2013). Briefly,
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48 AuNPs with an approximate diameter of 4 nm were functionalized with three different
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50 surface ligands: a negatively charged thiol, mercaptopropionic acid (MPA); a negatively
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52 charged antioxidant, citrate (Cit); or a cationic polyelectrolyte, polyallylamine
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54 hydrochloride (PAH). The fourth NP was created using a gold nanorod (AuNR) with an
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approximate dimension of 12 x 50 nm functionalized with the surfactant group cetyltrimethylammonium bromide (CTAB).

MPA-AuNPs (4.0 nm) were synthesized according to previously reported methods (Ackerson et al., 2005). Briefly, a growth solution of pH 8.5 was prepared containing 3.0 mM gold tetrachloroaurate (HAuCl_4) and 6.0 mM MPA dissolved in nanopure-deionized water. This solution was combined with 5.0 mM-aqueous sodium tetrahydroborate (NaBH_4) in a millifluidic reactor at 50 mL min^{-1} with a residence time of 3.0 min. The MPA-AuNP solution was then stirred for 3.0 hours and then concentrated and purified by centrifugation and removal of the supernatant. MPA-AuNPs were then concentrated and purified by diafiltration using 5 volume equivalents (Sweeney et al., 2006).

Cit-AuNPs (5.0 nm) were synthesized using previously reported procedures (Jana et al., 2001). In brief, 10.0 mM aqueous HAuCl_4 and 10.0 mM sodium citrate_(aq), were mixed with an aqueous solution of 5.0 mM NaBH_4 in a millifluidic synthesis reactor at a flow rate of 50 mL min^{-1} , and a residence time of 3.0 min. The Cit-AuNP solution was then stirred for 3.0 hours and concentrated and purified by centrifugation and the supernatant was removed. The Cit-AuNPs were then concentrated and purified by diafiltration using 5 volume equivalents (Sweeney et al., 2006).

PAH-AuNPs (5.0 nm) were created by wrapping the Cit-AuNPs with polyallylamine hydrochloride (PAH) as previously described (Gole and Murphy, 2004). Briefly, Cit-AuNPs were dissolved in 20.0 mL of a 1.0 mM aqueous sodium chloride (NaCl) solution. Then 500.0 μL of PAH (10 mg mL^{-1}) dissolved in 1.0 mM NaCl was added per every 20.0 mL of PAH wrapping solution. Then this solution was vortexed and

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4 left for 16 h. PAH-AuNPs were purified by centrifugation (55 min at 8,000 rcf) and rinsed
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6 in nanopure-deionized water.
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9 CTAB-stabilized AuNPs were synthesized as previously reported (Sau and
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11 Murphy, 2004). Briefly, 10.0 mL HAuCl₄ (0.1 M), 16.0 mL silver nitrate (AgNO₃, 0.01 M),
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13 and 11.0 mL ascorbic acid (0.1 M) were added to 1.0 L of a 0.1 M aqueous CTAB
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15 solution to prepare the growth solution. To prepare the seed solution, 2.4 mL of a
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17 previously prepared AuNP dispersion was added to 998.0 mL of a 0.1 M CTAB solution.
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19 The solutions were mixed in a flow reactor (flow rate = 50 mL min⁻¹) with a 15.0 min
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21 residence time. AuNRs were twice purified and concentrated by centrifugation (20 min
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23 at 11,000 rcf) and washing with nanopure deionized water.
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29 All AuNPs were verified for size and aggregation using transmission electron
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31 microscopy (TEM), ultraviolet-visible spectroscopy with a Cary 500 Scan UV-vis-NIR
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33 spectrophotometer, and dynamic light scattering (DLS) with a ZetaPALS phase-analysis
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35 light scattering system (Brookhaven Instruments, Holtsville). Analysis of ligand density
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37 on the surface of AuNPs was determined using a custom-built, ultra-high vacuum
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39 Physical Electronics XPS system equipped with aluminum K α source (~1487 eV x-ray
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41 energy) (Torelli et al., 2014).
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51 Adult *D. magna* used for this study were obtained from the culture maintained in
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53 the Klaper lab at University of Wisconsin-Milwaukee. Daphnids were kept at 20°C with a
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55 16:8 light/dark cycle in 500 ml of moderately hard reconstituted water (MHRW) (USEPA,
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2003). All daphnids were fed with *Raphidocelis subcapitata* at least three times per week. Before acute exposures, daphnids were fasted for at least 12 hours.

2.3 Exposures and dissection

Two separate exposures were conducted, one to examine ROS production within the enterocyte cells of the gut upon ingestion of AuNPs, and the other to collect gut tissue to determine gene expression patterns of key genes related to stress upon AuNP ingestion. For each exposure, adult female daphnids (~3 weeks old) were exposed for 24 hours to one of three different concentrations: 1, 10, and 50 µg/L of a functionalized AuNP suspension or 10 µg/L of each free ligand. The AuNP concentrations were chosen as a sublethal exposure based on results of a previous study published by our group (Bozich et al., 2014) plus a mid-range 10 µg/L concentration of free ligand that was higher than the total ligand existing in any of the AuNP suspensions, as previously measured in that same study. Three independent experiments with 3 replicates per concentration for each AuNP or free ligand were performed. Controls in MHRW were included in triplicate on each exposure date. During the acute exposure experiments mortality was only observed for the free ligand CTAB treatment at 10 µg/L concentration in both ROS and gene expression exposures. Due to the low survival rate, there is no gene expression data for CTAB in this study. However, guts from surviving daphnids exposed to free ligand CTAB were used for ROS assay. Dissection of the daphnid gut was performed under a stereomicroscope (Leica) after 24-hour acute exposure. Daphnids were individually placed in 1X phosphate buffered solution (PBS), and using a

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4 pair of 3c tweezers (Dumont) intact guts were carefully collected by removing the
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6 carapace and all soft tissue surrounding the gut.
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14 A total of nine daphnids were exposed per treatment for ROS quantification.
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16 Individual daphnids were placed in 20 mL of AuNP suspension or free ligand solution to
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18 determine an individual oxidative stress response in gut tissue using confocal
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20 fluorescent microscopy. An equivalent number of daphnids as negative controls were
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22 also included without AuNPs or free ligands. ROS activity was detected using the
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24 general oxidative stress cell-permeant chloromethyl-2', 7'-dichlorodihydrofluorescein
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26 diacetate (CM-H₂DCFDA) (Life Technologies). This dye passively diffuses into the cells
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28 and interacts with endogenous esterases, which cleave the diacetate groups. Then
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30 ROS present in the cell oxidizes the non-fluorescent H₂DCFDA into a highly fluorescent
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32 2',7'-dichlorofluorescein (DCF). After dissection, each gut was stained in 0.25 mL of 10
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34 μ M CMH₂DCFDA dissolved in PBS 1X for 20 min in the dark. Stained tissues were
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36 briefly transferred by pipet into fresh PBS 1X to wash off excess dye. Each gut was then
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38 individually mounted on a glass slide, 20 μ L Vectashield (Vector Laboratories) dropped
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40 onto the tissue to prevent photobleaching, and a cover slip was added to protect the
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42 stained tissue. Images of the stained guts were acquired using a laser scanning
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44 confocal mode with an Inverted Research Microscope Eclipse Ti (Nikon, Japan) with an
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46 excitation/emission 493-556 nm for fluorescein dye at 20X magnification.
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55 ROS activity in the gut cells of control and treated groups were quantified by
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57 measuring the intensity of the fluorescence from the tissues stained with the DCF. We
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quantified the number of fluorescent enterocytes from the confocal images using ImageJ software (Schneider et al., 2012). By employing the automatic nuclei counter plug-in, the total number of labeled cells per gut tissue was counted. Then the average number of fluorescent cells per gut for each concentration and for each functionalized AuNP, free ligand, or control group was calculated for statistical comparisons.

2.5 Gene expression

To measure the expression levels of key genes associated with oxidative and cellular stress in the *D. magna* gut three daphnids were placed in 60 mL of AuNP suspension or free ligand solution for 24 h. The number of daphnids for this assay was increased to obtain a higher RNA yield to be used for qPCR analysis. Triplicate samples were prepared per concentration for each AuNP or free ligand, and all treatments were replicated across three days to give a total of nine replicates. Triplicate controls were also conducted with MHRW with and without ligand. Gut tissues were removed from individual daphnids by dissection as described in section 2.3 after 24 h exposure to AuNPs or free ligands. Three of the replicates were pooled for per each AuNP treatment, free ligand, or control group, and immediately frozen in liquid nitrogen, and stored at -80°C until RNA extraction. RNA was isolated using Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA) and treated with DNase I before cDNA synthesis. Reverse transcription was performed using 0.25 µg RNA, 10 µl 2X RT reaction mix, 2 µl RT enzyme mix, and RNase free water in a total volume of 20 µl using SuperScript™ III First-Strand Synthesis Supermix (Life Technologies).

Quantitative real-time PCR (qRT-PCR) was performed using a StepOnePlus™ Real Time PCR System (Applied Biosystems) with 1 µl of cDNA for target gene in a volume of 20 µl containing 10 µl iTaq Universal SYBR® Green supermix 2X (Bio-Rad), 7 µl nuclease free water, 1 µl of forward primer (10 µM), and 1 µl reverse primer (10 µM). Real-time PCR conditions were 95°C for 10 min, 95°C for 15 sec and 62°C for 30 sec for 40 cycles. The mRNA expression levels of target genes: *glutathione S-transferase* (*gst*), *catalase* (*cat*), *heat shock protein 70* (*hsp70*), and *metallothionein1* (*mt1*) were normalized to corresponding endogenous control values, *actin* (*act*), and presented as fold expression change from control using the $\Delta\Delta C_t$ method. All primers used in the qPCR study are presented in Table 2.

2.6 Statistical analysis

SigmaPlot version 13.0 (Systat Software Inc., San Jose, CA) software for Windows was used for statistical analysis. Data generated from gene expression analysis was $\log_2$ transformed. Significant differences in ROS generation and gene expression were determined using One-way ANOVA with Tukey's multiple comparison tests among treatments after examination for normality and variance homogeneity ($p < 0.05$).

3. Results

3.1 Nanoparticle suspension characterization

The analysis of the diameter of spherical AuNPs by TEM revealed an average size around 4 nm. The mean core diameter of MPA-AuNPs, Cit-AuNPs and PAH-

AuNPs were 3.8 ± 1.1 nm, 4.9 ± 1.4 nm, and 4.7 ± 1.4 nm, respectively. The TEM study showed the CTAB-AuNRs to have a mean longitudinal diameter of ~ 50 nm and a mean transversal diameter of ~ 12 nm (Figure 2). The hydrodynamic diameter (D_h) values and surface charges for our AuNPs are summarized in Table 1. The D_h values measured by DLS indicated all particles as being below 20 nm. Although we reported the DLS values for CTAB AuNRs, it is important to bear in mind that most DLS software packages assume that the particles being measured are spheres. Therefore, the mean D_h for the CTAB AuNRs, which are anisotropic, cannot be accurately determined using the basic DLS fitting parameters. The ζ -potential analysis confirmed the predicted charges of all AuNPs. The ligand density evaluation of our AuNP library was performed using XPS (Table 1). The values of the two negatively charged AuNP are very similar. MPA-AuNPs had 5.6×10^{14} molecules cm^{-2} , and Cit-AuNPs had 4.7×10^{14} molecules cm^{-2} . PAH and CTAB are more difficult to estimate, as PAH is a polymeric free ligand, and CTAB coats the nanorods in a bilayer. However, they were estimated to be comparable to the other functionalized AuNPs.

3.2 Cellular ROS production

Our results demonstrated that all four functionalized AuNPs and all free ligands were capable of inducing ROS after 24 hours exposure (Figure 3). However, only the positively charged AuNPs, CTAB-AuNPs ($F=21.06$, $df=45$, $p<0.001$) and PAH-AuNPs ($F=6.54$, $df=46$, $p<0.001$), induced a significantly higher percentage of enterocytes to produce ROS compared to controls at all concentrations (Figure 4). This was not statistically different than the comparable positive free ligands for PAH at any

concentration, however the CTAB-AuNPs differed from CTAB ligand alone at 10 µg/L (Figure 4). Mortality (42.6%) was also observed in the free ligand CTAB treatment. Guts from surviving daphnids that had been exposed to free ligand CTAB produced a significant level of ROS compared to control ($F=21.06$, $df=45$, $p<0.001$).

The negatively charged AuNPs elicited a low percentage of enterocytes to generate ROS when compared to the positively charged AuNPs (Figure 4), and only MPA-AuNP was significant at 10 µg/L concentration ($F=6.48$, $df=48$, $p<0.05$). Neither 10 µg/L nor 50 µg/L MPA-AuNP were statistically different from the free ligand MPA control (Figure 4). Cit-AuNP was not significantly different from control. Surprisingly, free ligand citrate at 10 µg/L produced a significant ROS formation in daphnid guts compared to control and Cit-AuNP treatments ($F=9.59$, $df=48$, $p<0.001$; Figure 4). Overall, we were not able to determine a specific area in the *D. magna* gastrointestinal tissue that was particularly sensitive to any AuNPs or free ligand treatments.

3.3 Gene expression

Expression levels of *gst*, *cat*, *hsp70*, and *mt1* genes after exposure to 1, 10, or 50 µg/L of AuNPs showed differences in their expression patterns, and in some cases were significantly different than the comparable 10 µg/L free ligand exposure (Figure 5). Overall, positively charged AuNPs increased the expression of *gst*, where negatively charged NPs did not. However, PAH-AuNPs were the only treatment showing statistical significance. The level of *gst* expression was significantly upregulated by 50 µg/L PAH-Au with a 2.9-fold increase in expression over controls ($F=20.50$, $df=22$, $p<0.001$). In comparison, free ligand PAH exposure did not significantly increase *gst* expression in

daphnid guts (Figure 5A). None of the other positively or negatively charged AuNP concentrations produced a significant upregulation of *gst* expression ($p > 0.05$; Figure 5A), indicating a ligand specific AuNP impact rather than a charge specific impact. The negatively charged AuNPs, MPA-AuNP and Cit-AuNP, and the free ligand MPA did not elicit any substantial changes in *gst* expression (Figure 5A). However, free ligand citrate induced a significant -2.1-fold downregulation of *gst* expression ($F=15.18$, $df=35$, $p<0.001$) (Figure 5A).

The gene expression of *cat* varied in response to the different AuNPs and free ligand exposures. Negatively charged MPA-AuNP elicited a significant upregulation in *cat* expression (0.8-fold) at 10 $\mu\text{g/L}$ compared to other MPA-AuNP concentrations (Figure 5B), and this result correlates with ROS formation observed at the same concentration by MPA-AuNP as shown in Figure 4. Exposures to CTAB-AuNP (50 $\mu\text{g/L}$) and PAH-AuNP (10 $\mu\text{g/L}$) significantly reduced *cat* expression -0.8-fold and -0.9-fold ($F=6.49$, $df=35$, $p<0.001$), respectively. The citrate ligand was the only ligand to cause the downregulation of all genes (Figure 5).

The *hsp70* expression was significantly upregulated 1.7-fold at the highest concentration (50 $\mu\text{g/L}$) of PAH-AuNP ($F=7.22$, $df=32$, $p<0.001$). Ligand PAH also increased *hsp70* expression, but this was not statistically significant (Figure 5C). CTAB-AuNP had a significant effect on *hsp70* expression at the lowest concentration (1 $\mu\text{g/L}$) ($F=4.55$, $df=23$, $p<0.014$) (Figure 5C). Negatively charged MPA-AuNP treatment significantly induced expression of *hsp70* (0.8-fold) at 10 $\mu\text{g/L}$ ($F=3.32$, $df=35$, $p<0.022$). MPA, Cit-AuNP, and citrate did not significantly affect the levels of *hsp70* expression (Figure 5C).

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4 The *mt1* expression was significantly induced by all Cit-AuNP concentrations
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6 ($F=11.65$, $df=34$, $p<0.001$) (Figure 5D). MPA-AuNP also elicited a significant increase in
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8 *mt1* expression, but only at the lowest concentration (1 $\mu\text{g/L}$) (Figure 5D). PAH-AuNP,
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10 CTAB-AuNP, MPA, citrate, and PAH did not significantly impact expression of *mt1* at
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12 any concentration (Figure 5D).
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19 **4. Discussion**

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21 In this study we show that the surface charge of functionalized AuNPs plays an
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23 important role in the NP toxicity observed in the *D. magna* gut. Positively charged PAH-
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25 AuNPs and CTAB-AuNPs produce greater levels of oxidative stress, observed as ROS
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27 generation in the gastrointestinal tract of daphnids, when compared to controls and
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29 negatively charged NPs. Overall, using this generalized oxidative stress measurement;
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31 the impacts of the ligand-NP combinations were for the most part not distinguishable
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33 from the comparable free ligands. This indicates that the generation of oxidative stress
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35 may be a general response to exposure to a positively charged ligand whether it is
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37 attached to the AuNP or not. The results seen are also ligand dependent as the
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39 oxidative stress response of cells to the CTAB ligand were very different than PAH
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41 ligand. There was a smaller level of ROS production by free ligand CTAB exposure in
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43 comparison to the 10 $\mu\text{g/L}$ CTAB-AuNP exposure. We believe this result is due to the
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45 toxicity of the free ligand CTAB and that the results we see are more likely due to the
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47 increase in mortality in the ligand treatment and decrease in overall health condition of
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49 daphnids observed in the 50 $\mu\text{g/L}$ CTAB-AuNP treatment, which may have led to
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51 decrease feeding and changes in physiology of the gut.
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4 In contrast, gene expression patterns associated with stress including oxidative
5 stress, processing of metals, and other chemicals indicate unique ligand-AuNP impacts
6 versus ligands alone and also demonstrate that not only charge imparted by the ligand
7 attached to the NP, but also ligand type may influence gene expression patterns. In
8 previous nanotoxicity studies using metal NPs, gene expression has been shown to be
9 a good indicator of a toxic response and a way to separate the impacts of a particle
10 from its respective metal ion after NP dissolution (Griffitt et al., 2009; Poynton et al.,
11 2012; Poynton et al., 2011; Sabella et al., 2011; Vecchio et al., 2012). In this study,
12 PAH-AuNP caused the greatest upregulation in gene expression for all genes
13 considered and in our previous work was found to be the most toxic in both acute and
14 chronic studies (Bozich et al. 2014). Upregulation of *gst* at the highest concentration of
15 PAH-AuNP tested may be a result of an electrostatic effect between positively charged
16 PAH-AuNPs and the negatively charged cellular membranes in the daphnid gut causing
17 a stress response in the cell (Cho et al., 2009). The expression changes for the PAH-
18 AuNPs differ from the PAH ligand exposures. This may indicate a unique impact of the
19 ligand-AuNP when the ligand is bound to the AuNP, potentially resulting in a ligand
20 combination or a different ligand localization or interaction of particles versus the ligand
21 alone.
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47 The negatively charged AuNPs, MPA-AuNP and Cit-AuNP, have a smaller effect
48 on oxidative stress and gene expression with the exception of *mt1* expression, which is
49 significantly induced by Cit-AuNP exposure. This is an unexpected finding and may be
50 related to findings from our previous study, which documented a change from groups of
51 aggregated particles to larger agglomerates for Cit-AuNPs in suspensions during
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4 exposures (Bozich et al. 2014). These larger agglomerates, with a comparatively
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6 smaller surface area compared to the collective individual particles, may present less
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8 citrate to the guts of the daphnids. This could lead to a different gene expression and
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10 oxidative stress response between the citrate ligand and Cit-AuNPs.
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14 The increase in *mt1* gene expression may also indicate a mechanism for toxicity
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16 for the Cit-AuNPs. These AuNPs agglomerated in the lumen and therefore might not be
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18 completely covered in ligand. In this case the metals exposed may induce metal
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20 processing gene expression inside the organism. Similar results have been reported for
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22 negatively charged amino-coated AuNP exposures in golden clam *Corbicula fluminea*
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24 (Renault et al., 2008), where *mt1* gene expression was also found to be upregulated
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26 upon exposure. In fruit fly, Cit-AuNPs exposure has demonstrated important toxic
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28 impacts including reduction of life span and fertility, DNA fragmentation, and significant
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30 overexpression of stress proteins (Pompa et al., 2011; Sabella et al., 2011; Vecchio et
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32 al., 2012). Also, the high state of aggregation exhibited by Cit-AuNPs during their
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34 characterization in daphnid media (Bozich et al., 2014) may possibly explain the dose
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36 response of *mt1* expression. As agglomerates of Cit-AuNPs enter the lumen of the
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38 daphnid gut, it is likely that citrate may be stripped out from the surface of AuNPs by
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40 enzymatic action, which produces bare AuNP agglomerates. As the AuNP
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42 agglomerates oxidize as previously reported (Praharaj et al., 2007), it triggers a typical
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44 response that is indicative of metal exposure.
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53 Free ligand citrate also caused significant ROS production in the gut cells. A
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55 similar result has also been reported in yeast (Wang and Wang, 2012). However, citrate
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57 failed to induce expression of *gst*, *cat*, *hsp70*, and *mt1*. This lack of response in the gut
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cells may indicate that the concentration of citrate given to the daphnids was not sufficient to trigger the production of a specific ROS such as hydrogen peroxide, a common target of *cat*. Exposure of human gastric cells to citrate revealed destruction of cell populations by apoptotic cell death through the mitochondrial pathway (Liu and Ye, 2013). Thus, it is also possible that the exposure of daphnids to citrate may have initiated an apoptotic signal, and the expression of our target genes was superseded by the apoptotic signaling in gut cells.

The stability of functionalized AuNPs in *D. magna* media may also play a role in the observed effects. The positively charged AuNPs are more stable in suspension and cause greater oxidative stress. Stability in suspension could lead to greater uptake and availability of particles to the daphnids (Petersen et al., 2010). However, our previous data indicate that an equal amount of positively and negatively charged AuNPs are taken up by daphnids (Bozich et al., 2014). It is likely that the aggregation state could instead impact the amount of surface area available for interaction with cellular membranes when ingested by the *D. magna*. The considerable change in ζ -potential and D_h values of negatively charged AuNPs over time indicates significant aggregation/agglomeration and thus a larger particle size with less total surface area. These changes definitely may decrease the availability of Cit-AuNPs that interact with cellular membranes after being ingested by *D. magna* resulting in less ROS production. Overall, aggregation could play a role in the response due to surface area and therefore ligand availability.

5. Conclusion

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4 Our study provides evidence that the type of surface group of NPs has a
5
6 tremendous influence over toxicity in *D. magna* gut. This study reveals that positively
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8 charged AuNPs impact daphnid gut cells at a molecular level and that the impact is
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10 ligand specific and unique to the ligand-NP combination versus the ligand alone. In
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12 addition, negatively charged AuNPs can also cause stress and affect the generation of
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14 ROS, but to a lesser degree. This study reveals that it is possible to evaluate the
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16 interaction of functionalized NPs at molecular-level within the gut of an aquatic
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18 invertebrate such as *D. magna*, and that this approach provides detailed information of
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20 potential mechanisms of functionalized NPs toxicity. Our findings also expand the
21
22 knowledge on the likely impact of functionalized NPs on other aquatic fauna.
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Table 1**Characterization of AuNPs in this study**

AuNP name	d _{core} (nm)	D _h (nm)	ζ-potential (mV)	Ligand Density
MPA-Au	3.8 ±1.1	8.0 ±1.2	-18.5 ±1.3	5.6 x 10 ¹⁴ molecules cm ⁻²
Cit-Au	4.9 ±1.4	12.8 ±1.2	-15.3 ±1.5	4.7 x 10 ¹⁴ molecules cm ⁻²
PAH-Au	4.7 ±1.2	17.9 ±0.9	17.9 ±0.9	1.12 x 10 ¹⁵ formula units cm ⁻²
CTAB-Au	50 x 14	20.7 ±0.5	16.7 ±1.5	7.6 x 10 ¹⁴ and 1.39 x 10 ¹⁵ molecules cm ⁻²

Table 2**Primer sequences used for the qRT-PCR study**

Primer name	Primer sense	Size bp	Sequences from 5' to 3'	Accession No.
<i>gst</i>	Forward	20	CAACGCGTATGGCAAAGATG	ES408246.1
	Reverse	21	CTAGACCGAAACGGTGGTAAA	
<i>cat</i>	Forward	21	CAGCATCATCGGCAGTTAGTT	GQ389639.1
	Reverse	21	CTGAAGGCAAACCTGTCTACT	
<i>hsp70</i>	Forward	21	CCTTAGTCATGGCTCGTTCTC	EU514494.1
	Reverse	20	TCAAGCGGAACACCACTATC	
<i>mt1*</i>	Forward	20	TTGCCAAAACAATTGCTCAT	KF561474.1
	Reverse	19	CACCTCCAGTGGCACAAT	
<i>act</i>	Forward	21	CCTCCACCTCTTTGGAGAAAT	AJ292554.1
	Reverse	21	CAAGAATGAGGGCTGGAAGAG	

* Poynton et al., 2008

Figure captions

Figure 1. Library of functionalized gold nanoparticles used in this study. A) Mercaptopropionic acid gold nanoparticle (MPA-AuNP); B) Citrate gold nanoparticle (Cit-AuNP); C) Polyallylamine hydrochloride gold nanoparticle (PAH-AuNP); D) Cetyltrimethylammonium bromide gold nanoparticle (CTAB-AuNP).

Figure 2. Characterization of our library of functionalized gold nanoparticles. Shape and size distribution were determined by Transmission electron microscopy (TEM). Scale 50 nm.

Figure 3. Ingested functionalized gold nanoparticles and free ligands induced ROS activity in *D. magna* gut tissue. A) MPA-AuNPs and MPA. B) Cit-AuNPs and citrate. C) CTAB-AuNPs and CTAB. D) PAH-AuNPs and PAH. The presence of ROS is indicated by a bright green fluorescence in the cytoplasm of the enterocytes after 24 h exposure. Images were acquired by confocal microscopy at 20X magnification.

Figure 4. ROS levels in *D. magna* gut tissue exposed to functionalized AuNPs and free ligands. Data were graphed as mean $\pm$ SEM of labeled enterocytes by DCF after 24 h exposure. Letters represent significant differences among treatment groups after One-way ANOVA with Tukey's multiple comparison test statistical analysis ($p < 0.05$).

Figure 5. Expression levels of antioxidant and cellular stress genes in response to AuNPs and free ligands exposure after 24 h. (A) *glutathione-S-transferase* (*gst*), (B)

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4 catalase (*cat*), (C) *heat shock protein 70* (*hsp70*), and (D) *metallothionein1* (*mt1*). Data
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6 were graphed as mean $\pm$ SEM of fold expression change from control. Letters represent
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8 significant differences among treatment groups after One-way ANOVA with Tukey's
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10 multiple comparison test statistical analysis ($p < 0.05$).
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A

Mercaptopropionic Acid
(MPA) 4nm

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C

Polyallylamine hydrochloride
(PAH) 4nm

+

B

Citrate (CIT) 4nm

—

D

Cetyltrimethylammonium Bromide
(CTAB) 45x12 nm

+

Figure 2

Figure 3

Figure 4

Figure 5