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Surface functionalities of gold nanoparticles impact embryonic gene expression responses

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Abstract

Incorporation of gold nanoparticles (AuNPs) into consumer products is increasing; however, there is a gap in available toxicological data to determine the safety of AuNPs. In this study, we utilised the embryonic zebrafish to investigate how surface functionalisation and charge influence molecular responses. Precisely engineered AuNPs with 1.5 nm cores were synthesised and functionalized with three ligands: 2-mercaptoethanesulfonic acid (MES), N,N,N-trimethylammoniummethanethiol (TMAT), or 2-(2-(2-mercaptoethoxy)ethoxy)ethanol. Developmental assessments revealed differential biological responses when embryos were exposed to the functionalised AuNPs at the same concentration. Using inductively coupled plasma–mass spectrometry, AuNP uptake was confirmed in exposed embryos. Following exposure to MES- and TMAT-AuNPs from 6 to 24 or 6 to 48 h post fertilisation, pathways involved in inflammation and immune response were perturbed. Additionally, transport mechanisms were misregulated after exposure to TMAT and MES-AuNPs, demonstrating that surface functionalisation influences many molecular pathways.

Keywords

Gold nanoparticles; toxicity; zebrafish; nanoparticle–biological interactions

Introduction

Despite the rapid growth of the nanotechnology industry, research assessing the interaction of nanoparticles (NPs) and biological systems has not kept pace. At present, the mechanisms

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Declaration of interest

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of how NPs induce biological responses are poorly understood. It will be impossible to identify risk associated with NP exposure without evaluating NP interaction with biological systems. The use of an efficient and relevant toxicological model with a systematic approach to assess NPs can help fill these knowledge gaps.

Gold nanoparticles (AuNPs) have shown great potential to revolutionise therapeutics as delivery vectors (Kim CK et al. 2009; Kim CS et al. 2009) and as ultra sensitive probes for detecting proteins (Nam et al. 2003). A suite of AuNPs that are well-characterised and precisely engineered is ideal to systematically isolate the effects of individual physicochemical features, or a combination of multiple properties, on biological responses. AuNPs can be synthesised with precise control over size, shape, purity, surface charge, and functionalisation enabling the independent evaluation of each aspect (Shipway et al. 2000; Kim et al. 2005; Dahl et al. 2007). They are characterised using transmission electron microscopy (TEM) to determine shape and size, ultraviolet-visible (UV-Vis) absorption spectroscopy to assess core size and agglomeration state, and nuclear magnetic resonance ($^1\text{H-NMR}$) to confirm that the ligands are attached to the gold core and samples are free of other molecular impurities. In addition, AuNP dose can be quantified within complex biological systems by using either instrumental neutron activation analysis or inductively coupled plasma-mass spectrometry (ICP-MS). It is, therefore, critical to understand all aspects of how AuNPs interact with biological systems.

There have been many proposed biological models to screen for nanomaterial bioactivity, such as cell culture and rodent models. Cell culture-based approaches are rapid, cost effective, and amenable to high-throughput analysis, but lack the complexity of whole animal systems making it difficult to extrapolate to human safety (Teraoka et al. 2003; den Hertog 2005). Rodent models are the gold standard for safety prediction (Paigen 2003), but are labour and cost-intensive, and require hundreds of milligrams or larger quantities of test material, which is impractical for evaluating numerous types of precisely engineered NPs. A useful toxicological model must enable rapid assessment of the backlog of untested NPs and facilitate definition of the basic NP characteristics that drive the biological response.

An emerging model to investigate how NP physicochemical properties influence biological responses is the embryonic zebrafish (Usenko et al. 2007; Truong et al. 2011; Harper et al. 2011; Truong et al. 2011; Truong et al. In Press). Zebrafish have a high degree of homology to the human genome (~80%) (Barbazuk et al. 2000) and share many cellular, anatomical, and physiological characteristics with other vertebrates. The embryos are optically clear, small in size, and have a short life cycle (Dodd et al. 2000; Rubinstein 2003; Yang et al. 2009). Embryos develop externally (Kimmel et al. 1995), thereby allowing for non-invasive assessments of the embryo over time. One of the clear advantages of using the embryonic zebrafish to assess nanomaterial-biological interactions is that much less material is required compared to rodent-based studies.

The aim of this study was to isolate one feature – surface functionalisation – while keeping all the other factors (core size, composition, and shape) constant. We hoped to gain an insight into understanding the toxicity of these medically relevant NPs. In a previous study, three types of water soluble AuNPs were evaluated in the embryonic zebrafish (Harper et al. 2011). The AuNPs had similar core size (~1.5 nm) and were functionalised with different ligands: N,N,N-trimethylammoniummethanethiol (TMAT), 2-mercaptoethanesulfonic acid (MES), or 2-(2-(2-mercaptoethoxy)ethoxy)ethanol (MEEE). Differential biological responses were observed for each NP type. TMAT-AuNPs were lethal to embryos, MES-AuNPs induced sublethal malformations, and MEEE-AuNPs did not induce any *in vivo* biological response. The focus of this study was to evaluate how these NP physicochemical

properties induce differential biological responses at a molecular level by evaluating tissue concentration and gene expression profiling.

Materials and Methods

Materials

Hydrogen tetrachloroaurate ($\text{HAuCl}_4 \cdot \text{H}_2\text{O}$) was purchased from Strem (Newburyport, MA) and was used as received. Dichloromethane was distilled over phosphorous pentoxide prior to use. Chloroform was filtered through a plug of basic alumina prior to use to remove acid impurities. MEEE (Woehrle et al. 2004) and thiocholine (N,N,N-trimethylaminoethanethiol iodide) (Warner & Hutchison 2003) were synthesised according to known procedures. All other compounds were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and used as received.

Procedure for preparation of MES-, TMAT- and MEEE-AuNPs

Water soluble 1.5 nm particles were synthesised using known procedures (Woehrle et al. 2005).

Physicochemical characterisation of NPs

Proton NMR spectra were collected at 25 °C on a Varian Unity Inova 300 MHz spectrometer in D_2O . UV-Vis spectra were obtained on a Hewlett-Packard 8453 diode array instrument using 1-cm quartz cuvettes. TEM images were collected at 300 kV with an FEI Titan using a C_s aberration corrector. NP samples were prepared on amine functionalised SMART grids by soaking each in a dilute NP solution (0.2 mg/mL) and then in nanopure water for 2 min each. The grid was air dried. Zeta potentials were measured on samples diluted in reverse osmosis water (~2.5-9 ppm) using the ZetaPALs system (Brookhaven Instruments, Redditch, Worcestershire, UK). Each sample was diluted, vortexed, and read on the machine within 5 min. The zeta potentials for 1.5 nm MES-, TMAT-, and MEEE-AuNPs in fish water were -13.3, 8.71, and 2.91, respectively.

Zebrafish maintenance and exposure protocols

Tropical 5D zebrafish (*Danio rerio*) were reared in the Sinnhuber Aquatic Research Laboratory at the Oregon State University (OSU). Adults were kept at standard laboratory conditions of 28°C on a 14 h light/10 h dark photoperiod in fish water consisting of reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean®). Embryos were collected and staged (Kimmel et al. 1995) from group-spawned zebrafish. The chorion was enzymatically removed with pronase to increase bioavailability at 4 h post fertilisation (hpf) using protocols previously published (Truong et al. 2011). Dechorionated embryos were left to rest for at least 30 min prior to the initiation of NP exposure. Embryos were transferred to individual wells of a 96-well plate with 100 µL of prepared NP solution. A subset of embryos with intact chorions was kept to monitor inherent clutch quality. Exposure plates were sealed to prevent evaporation and wrapped with aluminum foil to guard against potential photo-oxidation of the NPs.

NP exposure

Embryos were exposed to seven concentrations and an embryo media (EM) control ($n = 12$, three replicates) with the highest concentration at 250 µg/mL (ppm) and five-fold dilutions down to 0.016 ppm. The static NP exposure continued under standard laboratory conditions in covered, sealed plates until 120 hpf. At 120 hpf, individual embryos were scored for mortality, then euthanised prior to evaluation of morphological malformations. For malformation statistics, only embryos that survived were considered. Fifteen morphological

malformations were evaluated. The percent mortality and total malformations were calculated and graphed as a mean of three replicates. EM consisted of 15 mM NaCl, 0.5 mM KCl, 1 mM MgSO₄, 0.15 mM KH₂PO₄, 0.05 mM Na₂HPO₄, and 0.7 mM NaHCO₃ (Westerfield 2000).

NP uptake by ICP-MS

Six hpf embryos were statically exposed to EM, 2, 10, 50, 250 ppm of each AuNP solution, and sampled at 24 and 48 hpf to quantify overall gold tissue concentration. Briefly, for each exposure group, three embryos were washed thoroughly with milli-Q water and then placed individually into 14 mL round bottom plastic tubes and stored at -20 °C until time to sample. Twelve hours prior to sampling, the embryos were digested using nitric acid, a final concentration of 1 ppb of internal standards (Indium, Rhenium and Bismuth) were added, and the samples were brought to a total volume of 5 mL with milli-Q water. Samples were vortexed, and then placed into the autosampler racks. A five-point calibration curve (0.01, 0.1, 1, 5, 10 ppb) was created using a purchased gold standard and had an R² value of 0.996. The number of gold particles per embryo was back-calculated using the assumption that a 1.5 nm AuNP core consists of 101 gold atoms.

NimbleGen zebrafish expression array

Zebrafish expression arrays were printed by Roche NimbleGen (Madison, WI) based on Ensembl Zv7 build. MES- and TMAT-AuNPs gene expressions were completed on the 385K format that had 37,157 genes with 12 probes per target (60 mer).

Gene expression exposure and RNA collection

Six hpf dechorionated embryos were exposed to 1.5 nm MES- and TMAT-AuNPs at 50 and 10 ppm, respectively, and to vehicle control (fish water) as described in the above exposure protocol. Embryos were pooled into three replicates of 40 embryos, euthanised using MS-222, and washed with milli-Q water. Once thoroughly washed, embryos were transferred to sterile 1.7 mL microcentrifuge tubes where excess water was removed from samples and 400 µL of TriReagent (Sigma Aldrich St. Louis, MO) was added to extract RNA. Samples were homogenised using a plastic pestle and battery operated mortar. After homogenisation, 600 µL of TriReagent was added and the samples were stored at -80 °C until processing. In all, 24 and 48 hpf samples were collected for 1.5 nm MES- and TMAT-AuNPs. Once all samples were collected, homogenates were thawed on ice and centrifuged at 12,000 *g* at 4 °C. The supernatant was removed and transferred to a new microcentrifuge tube, where 200 µL of chloroform was added. The samples were centrifuged and the clear aqueous layer was extracted and transferred to a new microcentrifuge tube. In all, 500 µL of isopropanol was added to each microcentrifuge tube. The samples were once again centrifuged, then all liquid was removed, and the RNA pellet was washed several times with 75% ethanol: RNase Free H₂O. The RNA pellets were air dried and then resuspended in 12 µL with RNase Free water. A small aliquot (1 µL) was removed and diluted in 3 µL of RNase Free water. The remaining RNA was stored at -80 °C. The aliquot was used to verify quality and quantity using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent BioAnalyzer 2100 (Palo Alto, CA) at the Center for Genome Research and Biocomputing at the OSU.

Nimblegen microarray processing

A total of 10 µg of total RNA was reverse transcribed using SuperScript III and oligo (dT) primer (Invitrogen, Carlsbad, CA), and double-stranded cDNA was synthesised and purified using Qiagen Minelute PCR Purification spin column. Double-strand cDNA was labelled with Cy5 dNTP, and then the samples were hybridised to 385K zebrafish gene expression

arrays (Roche Nimblegen, Madison, WI) and scanned using the Axon GenePix 4200A Pro scanner (Molecular Devices, Sunnyvale, CA) with a green laser (532 nm) and a hardware setting of 450 pmt, laser power of 100, and a pixel size of 5. Histogram analysis was performed to assure that the normalised counts lie between $1e-4$ and $1e-5$ at the signal intensity of saturation (65,000).

Data and pathway analysis

Raw data were extracted, background subtracted, and quantile normalised (Bolstad et al. 2003) using NimbleScan v2.5 software. Gene calls were generated using the Robust Multichip Average algorithm (Irizarry et al. 2003a; Irizarry et al. 2003b). Statistical analysis was performed by one-way ANOVA for unequal variance or by unpaired t-test with Tukey's *post hoc* test ($p < 0.05$) and 5% FDR in GeneSpring GX. Unsupervised hierarchical clustering was performed using Euclidean distance metric and centroid linkage clustering to group gene expression patterns by similarity. The clustering algorithms, heat map visualisations, and centroid calculations were performed in Multi-Experiment Viewer (MeV) (Saeed et al. 2003) software. Functional enrichment statistics and network analysis were determined using DAVID (<http://david.abcc.ncifcrf.gov/home.jsp>) (Dennis et al. 2003; Lempicki et al. 2007) and Metacore (GeneGo, St. Joseph, MI) to identify the most significant biological processes affected by NP treatment. The DAVID functional annotation tool utilises the Fisher exact test to measure gene enrichment in biological process. Gene ontology category terms for significant genes were compared to a background list, which included all genes on the zebrafish Nimblegen platform. Functional annotation clustering with high stringency was used to group similar annotations together into non-redundant functional groups. The statistical scores in MetaCore were calculated using a hypergeometric distribution, where the p value represents the probability of a particular mapping arising by chance for experimental data compared to the background. Networks were built in MetaCore for experimental data utilising the direct interactions algorithm.

Gene validation

RNA was isolated from embryos exposed to 50 $\mu\text{g/mL}$ MES-AuNPs and 10 $\mu\text{g/mL}$ TMAT-AuNPs, as described above. cDNA was synthesised from 1 μg of RNA in a 20 μL reaction following the SuperScript III First Strand Synthesis Kit Protocol (Invitrogen, Carlsbad, CA). After cDNA was synthesised, samples were diluted to 1:10 prior to storage. Six genes (PTRH1, HOXC9A, BTR29, PDE11A, KIF4A, and RPH3AL) were selected to represent both elevated and repressed transcripts from the microarray. Quantitative real time-PCR (qRT-PCR) was used to validate these genes. Briefly, a melt curve was generated for each primer set (Supplemental Table I) at six temperatures (55, 57, 58, 59, 60, and 61 $^{\circ}\text{C}$) to find an optimal temperature that worked for most primers. Using the Lonza (Basel, Switzerland) Flashgel system, the PCR products were assessed to ensure the product was the expected size. Each sample included three biological replicates for both the control and treated, along with a no template control (no cDNA) and adult zebrafish cDNA. For each sample, a reference gene was used (*beta actin*) for normalisation. CT mean values were used and normalised to *beta actin*, and then corresponding biological replicates were averaged. The average value of treated and controls were transformed to a ratio to determine the fold change magnitude of the treated sample compared to the control.

Statistics

All analyses were compiled using SigmaStat/Plot 11 (SPSS Inc, Chicago, IL). Dose response significance was determined using one-way ANOVA ($p < 0.05$) and Dunnetts *post hoc* test. A two-way ANOVA ($p < 0.05$) and Dunnetts *post hoc* test with time and concentration as factors were used to determine tissue concentration significance.

Statistically significant genes supporting microarray data were determined using a Student t-Test.

Results

Characterisation of functionalised 1.5 nm AuNPs

Previously, we developed a precisely engineered AuNP library to determine how the individual effects of core size and surface functionalisation impact biological responses (Harper et al. 2011). These AuNPs are rigorously purified by diafiltration and are thoroughly characterised using TEM, $^1\text{H-NMR}$, and UV-Vis spectral analysis. TEM was used to determine NP size and distribution for each formulation. As illustrated in Supplemental Figure 1a, d, g, the functionalised AuNPs were monodispersed and did not agglomerate. Size analysis of the TEM micrographs of MES- (Supplemental Figure 1a), TMAT- (Supplemental Figure 1d), and MEEE- (Supplemental Figure 1g) AuNPs revealed an average size of 1.6 ± 0.5 nm ($n = 250$), $D = 1.6 \pm 0.5$ nm ($n = 199$), and $D = 1.3 \pm 0.5$ nm ($n = 657$), respectively. Further characterisation using nuclear magnetic resonance ($^1\text{H-NMR}$) spectrometry (Supplemental Figure 1b, e, h) showed peaks at approximately 4.3-4.5, which illustrate the residual proton from the solvent used for NMR, while the other peak around 3.5 corresponds to the protons of the ligand shell. This confirms that the free ligand and other small molecular impurities were successfully removed.

Stability of 1.5 nm AuNPs in test media (EM)

The characterisation of these AuNPs in nanopure water illustrated that they are highly dispersed and were not agglomerated in solution. However, the EM used for these toxicological studies consisted of ions that buffer the pH. Those ions may cause the NPs to agglomerate and precipitate. Thus, prior to conducting toxicity studies, AuNPs were dispersed in EM and monitored with UV-Vis spectroscopy at least through the experimental duration (0-120 hpf) to determine whether the particles remained in solution. As Supplemental Figure 1c, f and i illustrate, when MES-, TMAT-, and MEEE-AuNPs were suspended in EM at 250 $\mu\text{g/mL}$, the absorbance patterns at 24 hpf, 120 hpf, 14 days, and 1 month were similar to when the first measurements were taken ($t = 0$). The surface functionalised 1.5 nm AuNPs were stable in the test media, and any responses observed in the assay would not be attributed to agglomeration.

Functionalised AuNPs induce differential biological responses

In a previous study conducted by our lab, MES-, TMAT-, and MEEE-AuNPs were assessed for developmental toxicity in the embryonic zebrafish model (Harper et al. 2011), but without controlling for factors that influence NP stability. Here, we have conducted all experiments with EM, a defined matrix with a known pH (7.4) buffering capacity, for at least 7 days. MES-, TMAT-, or MEEE-AuNPs were dispersed in EM, and embryos were statically exposed to seven concentrations (0–250 $\mu\text{g/mL}$) from 6 to 120 hpf. As illustrated in Figure 1A, at 120 hpf, TMAT-AuNPs induced 100% morphological malformations at 10 $\mu\text{g/mL}$, while MES-AuNPs induced 40% and MEEE-AuNPs did not. At 50 $\mu\text{g/mL}$, TMAT-AuNPs induced 80% mortality, while MES- and MEEE-AuNPs did not. MES-AuNPs induced statistically significant higher incidence of malformations compared to MEEE-AuNPs at 2 $\mu\text{g/mL}$. These results are comparable to our previous study using fish water confirming that the observed toxicological responses were influenced by NP surface functionalisation and not the pH or media composition.

AuNPs are bioavailable to embryonic zebrafish

The differential toxicity of the three AuNPs led us to ask whether responses were associated with differential bioavailability. ICP-MS was used to quantify the amount of gold tightly associated with the embryos after exposure (followed by careful washing to remove free particles and digestion in nitric acid) to 2-250 $\mu\text{g/mL}$ of MES-, TMAT-, or MEEE-AuNPs. As illustrated in Figure 1B, at both 24 and 48 hpf, and at the same concentration, the numbers of gold particles per embryo were similar for all three NPs. The one exception was at 250 $\mu\text{g/mL}$ exposure to TMAT-AuNPs resulted in no embryos surviving to the sample time point. The number of particles associated with an embryo increased with NP concentration in the water. The quantity of AuNPs associated with an embryo did not significantly change from 24 to 48 hpf for any exposure concentration or type of AuNP. We observed that the control embryos had detectable gold, which can be explained by our use of nitric acid, with its low level (ppb) of elemental gold. The use of nitric acid was unavoidable since it is the preferred acid for the ICP-MS. This finding suggested that: (1) the surface functionalisations studied did not differentially influence uptake into the embryo, (2) the uptake was rapid, and (3) none of the AuNP types accumulated over time.

Gene expression changes elicited by MES and TMAT at 24 and 48 hpf

To explore the mechanism of how surface functionalised AuNPs induce differential biological responses over time, we conducted global gene expression studies using embryos exposed to 50 $\mu\text{g/mL}$ of 1.5 nm MES- and 10 $\mu\text{g/mL}$ of TMAT-AuNPs from 6 to 24 and 6 to 48 hpf. Due to the lack of biological response in other assays by MEEE-AuNPs, this NP was not included in these experiments. At 24 hpf, exposure to MES-AuNPs led to the misexpression of more transcripts than TMAT-AuNPs (24 and 18, respectively). Fourteen misexpressed transcripts were common between both AuNPs (Figure 2A). By 48 hpf, the number of transcripts misexpressed by MES- and TMAT-AuNPs increased to 316 and 58, respectively. The number of transcripts common to both were 184 (Figure 2B). The statistically significant genes for all samples (606) were grouped using bi-hierarchical clustering by MeV to produce a heat map (Figure 2C). The heat map had a gene expression pattern that was consistent over time. By taking the generally elevated or repressed transcripts by AuNPs from the heat map, significant biological process networks were identified (Figure 2D). As Figure 2D illustrates, the most significant biological process networks relating to the elevated transcripts were inflammation (complement system, cell adhesion-leucocyte interactions, and immune response), phagocytosis, and signal transduction-nitric oxide signalling. While, the significant pathways for repressed transcripts were signal transduction-WNT and NOTCH signalling, inflammation-IL-12, 15, 18 signalling, cell cycle-G1-S growth factor regulation, muscle contraction, reproduction-gonadotropin regulation, inflammation-histamine signalling, and immune response-IL-5 signalling. There were more elevated transcripts than repressed; however, there were fewer statistically significant biological process networks (only four). These networks were involved in inflammation and immune response. Biological processes such as immune and inflammatory responses are elevated by both surface functional groups.

From this heat map, we used qRT-PCR to confirm that the changes in gene expression were similar to gain confidence that the networks identified were, in fact, a result of exposure to 1.5 nm MES- or TMAT-AuNPs. We validated six misregulated transcripts that were elevated or repressed. As illustrated in Table I, the direction and magnitude of the gene expression changes observed by qRT-PCR corresponded to those of the microarray, thereby confirming the changes caused by MES- and TMAT-AuNPs at 24 or 48 hpf.

Pathway analysis comparison of MES- and TMAT-AuNPs

Due to the differential biological responses caused by exposure to MES- and TMAT-AuNPs we specifically analysed the gene expression data to identify genes that are differentially expressed between MES- and TMAT-AuNPs. When the surface functional groups on the NPs, TMAT and MES were directly compared to one another, 512 and 1737 statistically significant differentially expressed transcripts were identified at 24 and 48 hpf, respectively. To accomplish this analysis, each surface functionality was normalised to its time matched control, and then a Student T-test was performed. As Figure 3A, B illustrates, there is a difference in response for each NP. However, the magnitude of response varied between one another. Figure 3C demonstrates that when comparing the gene lists generated for each time point, at 24 hpf there were 486 unique genes, while at 48 hpf there were 1711. There were 26 common genes between the two time points. This direct comparison of the TMAT- and MES-response provided evidence that as early as 24 hpf, the surface functional groups were already perturbing the embryo in different ways, and these events led to more molecular disruption at 48 hpf. Additionally, the surface functionalities are driving the gene expression changes and are undergoing differential mechanisms to induce these differential biological responses.

The significantly enriched biological processes at 24 hpf were associated with immune system, inflammation, protein folding, proliferation, and G-protein coupled receptor protein signalling (Table IIa). Immune response and inflammatory related biological processes were the most prevalent processes, which included three genes (ELF4, RUNX3, and c-Fos) that were elevated by TMAT compared to MES, and eight genes (STAT4, PAK2, PP2A cat (alpha), PP2A catalytic, p70 S6 kinases1, ubiquitin, NF-AT1, and ATF-2) that were repressed by TMAT versus MES. The G-protein coupled receptor signalling pathway was also elevated by TMAT-AuNPs, meaning TMAT-AuNPs cause an increase in cellular responses.

At 48 hpf, the significantly enriched biological pathways identified by Metacore were mainly related to G-protein coupled receptor protein signalling pathways, transport, proliferation, and responses to protein stimulus (Table IIb). The proliferation pathway was one of the largest biological pathways and includes a large number transcripts that were repressed by TMAT versus MES (PKR, NDPK A, NF2, GRB2, KLF4, and PAX6) compared to those that were elevated (VEGF, CDK2m TGFB-1, and TGFB-3).

Discussion

In this in vivo study, we report that exposure to 1.5 nm AuNPs functionalised with TMAT, MES, and MEEE induced differential biological responses in dechorionated embryonic zebrafish. TMAT functionalised AuNPs induced embryo lethality, while mortality was not observed after exposure to MES- and MEEE-AuNPs. MES functional group caused sublethal malformations to the embryos. No adverse responses were observed after exposure to MEEE-AuNPs. The differential biological response was not due to a difference in the ability of the embryos to uptake certain AuNPs. Here, we report that the different adverse responses observed can be attributed to the surface functional groups. We found that surface functionality influenced the gene expression profile when the two surface groups (MES and TMAT) were directly compared to one another.

In a previous study (Harper et al. 2011), we used these same functionalised AuNPs dissolved in fish water, which is made up of reverse osmosis water and Instant Ocean and found that each AuNP induced differential biological response in the zebrafish. However, since the medium used for the toxicological study consists of Instant Ocean, which has a proprietary recipe, it makes it near impossible to control or predict how the NPs will behave

in repeated studies. Characterisation of AuNPs in ion-rich versus low ion media often yields different agglomeration and precipitation properties. Ions in media are known to cause NPs to agglomerate and display unpredictable surface area, charge, and size characteristics compared to the original synthesised particles (Saleh et al. 2008; Liu et al. 2009). These changes in parameters influence the toxicological outcome (Truong et al. In Press). Although agglomeration did not occur for MES-, TMAT-, and MEEE-AuNPs in ion-rich media, it is critical to evaluate the stability of the NPs over time to ensure that the biological responses observed are ascribed to a known NP formulation.

There is great interest in efficiently assessing toxic potential of NPs and in understanding the physicochemical properties that elicit the toxic response. The effects of core materials and surface functionalisation have been assessed for gold, silver, and titanium NPs (Euliss 2005; Sayes et al. 2006; Pan et al. 2007; Furgeson et al. 2009; Li et al. 2010; Park et al. 2010). Under certain circumstances, AuNPs cause cytotoxicity (Pan et al. 2007), cell death (Homburger & Simon 2010), and immune responses (Karthikeyan et al. 2010). While NP size is an important determinant of biological response (Pan et al. 2007; Liu et al. 2010), so is the NP surface functionalisation (Toru et al. 2004). In other studies, cationic AuNPs exposed to Japanese medaka fish resulted in mortality in less than 24 h (Zhu et al. 2010), which is similar to what we observed in our cationic AuNPs (TMAT-AuNPs) (Harper et al. 2011). Our studies indicate that alteration of a NP physical property changes the biological response.

Understanding the differential toxicities is a challenge. Differential uptake was hypothesised, but this study suggests it is not a factor. We demonstrated that the uptake is not predictive of toxicity, which is consistent with other studies. In a study using *Daphnia magna*, exposure to 13-17 nm AuNPs did not cause toxicity at low concentrations, even though the NPs accumulated over time in the gut (Lovern et al. 2008). The *D. magna* observation supports our finding that a lack of biological response to AuNPs cannot be attributed to low or no uptake. From this study alone, we conclude that at 24 and 48 hpf, regardless of surface functionalisation on the AuNPs, gold was detected in exposed embryos and that the uptake level is not directly correlated to any specific physical parameter.

Our gene expression profiling is not the first study to identify misregulation in pathways related to inflammation and immune responses. A study using AuNPs found that when macrophages were exposed to 35 nm AuNPs *in vitro*, some toxicity was observed (Shukla et al. 2005). Silica particles stimulated inflammatory protein and induced macrophage cytotoxicity (Waters et al. 2009). These studies used NPs that varied in size, core material, and surface functionalisation to ours, but illustrate that a general immune response can be initiated by varying NP structural attributes.

NP effects on cell cycle control and proliferation are not without precedent. AuNPs with a diameter of 300 nm inhibited VEGF and induced cell proliferation and migration (Karthikeyan et al. 2010). It is likely that when such pathways are impacted during development there will be deleterious consequences. Transport mechanisms were more elevated by TMAT than MES, including metal ion transport. The ion transport process was also repressed by TMAT. In general, transport channels are always open, but they are highly selective and will only allow specific molecules through (Zilman et al. 2010). Zilman et al (2010) demonstrating that certain particles can be strongly trapped and have an enhanced presence for transport regardless of the presence of other particles. However, there is a certain range of intermediate trapping strength for the ion transporters that allows the particles to penetrate the channel to a certain degree, and mostly accumulate near the entrance (Zilman et al. 2010). This clogging causes a change of particle density inside the channel. It could be that TMAT-AuNPs have a high trapping strength for metal ion

transporter and are able to get into the cell readily at 24 hpf, resulting in the perturbation of the G-coupled protein receptor signalling pathway and, in the end, various transport mechanisms within the cell. In comparison, it is possible that MES-AuNPs have an intermediate trapping strength, which causes a blockage of the channel, resulting in a disruption of the metabolic processes at 24 and 48 hpf, due to the imbalance of ions inside and outside the cell. This identification of affected pathway candidates has provided a first pass at understanding the molecular mechanism by which MES- and TMAT-AuNPs induce differential biological responses at both the molecular and phenotypical levels.

In summary, surface functionalisation of AuNPs influences the biological response at the phenotypical and molecular levels. From this study, we have identified that inflammation and immune response are relatively general responses to NP exposure. Additionally, transport mechanisms were misregulated after exposure to different surface functional group AuNPs. Further studies using different surface functional groups can help identify pathways that are driving these adverse responses at the mRNA level. Collectively, these results suggest that surface functionalisation plays the largest role in producing differential responses. We believe that this and other recent NP toxicity studies demonstrate efficacy of systematic toxicology studies to establish structure-activity relationships among size, surface functionalisation and charge, and the biological response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Differential biological responses induced by AuNPs. (A) Embryos were dechorionated and exposed from 6 to 120 hpf. 1.5 nm TMAT-AuNPs were embryo lethal, causing mortality in 100% of exposed embryo at 250 μg/mL, while MES-AuNPs induced little mortality, but did induce malformations at concentrations of 2 μg/mL and above. MEEE-AuNPs did not induce any differential biological response. (B) Tissue concentration of embryos exposed to 2-250 μg/mL of MES-, TMAT-, and MEEE-AuNP from 6 to 24 and 6 to 48 hpf. Error bars are standard error. Data with * denotes statistical significance ($p < 0.05$). AuNP, gold nanoparticle; hpf, hours post fertilisation; MEEE, 2-(2-(2-mercaptoethoxy)ethoxy)ethanol; MES, 2-mercaptoethanesulfonic acid; TMAT, N,N,N-trimethylammoniummethanethiol.

Figure 2.

Venn diagram of misregulated transcripts after exposure to MES- and TMAT-AuNPs at (A) 24 and (B) 48 hpf. (C) Hierarchical clustering and (D) functional enrichment of statistically significant genes ($p < 0.05$) elevated or repressed by MES- and TMAT-AuNPs at 24 and 48 hpf. Orange bars represent significant biological process networks (MetaCore, GeneGo) for elevated transcripts and blue bars for the repressed transcripts compared to the control. AuNP, gold nanoparticle; hpf, hours post fertilisation; MES, 2-mercaptoethanesulfonic acid; TMAT, N,N,N-trimethylammoniummethanethiol.

Figure 3. Direct comparison of genes differentially expressed by TMAT- vs. MES-AuNPs. Heat map of significantly different genes ($p < 0.05$) at 24 hpf (A, 512 genes) and 48 hpf (B, 1737 genes). A venn diagram comparing the gene lists for each time point at 24 and 48 hpf (C). AuNP, gold nanoparticle; hpf, hours post fertilisation; MES, 2-mercaptoethanesulfonic acid; TMAT, N,N,N-trimethylammoniummethanethiol.

Validation of statistically significant genes identified by the microarray using qRT-PCR. Genes were selected from the statistically significant gene lists generated from the heat map to determine if the misregulation identified by the microarray was valid. These transcripts were selected to represent both elevated and repressed transcripts. qRT-PCR was used to confirm that the direction of the misregulation is consistent but not necessarily that the magnitude of the change is not identical.

Table 1

Gene symbol	Gene accession ID	Gene name	Fold changes of MES			
			24 hpf		48 hpf	
			Microarray	qPCR	Microarray	qPCR
PTRH1	NM_001013330	Peptidyl-tRNA hydrolase 1 homolog	2.01↓	2.68↓		
HOXC9A	NM_131528	Homeobox C9a	2.11↓	1.43↓		
BTR29	NM_001166134	Bloodthirsty-related gene family, member 29	3.02↓	2.20↓		
PDE11A	NM_201102	Phosphodiesterase 10A			2.71↓	1.43↓
KIF4A	-	-			2.76↓	1.63↓
RPH3AL	-	*B8k0F3_DANRE			2.36↓	1.55↓

* Denotes a gene identified using Ensembl database and ± from TIGR; MES, 2-mercaptoethanesulfonic acid; qPCR, quantitative PCR; qRT-PCR, quantitative real time-PCR.

Table II

Functional enrichment of biological processes at (a) 24 and (b) 48 hpf. Significantly ($p < 0.05$) enriched biological network processes (Metacore, GeneGo) and biological process GO terms for genes differentially expressed at (a) 24 and (b) 48 hpf.

Biological process network	<i>p</i> value	Count (%)
a.		
Translation elongation - Termination	4.10E-03	9/115 (7.8%)
Immune response - TCR signaling	8.33E-03	8/106 (7.6%)
Inflammation - IgE signaling	8.47E-03	6/65 (9.2%)
Protein folding - Protein folding nucleus	1.34E-02	4/34 (11.8%)
Regulation of cellular metabolic process	2.18E-02	45/135 (33.3%)
G-protein coupled receptor protein signaling pathway	2.25E-02	14/97 (14.4%)
Immune Response - T helper cell differentiation	2.34E-02	6/81 (7.4%)
Protein biosynthesis	2.35E-02	7/172 (4.1%)
Protein folding - Folding in normal condition	3.03E-02	6/86 (7%)
Proliferation - Lymphocyte proliferation	3.42E-02	8/137 (5.8%)
Organ regeneration	3.68E-02	3/135 (2.2%)
Protein folding - Response to unfolded proteins	4.49E-02	4/49 (8.2%)
Cell cycle - S phase	4.80E-02	6/96 (6.3%)
b.		
G-protein coupled receptor protein signaling pathway	7.34E-08	59/385 (15.3%)
Transport - Synaptic vesicle exocytosis	6.93E-03	15/94 (15.9%)
Regulation of vesicle-mediated transport	1.38E-02	8/304 (2.6%)
Metal ion transport	1.77E-02	22/304 (7.2%)
Proliferation - Negative regulation of cell proliferation	2.00E-02	17/125 (13.6%)
Bone remodeling	2.25E-02	4/340 (1.2%)
Response to protein stimulus	2.26E-02	8/340 (2.4%)
Neurophysiological process - Transmission of nerve impulse	2.47E-02	17/128 (13.3%)
Regulation of nucleotide biosynthetic process	2.84E-02	10/385 (2.6%)
Regulation of catecholamine secretion	3.37E-02	4/385 (1.0%)
Protein folding - Response to unfolded proteins	3.83E-02	8/49 (16.3%)
Biogenic amine metabolic process	4.57E-02	8/385 (2.1%)
Cellular macromolecular complex assembly	4.78E-02	15/330 (4.5%)
Signal transduction - CREM pathway	4.96E-02	10/70 (14.3%)

GO, gene ontology; hpf, hours post fertilisation.