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US ARMY INSTITUTE OF PUBLIC HEALTH
5158 BLACKHAWK ROAD
ABERDEEN PROVING GROUND MARYLAND 21010-5403

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MEMORANDUM FOR Department of Defense Chemical, Biological, Radiological, and Nuclear (CBRN) Defense Program (DASG-HCZ/LTC Ricardo Reyes), U.S. Army Medical Command/Office of the Surgeon General, 7700 Arlington Boulevard, Falls Church, VA 22042-5143

SUBJECT: Toxicology Study No. 87-XE-0EJ5-11 (FY12 Continuation), Protocol Development and Preliminary Toxicity Study of CBRN Nanomaterials, September 2012

1. An electronic copy of the final report is enclosed.

2. The U.S. Army Public Health Command, Army Institute of Public Health point of contact is Dr. Cheng J. Cao. She may be contacted at DSN 584-3980, commercial 410-436-3980, or electronic mail at usarmy.apg.medcom-phc.mbx.tox-info@mail.mil

FOR THE DIRECTOR:

Encl

MARK S. JOHNSON
Portfolio Director, Toxicology

USAPHC
U.S. ARMY PUBLIC HEALTH COMMAND
5158 Blackhawk Road, Aberdeen Proving Ground, Maryland 21010-5403

Toxicology Study No. 87-XE-0EJ5-11 (FY12 Continuation)

Protocol Development and Preliminary Toxicity Study of CBRN Nanomaterials

Prepared by Dr. Cheng J. Cao

Toxicology Portfolio
Health Effects Research Program
Army Institute of Public Health

Specialty: 500C, Toxicity Tests

Toxicology Study No. 87-XE-0EJ5-11 (FY12 Continuation)

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14. ABSTRACT

This project uses fast, high-throughput in vitro approach to study new nanomaterials applicable to Chemical Biological Defense (CBD) for their environmental and human related toxicity. Four CBD nanomaterials were evaluated for their aquatic toxicity by Microtox test and human cytotoxicity by NRU assay. These studies fill the data gaps and provide toxicity information useful in risk assessment when the environmental and human exposure to these new CBD nanomaterials. In addition, Transepithelial Electrical Resistance (TER) and Transepithelial Permeability (TEP) assays were developed and tested on EpiAirway, a 3-D human tracheal/bronchial epithelial equivalent. Further evaluation of the effects of these CBD nanomaterials on tissue tight junction and barrier function is the on-going study. This project supports the efforts of USAPHC/OTSG in CBRN incident response for protecting the work force, civilian and military, from the unintended consequences of nanotechnology processes and materials exposure.

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MCHB-IP-THE
5158 Blackhawk Road
Aberdeen Proving Ground, MD 21010-5403

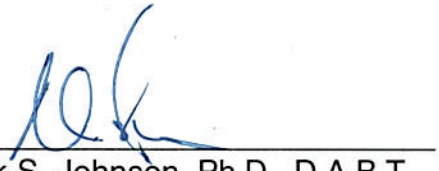
Prepared by:

10-16-2013

Cheng J. Cao, M.P.H, Ph.D.
Toxicologist
Army Institute of Public Health (AIPH)
Health Effects Research Program

Date

Approved by:



12-5-2013

Mark S. Johnson, Ph.D., D.A.B.T
Portfolio Director, Toxicology
Army Institute of Public Health (AIPH)

Date

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September 2012

1 Summary

1.1 Purpose

Nanomaterials are gaining in commercial and military applications. Nanotechnologies also pave the way for new weapons. U.S. Army Public Health Command (USAPHC) and Medical Command (MEDCOM) Office of the Surgeon General (OTSG) have made efforts in chemical, biological, radiological and nuclear (CBRN) incident response for protecting the work force, civilian and military, from the unintended consequences of nanotechnology processes and materials exposures. This project uses fast, high-throughput *in vitro* approach to study new nanomaterials applicable to Chemical Biological Defense (CBD) for their environmental and human related toxicity in address of the data gaps and support of the ongoing USAPHC/OTSG efforts.

1.2 Conclusions

- The Microtox study fills the data lack of aquatic toxicity for these new CBD nanomaterials and provides ecologically relevant information of risk when these nanomaterials are environmentally exposed.
- The Neutral Red Uptake (NRU) study in human liver cells provides cytotoxicity data for these new nanomaterials which is complementary for the *in vivo* animal studies with additional human related toxicity information.
- Transepithelial Electrical Resistance (TER) and Transepithelial Permeability (TEP) assays were developed and performed on EpiAirway, a 3-D human tracheal/bronchial epithelial equivalent, showing that both TER and TEP methods work well on the 3-D tissue model. Further systematical testing on the 3-D human tissue models needs to be performed in order to evaluate the effects of these CBD nanomaterials on tissue tight junctions/barriers.

1.3 Recommendations

The study using human tissue models for risk assessment of CBRN nanomaterials should be continued. Completion of the proposed study will provide human-related toxicity data of the CBRN nanomaterials in support of the ongoing USAPHC/OTSG efforts for safety development and applications of nanomaterials and nanotechnologies.

2 References

See Appendix A for a list of references.

3 Authority

This assessment addresses, in part, the environmental safety and occupational health requirements outlined in Army Regulations (AR) 200-1; AR 40-5; AR 70-1; Department of Defense Instruction (DODI) 4715.4, and Army Environmental Research and Technology Assessment Requirement PP-3-02-04. It was performed as part of an ongoing effort of MEDCOM, OTSG in CBRN incident response for protecting the work force, civilian and military, from the unintended consequences of nanotechnology processes and materials exposures. This program is under the direction of the MEDCOM/OTSG (LTC Ricardo Reyes) and the USAPHC (Dr. Laurie E. Roszell).

4 Background

Nanotechnology (nanotech) is a revolutionary new technology now at an explosive stage where it could significantly drive the 21st century's economy. There are now an estimated 700 products on the market and by 2015 nanotech will have a $1 trillion impact on the global economy and employ two-million workers. Nanotech is emerging as a positive, powerful tool for improving science, economics, and the environment. Nanomaterials are also being developed for a myriad of military applications, such as sensors, coatings, electronics, textiles, pharmaceutics, and medical devices. Products made using nanomaterials are already a part of our lives. However, the comparative lack of scientific knowledge on the effects of nanomaterials has led to concern regarding the environmental health and safety risks potentially associated with nanotech and its products. Recently, calls from government, industry, academic, and environmental leaders has demanded dramatic increases in funding to study the health and environmental effects of nanotechnology. The need to develop a rational, science-based approach to nanotoxicology is urgent to ensure the safety of manufacturing, applications, and environmental exposures and to protect the warfighter, worker, and civilian from potential exposures.

Nanotech has produced increasingly smaller particles. From a physicochemical viewpoint, these fine (less than 2.5 micrometers (µm) in size) and ultrafine (less than 0.1µm) particles can be potentially injurious to human populations, because they behave aerodynamically like gas molecules and have a larger and much more chemically active surface area per unit mass than larger particles. Thus, these tiny airborne particles cannot only be inhaled into the lungs, but can also cross into the blood stream and disseminate deeper inside the body to threaten the brain, liver, kidney, and other tissues/organs. These particles may also pose a skin and eye contact hazard as they can directly traverse these tissues. Since 2006, increased studies and reports, including agency publications by the U.S. Environmental Protection Agency, the Office of the Inspector General, and the U.S. Government Accountability Office acknowledge significant data gaps concerning nanomaterials' potential effects on humans and the environment. Most troubling are the studies using mice that show nano-titanium dioxide when inhaled and when eaten can cause changes in DNA that affect brain function, and may cause tumors and developmental problems in offspring. One study found titanium dioxide (TiO_2) nanoparticles were present in the placenta, fetal liver and fetal brain. This is quite different than for micro-sized TiO_2 particles, which are considered inert and relatively non-toxic to experimental animals and humans. Nanoparticles are fundamentally different substances from their larger scale cousins, and nanomaterials can create new and unique health and environmental risks that need new forms of safety testing.

Often, toxicity and other physical parameters cannot be predicted from chemical composition as they behave differently than larger sized particles of the same material. Because such a large percentage of atoms lie on their surface, these tiny sized particles can be highly reactive and potentially harmful to human health. There is almost unanimous opinion among proponents and skeptics alike that the full potential of nanotech requires attention to safety issues. In 2006, six

people were hospitalized with serious (but nonfatal) respiratory problems after using a German household cleaning product called Magic Nano. Indeed, some studies have suggested that nanomaterials are not inherently benign, but they affect biological behaviors at cellular, subcellular, and molecular levels. Moreover, some nanoparticles readily travel throughout the body, deposit in target tissues/organs, penetrate cell membranes, lodge in mitochondria, and may trigger injurious responses. As we understand that the Gulf War Syndrome is not limited to pulmonary illness, it is indisputable that tissue samples from Soldiers from the Gulf War and the Yugoslavian War contain inorganic micro- and nano-particles produced by the high temperature explosions of shells (Montanari, S. 2004 & Zimmerman, P. 2009).

Human toxicity data about exposure to and application of nanomaterials are lacking, and are especially insufficient to conduct epidemiological studies for evaluation of the impact of nanomaterials on human health. A report of carbon nanotube (CNT) toxicity and assessment of potential occupational and environmental health risks was based on a review of several rodent studies. In these animal studies, test dusts were administrated intratracheally or intrapharyngeally to assess the pulmonary toxicity of the manufactured CNTs. A few *in vitro* studies evaluated biomarkers of toxicity in CNT-treated skin cell cultures. Tests in human cells/tissues are limited but their numbers have increased. A recent article presented at the Society of Toxicology Meeting (2004) highlighted the use of MatTek's EpiAirway *in vitro* tracheal/bronchial human tissue construct in evaluating the role of particle size as a factor in predicting the translocation potential of nano-sized particles. The *in vitro* experiments, using EpiAirway tissue constructs, were observed for membrane/cell/particle interactions. Histological evaluations were also performed on these tissues 2, 8, and 24 hours post exposure. More interestingly the EpiAirway *in vitro* data yielded similar findings, indicating particle size is a factor in predicting translocation potential of nano-sized particles as *in vivo* (Carter J.M. etc. 2004). (EpiAirway™ is a trademark of the MatTek Corporation; Ashland, MA).

Biological and toxicological tests can be conducted *in vivo* and *in vitro*. Traditional whole animal testing is expensive, time-consuming, and often unsuited for the study of the mechanism of toxic action at cellular and molecular levels. Thus, animal tests are unable to meet the testing demanded by increased numbers and types of the novel nanomaterials. Results from animal *in vivo* studies can be very variable between species. For example, oral LD_{50} microgram/kilogram (µg/kg) of Dioxin is ranked as 2 (guinea pig), 4 (mink), 50 (rabbit), 70 (monkey), 200 (mouse), 350 (rat), and 2000 (hamster). The uncertainty due to species difference has often created a dilemma for scientists in the use of the *in vivo* data in prediction or assessment of human consequence. Another example is that the FDA figures show a 92 percent failure rate for drugs that pass preclinical trials which are based on animal experiments (Lovell-Badge R. 2013). *In vitro* technology and methodology are increasingly being developed and used to address these challenges. *In vitro* cellular assays have the advantage of being relatively inexpensive, high-throughput, and capable of addressing many mechanistic issues at the cellular and molecular level. Specifically for the newly developed materials, the *in vitro* tests are the most suitable and effective in providing baseline toxicity data, given that often very limited amounts of test substances are available. *In vitro* testing can be performed using human cells, transgenic cells carrying human genes, and tissues.

The DOD has emphasized that alternative methods to animal species, such as cell culture techniques, shall be considered whenever possible if such alternatives produce scientifically valid or equivalent results to attain our research testing and training objectives. In 2004, the National Toxicology Program (NTP) of the National Institute of Environmental Health Sciences (NIEHS) released its vision and roadmap for the 21st Century (Tox 21st), which established a high-throughput screening (HTS) initiative to focus on integrating HTS and other nonrodent screening assays into its testing program. In 2005, the U.S. Environmental Protection Agency (EPA) established the

National Center for Computational Toxicology (NCCT). Through these initiatives the NTP and EPA, along with the National Institute of Health Chemical Genomics Center, are promoting the evolution of toxicology from a predominantly observational science at the level of disease-specific models *in vivo,* to a predominantly predictive science focused on broad inclusion of target-specific, mechanism-based, biological observations *in vitro*.

Since the development and application of nanomaterials and nanotechnologies has been so rapid, largely funded from government and industry, driven by factors other than for safety development, their biological activities and potential toxicities are poorly known. To address the urgent need of knowledge and data of the side effects and level of toxicity, an *in vitro* approach is most suitable and feasible under such circumstances. The proposed study focuses on the use of human cells-derived tissue equivalent to study toxicity of CBRN nanomaterials. The human-related toxicity data will be useful in risk assessment of human health in support of the safety of Soldiers, workers, and Civilians in application and the environmental exposure of these nanomaterials.

5 Materials and Methods

5.1 Microtox Assay

Microtox is a standardized toxicity test system which is rapid, sensitive, reproducible, ecologically relevant and cost effective. This *in vitro* toxicity testing system uses a strain of naturally occurring luminescent bacteria, *Vibrio fischeri*. The marine bacterial bioluminescence is tied directly to cellular respiration which is fundamental to cellular metabolism and associated life processes. These non-pathogenic, marine, luminescent bacteria are sensitive to a broad range of toxicants resulting in a decrease rate of respiration and a corresponding decrease in the rate of luminescence. Reduction of the microorganism light emission is proportional to the toxicity expressed as EC_{50} (the midpoint of the effective concentration). Microtox test has been shown to be an effective screening tool in assessing toxicity to aquatic organisms. This test has also been evaluated by the industrial, academic, and governmental testing communities and achieved official "Standards Status" in several countries. It is recognized and used throughout the world, such as Canada, the United Kingdom, Germany, Sweden, the Netherlands, and Australia as a standard test for aquatic toxicity testing. In the United States it is a recognized test method in a number of federal programs. The EPA has adopted Microtox as a standard test in an ongoing program of Assessment and Remediation of Contaminated Sediments. Microtox has been adopted by the U.S. Fish and Wildlife Service (USFWS) as a screening test at the National Fisheries Contaminant Research Center. Currently, Microtox is the only product of its kind designated as antiterrorism technology and given a Safety Act Certificate by the U.S. Department of Homeland Security. The PI's lab has used the Microtox to evaluate numerous new munition compounds for their aquatic toxicity and provided risk assessment to munition scientists in making environmental health-based decisions regarding the design and selection of new formulas and materials (Cao, J. J., Johnson, M.S. and Klapötke, T.M. 2012).

5.1.1 Organisms

Microtox Reagent, the microorganisms (AZF686018A) purchased from Modern Water Inc., is the test system for this method. The reagent is a freeze-dried preparation of a selected special strain of the marine bacterium, *Vibrio fischeri* (NRRL-B-11177). The Microtox Reagent is specially formulated for bioreactivity testing with sensitivity to a broad range of toxicants. A vial of reagent contains roughly one million test organisms. The Microtox assay also utilizes the manufacturer prepared Reconstitution Solution (AZF686016) and Diluent (AZF686011), (Modern Water Inc.; New Castle, DE).

5.1.2 Positive Control

Zinc sulfate is recommended as a standard or positive control of the Microtox assay by the supplier. The zinc standard ($ZnSO_4 \cdot 7H_2O$) was purchased from Sigma-Aldrich (St. Louis, MO).

5.1.3 Test Nanomaterials

- **FAST ACT®** is a proprietary formulation based on nanocrystaline metal oxides used for neutralizing a wide range of toxic chemicals and Chemical Warfare Agents (CWAs) and was purchased from NanoScale Corp (Manhattan, KS).

- **RNP-212** is an earlier developed metal oxide formulation similar to the FAST-ACT production and also purchased from NanoScale Corp (Manhattan, KS).

- **PhotoScrub™** is a fiberglass cloth coated with TiO2 that uses UV light induced catalytic ionization of TiO2 to destroy Chemical Warfare/Biological Warfare (CW/BW) agents and purchased from Applied Nanotech Holdings Inc.(Austin, TX).

- **Nano Sodium Bicarbonate** is a component in the formulation being investigated as a replacement fire extinguishing agent for the Halon 1301 currently used in hand held fire extinguishers (HHFEs) of Army Aviation Weapons Systems. It was provided by Aviation Ground Support Equipment (Huntsville, AL).

5.1.4 Equipment

The assays were carried out in the Microtox Model 500 Analyzer® with MicrotoxOmni™ software. The analyzer is a self-calibrating, temperature controlled photometer. (Microtox Model 500 Analyzer® and MicrotoxOmni™ software are initially registered trademarks of AZUR Environmental and currently owned by Modern Water Inc.).

5.1.5 Procedures

The Microtox Analyzer was turned on and allowed to reach proper temperature before performing assay. The “100% toxicity test” protocol was selected. Appropriate parameters, such as number of controls, samples, and dilutions, dilution factor, initial concentration and replicates of control/sample, were inputted into General Parameters of the program. More dilutions were used in order to smooth or refine a dose-response curve. Cuvettes were placed in their designated wells in the machine. Reagent (a freeze-dried preparation of the *Vibrio fischeri*) was prepared freshly using reconstituted solution avoiding use of a pipette or a vortex mixer for the bacteria reconstitution and placed in the reagent well to maintain it at a designated temperature ($5.5^{o}C \pm 1.0^{o}C$). For example of a four-dilution scheme, cuvettes #1-4 were filled with 1 milliliter (mL) of diluent. The last cuvette #5 was filled with 2 mL of the test chemical at the highest concentration. Several serial dilutions were made by decanting at 1:1 from cuvette # 5 to # 2 (e.g., remove 1 mL test solution from cuvette #5 and add it to cuvette #4, repeat through cuvette #2). One mL of the test chemical from cuvette #2 was discarded. Cuvette #1 was the control containing no test article, just the diluent. Each

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Sponsor

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Falls Church, Virginia

Study Title

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Protocol Development and Preliminary Toxicity Study of CBRN Nanomaterials

Author

Cheng J. Cao

Study Completed

September 2012

Performing Laboratory

Army Institute of Public Health
Toxicology Portfolio
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Aberdeen Proving Ground, MD 21010-5403

dilution was mixed completely. The reagent was decanted from the reagent well into a repeating pipette with 10 microliter (µL) scales. Once the software was initiated, 10 µL of the reagent was added to each cuvette (from cuvette #1 to #5). All cuvettes were mixed during this process. After 5-minute incubation (the 1st reading cycle), cuvette #1 was placed into the "Read well" for analysis until all samples were analyzed. This process was repeated for the 15 and 30 minutes readings as the 1st cycle reading.

5.1.6 Data Analysis

Raw luminescence data were recorded at 5, 15, and 30 minutes by the Microtox analyzer. The EC_{50} values at 5, 15, and 30 minutes were given by the MicrotoxOmni software and further fitted to Hill Function using GraphPad PRISM 4®, a registered trademark of GraphPad Software, Inc. (San Diego, CA). All data (prints and files) were archived.

5.2 Neutral Red Uptake Assay

The Neutral Red Uptake (NRU) cytotoxicity assay is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. Healthy mammalian cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical can interfere with this process and result in a reduction of the growth rate and/or a production of unhealthy cells. Alterations of the cell surface or sensitive lysosomal membranes lead to lysosomal fragility and other changes that gradually become irreversible. The cytotoxicity is expressed as a concentration dependent reduction (i.e., IC_{50}, the half maximal inhibitory concentration) of the uptake of the NR after chemical exposure; thus, providing a sensitive, integrated signal of both cell integrity and growth rate. The NRU method has recently been validated in a three-phase study (2002–2005) sponsored by the National Institute for Environmental Health Sciences, EPA, and Interagency Coordinating Committee on the Validation of Alternative Methods and accepted as an alternative test method for acute oral systemic toxicity in U.S. 2008 and international Organization for Economic Co-operation and Development (OECD) 2010. The NRU is now used as an *in vitro* method useful in reducing the use of animals, particularly in rodent lethality testing. The PI directed the Army's lab to participate in this validation study with international collaborations (Cao, C.J., Madren-Whalley, J., Chundakkadu, K. and Valdes, J.J. 2003) and recently used the NRU assay to assess new munition compounds for their cytotoxicity in human cells in the ongoing OEP program since 2006. (Cao, C.J., Johnson, M.S. and T. M. Klapötke. 2012).

5.2.1 Cell Line

Human liver cell line (CCL-13) purchased from American Tissue Culture Center (ATCC; Manassas, VA). This cell line is a frozen cell preparation originally derived from human normal liver tissue (Chang Liver). The Chang liver cells have been extensively used in investigations of virology, biochemistry, pharmacology and toxicology. We have used this cell line to evaluate many new munition formulas/compounds for their basal cytotoxicity potentially to human by NRU assay. A vial contains roughly one million of the test cells.

5.2.2 Culture Media

Eagle's Minimal Essential Medium (EMEM) and Fetal Bovine Serum (FBS) were purchased from ATCC and used for growing cells.

5.2.3 Positive Control

The positive control, sodium lauryl sulfate (SLS), (Cat. # 71736 for 10% solution) and other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO).

5.2.4 Test Nanomaterials

Test nanomaterials are the same as the ones used in the Microtox assay (5.1.3).

5.2.5 Equipment

The NR absorbance is measured on Synergy™ HT Multi-Detection Microplate Reader (Model SIAFRTD) with Gen5™ software (both are trademarks of BioTek Instruments, Inc.; Winooski, VT).

5.2.6 Procedure

Initially, human liver cells were seeded into 96-well plates (regular clear plate) at $5.0x10^4$ cells/mL (e.g., $5.0x10^3$ cells/well/0.1 mL) as the plate configuration and maintained in culture with the routine culture medium (e.g., EMEM-10% FBS) in a 37°C, 5 percent CO_2 incubator for 24 ± 1 hr to form a semi-confluent monolayer. On the following day, cultures were observed under the microscope for contamination and cell confluence prior to performing the chemical treatment. The cells were treated with test compounds by adding 0.1 mL of each concentration in serum-free EMEM medium per well and 0.1 mL of the medium per vehicle control well. The cultures were exposed to the test chemicals over a range of eight concentrations (6 wells per concentration) for 48 ± 1 hr in 37°C, 5 percent CO_2 incubator. Next, cultures were observed under microscope for contamination or precipitates. Treatment medium was removed and the cultures were washed once with phosphate buffered saline (PBS). Neutral red medium (NRM) containing 33 micrograms (µg) dye /mL was added to each well (0.2 mL/well). After a 3-hr incubation, NRM was discarded and the cultures were washed once with PBS and received 0.1 mL of NR desorbing fixative per well. The plates were placed on a shaker for 20 min at room temperature (24 ± 2 degrees Celsius (°C). NR absorption was detected at optical density 540 nanometers (nm) in the Synergy HT Multi-Detection Microplate Reader.

96-Well Plate Configuration

	1	2	3	4	5	6	7	8	9	10	11	12
A	VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb
B	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
C	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
D	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
E	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
F	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
G	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
H	VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb

VC1 and VC2 = VEHICLE CONTROL
$C_1 – C_8$ = TEST CHEMICALS at eight concentrations (C1 = highest, C8 = lowest)
C_1b – C_8b = CHEMICAL BLANKS (test chemical or PC, but contain **no** cells)
VCb = VEHICLE CONTROL BLANK (contain **no** cells)
A – D = SAMPLE 1
E – H = SAMPLE 2

5.2.7 Data Analysis

Raw optical density data were recorded by the Synergy HT Multi-Detection Microplate Reader and transferred to Excel spreadsheet for initial data analysis. The IC_{50} values were determined by Hill Function using PRISM 4 (GraphPad Software, Inc.; San Diego, CA). All data (prints and files) were archived.

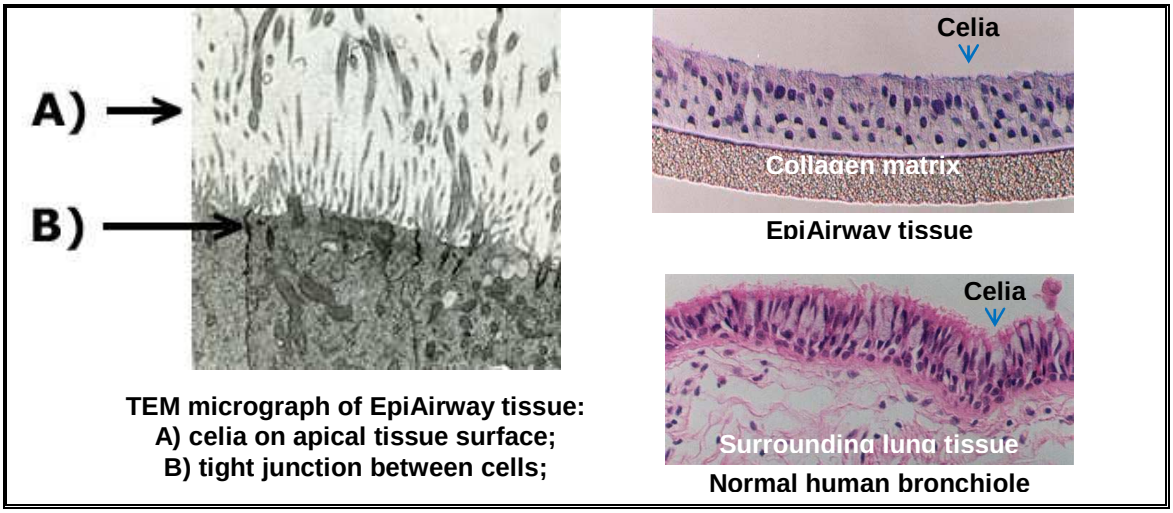
5.3 Measurement of Transepithelial Electrical Resistance

Transepithelial electrical resistance (TER) assay is a widely used method to functionally analyze tight junction dynamics in cell culture models of physiological barriers. Tissues, such as tracheal/bronchial, corneal, or blood vessel endothelium, produce tight junctions between cells which inhibit the permeation of low molecular weight solutes across the tissue. Formation of tight junctions also inhibits the ability of electric current to flow across tissue, conferring an electrical resistance property upon these tissues. Therefore, measurement of TER provides a convenient indicator of tight junction development and function. A study by Gillette Medical Evaluation Laboratory reported that the TER response appears to be a more sensitive parameter of barrier function alternation in the human corneal tissue model, especially more useful in discriminating mildly irritating substances. The PI has the knowledge and experience in use of the TER

technology from participation in a validation study of human corneal epithelial cell line as an *in vitro* model for assessing ocular irritation (Cao, C.J. Heroux, K. and Valdes, J.J. 2001).

5.3.1 Tissues

EpiAirway System (MatTek AIR-100) is a human tracheal/bronchial epithelial equivalent originated from normal, human-derived tracheal/bronchial epithelial cells. This system has a 3-D, pseudo-stratified highly differentiated structure closely resembling the epithelial tissue of the respiratory tract. Histological cross-sections of both the *in vitro* tissue and a normal human bronchiole reveal a pseudo-stratified mucociliary phenotype. Transmission electron microscopy shows numerous microvilli and cilia on the apical surface of the cultures and confirmed the presence of tight junctions. In addition, secretions from the apical surface of the cultures were analyzed using immuno-dot blot procedures to quantify mucin secretion. EpiAirway "ready-to-use" tissues are grown on cell culture inserts at the air-liquid interface allowing for gas phase exposure of volatile materials in airway inflammation and irritancy studies, as well as in nanotoxicology applications (Hayden, P., Kaluzhny, Y. etc. 2011). This convenient format also allows the facile measurement of transepithelial permeability for inhaled drug delivery studies. The ability to grow the tissue without antibiotics can be used to investigate the mechanisms of bacterial infection of the respiratory tract along with the possible pharmaceutical prevention thereof. It is an ideal platform to evaluate inhalation toxicity of nanoparticles (Carter, J.M. 2004).



TEM micrograph of EpiAirway tissue:
A) celia on apical tissue surface;
B) tight junction between cells;

Figure 1. EpiAirway Human Tissue Features

5.3.2 Culture Medium

AIR-100-MM Maintenance Media (Serial/Lot # 082412JMD) was purchased from MatTek Corp. (Ashland, MA).

5.3.3 Positive Control

The positive control, SLS (Cat. # 71736 for 10% solution), was purchased from Sigma-Aldrich (St. Louis, MO).

5.3.4 Test Nanomaterials

Test nanomaterials are the same as the ones used in the Microtox assay (5.1.3), but RNP-212 was not included in this pre-test because this compound is an earlier developed formulation of the FAST-ACT product.

5.3.5 Equipment

EVOM™ Epithelial Voltohmmeter and Endohm™ Tissue Resistance Measurement Chamber, Endohm-6, were manufactured by World Precision Instruments (Sarasota, FL).

5.3.6 Procedures

5.3.6.1 Preparation of Tissues

Using sterile technique in a tissue culture hood, 0.5 ml of AIR-100 medium was dispensed into each well of the 24-well plate(s). Using a sterile forceps, each tissue insert was transferred to a well of the 24-well plate(s). Make sure that no agarose adheres to the insert. Also make certain that the media makes full contact with the underside of the tissues insert membrane (no air bubbles under the insert). The apical surface of the tissue should remain exposed to air (i.e. media should *not* be added to the inside of the insert). The tissues in the 24-well plates was equilibrated at 37°C, 5 percent CO_2 overnight (16-18 hr) and ready for the experimental use. If longer culturing times are desired, feed the tissues again with 0.5 ml of media each day.

5.3.6.2 Treatment of Test Nanomaterials

At the end of the equilibration period, aspirate the media from each well of the 24-well plate(s) containing the tissue inserts and replace with fresh, pre-warmed media. All stocks and dilutions of the test nanomaterials were prepared with the culture medium AIR-100. The tissues were treated with test nanomaterials by adding 0.1 mL of each dilution (a range of three concentrations of each test nanomaterial) per insert, 0.1 mL of the medium per vehicle control insert and 0.1 mL of 0.1 percent SDS per positive control insert. The tissues were exposed to the treatment for 48 ± 1 hr in 37^oC, 5 percent CO_2 incubator.

5.3.6.3 Performance of TER

Charge the $EVOM^2$ battery (12 hours charge for ~8 hours of a normal battery run time) prior to the use. Do not take resistance or voltage measurement with the charge connected.

Connect Endohm Tissue Resistance Measurement Chamber to instrument with electrode leads (longer electrode lead wire should attach to bottom portion of chamber to obtain correct polarity). If electrodes have been stored dry, fill chamber with PBS deep enough to immerse top electrode and equilibrate electrodes for about 20 minutes with the power off prior to checking calibration. To check calibration, turn Function Switch to Millivolts position and turn power on. Adjust potentiometer with a screwdriver to obtain a zero reading if necessary. Next, turn Function Switch to Ohms position. Adjust potentiometer with a screwdriver to obtain a zero reading if necessary.

With a small amount of PBS in the Endohm Tissue Resistance Measurement Chamber (1.5 ml for Endohm-6), place a blank insert into the chamber, add 0.2 ml PBS to the top surface of the insert to completely cover the membrane surface to a depth of 4-5 mm and adjust the top electrode so that it

is close to, but not making contact with, the top surface of the insert membrane. Background resistance reading of the blank insert should remain consistently low (generally < 50 $\Omega \cdot cm^2$). Instrument is now ready for tissue measurements.

By the end of treatment, carefully remove test articles by aspirating and gently rinse top surface of the tissues by adding 0.2 ml of PBS per insert and aspirating twice. Transfer inserts to be measured to a standard 24-well plate containing room temperature PBS (1 mL/well). Then add 0.2 ml PBS to the top of each insert to completely cover the surface of the tissue and submerge the top electrode (4 - 5 mm). Transfer individual tissues (the inserts) into Endohm™ Tissue Resistance Measurement Chamber, and replace top electrode (make certain that the electrode does not touch the tissue). With Mode knob set to R, push measure button and record resistance.

Decant PBS from top surface of tissue insert and return the tissue to culture vessel for the following TEP assay.

5.3.7 Data Analysis

TER is calculated as follows:

Ω x cm^2 = (Resistance of tissue – Resistance of blank insert) X 0.6 cm^2

Note: The insert used here has a growth area of 0.6 cm^2

5.4 Transepithelial Permeability Assay

TEP assay is an *in vitro* test method that has been widely used in toxicological and pharmacological studies. It is simple and meaningful, not only useful in collection of fundamental toxicity information of chemicals/drugs, but also valuable in assessment of their toxic/therapeutic pathways or mechanisms. The TEP assay has special usefulness in evaluation of the function of barriers/junctions in epithelial and endothelial cells or tissues. Engineering tissue models, such as a 3-D human corneal epithelial (HCE-T) model and human tracheal/bronchial epithelial equivalent (EpiAirway), possess the structures of tight junctions with barrier function. Therefore, the TEP assay is a valuable tool and often performed on these *in vitro* tissue models to evaluate the effects of chemicals/drugs on the tight junction integrity. A validation study (2000 – 2001) sponsored by Gillett Medical Evaluation Laboratories validated the TEP method on the HCE-T model by evaluating of 35 cosmetic compounds for their ocular toxicity potentially to human (Cao, C.J., Heroux, K. and Valdes, J.J. 2001). TEP is expressed as percentage retention of sodium fluorescein relative to the negative control. Increased permeability will indicate cytotoxicity/tissue damage induced by the test articles.

5.4.1 Tissues

The TEP assay was conducted on EpiAirway System (MatTek Corp.), the same tissue used in TER assay (5.3.1).

5.4.2 Culture Medium

AIR-100-MM Maintenance Media (Serial/Lot # 082412JMD) was purchased from MatTek Corp. (Ashland, MA).

5.3.3 Positive Control

The positive control, SLS (Cat. # 71736 for 10% solution), and other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO).

5.4.4 Test Nanomaterials

Test nanomaterials are the same as the ones used in the TER assay (5.3.4).

5.4.5 Equipment

The fluorescein absorbance is measured on Synergy HT Multi-Detection Microplate Reader (Model SIAFRTD) with Gen5 software.

5.4.6 Procedures

5.4.6.1 Preparation of Tissues

Preparation of tissues was the same as that in TER assay (5.3.6.1).

5.4.6.2 Treatment of Test Nanomaterials

Treatment of test nanomaterials was the same as that in TER assay (5.3.6.2).

5.4.6.3 Performance of TEP

- Prepare 0.02 percent fluorescein sodium salt using phenol-free MEM medium (GIBCO#51200-038) containing 1 mM calcium. Protect the fluorescein solution from light exposure and warm it at 37^{o}C water bath or incubator prior to the use of TEP assay.

- Use additional 24-well plate(s) for receiving the fluorescein permeated through the membrane of inserts. Dispense 1 mL of 37^{o}C-warmed phenol-free MEM into the well(s) of the 24-well plate. Place empty insert(s) into the designed well(s) of this plate as maximal leakage controls. Place the plate(s) in 37^{o}C and 5 percent CO_2 incubator if the following performance is not followed immediately.

- Transfer the tissues of inserts from the plate(s) used for TER measurement into the new 24-well plate containing MEM in each well.

- Dispense 200 µL of the 0.02 percent fluorescein solution into all inserts, including the empty inserts, to completely cover the surface of the tissues. Place the plate in incubator at 37^{o}C and 5 percent CO_2 for 30 min.

- Remove inserts in the same order as adding of the fluorescein into vehicle control, positive control, maximal leakage and samples of the test articles. Discard the inserts.

- Transfer 200 µL of the medium from each well in to a standard 96-well plate. Do duplicate of the transfer for each well.

- Read O.D. at 490 nm on Synergy HT Multi-Detection Microplate Reader. All raw data

(prints and files) were archived

- Perform data analysis as followings.

5.4.7 Data Analysis

- % Retention for each test material dilution or control will be calculated as follows:

$$\textbf{\% Retention} = \left[1 - \frac{\text{Mean } OD_{490} \text{ of culture medium}}{\text{Mean } OD_{490} \text{ of maximal leakage medium}}\right] \times 100$$

- Relative Retention is expressed as % retention relative to the control (% of control) and will be calculated as follows:

$$\textbf{\% of control} = \frac{\text{\% Retention at each treated culture at each dilution}}{\text{Mean \% retention of negative control cultures}} \times 100$$

6 Results and Discussion

6.1 Microtox Toxicity and Risk Assessment

Toxicity of FAST-ACT, RNP-212, Nano SBC and NanoScrub to marine bacteria, *Vibrio fischeri*, was measured by Microtox test system at 5, 15, and 30 min. Table 1 presents the toxicity data (IC_{50} Mean ± SEM) collected from 2-3 individual experiments per test compound and risk assessment. The EC_{50} values at 5 min, 15 min, and 30 min were initially determined by MicrotoxOmni™ software from concentration-dependent response. Data were further analyzed using Hill function (PRISM 4; GraphPad software) and presented in Appendix B-1: Figures - Microtox. X and Y axis represent log concentrations of the test article and the percentage of the effect bacteria of the control, respectively.

Microtox toxicity ($EC_{50\ 15min;}$ mg/L) shows FAST-ACT (13.78) > RNP-212 (362) > Nano SBC (1394) > PhotoScrub (>2500). Comparisons of toxicity results using these methods for a variety of compounds found that *Vibrio fischeri* were, in most cases, more sensitive than other aquatic organisms (Dutka et al. 1983; McFeters et al. 1983, Riva et al. 2007). Thus, the results with Microtox tests are often useful screens in the assessment of relative toxicity to aquatic organisms. We use the aquatic toxicity criteria of USF&WS and OECD to categorize the potential ecotoxicity of these four nanomaterials (Table 2). This evaluation suggests that RNP-212 is considered "Practically Nontoxic" and both Nano SBC and PhotoScrub are considered "Relatively Harmless", e.g. all three are not toxic to aquatic life; only FAST-ACT is considered "Slightly Toxic" or "Harmful to aquatic life" (Table1).

No such ecotoxicity data are found for these four new CBD nanomaterials from literature. This Microtox study fills the data lack and provides ecologically relevant information of risk when these nanomaterials are environmentally exposed.

Table 1. Microtox Toxicity and Risk Assessment

Compound	Microtox EC50 (mg/L) Mean ± SEM			Hazard Categories (USFWS 1984)	Hazard Classes (OECD 2001)
	5 min	15 min	30 min		
FAST-ACT	14.70 ± 1.049	13.78 ± 1.041	13.48 ± 1.048	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)
RNP-212	1124.5 ± 164.58	361.5 ± 33.41	151.7 ± 13.37	Practically Nontoxic	---
Nano SBC	4025 ± 963.7	1394 ± 120.0	1044 ± 137.9	Relatively Harmless	---
PhotoScrub	>2500	>2500	>2500	Relatively Harmless	

Table 2. Ecotoxicity Assessment Scale

LC_{50} or EC_{50} Concentration Range (mg/L)	Hazard Categories (USFWS, 1984)	Hazard Classes (OECD, 2001; Pratt, 2002)
< 0.01	Super Toxic	Acute Toxicity I (very toxic to aquatic life)
0.01 to 0.1	Extremely Toxic	
0.1 to 1	Highly Toxic	
1 to 10	Moderately Toxic	Acute Toxicity II (toxic to aquatic life)
10 to 100	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)
100 to 1000	Practically Nontoxic	---
> 1000	Relatively Harmless	---

6.2 NRU Cytotoxicity and Risk Assessment

Toxicity of FAST-ACT, RNP-212, Nano SBC and PhotoScrub to human liver cells was evaluated by NRU assay. The cultures were exposed to 8 concentrations of each test nanomaterial for 48-hrs. FAST-ACT, RNP-212 and PhotoScrub were insoluble in medium at higher concentrations. To avoid significant interference of the precipitates with the optical measurement the highest concentration tested for these three nanomatrials was 1250 µg/mL, instead of 2500 µg/mL as tested for Nano SBC, which was soluble in medium at this concentration. Neither FAST-ACT, RNP-212, PhotoScrub at 1250 µg/mL, nor Nano SBC at 2500 µg/mL were found toxic to the human cells (Table 3).

Table 3. NRU Cytotoxicity

Sample	Range of Concentration (µg/mL)	No. of Dilutions	$IC_{50\ 48hr}$ (µg/mL)
FAST-Act	1250 – 9.8	8	ND*
RNP-212	1250 – 9.8	8	ND*
PhotoScrub	1250 – 9.8	8	ND*
Nano-SBC	2500 – 19.5	8	ND*
PC (SLS)	100.0 – 26.3	8	34.05

*Not Detected.

The following rodent predictive model can be used to predict acute rodent toxicity based on the cytotoxicity data.

$$\log LD_{50}\ (mg/kg) = 0.372 \log IC_{50}\ (\mu g/mL) + 2.024$$

Furthermore, the predicted acute toxicity can be categorized for hazard potentials using acute mammalian toxicity criteria for hazard designation derived from a Globally Harmonized System (GHS) (Table 4).

Table 4. GHS Acute Mammalian Toxicity Criteria

Acute Mammalian Toxicity	Very high	High	Moderate	Low
Oral LD_{50} (mg/kg)	$\leq$ 50	>50 – 300	> 300 – 2000	>2000

We estimated the predicted LD_{50} to be >1500 mg/kg for FAST-ACT, RNP-212 and PhotoScrub and >1800 mg/kg for Nano SBC. All four nanomaterials, FAST-ACT, RNP-212, PhotoScrub and Nano SBC, appear to be low hazard potentials. This *in vitro* toxicity evaluation is comparable with the following toxicological information from animal studies: FAST-ACT: LD_{50} oral (rabbit): >2000 mg/kg; PhotoScrub: LD_{50} oral (rats): >15,000 mg/kg; For RNP-212 and Nano SBC, no LD_{50} data were obtained and no remarkable toxicity were found either from rodent studies. Taking together, both *in vitro* and *in vivo* studies suggest that the low acute mammalian toxicity of the four nanomaterials tends to be low hazard to human health too (U.S. EPA Alternatives Assessment Criteria Quick Reference, 2010).

6.3 TER and TEP Pre-Testing on Human Airway Tissue Model

Two assays, TER and TEP, were performed on the EpiAirway, a human tracheal/bronchial epithelial equivalent. Protocols/procedures of both methods worked well as the positive control met the criteria. Three CBRN nanomaterials, FAST-FACT, PhotoScrub, and Nano SBC, were tested for their effects on the tight junctions/barriers of this tissue with a dosing range of 100 – 2500 µg/mL. No significant adverse effects were found for each test nanomaterial in both TER and TEP assays. Further more tests need to confirm the preliminary finding and perform risk assessment.

7 Conclusions

- The Microtox study fills the data lack of aquatic toxicity for these new CBD nanomaterials and provides ecologically relevant information of risk when these nanomaterials are environmentally exposed.
- The Neutral Red Uptake study in human liver cells provides cytotoxicity data for these new nanomaterials which is complementary for the *in vivo* animal studies with additional human related toxicity information.
- Combining use of the two methods as a panel of *in vitro* assays allows evaluation of both aquatic and mammalian toxicities to assess the potential for adverse effects to the ecology, environment, and human health due to the chemical exposure.
- Transepithelial Electrical Resistance (TER) and Transepithelial Permeability (TEP) assays were developed and performed on EpiAirway, a 3-D human tracheal/bronchial epithelial equivalent, showing that both TER and TEP methods work well on the 3-D tissue model. Further systematical testing on the 3-D human tissue models needs to be performed in order to evaluate the effects of these CBD nanomaterials on tissue tight junctions/barriers.

8 Recommendations

The study using human tissue models for risk assessment of CBRN nanomaterials should be continued. Completion of the proposed study will provide human-related toxicity data of the new CBRN nanomaterials in support of the ongoing USAPHC/OTSG efforts for safety development and applications of nanomaterials and nanotechnologies.

Appendix A

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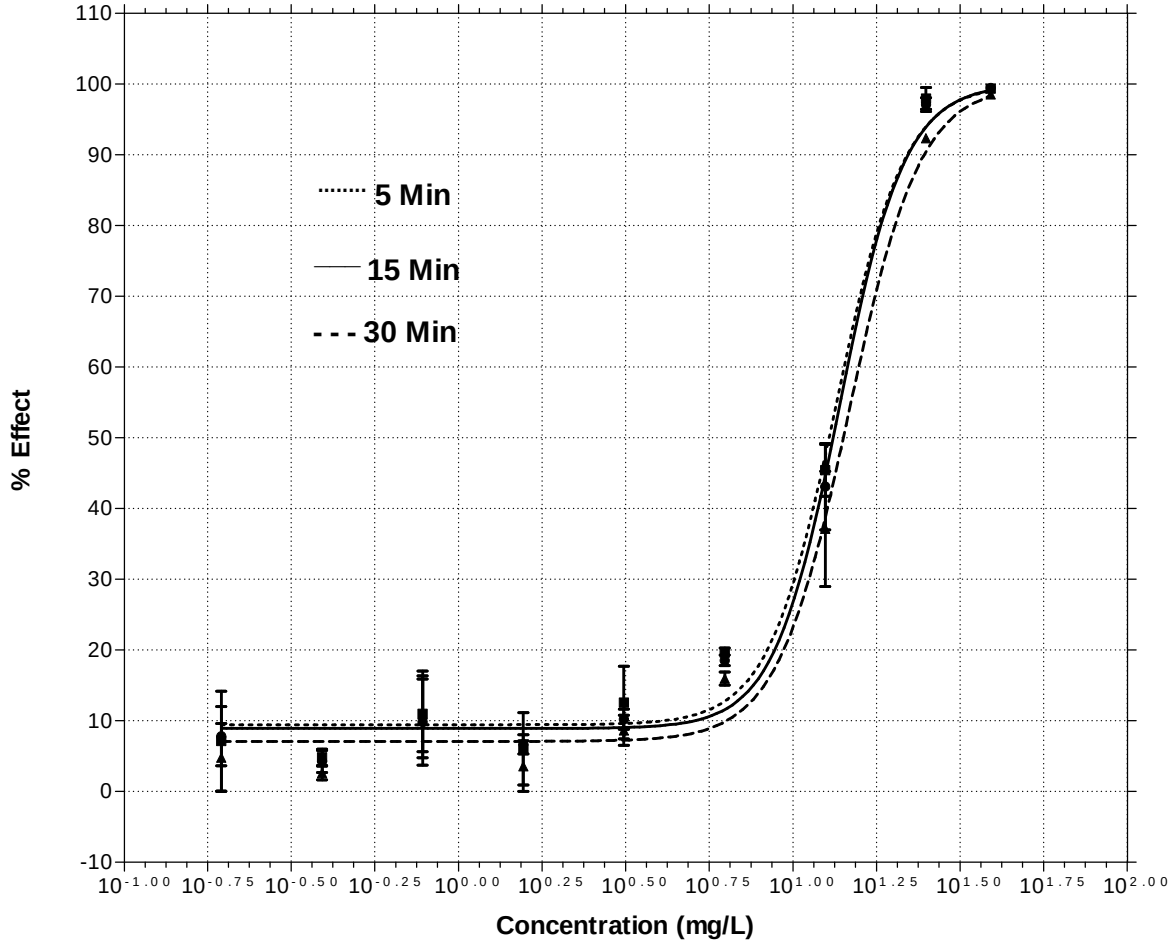
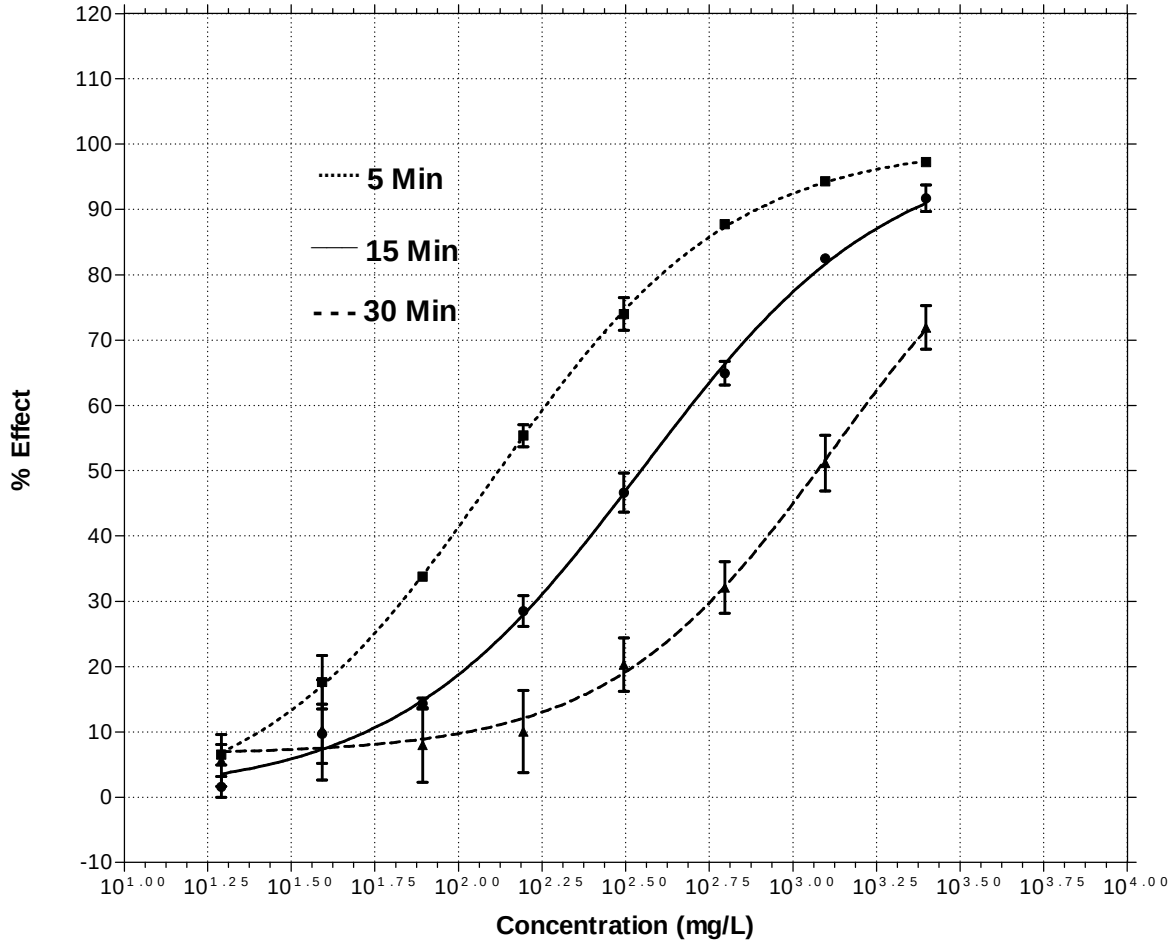
Appendix B - Figures

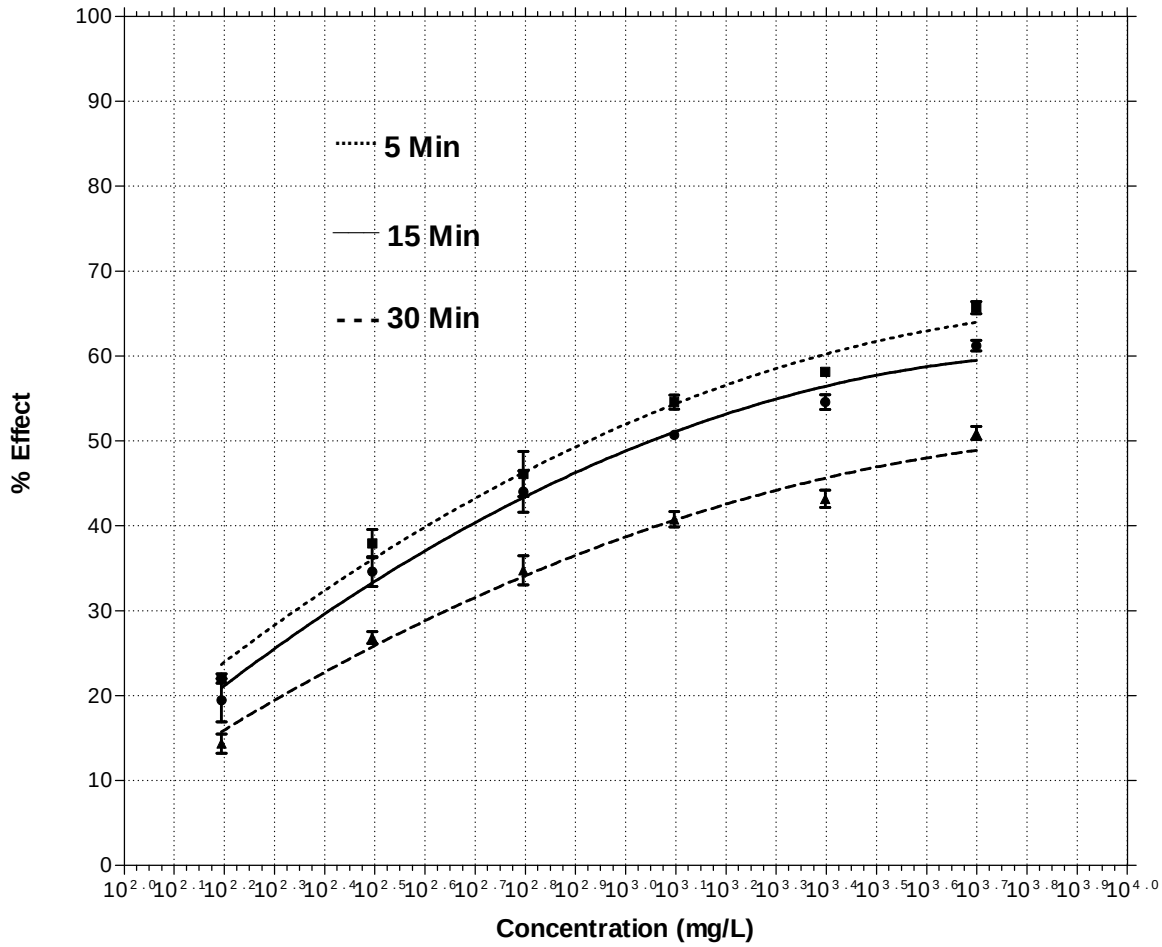
Aquatic Toxicity of FAST-ACT

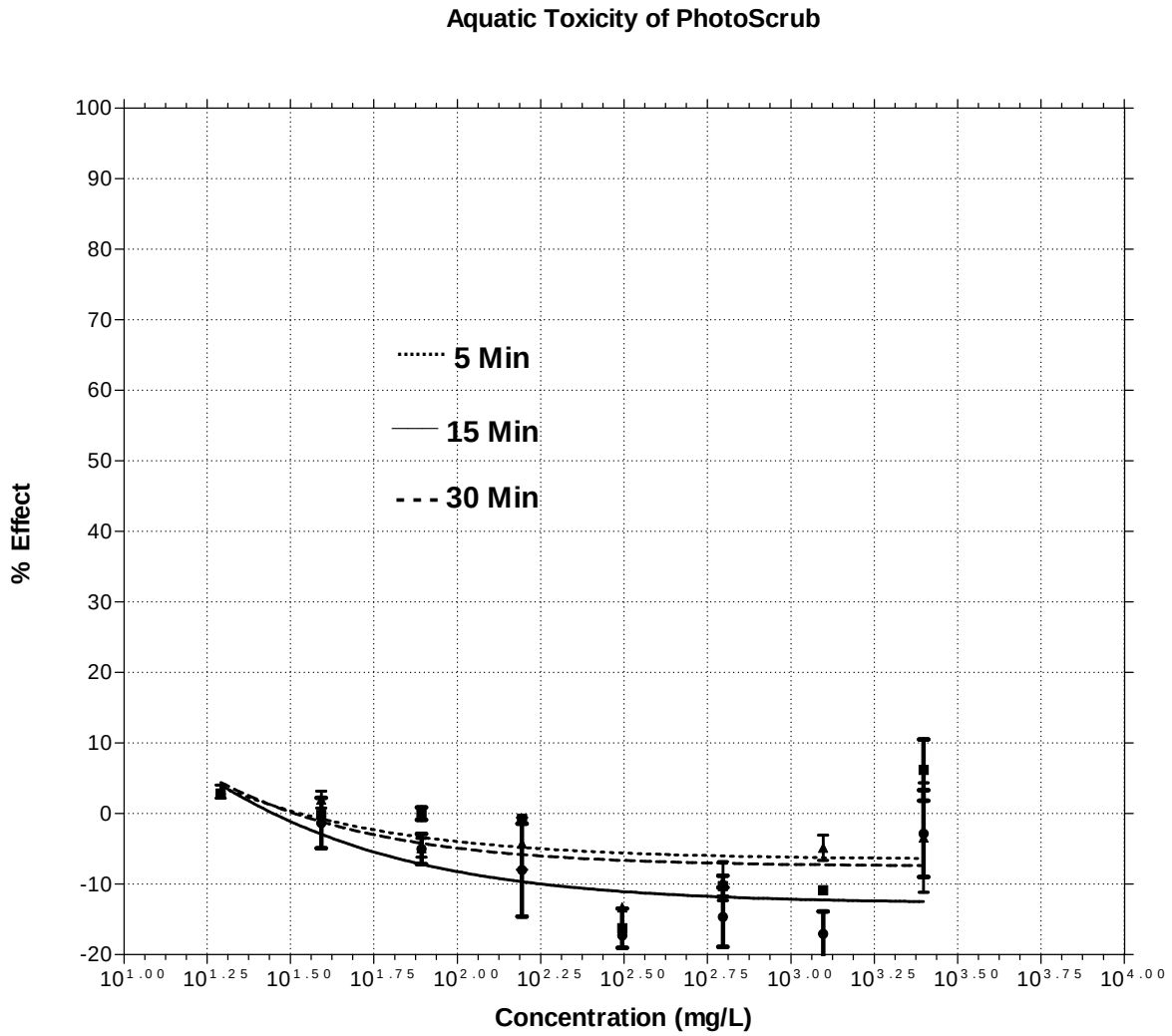


Aquatic Toxicity of RNP-212



Aquatic Toxicity of Nano Sodium Bicarbonate




Aquatic Toxicity of PhotoScrub
% Effect
Concentration (mg/L)
5 Min
15 Min
30 Min
100
90
80
70
60
50
40
30
20
10
0
-10
-20
10^1.00
10^1.25
10^1.50
10^1.75
10^2.00
10^2.25
10^2.50
10^2.75
10^3.00
10^3.25
10^3.50
10^3.75
10^4.00