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High-Sensitivity Real-Time Analysis of Nanoparticle Toxicity in Green Fluorescent Protein-Expressing Zebrafish

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Gold nanoparticles (AuNP) show great potential for diagnostic and therapeutic application in humans. A great number of studies have tested the cytotoxicity of AuNP using cell culture. There is, however, an urgent need to test AuNP in vertebrate animal models that interrogate biodistribution and complex biological traits like organ development, whole body metabolism, and cognitive function. The sheer number of different compounds precludes the use of small rodent model for initial screening. The extended fish embryo test (FET) is used here to bridge the gap between cell culture and small animal models. A study on the toxicity of ultrasmall AuNP in wild type and transgenic zebrafish is presented. FET faithfully reproduce all important findings of a previous study in HeLa cells and add new important information on teratogenicity and hepatotoxicity that could not be gained from studying cultured cells.

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1. Introduction

The physicochemical properties of nanoparticles are often markedly different from bulk materials owing to their surface reactivity. Nanoparticles can penetrate biological barriers because of their small size. They exhibit high surface-to-volume ratio, enhancing dissolution and surface reactivity thus causing biological interaction.[1–5] Gold nanoparticles (AuNP) in particular differ from chemically inert bulk gold in that AuNP become cytotoxic depending on size and ligand composition.[6,7] AuNP are currently being evaluated for therapeutic and diagnostic purposes in vitro[8–10] and in vivo.[11] Previously, we showed that triphenylphosphine monosulfonate (TPPMS) capped 1.4 nm gold nanoparticles (Au1.4MS) were toxic in HeLa cells while smaller and larger TPPMS capped and glutathione (GSH) or thioglucose (used as ligand in the commercial product Aurovist) capped AuNP of similar size were much less toxic.[7] In this cell test, the toxicity of Au1.4MS was caused by oxidative stress emanating from the nanoparticles that was much exacerbated by cellular ROS production causing apoptosis and necrosis.[6] In addition, the toxicity of the Au1.4MS was inhibited by the addition of GSH.

AuNP toxicity was mainly studied in cell based in vitro experiments. Translocation of nanomaterials across biological barriers into the intact organism, organ specific toxicity, the interference of nanoparticles with embryonic development

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and reproductive toxicity can not however, be addressed in cell-based experiments. The European Community legislative framework REACH for the Registration, Evaluation, Authorisation and Restriction of Chemicals and its regulatory annex for nanomaterials call for modified test guidelines to determine specific hazards associated with nanomaterials.[12] Studies in vertebrates are indispensable to obtain a comprehensive toxicity profile of chemicals. The German Federal Institute for Risk Assessment estimated that 45 million laboratory animals will be required to meet the REACH legislation.[13] Ethical issues and cost preclude this excessive use of higher vertebrates. We propose that the zebrafish embryo test may be ideally suited as a complex vertebrate test to study general toxicity, organ toxicity and teratogenicity of nanoparticles. Furthermore, transgenic zebrafish may be employed to interpret specific defense or detoxification pathways triggered by nanoparticles. Zebrafish have a large number of progeny and are a successful refinement of conventional rodent animal models for toxicity assessment.[14,15] 25% of the zebrafish genes are known to be essential for early development and 99% of these genes are homologous to human genes, suggesting that results obtained in zebrafish may be transferable to humans.[16] Besides acute toxicity, the zebrafish embryo test adapts well for kinetic measurements,[17] functional gene analysis, investigation of immune response in the presence of nanoparticles,[15,18] and prediction of long-term effects.[18,19] Zebrafish are small, easy to maintain, transparent, and require low amounts of testing compounds, all of which suggest the use of zebrafish embryos for nanoparticle toxicity screening as proposed by a OECD draft guideline.[20]

We previously reported that a robust heat shock proteins (HSP) stress response was observed in HeLa cells treated with Au1.4MS.[6] Therefore, we deemed transgenic heat shock reporter zebrafish[21] ideal to test toxicity, teratoginicity and upregulation of defense pathways in a complete vertebrate animal model. The activation of HSP expression in the presence of toxic compounds was also reported by others.[6,22–27] We asked if FET would be sensitive and specific enough to recapitulate the toxicity profile of ultrasmall AuNP differing in ligand composition. In particular, we asked if the transgenic zebrafish expressing green flouorescent protein (GFP) under the control of the heat shock protein 70 kDa (hsp70) promoter Tg(hsp70: GFP) were suited to report the robust stress response triggered by Au1.4MS in HeLa cells. We report faithful replication in FET of the toxicity profile previously determined in HeLa cells, both regarding size and ligand chemistry of AuNP. Furthermore Tg(hsp70: GFP) zebrafish reported teratogenicity and hepatotoxicity at AuNP concentrations lower than the LD_{50} concentration observed in HeLa cells and zebrafish.

2. Results and Discussion

We used Au1.4MS, Au1.4GSH and Aurovist as model AuNP of similar size, yet different toxicity in HeLa cells to test toxicity in zebrafish embryos. The stability of AuNP following incubation in embryo medium at 28 °C for 24 h was measured by transmission electron microscopy. All AuNP were stable in embryo medium showing similar size distributions like freshly synthetized compounds (Figure S1). Most importantly there was no hint of AuNP aggregation in fish embryo medium that would explain the relative lower toxicity of Au1.4GSH and Aurovist. To exclude adverse effects derived from ligand composition, the toxicity of TPPMS and GSH ligands was tested separately. We also asked if GSH could abolish the toxicity of AuNP in zebrafish like it did in HeLa cells. In short, we asked whether the results obtained in cell culture could be faithfully reproduced in zebrafish embryos, a complex vertebrate animal model. **Figure 1**A shows that 400 µM

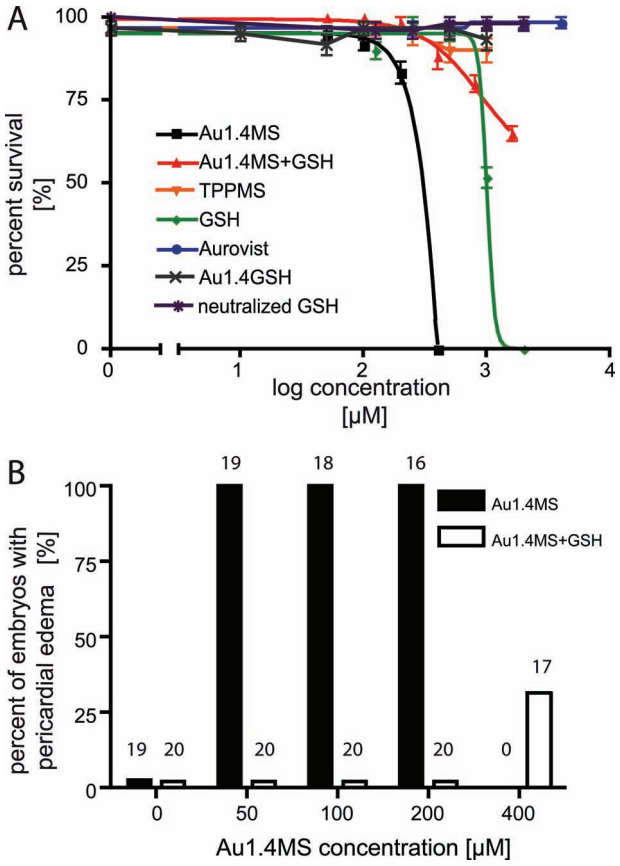

Figure 1. AuNP toxicity in developing zebrafish embryos. (A) LD_{50} of AuNP and ligand chemicals in zebrafish embryos scored at 72 hpf. The zebrafish had been continuously treated from 4 hpf with TPPMS, GSH, Aurovist, Au1.4GSH and Au1.4MS alone or in combination with GSH. One hundred percent of embryos died in the presence of 400 µM Au1.4MS. Adding 500 µM GSH to 400 µM Au1.4MS increased survival from 0% to 88%. 95% embryos survived in the presence of 500 µM either TPPMS (orange) or GSH (green). 98% embryos survived in the presence of up to 4 mM Aurovist (blue). 93% embryos survived in the presence of up to 1 mM Au1.4GSH (dark grey). 100% embryos survived in the presence of up to 2 mM neutralized GSH (dark blue). 65% embryos survived in the presence of up to 1600 µM Au1.4MS in combination with 500 µM GSH (red). (B) Pericardial edema scored at 72 hpf in zebrafish embryos that had been continuously exposed from 4 hpf onward to 0 to 400 µM Au1.4MS with or without 500 µM GSH. Pericardial edema was absent in embryos kept in embryo medium or in buffered GSH. In the absence of GSH, 100% of the embryos developed pericardial edema in exposure to 50 to 200 µM Au1.4MS and all embryos died after exposure to 400 µM Au1.4MS. In the presence of additional GSH, none of the embryos developed pericardial edema after exposure to up to 200 µM Au1.4MS and 30% of the surviving embryos developed pericardial edema in exposure to 400 µM Au1.4MS. Percentage pericardial edema was scored in surviving embryos out of 20 embryos. Number of surviving embryos is given above each bar.

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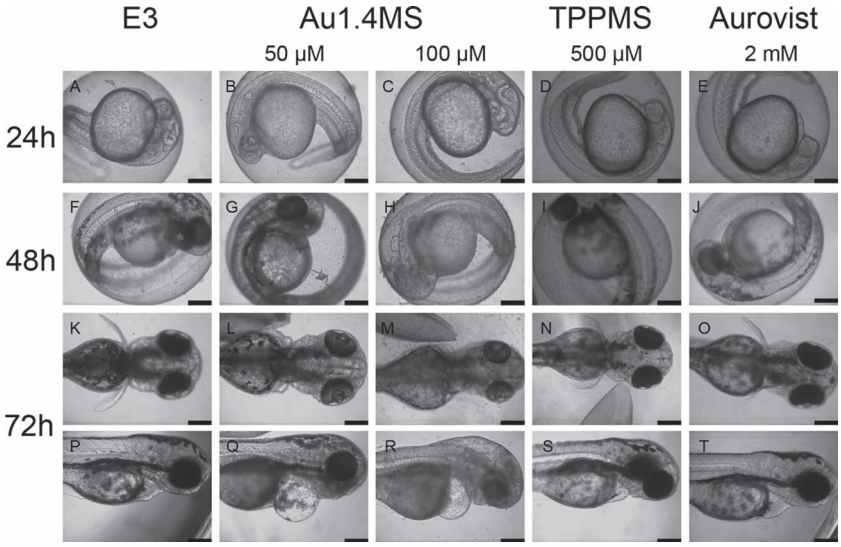

Figure 2. Malformations due to AuNP toxicity in developing zebrafish embryos. Zebrafish at 24 hpf (A–E), 48 hpf (F–J), and 72 hpf (K–T) after exposure to embryo medium E3 (A,F,K,P), 50 µM Au1.4MS (B,G,L,Q), 100 µM Au1.4MS (C,H,M,R), 500 µM TPPMS (D,I,N,S) and 2 mM Aurovist (E,J,O,T). Embryos were scored for number of somites, tail detachment, normal yolk body size, yolk extension and sculptured brain and eyes at 24 hpf (A,B,C,D,E). (F) Embryo showing normal morphology and pigmentation in embryo medium at 48 hpf. (G,I,J) At 48 hpf embryos exposed to 50 µM Au1.4MS, 500 µM TPPMS and 2 mM Aurovist, had similar morphology compared to the untreated control. (H) Embryos exposed to 100 µM of Au1.4MS were less pigmented than untreated embryos. (K,P) At 72 hpf embryos in embryo medium showed normal development regarding pigmentation, head-tail angle (HTA), protruding mouth, pectoral fin and looped heart morphology (K dorsal, P dorsal view). (L,Q) In contrast embryos exposed to 50 µM Au1.4MS had a string-like heart and pericardial edema (L dorsal, Q lateral view). Embryos exposed to 100 µM of Au1.4MS had an increased HTA, hypopigmentation, pericardial edema, string-like heart and a flattened yolk sac with increased width (ventral-trunk orientation; M dorsal R lateral view). Embryos exposed to 500 µM TPPMS (N,S) and 2 mM Aurovist (O,T) had no malformation at 72 hpf. Scale bar = 200 µm.

Au1.4MS (black curve) caused 100% lethality at 72 hour post fertilization (hpf). The LD_{50} of Au1.4MS in zebrafish embryos was 234.1 µM. In contrast, up to 1 mM Au1.4GSH (dark grey) and 4 mM Aurovist (blue curve) did not cause lethality in zebrafish embryos. Also TPPMS alone did not decrease the survival rate of embryos up to 1 mM (orange curve). Fifty percent of the embryos died in 1 mM GSH at 72 hpf (green curve) This unexpected toxicity of GSH was due to a drop in pH to 3.5 in the embryo medium. Adjusting the pH of a 1 mM GSH solution in embryo medium to pH 7.4 by adding $NaHCO_3$ completely abrogated the lethality as shown in the dark blue curve illustrating the effect of up to 2 mM neutralized GSH. Thus it is important to check the pH of the complete test solution and to neutralize if necessary. Cell culture media commonly contain a bicarbonate/CO_2 buffer and are thus protected against toxic pH change that may be introduced with test compounds. We had similar experience when we tested large protein-stabilized AuNP that were non-toxic in cell culture, but proved toxic in zebrafish embryos (results not shown). In HeLa cells, GSH abrogated the toxicity of Au1.4MS. Similarly in FET, 400 µM Au1.4MS toxicity was reduced from 100% to 12% after addition of 0.5 mM GSH and 65% of the embryos survived upon to 1600 µM Au1.4MS co-incubation with GSH (red curve). Figure 1B reports the percentage of cardiac malformation in response to nanoparticle treatment. Pericardial edema occurred in all zebrafish embryos treated with 50 µM or more of Au1.4MS. The rate of pericardial edema decreased from 100% to 0% in medium containing 500 µM GSH with up to 200 µM Au1.4MS. 88% of the embryos even survived exposure to 400 µM Au1.4MS in the presence of GSH and 70% of the embryos survived without pericardial edema. Thus, like in HeLa cells, GSH abrogated the toxicity of Au1.4MS most likely by a combination of ligand exchange to the nontoxic compound Au1.4GSH and by reactive oxygen species (ROS) scavenging.

Figure 2 shows zebrafish from 24 hpf to 72 hpf that were continuously exposed to AuNP from 4 hpf. At the sublethal concentration, Au1.4MS induced prominent hypopigmentation, the extent of which was dose dependent. Compared to untreated embryos (Figure 2F,K,P), pigmentation was lacking after treatment with 100 µM Au1.4MS at 48 hpf (Figure 2H) while embryos cultured in 50 µM Au1.4MS showed normal pigmentation (Figure 2G). In addition, pericardial edema developed upon treatment with Au1.4MS at up to 50 µM (1/5 LD_{50}) dose. Pericardial edema as a result of nanoparticles exposure was also frequently reported in several other studies concerning nanoparticle toxicity.[28–31] Therefore, pericardial edema may be regarded as a common developmental defect triggered by toxic compounds including toxic nanoparticles. In addition to hypopigmentation and pericardial edema, embryos treated with Au1.4MS at 100 µM (2/5 LD_{50}) (supplemental Figure S2B) developed an abnormal curvature of the body axis. TPPMS up to 1 mM did not cause any of the malformations observed in zebrafish treated with Au1.4MS alone (Figure 2D,I,N,S). Therefore, like in HeLa cells the Au1.4MS nanoparticles, but not the ligand were toxic. Aurovist up to 2 mM also did not cause malformation or lethality (Figure 2E,J,O,T).

The malformations observed in response to Au1.4MS at an acute lethal dose (400 µM) were prevented by adding reducing agents and ROS scavengers including GSH, N-acetylcysteine (NAC), and sodium thiosulfate (STS). **Figure 3** shows that the embryos cultured in embryo medium, Aurovist, GSH, and TPPMS all had normal pigmentation and cardiac morphology (Figure 3A–D). In contrast, Au1.4MS caused severe pericardial edema and cardiac malformation resulting in a string-like morphology of the heart at 50 µM (Figure 3E), and complete embryo death at 400 µM (Figure 3G). With added GSH the percentage of surviving embryos increased from 0% to 88%. Most of the surviving embryos had normal pigmentation and morphology (Figure 3F,H) suggesting that the toxicity of Au1.4MS was due to oxidative stress, which could be abrogated by ROS scavengers.

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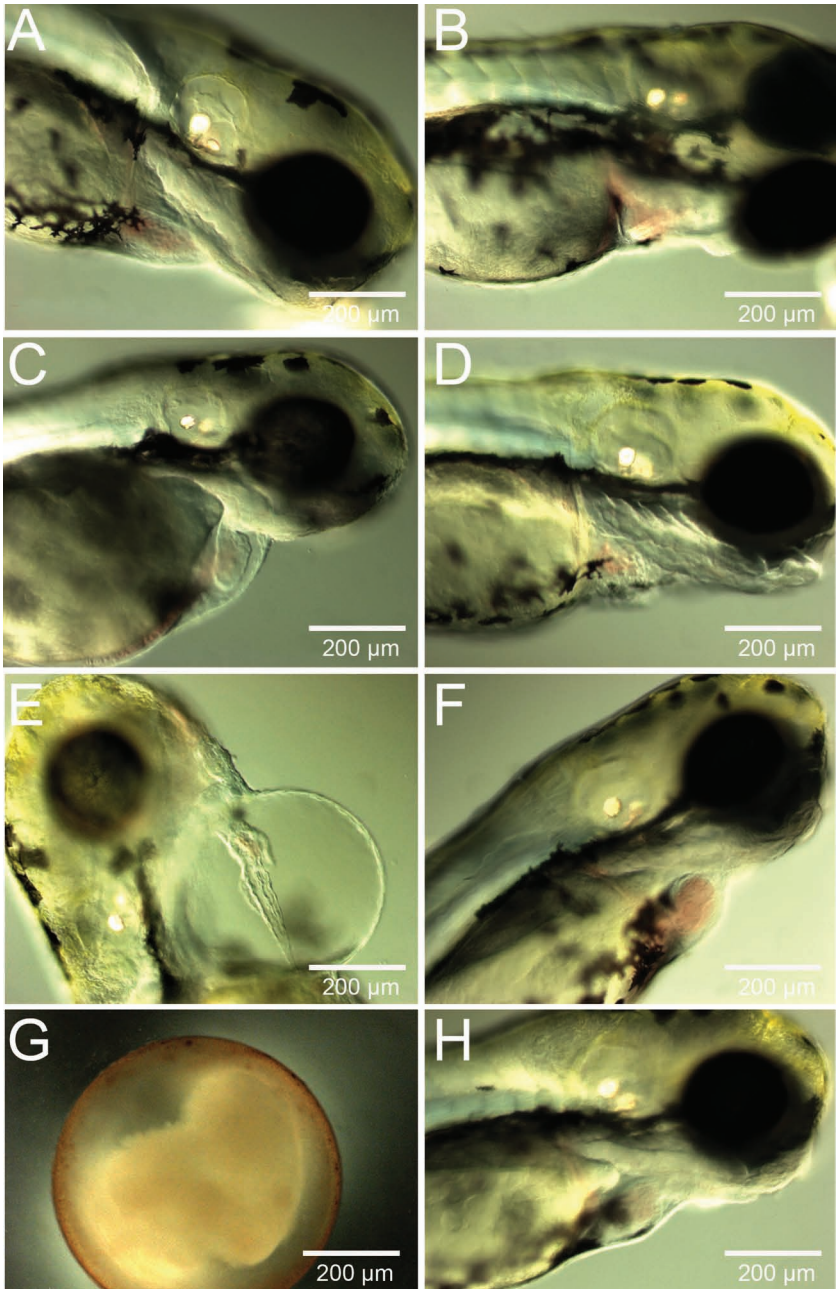

Figure 3. Zebrafish embryo morphology at 72 hpf following AuNP treatment. Zebrafish embryos were kept in (A) embryo medium, (B) 3 mM Aurovist, (C) 0.5 mM GSH, (D) 0.5 mM TPPMS, (E) 50 µM Au1.4MS, (F) 50 µM Au1.4MS + 0.5 mM GSH mixture (G) 0.4 mM Au1.4MS, (H) 0.4 mM Au1.4MS + 500 µM GSH mixture. Note severe pericardial edema (E) and embryo coagulation (G) in Au1.4MS treated zebrafish embryos that are normalized by the addition of GSH (F,H).

We determined AuNP uptake by fish embryos using inductively coupled plasma mass spectrometry ICP-MS. **Figure 4** shows that fish embryos cultured in AuNP for 48 hours with their chorion still attached, and hatched fish larvae that had been cultured in AuNP for 72 hours contained similar amounts of internalized Au1.4MS, Au1.4GSH, and Aurovist. Therefore the different toxicities observed are most likely due to the AuNP characteristics, not barely due to lower internalization.

We previously showed that the protective effect of GSH in Au1.4MS toxicity was due to ligand exchange of GSH for TPPMS resulting the non-toxic compound Au1.4GSH.[6] Häkkinen and colleagues have determined by density functional theory (DFT) that the binding energy of the gold phosphine (Au-PH_3) bond is 0.93 eV, compared to 2.45 eV for a gold thiolate (Au-SCH_3) bond.[32] The potential interaction between the bare gold atoms and biological targets especially thiol containing targets may thus be an additional reason for the toxicity of Au1.4MS. Once the weaker binding TPPMS was replaced with GSH, the stronger gold thiolate bond would prevent further interaction between the unprotected, highly electron-affine gold atom core and biological targets, thus abrogating toxicity.

Conventional FET tests report as primary readout phenotypic changes or death. These two criteria are insufficient to evaluate sublethal toxicity of test material. In contrast, the latent organ specific toxicity caused by nanoparticles at low dose (1/10 LD_{50}) is hard to detect using the conventional FET test. We asked if the sensitivity of FET could be improved by using transgenic zebrafish reporting specific stress pathways. Our previous study showed that HSP70 was upregulated in response to Au1.4MS in HeLa cells.[6] Therefore, we employed transgenic zebrafish expressing GFP under the control of the hsp70 promoter[21] to evaluate the response to sublethal doses of AuNP. The transgene performance was validated by keeping embryos either at 28 °C or at heat shock 37 °C. GFP was constitutively expressed in the eye lens even at ambient temperature (**Figure 5**A, E). Zebrafish lacking lens expression of GFP were excluded from the tests and from further breeding. Figure S3 shows that heat shock for 1 hour at 37 °C from 24 hpf induced robust GFP expression. Exposure to toxic compounds before 24 hpf may therefore fail to induce GFP reporter gene expression in these fish. Nevertheless, we maintained the wild type zebrafish FET protocol and continuously exposed the Tg (hsp70: GFP) transgenic zebrafish to AuNP from 4 hpf.

The GFP expression in response to Au1.4MS was dose and time dependent. Figure 5C shows strong up-regulation of the HSP70 protein after 48 h of treatment with 400 µM Au1.4MS. Weaker GFP signals were observed after treating with 200 µM Au1.4MS for 48 hours (Figure 5B). Strong GFP expression was equally present in the nervous system, brain and muscles in the embryos treated with 200 µM Au1.4MS for 72 hpf (Figure 5D). Au1.4MS up to 50 µM did not induce GFP expression in the transgenic animals in the first 72 hpf.

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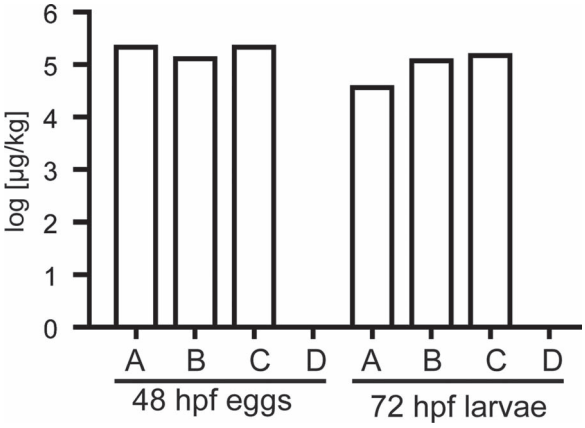

Figure 4. AuNP uptake measured by inductively coupled plasma mass spectrometry ICP-MS. Fish eggs were harvested after culture in 100 µM AuNP or in embryo medium for 48 h, and hatched fish larvae were harvested after 72 h. Fish embryos contained similar amounts of internalized AuNP. A, Au1.4MS; B, Au1.4GSH; C, Aurovist; D, embryo medium without AuNP.

The liver is an important detoxification organ. Liver morphogenesis in zebrafish starts with a budding phase shortly after 24 hpf, proceeds with the formation of a hepatic duct at 50 hpf and ends around 96 hpf with a fully developed liver.[33] Thus liver function or hepatotoxicity can only be interrogated from 96 hpf onward. In addition, zebrafish have enhanced uptake due to direct exposure to the test compound after hatching around 60–72 hpf. A steadily increasing GFP signal reporting hsp70 stress gene induction was observed in the liver of transgenic zebrafish even in the presence of a mere 13 µM Au1.4MS (1/20 LD_{50}) at 120 hpf (**Figure 6**B), yet all these zebrafish with GFP expression in liver had completely normal morphology. Thus Tg(hsp70: GFP) reporter zebrafish were 20-fold more sensitive in reporting toxicity than wild type zebrafish.

3. Conclusion

We have successfully used the zebrafish embryo test to test the developmental toxicity of gold nanoparticles. Like in cell culture toxicity depended on size and ligand chemistry. At similar size, AuNP carrying ligands with high affinity to the gold atomic core were less toxic than AuNP with more labile ligand. Transgenic Tg(hsp70: GFP) zebrafish had similar responses to Au1.4MS like wild type zebrafish in terms of teratogenicity, but were 20-fold more sensitive than wild type in reporting hepatotoxicity of Au1.4MS.

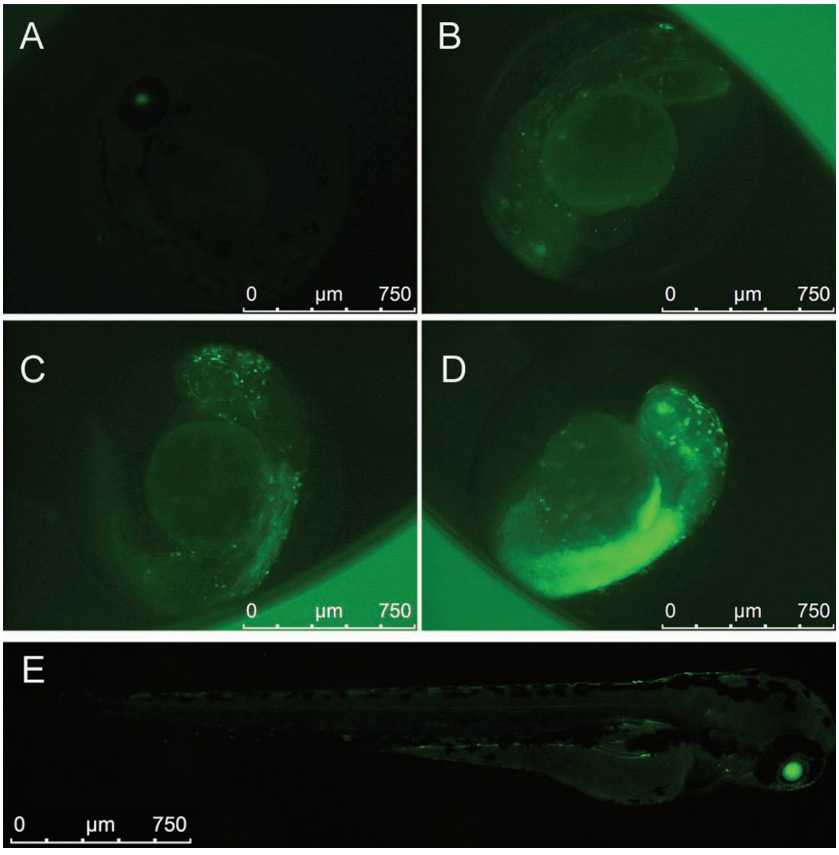

Figure 5. Rapid induction of GFP expression in Tg(hsp70: GFP) zebrafish triggered by toxic doses of Au1.4MS. Transgenic zebrafish embryos expressing GFP under the control of the heat shock protein 70 promoter Tg(hsp70: GFP) were exposed to embryo medium (A,E), 200 µM (B,D) and 400 µM Au1.4MS (C). Spotty GFP signal was seen at 48 hpf in the presence of 200 µM Au1.4MS (B). The GFP signal increased with the toxic dose (C) and with time (D). In addition to the spotty GFP signal observed in the head region, strong GFP expression was present along the trunk at 48 hpf and was further enhanced at 72 hpf (D).

4. Experimental Section

Synthesis of AuNP: Aurovist® was purchased from Nanoprobes. L-glutathione (reduced) is purchased from Fisher BioReagents. HAuCl4. $3H_2O$ (Sigma-Aldrich, ACS reagent), triphenylphosphine (Alfa Aesar, 99+%), $NaBH_4$ (Aldrich, purum p.a.), BF_3 . OEt_2 (Aldrich), H_2SO_4 (Grüssing, p.a.), GSH (Fisher BioReagents, ≥98.%), bis(2-methoxyethyl)ether (Acros, 99%), benzene (AppliChem, p.a.) dichloromethane (VWR, p.a.), methanol (VWR, p.a.). Water was deionized using an ELGA Purelab Ultra water purification system. TPPMS was synthesized as described.[34] 1.4 nm sized, triphenylphosphine-stabilized AuNP were synthesized as described and transferred to the water phase via a two-phase ligand exchange reaction to yield TPPMS-stabilized Au1.4MS[35] Au1.4GSH was synthesized following a published protocol.[36] The AuNP were characterized by scanning transmission electron microscopy, elemental analysis, and UV/VIS spectroscopy. Concentrations of AuNP solutions were determined by atomic absorption spectroscopy.

Size Distribution of AuNP in Embryo Medium Determined by Electron Microscopy: Au1.4MS, Au1.4GSH and Aurovist at stock concentrations were diluted in embryo

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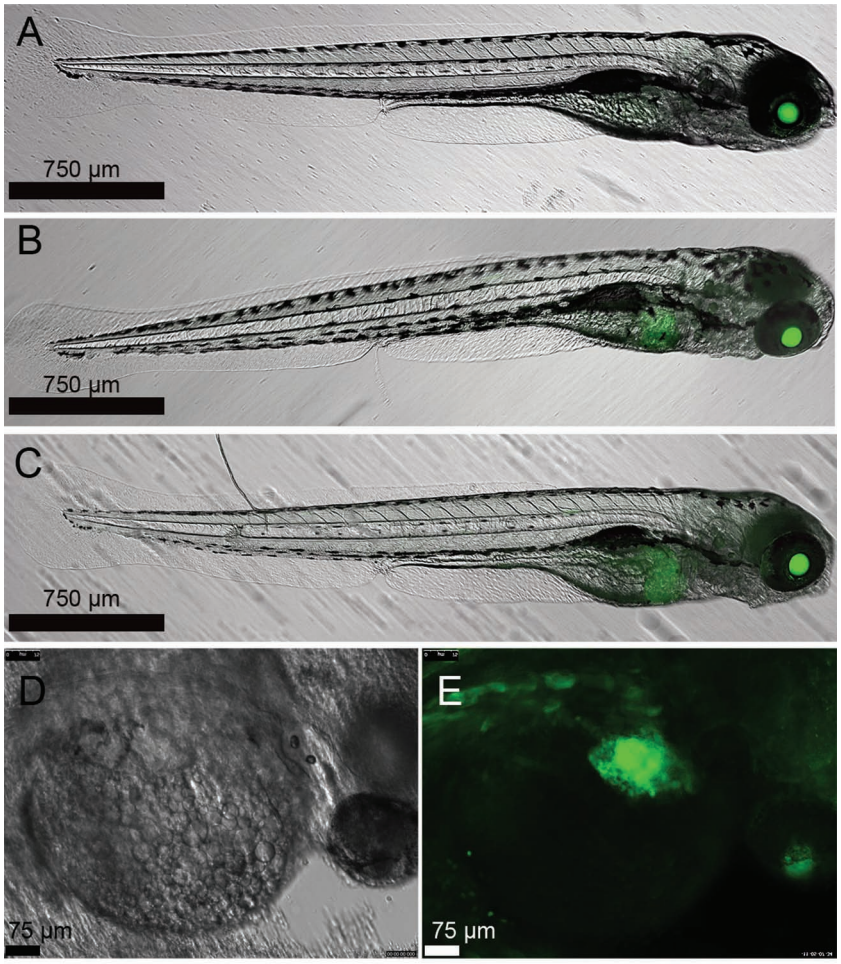

Figure 6. Delayed hepatic GFP expression in transgenic Tg(hsp70: GFP) zebrafish triggered by low doses of Au1.4MS. Untreated embryos kept in embryo medium did not express HSP in the liver (A). HSP induction was strongly upregulated in liver in response to sub-lethal dose Au1.4MS 13 µM (B) and 25 µM (C). Note that no morphological malformations were present at this low concentration. 200 µM Au1.4MS causes severe malformation and high expression of HSP in liver (D,E).

medium to 500 µM and incubated at 28 °C for 24 h. Five microliter droplets of sample were dried on carbon coated copper foil. The specimens were treated for 10 s using Argon plasma activation to remove organic contaminants.

Zebrafish Raising and Breeding: Wild type and Tg(hsp70: GFP) transgenic zebrafish[21] were maintained at 26.5 °C on a 14 h/10 h light dark cycle in an aquarium (aquaPP-Module, Aqua-Schwarz). Embryos were obtained from individual fish by pairwise breeding as described.[37] The onset of daylight is the major stimulus for zebrafish to spawn. The embryos were collected one hour after the light was turned on in the fish rack and the fertilized embryos were harvested using a dissecting microscope.

LD_{50} of AuNP and Compounds in Zebrafish Embryos: Assays were performed in U-shape bottom 96-well plates. AuNP and compound stock solutions were diluted in embryo medium to prepare serial working concentrations. 200 µL of test solutions were added into each well. One fertilized embryo was added into each well using a wide bore plastic pasteur pipette, 10 embryos were plated for each concentration. Embryos exposed to embryo medium served as control. All embryos were cultured at 28 °C. The morphology of embryos was observed directly after seeding and at 24, 48, and 72 hpf, respectively. The percentage of viable, dead and hatched larvae (72 hpf) was recorded as described.[38,39] The mortality was calculated from three independent experiments.

Inductively Coupled Plasma Mass Spectrometry ICP-MS: Measurements were done according to DIN ISO 11466 guidelines for environmental sample analysis. Zebrafish embryos were incubated in AuNP at 100 µM. For each AuNP tested, 50 embryos with chorion still attached were collected at 48 hpf and 80 hatched larvae were collected at 72 hpf. Embryos were weighed, transferred into 100 ml-PTFE-Tubes and digested in a mixture of 1 mL aqua regia and 1 mL 30% H_2O_2 for 1 h at 210 °C using a microwave digestion oven (MLS-Ethos plus). The digested samples were diluted tenfold with deionized water. Gold content was measured via ICP-MS (Elan-DRC2) in ppb (µg/Kg).

Induction of the Heat Shock Protein in Tg(hsp70: GFP) Zebrafish: Transgenic zebrafish, one embryo per well, were seeded in 96-well plates. The 96-well plate was incubated at 37 °C for 60 min to induce heat shock. Following the induction, the embryos were further cultured at 28 °C. To validate the capability of the transgenic strain as a reporter, embryos at stages 4, 8, 12, 24, 48 and 72 hpf were exposed to heat shock. GFP expression was continuously monitored by time-lapse video microscopy using a Leica DMI 6000B inverted microscope equipped with TOKAI HIT heated stage. No GFP signal was observed when the heat shock was applied before 12 hpf. Thus full strength GFP expression can be induced from 24 hpf onward.

Induction of HSP Promoter by AuNP in Tg(hsp70: GFP) Zebrafish: 200 µL per well freshly prepared AuNP at serial concentrations in embryo medium were added to the 96-well plates. The transgenic zebrafish embryos, one embryo per well, were added. All embryos were continuously exposed to the nanoparticles throughout the test.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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