Size matters: The outer diameter of multiwalled carbon nanotubes (MWCNTs) affects gene expression in female fathead minnows (*Pimephales promelas*)

Barbara J. Carter[*], Robert J. Griffitt[**], Nancy J. Brown-Peterson[**], Heather R. Hammers[*] and David S. Barber[***]

[*]EcoArray, Inc., Interstate Office Park, Suite 50, 4949 SW 41st Boulevard, Gainesville, FL, 32608-5061, bcarter@ecoarray.com and hrhammers@ecoarray.com
[**]Department of Coastal Sciences, University of Southern Mississippi, Ocean Springs, MS, 39564, joe.griffitt@usm.edu and Nancy.Brown-Peterson@usm.edu
[***]Center for Environmental and Human Toxicology (CEHT), University of Florida, Gainesville, FL, 32611, BarberD@vetmed.ufl.edu

ABSTRACT

We used oligonucleotide microarrays to examine the gene expression patterns in liver, gonad and gill tissues from adult female fathead minnows (FHM, *Pimephales promelas*) exposed to two different sizes (< 8 nm and 50-80 nm outer diameter) and multiple doses of multiwalled carbon nanotubes (MWCNTs) under aerated static renewal conditions. We observed significant ($p \leq 0.05$ and fold change ≥ 2) differential gene expression in each of the three tissues due both to size and exposure dose. All three tissues express more genes (regardless of dose) after exposure to the larger size MWCNTs. The liver shows a higher percentage of genes up-regulated in response to exposure to MWCNTs regardless of size. The ovary shows more genes down-regulated after exposure to both sizes. Forty-eight genes were differentially expressed in gills for both sizes of MWCNT although significant histopathological results (fused and shortened gill lamellae) were only observed for the smaller MWCNTs. We conclude that, under some conditions, size does matter in regard to MWCNTs.

Keywords: MWCNTs, carbon nanotubes, microarrays, gene expression, fathead minnow

1. INTRODUCTION

The use of nanoparticles in manufacturing, industry, and commercial products has increased over the past two decades. Since industrial products and wastes tend to end up in waterways despite safeguards, it is inevitable that nanoscale products and by-products will also enter aquatic environments as nanotechnology industries scale up production [1, 2]. The consumer public wants assurances that nanotechnologies are safe [3] with minimal impact on the environment and human health.

However, assessing the potential direct effects of nanoparticles on human health is not an easy task. The properties of nanoparticles depend not only on the size of the particle, but also on the structure, microstructure, and surface properties (coating) [1, 4, 5]. Many times, animal models are used as a surrogate for humans, and the results extrapolated. Additionally, nanoparticles may access the body through multiple routes including direct ingestion; entry across epithelial boundaries such as gills, olfactory organs, or body wall; or through phagocytosis or endocytosis [1]. These processes are integral to key physiological functions such as cellular immunity and intracellular digestion.

Increasing concerns about environmental contaminants that adversely affect health, development and reproduction of exposed fish and wildlife have begun to be addressed only recently through toxicological testing, and data are extremely limited. Such research has led to the development of both specific *in vitro* and *in vivo* assays to test for these effects. Gene microarrays integrate *in vivo* exposures with mechanistic outcomes. Using this technology, we can test thousands of genes at one time with mRNAs isolated from tissues of exposed animals.

This research uses gene expression data from 60-mer oligonucleotide microarrays and histopathological anomalies to examine the effects of 48 hr. exposure to two different sizes of multiwalled carbon nanotubes (MWCNTs) on female fathead minnows (FHM, *Pimephales promelas*).

2. MATERIALS AND METHODS

2.1 Nanotube suspension

We purchased two dry nanotube powder samples, <8 nm and 50-80 nm outer diameter (O.D.), from Cheaptubes (www.cheaptubes.com, Brattleboro, VT). We suspended each sample in water containing 10mg/ml sodium dodecylbenzene sulfonate (NaDDBS), at a nominal concentration of 10 mg/ml. We bath-sonicated the nanotubes suspensions for four hours, and then centrifuged at 600 rcf for 30 minutes. We retained the supernatant, and calculated that between 35-50% of the nanotubes remained in suspension. That is approximately what was expected according to the method described by Attal *et al*. [6].

SMALL	PARAMETER	LARGE
<8 nm	Outer diameter	50 - 80 nm
2 - 5 nm	Inner diameter	5-10 nm
<1.5 wt%	Ash	<1.5 wt%
10-30 nm	Length	10-20 nm
>97 wt% (1% Cl and Co; trace S and Al)	Purity Energy Dispersive X-ray Spectroscopy	>97 wt% (~2% Ni; trace Fe, Cl, S)
500 m^2/g	Surface area	60 m^2/g
$>10^{-2}$ S/cm	Electrical conductivity	$>10^{-2}$ S/cm

Table 1. Comparison of the characteristics of the small and large size (based on O.D.) MWCNTs. Note the differences in surface area.

2.2. Nanotube exposures/tissue collection

We performed all exposures as aerated 48 hour static bioassays in 2L beakers, with 4 replicate beakers per concentration, and 3 adult female fathead minnows per beaker. For each size of nanotube, we used five different exposure conditions: (water) control, carrier control (1.2 mg/L NaDDBS for the 8 nm MWCNT, 2.2 mg/L for the 80 nm), 0.1 mg/L, 0.3 mg/L, and 1.0 mg/L MWCNTs. After 48 hours, we euthanized two fish from each beaker by immersing them in 100 mg/L MS-222 (Tricaine) buffered with 10 mg/L NaHCO3 for five minutes. We opened the carcass by ventral incision, removed the liver (partial), ovary (left horn), and gill (left), and immediately placed the tissues in 1 ml RNALater (Ambion, Inc., Austin, TX), storing samples at -20^{o}C.

2.3. Hybridization of microarrays

We isolated total RNA using the RNEasy Plus Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. We determined the quality of the RNA by running a 1.0 μL aliquot on a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). As this was a reference design experiment, we labeled the exposed samples with Cy-5-CTP (Perkin Elmer, Wellesley, MA, USA) and the reference sample with Cy-3-CTP. We labeled, hybridized, and washed the arrays according to Agilent's Two-Color Microarray-Based Gene Expression Analysis (Quick Amp labeling) Protocol (version 5.7, March 2008). The FHM microarrays used in this experiment were developed by EcoArray and manufactured by Agilent Technologies, Inc. Each array contains probes for 15,208 well-annotated (e-value $\leq$ 1e-^{5}) gene sequences; eight arrays per glass slide. We scanned the slides with an Agilent DNA microarray scanner, which processed the raw images and converted the data into text files using Agilent's Feature Extraction Software, Version 9.5.3.

2.4. Histopathological analysis

We took samples of liver, gill, ovary, digestive tract and heart from each fish sampled for gene expression for histopathological analysis. Tissues were placed into labeled cassettes and fixed in 10% neutral buffered formalin for a minimum of 1 week. Following fixation, gill tissues were decalcified (Cal-Ex, Fisher Scientific) for 6 hours. All tissues were rinsed, dehydrated, embedded in paraffin, sectioned at 4μm and mounted on glass slides following standard histological techniques. Tissues were stained with hematoxylin and eosin and each tissue was examined for histopathological anomalies (Table 2). Results were expressed as percent occurrence for each treatment.

TISSUE	HISTOPATHOLOGY EVALUATED
Gill	Clubbed, fused or shortened lamellae, hemorrhage, telangiectasis, presence of many mucous cells
Digestive tract	Abnormal cilia, macrophage aggregate, increased number of goblet cells, vacuolated goblet cells
Heart	Macrophage aggregate, cysts, vacuoles
Liver	Cysts, spongiosis, vacuoles, macrophage aggregate, granuloma
Ovary	Oocyte type (primary growth, corticeolar alveolar, early vitellogenic, late vitellogenic, alpha and beta atretesia), POF $\leq$ 24 h

Table 2. Histopathological observations evaluated for each tissue type.

2.5. Statistical analysis

The resultant gene expression data were analyzed using Gene Spring version 9.0.3 (Agilent Technologies, Inc., Santa Clara, CA). For this project we considered the two sizes of nanotubes as different experiments, and we analyzed the tissues, gill, liver, and ovary, within each size category independent of each other. We accepted as differentially regulated all genes with $p \leq 0.05$ and a fold change ≥ 2.0. Histopathological anomalies were tested for significant differences among treatment groups using the Kruskal-Wallace test and the chi-square statistic (SPSS version 11.5, Chicago, IL); anomalies were considered significant if $p \leq 0.05$.

3. RESULTS

We observed no mortality in adult female FHMs exposed for 48 hours to the dispersed suspensions of two sizes of MWCNT at concentrations up to 1 mg/L. At necropsy, we did not note any gross pathology in any of the

organs examined. We observed more fused lamellae (p=0.014) in the gill of FHMs exposed to <8 nm O.D. MWCNTs (Figure 1), but no other histopathologies were statistically significant.

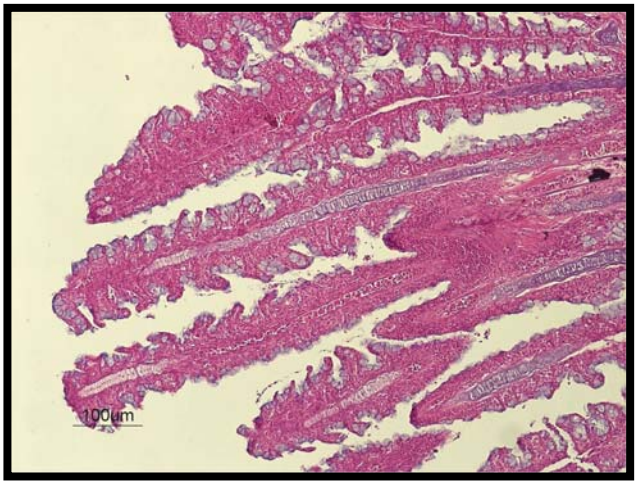

Figure 1. Fused and shortened lamellae in gill of adult female FHM after 48hr. exposure to < 8 nm O.D. MWCNTs. 10x magnification.

We used gene expression analysis to investigate whether MWCNT with different diameters produced different responses in each of the tissues. We observed a substantial transcriptional response to exposure in each of the tissues, with over 400 genes exhibiting altered expression in each of the tissues. A greater percentage of genes were differentially expressed in female FHMs after 48 hr exposure to the larger size of MWCNTs (Figure 2). However, there was very little commonality in the transcriptional response of the three tissues to a given size of MWCNT.

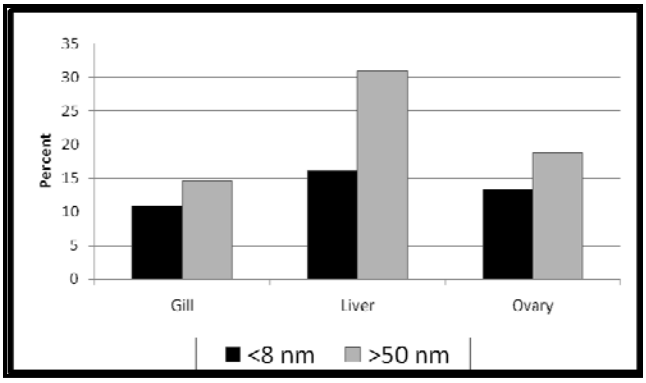

Figure 2. Percent of genes differentially expressed in common by both doses in female fathead minnows after 48 hr exposure to two sizes of MWCNTs.

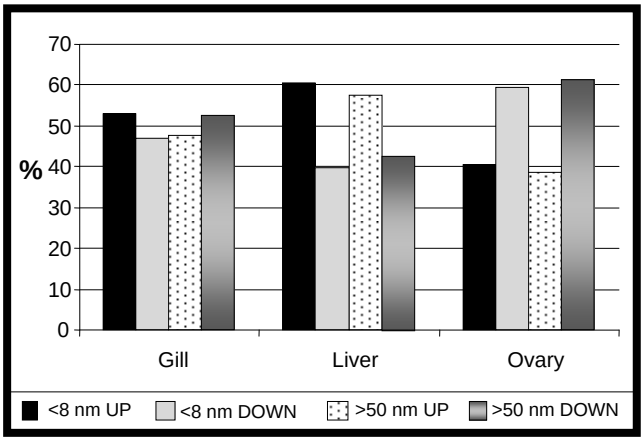

Figure 3. Percent of genes up- or down-regulated in female fathead minnows after 48 hr exposure to low dose (0.3 ng/µl) of two different sizes of MWCNTs.

Figure 3 depicts the percentage of differentially expressed genes that are up or down regulated after exposure to a low dose (0.3 ng/µl) and two different sizes of MWCNTs. The liver shows a higher percentage of genes up-regulated in response to exposure to MWCNTs regardless of size. The ovary shows more genes down-regulated after exposure to both sizes; and we find opposite patterns in the gene expression of the gill. After exposure to the smaller MWCNTs, more genes are up-regulated than down-regulated in gill. However, after exposure to the larger MWCNTs, more genes are down-regulated in the gill, but the numbers are not significantly different.

We then looked more closely at the transcriptional response in the gill. Our analysis found 48 genes whose expression was significantly altered that were common to exposures of both sizes of carbon nanotubes (Figure 4). Analysis of this subset of genes reveals some genes (n=6) with mixed regulation (Figure 4A).

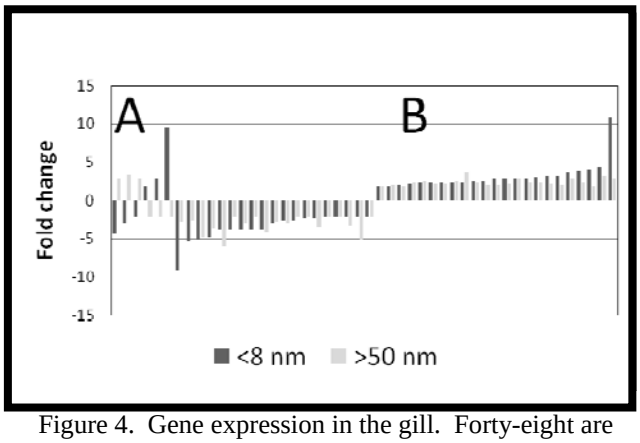

Figure 4. Gene expression in the gill. Forty-eight are significantly, differentially expressed and common to both sizes, regardless of dose. (A) Genes with mixed patterns of regulation. (B) Gene expression patterns that are similar in direction and magnitude.

4. DISCUSSION

Exposure to MWCNT produced significant ($p \leq 0.05$, fold change ≥ 2) transcriptional effects on gill, liver, and ovary of adult female FHMs. The responses in these organs were quite different, suggesting that the tissues are responding differently, though it is unclear whether MWCNT were absorbed and reached internal organs or if these are secondary responses of the liver and the ovary to physiological stress due to effects on gill. We do know that exposure to the smaller nanotubes (<8 nm O.D.) resulted in significantly more fused lamellae in gill.

Comparing the response of a specific tissue, in this case, gill, to different sizes of MWCNT suggests that nanotubes of different diameters can cause different responses. Six genes showed the opposite response after exposure to either small or large MWCNT. These include (from left, Figure 4A) genes coding for zebrafish clone CH211-191A16, Kallmann Syndrome 1a sequence, putative reverse transcriptase, Zgc:101132, Zgc:91911 and Serpin Peptidase Inhibitor. Some of the genes that responded similarly to both sizes of MWCNTs (Figure 4B) are involved in the biological processes of protein modification (GO:0006464; e.g., palmitoyl-protein Thioesterase-1), transcription (GO:0006810;e.g., uncoupling protein 2), and glycoprotein catabolism (GO:0006516; e.g., meningioma expressed antigen 5). The differences and similarities in gene response may be due to size alone, but as previously stated, the properties of nanoparticles depend not only on the size of the particle, but also on the structure, microstructure, and surface properties (coatings) [1, 4, 5]. The important finding from this research is that fathead minnows do show differential gene expression after exposure to MWCNTs.

These findings are not unique. Other researchers [7] have found that chemicals emerging from nanotechnology may pose a risk to aquatic life and encourage further studies on their adverse effects.

5. CONCLUSIONS

This study showed that microarrays can be useful in testing the effects of nanoparticles on aquatic organisms. These tools provide more precise, quantifiable data than existing assays, and they are cost-effective. In addition, microarrays offer the advantage of being able to provide biologically relevant, mechanistically based data in a short period of time. Fathead minnow microarrays and the gene expression data they generate can relate to ***human health***, which is the ultimate target of testing.

The results of this study suggest that while acute exposure to MWCNTs is not toxic to FHMs at concentrations up to 1.0 mg/L, such exposure does result in differential gene expression in gill, liver and ovary tissue. Many of the genes that exhibit a change are involved in regulating transport, transcription and protein functions. Larger MWCNTs (outer diameter >50 nm) elicit more differentially regulated genes in FHM after 48 hr exposure than smaller (<8 nm O.D.) MWCNTs. However, this is a preliminary examination of the data, and we will be undertaking a much more detailed analysis. We conclude that, under some conditions of exposure to MWCNTs, size does matter.

REFERENCES

[1] M. N. Moore, "Do nanoparticles present ecotoxicological risks for health of the aquatic environment?" Environment International 32, 487-976, 2006.

[2] P. JA. Borm, D. Robbins, S. Haubold, T. Kuhlbusch, H. Fissan, K. Donaldson, R. Schins, V. Stone, W. Kreyling, J. Lademann, J. Krutmann, D. Warheit and E. Oberdorster. "The potential risks of nanomaterials: a review carried out for the ECETOC". Particle and Fibre Toxicology 3: (35 pages), 2006.

[3] J. Macoubrie, "Informed public perceptions of nanotechnology and trust in the government." Report provided to the Project on Emerging Technologies, Woodrow Wilson International Center for Scholars and the PEW Charitable Trusts. Washington, DC, 2005.

[4] H. Yin, H.P. Too, and G. M. Chow. "The effects of particle size and surface coating on the cytotoxicity of nickel ferrite." Biomaterials 26 (29): 5818-26, 2005.

[5] D .J. Burleson, M.D. Driessen, and R.L. Penn. "On the characterization of environmental nanoparticles." Environ Sci Heath A Tox Hazard Subst Environ Eng 39 (10):2707-53, 2004.

[6] S. Attal, R. Thiruvengadathan, O. Regev**,** **"**Determination of the Concentration of Single-Walled Carbon Nanotubes in Aqueous Dispersions Using UV-Visible Absorption Spectroscopy**."** Anal. Chem**.** 78 (23) : 8098-8104, 2006.

[7] Blaise, C., F. Gagné, J. F. Férard , P. Eullaffroy. "Ecotoxicity of selected nano-materials to aquatic organisms." Environ Toxicol. Jun 4, 2008. [Epub ahead of print].

Acknowledgements: This research was funded wholly or in part by the U.S. EPA through a Phase 1 SBIR grant EP-D-08-026. The article has not been subject to the EPA's required peer and policy review and therefore does not necessarily reflect the views of the Agency. No official endorsement should be inferred.