Bio-compatibility of silver nanomaterials using the embryonic zebrafish bioassay

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ABSTRACT

The bio-compatibility of silver nanomaterials (AgNP) is currently of great interest to regulators and health and safety professionals because it is a material that has the potential to reach mass populations. This study reports on the development and implementation of a heart rate assay that requires only 30 seconds per animal using this semi-automated technique. Zebrafish embryos were exposed to four AgNP (20 nm Citrate, 20 nm PVP, 110 nm Citrate, 110 nm PVP), at five low exposure concentrations (<= 10 ug/mL), across two exposure media (EM, 62.5 uM $CaCl_2$). The heart rate assay at 6 days post fertilization (dpf) detected a significant concentration and media effect. Experimenters who alter exposure media must not overlook other potential biological effects that could influence or confound data interpretation. The development and implementation of this sensitive heart rate assay adds to the versatile well-established zebrafish bioassays, and is able to detect affects not identified with other assays.

Keywords: zebrafish, silver, nanomaterial, heart, physiology, toxicology

1 BACKGROUND

The bio-compatibility of silver nanomaterials (AgNP) is currently of great interest to regulators and health and safety professionals because it is a material that has the potential to reach mass populations. A prerequisite to any nanomaterial-biological interaction study is well characterized material [1]. Four well characterized AgNP were obtained in order to investigate low-concentration biological effects using three well-established zebrafish (*Danio rerio*) bioassays. The assays measure mortality and morbidity [2], and behavior [3]. Here we report the development and implementation of a heart rate assay that requires only 30 seconds per animal using this semi-automated technique. The nanomaterials were obtained from the NIEHS Centers for Nanotechnology Health Implications Research (NCNHIR) Consortium and characterized by the National Characterization Laboratory (NCL). The same nanomaterials were provided to numerous laboratories conducting parallel in vivo and in vitro studies allowing for comparative analysis. The nanomaterial sizes of 20 nm, 110 nm, and capping surfaces of Citrate and PVP were provided to the Oregon State University (OSU) Sinnhuber Aquatic Research Laboratory (SARL) in a liquid medium formulation for the experiments. All exposures were done with dechorionated embryos [4] to reduce potential confounding barrier effects. Two distinct exposure media formulations including the traditional 1x zebrafish embryo media (EM) [5] and a lower ionic strength 62.5 uM $CaCl_2$ solution were used. The low concentration $CaCl_2$ media formulation was chosen as it produces dispersed nanomaterials and normal embryos [2,6]. Five low-concentration AgNP exposure levels [0, 0.01, 0.1, 1, 10 ug/mL] were tested to investigate differential responses [7] at up to 6 days post fertilization (dpf).

2 RESULTS AND DISCUSSON

Before beginning to report on the heart rate assay, it is necessary to present the results from the mortality and morbidity assay [2]. Only three of the forty experimental combinations of agent (4-levels), concentration (5-levels), and media (2-levels), resulted in any significant adverse effects. These mortality and morbidity effects were all at the highest concentration level of 10 ug/mL and included: 20 nm AgNP PVP $CaCl_2$ (68% effected), 20 nm AgNP Citrate $CaCl_2$ (62% effected), and 20 nm AgNP Citrate EM (56% effected). Embryos exposed to the 10 ug/mL 20 nm AgNP PVP EM were not affected, nor were any of the embryos exposed to the 110 nm AgNP. $AgNO_3$ was included as a positive control, and at the four lowest concentrations resulted in three of the eight being effected: 1 ug/mL $AgNO_3$ $CaCl_2$ (75%), 0.1 ug/mL $AgNO_3$ $CaCl_2$ (56%), and 1 ug/mL $AgNO_3$ EM (56%). None of the other $AgNO_3$ concentrations impacted the zebrafish.

Behavioral based photomotor response movement and physiological based heart rate assays were both conducted at 6 dpf for animals displaying no mortality or morbidity. An exploratory analysis (Fig. 1-2) revealed a probable media effect for the heart rate assay and a possible media effect for the photomotor response movement assay. The median heart rate results included; EM (178.3 bpm) and $CaCl_2$ (162.8 bpm) and the photomotor response median movement results included; EM (23.4 sec/min) and $CaCl_2$ (22.1 sec/min) over the four agents and five concentrations. A tabular summary of the average heart rates and 95% confidence intervals (Tab. 1) revealed that while no significant differences could be found between AgNP and controls, AgNP heart rates were significantly lower in

$CaCl_2$ media versus EM media. The differences between utilizing 62.5 uM $CaCl_2$ media and EM media consisting of several ingredients (1 mM $CaCl_2$, 15 mM NaCl, 1 mM $MgSO_4$, 0.7 mM $NaHCO_3$, 0.5 mM KCl, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4) must influence the different heart rates. This reduced heart rate may be attributed to an ionic imbalance or reduced sodium (Na) pump [8].

These results led to a more robust analysis using linear mixed effects regression for both the photomotor response movement and physiological based heart rate assays. None of the AgNP materials, at any of the tested concentrations, in either of the media solutions, identified significant effects in the behavioral assay at the tested epoch. However, using the heart rate assay there was a significant media effect resulting in a heart rate reduction of 16.6 bpm (9.2%) [$t(11) = 5.15$, $p = 0.0003$] for $CaCl_2$ media and a segmented concentration effect; - 3.4 bpm [range 0 to 0.1; $t(1102) = 2.11$, $p = 0.035$] and + 4.9 bpm [range 0.1 to 10; $t(1104) = -2.95$, $p = 0.003$] (Fig. 1-3, Tab. 1). The 9.2% reduction in heart rate by media may also be clinically significant.

This work further explores prior laboratory work [6] by adding the heart rate assay and by implementing linear mixed effects statistical methods to provide more understanding of the fixed (agent, media, concentration) and random (plate, day) inferences. The motivation for the work was to add an additional biological assay to the versatile zebrafish model and to explore the sensitivity beyond the well-established assays. In addition, the need to adjust the environment or exposure medium to accommodate test agents may be necessary. Experimenters who alter exposure media must not overlook other potential biological effects that could influence or confound data interpretation. The development and implementation of this sensitive heart rate assay adds to the versatile well-established zebrafish bioassays.

3 METHODS

The four AgNP (20 nm Citrate, 20 nm PVP, 110 nm Citrate, 110 nm PVP) were each individually tested commencing on four consecutive days. The positive control of greater than 99% purity $AgNO_3$ (Sigma-Aldrich) was also included. During each day, one material was tested with the two media solutions (EM, 62.5 uM $CaCl_2$). The 96-well microtiter plate contained five AgNP low exposure concentration levels (0, 0.01, 0.1, 1, 10 ug/mL) with 16 animals per level or a total of 80 animals per plate. Each animal is individually exposed and individually contained in 100 uL of liquid exposure media within a single well of the microtiter plate. A biological replicate or second plate was added to increase the sample size to 32 for each level. The positive control $AgNO_3$ was included. The experiment began when animals were loaded into microtiter plates at 6 hpf, and ended when the assays were complete at 6 dpf.

3.1 Heart Rate Assay

Embryos were assayed using the authors protocol at 6 dpf. No form of anesthesia or immobilization were utilized. First heartbeats commence at approximately 24 hpf and increase to a peak rate above 200 bpm by 4 to 5 dpf. At 6 dpf and 28C the normal embryo heart rate reduces to approximately 180 bpm (3 Hz). Like the development of the human fetal heart during the first trimester, testing the zebrafish heart rate at 5 dpf is more difficult than 6 dpf because the mean and variance are both larger. The zebrafish heart rate at the 6 dpf timepoint can be reliably counted by a human with an inverted light microscope. The heart rate assay measures embryo heartbeats using a microscope with 2x objective and a Bluetooth enabled millisecond event logger. Real time display on a computer screen proceeds by counting 50 consecutive beats for each animal in each well of the microtiter plate. The combination of microscope-eye-brain detection of a zebrafish heartbeat is influenced more by the larger ventricle beat than the atrium beat. Total cycle time per well for this semi-automated technique is approximately 30 seconds for counting and movement to the next well; resulting in a total assay time of 48 minutes for a 96 well plate.

3.2 Animals

Adult tropical 5D strain (wild type) zebrafish were raised at the Sinnhuber Aquatic Research Laboratory (SARL) in the Aquatic Biomedical Models Facility Core of the Environmental Health Sciences Center at Oregon State University under standard conditions (28C, 14h light/10h dark cycle) on a recirculating water system. Zebrafish husbandry and testing was conducted in compliance with approved Oregon State University Institutional Animal Care and Use Committee protocols.

3.3 Data Management and Exploration

Data management, exploration, and analysis was conducted with Open Source and Public Domain software tools. Data from the three biological assays were collected, verified for accuracy, and stored in a ACID-compliant SQL transactional database. The Zuur protocol [9] was used as a guideline for data exploration. All statistical analysis and graphical representations were conducted using the R programming language (R 2.15.x). R packages were utilized as needed. Graphical R tools used were primarily from the base and lattice packages 'graphics::' and 'lattice::'. The primary analysis methods used were linear mixed effects regression from the R packages 'nlme::lme()' [10] and 'lme4::lmer()' [11], to conduct the regression analysis for the continuous response variable assays. The models included a single response variable representing the assay endpoint, fixed plus random intercepts and slopes, predictor variables, and a nesting group variable. A statistical significance ($p < 0.05$) was selected in advance and clinical

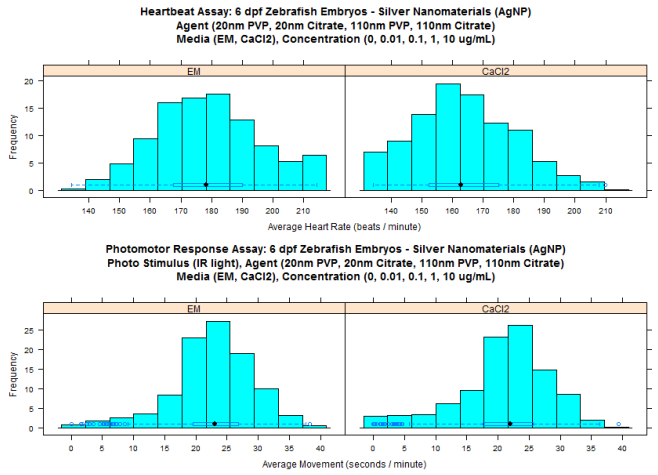

Figure 1. Heartbeat and Photomotor Response Assay: Median differences in heart rate and movement. Both median $CaCl_2$ heart rate and movement are lower than EM rates. Average movement measured in IR light (darkness) is for the first 5 minutes of darkness after 10 minutes of light. Samples: N=568 for EM and N=554 for $CaCl_2$.

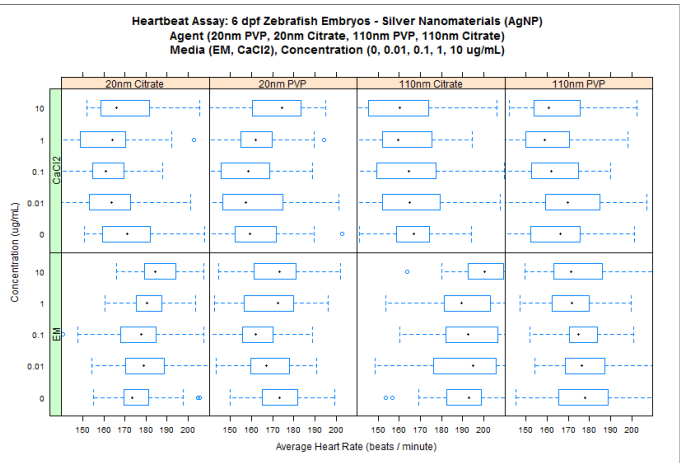

Figure 2. Heartbeat Assay: Median differences in heart rate. All $CaCl_2$ median heart rates are lower than EM rate. Heart rate averages taken from approximately 50 beats per animal. Samples: Total N=1122 (N=568 for EM and N=554 for $CaCl_2$). Per level (min N=10, max N=32, avg N=28). Notable differences in sample size all at 10 ug/mL (N=10 for 20nm AgNP PVP $CaCl_2$, N=11 for 20nm AgNP Citrate $CaCl_2$, N=14 for 20nm AgNP Citrate EM).

Date	Media	Agent	Samples	Average Heartbeat (bpm) Concentrations (0.01, 0.1, 1, 10 ug/mL) All AgNP Agents (20 nm, 110nm) All Capping (Citrate, PVP)		Media	Agent	Samples	Average Heartbeat (bpm) Concentrations (0) Vehicle	
All	EM	All	456	179.9 (178.2 to 181.7)		EM	None	112	179.8 (176.4 to 183.2)	
All	CaCl2	All	442	162.6 (160.9 to 164.3)		CaCl2	None	112	165.6 (162.7 to 168.5)	
20120626	EM	110 nm Citrate	116	195.9 (192.3 to 199.5)		EM	None	28	191.7 (182.7 to 200.8)	
20120627	EM	110 nm PVP	116	175.6 (172.8 to 178.3)		EM	None	29	177.7 (171.9 to 183.5)	
20120628	EM	20 nm Citrate	104	180.6 (177.7 to 183.4)		EM	None	28	176.8 (172.2 to 181.4)	
20120629	EM	20 nm PVP	120	168.1 (165.6 to 170.6)		EM	None	27	172.9 (168.1 to 177.7)	
20120626	CaCl2	110 nm Citrate	123	162.5 (158.8 to 166.2)	"lower"	CaCl2	None	30	166.1 (161.8 to 170.5)	"lower"
20120627	CaCl2	110 nm PVP	118	164.8 (161.6 to 168.0)	"lower"	CaCl2	None	30	164.9 (159.5 to 170.3)	"lower"
20120628	CaCl2	20 nm Citrate	100	162.1 (158.5 to 165.8)	"lower"	CaCl2	None	23	171.5 (165.5 to 177.5)	
20120629	CaCl2	20 nm PVP	101	160.6 (157.3 to 164.0)	"lower"	CaCl2	None	29	161.1 (154.1 to 168.2)	

Table 1. Heartbeat Assay: Summary of average heart rate (bpm) and 95% confidence intervals comparing media types and AgNP agent versus control. All AgNP heart rates are significantly lower in $CaCl_2$ versus EM (bottom to top comparison). There is no significant difference between combined AgNP heartbeats and controls (left to right comparison). Samples: Total N=1122 after 478 lost due to mortality and morbidity. An experimental error occurred on day 1 (20120626) when animals were tested at 5 dpf instead of the planned 6 dpf. All other animals were tested at 6 dpf.

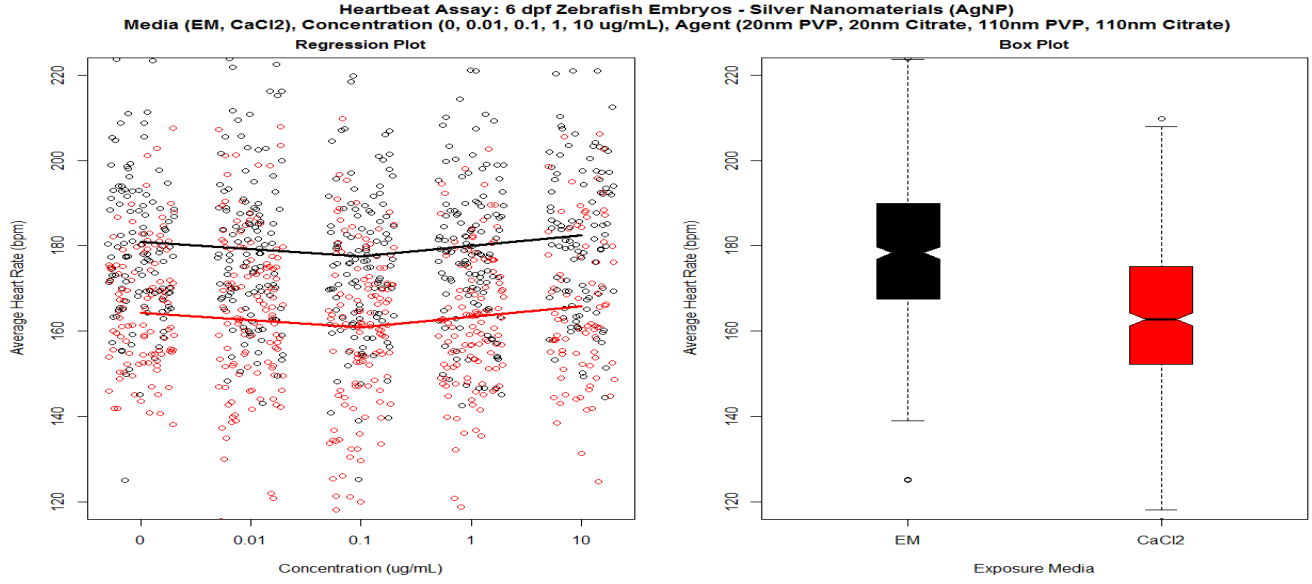

Figure 3. Heartbeat Assay: Mixed effects regression analysis for all factors – Fixed Effects (agent, media, concentration) and Random Effects (plate, day) with Nesting (plate within day) identified a significantly changed heart rate based on media and concentration. Media differences (EM to $CaCl_2$) reduce heart rate by 16.6 bpm (9.2%) [t(11) = 5.15, p = 0.0003]. Concentration differences (0 to 0.1) reduce heart rate by 3.4 bpm [t(1102) = 2.11, p = 0.035] and differences (0.1 to 10) increase heart rate by 4.9 bpm [t(1104) = -2.95, p = 0.003]. Y = 180.94 – [EM, $CaCl_2$] 16.6 * (0,1) – [range 0 to 0.1] 3.4 * (1) + [range 0.1 to 10] 4.9 * (1). Samples: Total N=1122. Note: Points are *Jittered* along the x-axis for display purposes.

significance was considered along with statistical significance. Model building proceeded using backward elimination from the most complex to the least complex until a satisfactory model was obtained. Significance values and the Akaike's Information Criteria (AIC) which have been demonstrated to be asymptotically equivalent to cross validation, were used as a selection criteria as well as guidelines from the Zuur protocol [9]. Pooled results are presented (Fig. 3).

3.4 Agent: Silver Nanoparticles

Silver nanoparticles (AgNP) used in this study were obtained from the NIEHS Centers for Nanotechnology Health Implications Research (NCNHIR) Consortium and characterized by the National Characterization Laboratory (NCL). The materials were manufactured by a commercial provider (nanoComposix) from their product line. Sizes include 20 nm and 110 nm and surfaces or capping agents include PVP and Citrate. As received, the stock solution has a concentration of 1 mg/mL in water. Samples are stored in the dark at 4C until used.

4 ADDITIONAL MATERIAL

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Authors contributions and competing interests

JAF prepared the manuscript, experiment, and conducted all the analysis. KTK [6] prepared the agent formulations. RLT reviewed and edited the manuscript. The authors declare that they have no competing interests.

REFERENCES

[1] Harper SL, Carriere JL, Miller JM, Hutchison JE, Maddux BL, Tanguay RL (2011) Systematic Evaluation of Nanomaterial Toxicity: Utility of Standardized Materials and Rapid Assays. ACS Nano 5:4688–4697. doi: 10.1021/nn200546k.

[2] Truong L, Harper SL, Tanguay RL (2011) Evaluation of Embryotoxicity Using the Zebrafish Model. In: Gautier J-C (ed) Drug Safety Evaluation. Humana Press, pp 271–279. doi: 10.1007/978-1-60761-849-2_16.

[3] Saili KS, Corvi MM, Weber DN, Patel AU, Das SR, Przybyla J, Anderson KA, Tanguay RL (2012) Neurodevelopmental low-dose bisphenol A exposure leads to early life-stage hyperactivity and learning deficits in adult zebrafish. Toxicology 291:83–92. doi: 10.1016/j.tox.2011.11.001.

[4] Mandrell D, Truong L, Jephson C, Sarker MR, Moore A, Lang C, Simonich MT, Tanguay RL (2012) Automated Zebrafish Chorion Removal and Single Embryo Placement Optimizing Throughput of Zebrafish Developmental Toxicity Screens. Journal of Laboratory Automation 17:66–74. doi: 10.1177/2211068211432197.

[5] Westerfield M (2000) The zebrafish book: A guide for the laboratory use of zebrafish, Ed. 4. M. Westerfield. Univ. of Oregon Press, Eugene.

[6] Kim K-T, Truong L, Wehmas L, Tanguay RL (2013) Silver nanoparticle toxicity in the embryonic zebrafish is governed by particle dispersion and ionic environment. Nanotechnology 24:115101. doi: 10.1088/0957-4484/24/11/115101.

[7] Truong L, Moody IS, Stankus DP, Nason JA, Lonergan MC, Tanguay RL (2011) Differential stability of lead sulfide nanoparticles influences biological responses in embryonic zebrafish. Arch Toxicol 85:787–798. doi: 10.1007/s00204-010-0627-4.

[8] Cibrian-Uhalte E, Langenbacher A, Shu X, et al. (2007) Involvement of zebrafish Na+,K+ ATPase in myocardial cell junction maintenance. J Cell Biol 176:223–230. doi: 10.1083/jcb.200606116.

[9] Zuur AF, Ieno EN, Elphick CS (2010) A protocol for data exploration to avoid common statistical problems. Methods in Ecology and Evolution 1:3–14. doi: 10.1111/j.2041-210X.2009.00001.x.

[10] Jose Pinheiro, Douglas Bates, Saikat DebRoy, Deepayan Sarkar and the R Development Core Team (2012). nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-106.

[11] Douglas Bates, Martin Maechler and Ben Bolker (2012). lme4: Linear mixed-effects models using S4 classes. R package version 0.999999-0.