DEVELOPMENT OF A DROSOPHILA MELANOGASTER
MODEL SYSTEM FOR NANOPARTICLE
TOXICITY ASSESSMENT

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DEVELOPMENT OF A DROSOPHILA MELANOGASTER MODEL SYSTEM FOR NANOPARTICLE TOXICITY ASSESSMENT

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ABSTRACT

DEVELOPMENT OF A DROSOPHILA MELANOGASTER MODEL SYSTEM FOR NANOPARTICLE TOXICITY ASSESSMENT

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Nanoparticles (NPs) are a growing facet of our industrial, medical and environmental economy. Toxicity research has focused on acute exposures both in vitro and in vivo. Few in vivo studies on chronic lifetime effects of NP exposure are available.

*Drosophila melanogaster* provides a powerful model for investigating human health and nanotoxicity. Counterparts of genes responsible for more than 700 different human genetic diseases, including neurological, immunological, cardiovascular, auditory, visual, developmental and metabolic disorders, are found in *Drosophila* (Koh
et al. 2006; Wolf et al. 2006; Rieter et al. 2001, Sykiotis and Bohmann 2008). The cost
effectiveness, experimental flexibility, and short generation time of *Drosophila* permit
rapid assessment of the vast number of NPs being produced, including chronic and
reproductive effects, thus providing a first tier assessment.

We have developed an in vivo chronic nanotoxicity model using *Drosophila*
melanogaster. The effects of different nanoparticles (silver and titanium) exposure on
*Drosophila* reproduction, development, and survivorship, were assessed based on
different sizes, and coatings. We’ve found that chronic exposure to silver NPs via
ingestion has toxic effects on fly viability and reproductive effort. Conversely, titanium
oxide has no effect on fly life history, and serves to verify the ability of our model to
discriminate among nanoparticle toxicity.

We also demonstrate the reversal of NP silver toxicity through diet
supplementation with vitamin C. By including vitamin C in NP treated fly food, the flies
were protected from the toxic life history effects of nanosilver ingestion. This
corroborates previous results that implicate oxidative stress as the primary contributor
to silver toxicity and provides a potential antioxidant-based strategy for the
development of prophylactics to NP exposure.
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<tr>
<td>ENM</td>
<td>engineered nanomaterials</td>
</tr>
<tr>
<td>NP</td>
<td>nanoparticles</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>UV-vis</td>
<td>UV-visible spectroscopy</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Ag</td>
<td>silver</td>
</tr>
<tr>
<td>CNT</td>
<td>carbon nanotubes</td>
</tr>
<tr>
<td>TiO₂</td>
<td>titanium oxide</td>
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<tr>
<td>vit C</td>
<td>ascorbic acid</td>
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CHAPTER 1

LITERATURE REVIEW

Introduction

The overall goal of this dissertation project was to develop Drosophila melanogaster as a model in vivo system for the systematic study of engineered nanomaterial toxicity. In order to establish D. melanogaster as a toxicity model, I created a novel nanomaterial inhalation delivery technique as well as devised an ingestion method to assay the effects of nanomaterial exposure on fly life history traits and molecular stress responses. Also, I explored the health implications due to the potential effects of nanomaterial anti-microbial properties on commensal gut microflora when ingested. This chapter describes the background and lays the groundwork explaining the significance of engineered nanomaterial technology (nanotechnology), the importance of the emergent field of nanomaterial toxicology (nanotoxicology), and the place that the Drosophila melanogaster model has in furthering our understanding of the interaction of nanomaterials with biological systems.
Nanoparticles

Properties

As defined in a 2010 report by the European Commission, a nanoparticle is “a material that consists of particles with one or more external dimensions in the size range 1nm – 100nm for more than 1% of their number” (Lovestam et al 2010). Materials at the nanoscale have unique properties that differentiate them from the bulk forms (greater than 100 nm). This is because the behavior of particles at the nanoscale is more closely governed by quantum physics than by classical Newtonian physics. Nanomaterials often exhibit distinctive electrical, chemical, and optical properties. For example, while bulk sized gold is inert, nanogold can act as a catalyst to promote chemical reactions. As another example, titanium oxide (TiO$_2$) is typically opaque but becomes increasingly transparent when it approaches the nanolevel.

Nanotechnology

Engineered nanomaterials (ENMs) are nano-sized particles that have been produced as a direct result of human activity. Recent advances in materials synthesis and characterization technology have led to an explosion in the production of ENMs. This new nano-industry has been driven by the seemingly limitless potential of nanotechnology in a diverse variety of fields, including: biomedical, industrial, electronics, sensors, and military defense, just to name a few. The “limitless potential” of nanoparticles is due to the exploitation of their small size and the novel properties
that materials exhibit at the nanometer scale. Nanoparticles have an extremely high surface area to volume ratio and, as a result, are more reactive compared to bulk materials. The increased surface area is used for carbon based high surface area electrodes to make more stable, higher density energy supercapacitors (Hiralal et al 2011). The large surface area to volume ratio also benefits chemical catalysis systems ranging from fuel cells and catalytic converters to photocatalytic devises (Wieckowski et al 2003). Nanomaterials can be stronger and more ductile than their bulk counterparts, too. Nanophase ceramics are more ductile than coarse grained ceramics and nano-impregnated steel is stronger and has higher sheer (Lojkowski & Blizzard 2002). These are just some examples of how the novel properties of nanomaterials are currently being.

Nanoparticles (NPs) in and of themselves are not novel entities. (For the purpose of this work, the terms “engineered nanomaterial" and “nanoparticle” are used interchangeably.) Naturally occurring nanoparticles can be found in volcanic soot, forest fire ash, clay, ocean spray, and plant phenols, among others (Oberdörster et al. 2005). There are even magnetic NPs endemic to the human body which, at abnormally high levels, have been associated with increased cancer risk (Binhi 2008).

However, the advent of the industrial revolution and the rapidly expanding field of nanotechnology have led to historically unprecedented, and ever increasing, levels of human exposure to nanomaterials. Anthropogenic NPs production occurs unintentionally by power plants and fuel combustion, as well as intentionally for applications in jet fuel additives, biomedicine, electronics, optics, and cosmetics.
Nanomaterials are being embedded into clothing to enhance stain resistance, used as polymer dispersions in paints and adhesives, added to rocket propellant and jet fuel for more efficient combustion, and incorporated into electron micrographs to improve conductivity. Commercial use of NPs will continue to rise for the foreseeable future, as well. There are over 1,300 nanoparticle containing commercial products currently available on the market worth more than $247 billion (The Woodrow Wilson’s Center Project on Emerging Nanotechnologies Consumer Products Inventory, http://nanotechproject.org/inventories/consumer/), accessed July 2012), and worldwide commercial sales of nano-enabled products are projected to reach over $ 1 trillion by 2015 (Lux Research, 2009).

Nanotoxicology

The same properties of nanoparticles that offer such immense promise for the development of new technologies also pose an unknown risk to both human and environmental health and safety. Concern about the risk associated with nanoparticle exposure has led to the emergence of a new field of research known as “nanotoxicology”. The broad aims of this new discipline are to understand the toxicological properties that are specific to nanoparticles and how these particles interact with living organisms and the environment.

The diameter of a typical human cell is in the range of 10 – 100 micrometers. Many of the organelles that make up an average cell are on the scale of nanometer sized. It is not surprising then that engineered nanomaterials directly interact, and
potentially interfere with biological machinery (Hussain et al 2006, Natvio et al. 2008, Arvizo et al. 2010, Samberg et al 2010). In order to evaluate the safety of nanomaterials, it is important that we have a firm understanding how these particles behave within a biological system (Nel et al 2006).

*Nanoparticle Characterization*

One of the most critical issues in any nanotoxicity assay is the thorough characterization of the material. In order for nanoparticle toxicity data to be meaningful and reproducible it is essential to understand the state of the material that is being studied as well as possible (Powers et al 2006, and Jiang et al 2008, Montes-Burgos et al 2009). Poor materials characterization due to lack of standardized characterization requirements threaten to undermine the work of the entire nanotoxicology field (Warheit 2008). It is impossible to overstate the importance of sound and thorough nanoparticle characterization.

In order to address this issue, a “bare-minimum” level of materials characterization has been adopted. The suggested minimum materials characterization prior to beginning a nanotoxicity experiment includes: particle size, size distribution, particle morphology, particle composition, surface area, surface chemistry, and particle reactivity (Murdoch et al 2007). In order to ensure the highest level of material characterization, a variety of state of the art characterization methods can be used to evaluate the condition of nanoparticles, as seen in Table 1.1.
Mechanisms of toxicity

While much remains unknown about the way through which nanoparticles exhibit toxicity, it is apparent that there is no single over-arching mechanism of toxicity inherent to all nanoparticle types. Toxicity of carbon nanotubes (CNTs) is generally due to their durability and the high aspect ratio of their shape. The fiber-like properties of CNTs induce toxicity through hindrance of phagocyte mediated clearance mechanisms and through the aggravation of proximal cells via mechanical interactions (Lam et al 2006, Kostarelos 2008). In comparison, metallic nanoparticles tend to disassociate and release ions when exposed to a biological environment, resulting in reactive oxygen species (ROS) production and initiation of the cellular oxidative stress response system (Donaldson & Stone 2003, Svedova et al 2005, Haase et al 2012, Mei et al 2012). Oxidative stress occurs due to an imbalance in the pro-oxidant/antioxidant homeostasis. The relationship between nanoparticle exposure and oxidative stress is an area of intensive research especially in consideration of increasing evidence that oxidative stress is involved in the development of various diseases, cancer, and aging (Kurz 2008, Sotgia et al 2011, Lee et al 2012).

Silver Nanoparticles

The focus of much of my research is directed towards silver nanoparticles (AgNPs). Silver is the most abundantly used metallic nanomaterial in both commercial and consumer products due to its antimicrobial properties and beneficial application in the fields of chemistry, physics, and materials science (The Woodrow Wilson’s Center
Project on Emerging Nanotechnologies Consumer Products Inventory, http://nanotechproject.org/inventories/consumer/, accessed July 2012). The antimicrobial properties of AgNPs are now being taken advantage of through use in medical devices, water treatment, wound dressings, and food packaging. They are incorporated into plastics, composites, and used to print flexible electronics due to their enhanced electrical conductivity, and the plasmonic properties are finding applications in biomedical labels, sensors, and detectors.

The rapidly rising use of AgNPs raises the concern of increased incidence of human and environmental exposure. Due to its widespread use, nano-silver has been the subject of much recent research. When introduced into rats via inhalation, ingestion, or injection, AgNPs are capable of translocation to various organs, including: lungs, kidneys, liver, spleen, olfactory bulb, and the brain where they dissociate and become toxic due to the release of silver ions (Ji et al 2007, Kim et al 2009, Johnston et al 2010). AgNPs have been shown to interfere with development by inducing morphological malformations in zebrafish, reducing fecundity in fruit flies, and impairing implantation of mouse blastocysts (Bar-Ilan et al 2009, Li et al 2009, Posgai et al 2011). AgNP toxicity has also been demonstrated in vitro. AgNPs induced toxic responses and ROS production have also been shown in various cell lines (Lesniak et al 2005, Braydich-Stolle et al 2005, Hussain et al 2005, Arora et al 2008).

While many studies have investigated the toxic effects of AgNPs, the mechanism of nano-Ag toxicity is unclear. As a metal oxide, much of the toxicity from AgNPs is associated with ion dissolution. The charged ions released from the Ag particles interact
with and damage cellular components resulting in the generation of ROS (Ivask et al. 2010). However, studies have shown that Ag ions alone cannot fully account for the observed toxicity (Navarro et al. 2008, Fabrega et al. 2009, Park et al. 2011). There is a definitive Ag nanoparticle effect. This effect may result in ROS generation through direct interaction of the particle surface with the mitochondria or through catalysis of redox reactions by the reactive surface of the Ag particle.

**Drosophila melanogaster as a model organism**

**History**

All living organisms essentially function using the same hardware. This basic set of signaling systems is in charge of building the hand of a human, the tail of a mouse, or the wing of an insect. Genetic research is in effect the study of how these signaling systems function together to build a complex living being. To understand such a complex problem requires many tools and there is there is no larger tool box available than that of *Drosophila melanogaster*.

It is nearly impossible to overstate the importance of *Drosophila melanogaster*’s contributions to the scientific research community. Since Thomas Hunt Morgan began using the fruit fly *D. melanogaster* to study genetics in the early 1900’s, the scientific community has accumulated over 100 years’ worth knowledge about practically every aspect of fruit fly biology. During the last century, *Drosophila* genetics research has contributed invaluable information towards the understanding of genetic linkage,
chromosomal basis of sex determination, and the behavior and mechanics of chromosomes (Rubin & Lewis 2000). The significance of fruit fly research isn’t limited to the past, either. In 2000, the fruit fly genome was the second multicellular organism, with *Caenorhabditis elegans* being the first, to have its genome fully sequenced. When the human genome was completed three years later, it served to highlight the great number of homologous genes shared between the two genomes, further strengthening the fly as a relevant human health model. Even more recently, in 2011, Jules A. Hoffman received the Nobel Prize in Physiology or Medicine for his work using the fruit fly to discover of the importance of the *Toll* gene in the innate immune response. As it has in the past, the fruit fly continues to demonstrate its relevance to biological research into the 21st century.

*Why the fly?*

The fruit fly offers many advantages for its use as a model organism. Its short life cycle, ease of rearing and reproduction, and low cost relative to other models are unquestionable advantages. However, the fly’s true scientific power lies in its genes. As previously mentioned, despite being a non-vertebrate organism that diverged from the vertebrate lineage approximately 700 million years ago, there is significant conservation of genetic and signaling pathways with humans.
Drosophila as a human disease model

Often referred to as the “workhorse” for genetic studies, comparison of *D. melanogaster* and human DNA and protein sequences has been an important source of information regarding the function of human genes. For example, in the study of human disease, the role of many genes has been elucidated using the fly. Feany and Bender (2000) confirmed the role of the gene *α-synuclein* in the development of Parkinson’s disease. They showed that mutations in the gene for *α-synuclein* resulted in loss of dopaminergic neurons, locomotor dysfunction, and formation of filamentous intraneuronal inclusions in the fly, all of which are hallmarks of Parkinson’s disease in humans. Fruit flies have also been used to answer important questions about the onset of Alzheimer’s disease. McBride and associates (2010) showed that *presenilin*, a gene associated with Alzheimer’s, mutant flies developed age related memory and learning issues and that these problems could be reversed through treatment with therapeutic drugs. From cancer (Rudrapatna et al 2012) and drug addiction (Bainton et al 2000) to sleep problems (Huber et al 2004), knowledge about almost any human disease condition of significance has benefited from *Drosophila* research.

Drosophila – Beyond human disease

Outside of its role in studying human disease, *Drosophila* has had an equally important role in the advancement of knowledge in many other scientific fields. The fly has been used to understand animal growth and development through studying cellular patterning, cell specification, cell death and survival, and polarity (Lawerence et al 1999,
Denton et al 2009, Singh et al 2012). As a tool to study learning and behavior, Bolduc and associates (2008) showed that genetically disrupting the gene FMR1 in the fruit fly’s brain eliminates its long term memory, while Baier et al (2002) demonstrated that alterations to the mushroom bodies in the fly brain dramatically effect aggressive behavior.

In the discipline of evolutionary research, the fly has found broad application. It has been integral in understanding the evolution of genes (Domazet-Loso & Tautz 2003); the evolution of morphological, physiological, and molecular traits (Nielsen et al 2006, Burke & Rose 2009, Yeh et al 2012); as well as mechanisms of speciation (Michalak et al 2001). Drosophila has found use in non-traditional fly research areas, as well. Examples include the field of pathogenesis through improvement of industrial scale insect repellents (Miller et al 2012) and the development of novel antifungal compounds (Lionakis & Kontoyiannis 2012).

The above examples are but a small fraction of the contributions that Drosophila melanogaster has made towards the enhancement of our knowledge about the world around us and serves to further illustrate the wide-ranging utility of the fly model.

Other model organisms: Drosophila in context to zebrafish and C. elegans

Drosophila is not alone as a non-mammal model research organism. The two most prominent alternative non-mammal model organisms are the nematode Caenorhabditis elegans and the zebrafish Danio rerio. Each of these organisms has its own unique advantages. Selection of which model to use depends upon the type of
research and scientific questions being studied. The strength of *C. elegans* as a model organism is in its simplicity. *C. elegans* has exactly 959 somatic cells. The growth and development of the 959 cells can be tracked from the moment of self-fertilization until full cell maturity. Despite being a more complex organism, *Drosophila* actually has fewer genes than *C. elegans* but twice as many human homologs (Friedman & Hughes 2001). Remarkably, over 70% of human disease genes have homologs in *Drosophila* and these genes often share greater than 90% nucleotide sequence identity (Bier 2005, Lloyd & Taylor 2010). In comparison, *C. elegans* shares only about 50% homology with human disease genes (Harris et al 2004). While the exact number of zebrafish genes is not yet known, as a vertebrate it is expected to have homologs for most human genes (Langheinrich 2003). The zebrafish is also considered a strong developmental model because embryogenesis is similar to higher vertebrates and is a highly observable process which occurs outside of the adult in a transparent egg. However, the relative paucity of zebrafish genetic data in comparison to the vast resources available for *Drosophila* and *C. elegans* limit the usefulness of zebrafish as a genetic research tool.

**Background of Drosophila as a toxicity model**

Despite the aforementioned advantages of the fly model across a wide spectrum of research fields, it has been used sparingly as a tool to assay toxicity. Toxicologists have traditionally used rodents or zebrafish as *in vivo* animal models and have almost completely ignored the fly. Historically, the fly has found some limited use in the study of herbicide toxicity and to research the mechanisms of drug toxicity (Kaya et al 2000,
McClung & Hirsh 1998) However, there has been a growing recognition of the need for expanding the realm of traditional toxicity research. The National Research Council Committee on Toxicity Testing and Assessment of Environmental Agents recently produced a report that identified the need to integrate high-throughput screening assays into toxicity testing programs (NRC 2007). The Drosophila model is considered a high throughput assay due to its low maintenance cost, rapid generation time, easily identifiable mutant phenotypes, and ability for high order screening.

**Advantages of Drosophila toxicity model**

The life cycle of a fly has four discrete parts: embryonic, larval, pupa, and adult. Each of these life cycle stages poses unique opportunities to assess toxicity. In comparison with mouse or zebrafish toxicity models, *D. melanogaster* has proven to be a powerful *in vivo* model because it has less redundancy in the genome, facilitating the rapid analysis of gene functions (Hsu & Schulz 2000). Another major advantage of the fly is that it can be manipulated experimentally much more readily than vertebrate models due to both ethical and technical issues. Restricting *in vivo* toxicity research to vertebrates is unreasonable if for no other reason than the fact that the sheer amount of time and resources required for rodents and zebrafish studies make it impossible to adequately address many important questions.
Drosophila as a nanotoxicity model

The similarity between mode of nanoparticle response, behavior, and gene response in D. melanogaster and mammalian systems, combined with the power of Drosophila genetics, have recently made the fly a very attractive system to study nanoparticle toxicity. As the fly nanotox model is in the early establishment phase, the majority of the initial studies to date have focused on the effects of nanoparticle exposure on life history traits. The most commonly used method of nanoparticle delivery to the fly is via ingestion. In one of the earliest Drosophila nanotox studies, Liu and associates found that fly larvae fed four different types of carbon nanotubes (CNTs) showed no physical or reproductive effects. Yet adult flies exposed to dry powder forms of the two of the same CNTs experienced impaired locomotor function and mortality. This seminal research was important both because it demonstrated that flies were susceptible to nanoparticle exposure and that there was a differential particle specific response. On the heels of this work, there has been a great deal of research showing ingestion of other types of nanoparticles is not nearly as benign as CNTs. In particular, for metal oxide nanoparticles there is a clearly size, dose, and material dependent toxicity. Silver nanoparticle fed fly larvae have decreased survivorship and fecundity, developmental delay, and a distinctive low-pigment “pale” adult phenotype (Gorth et al 2011, Key et al 2011, Panacek et al 2011, Philbrook et al 2011, Posgai et al 2011).

Taking advantage of the wealth of genetic and physiological tools available in Drosophila, a number of studies have assayed the genotoxic, subacute, and chronic effects of nanomaterial exposure. Through use of the wing somatic mutation and
recombination test, metal nanoparticles have been proven to be genotoxic, in addition to affecting life history traits (Demir et al 2010, Vales et al 2012). Noteworthy and possibly a bit alarming, researchers have been able to isolate and maintain the first nanomaterial-mutated organism named “nanomaterial-mutated” (NM-mut). After treatment with citrate-capped 15 nm Au NPs, Vecchio and associates induced mutagenic effects that were transferred to the next generation. The NM-mut has a significant phenotypic modification resulting in a bisected eye with the abnormal presence of an extraneous structure in the middle (Vecchio et al 2012).

As the fly progresses as a nanotoxicity model organism, even more sophisticated and creative research is emerging. Barandeh et al (2012) explored the fly model as a tool to evaluate the development of nanoparticle-based drug or gene delivery systems by showing that a medically relevant novel silica based nanoparticle (organically modified silica, ORMOSIL) has no effect on viability despite penetrating into the fly’s brain, neuronal cell bodies and axonal projections. Notably, even when the ORMOSIL nanoparticles were incorporated into the brain they did not induce neuronal death or interfere with regular neuronal processes. Huang et al (2012) investigated the effects of nano-alumina on the central nervous system. Using patch clamps to monitor the rhythmic and electrophysiological activities in the antennal lobe of the fly, they found that 15 minutes after ingestion of aluminum nanoparticles the average neuronal activity of exposed flies was significantly decreased compared with controls. These results indicate potential for adverse effects of aluminum nanoparticles on the central nervous system.
Other indicators of nanoparticle toxicity have been assessed in *Drosophila* through the presence of DNA fragmentation, as well as a modification in the expression levels of genes and proteins involved in stress responses, DNA damage recognition and apoptosis pathways (Ahamed et al 2010, Pompa et al 2011, Vecchio et al 2012).

*Using Drosophila to study nanoparticle inhalation effects*

Perhaps a bit non-intuitively, the fly holds a great deal of potential as a model to study the effects of nanoparticle toxicity to the respiratory system. The mode of delivery is an important consideration when exposing nanoparticles to cells, tissues, or organs and one of the most sensitive targets of nanomaterials is the respiratory pathway via inhalation of airborne particles. Historically, toxicologists have relied primarily on mouse or rat models to study the toxicity of inhaled particles (Warheit et al 2004, Oberdörster et al 2005). Considering the sheer volume and rapid development of novel manufactured nanomaterials, there is a need for a faster and cheaper screening method to serve as a triage for the more time and cost laborious mammal systems. Zebrafish are not a viable alternative because they do not have lungs nor do they have a respiratory system that has an air-to-blood oxygen exchange interface structure. Among the non-vertebrate model organisms, only *Drosophila* shares a similar organ composition and a lung equivalent. The fly may not have lungs per se, but its airway system has many distinct similarities with the respiratory system of mammals regarding its physiology and reaction towards pathogens (Roeder 2011). The systems have enough similarities that the fly is considered a model organism for asthma research
Drosophila is particularly suited for the study of aerosolized nanoparticles because its spiracles (external tracheal openings) are multiple microns in diameter, which is large enough to allow for nanoparticle entrance. As proof of this concept, I have shown through my research that airborne nanoparticles can be delivered to the respiratory system of drosophila (Posgai et al 2010).

Drosophila in comparison to in vitro nanotoxicity studies

Over the last decade there has been a persistent push to complement or even replace many types of animal experiments with cell culture studies. Cell lines offer a number of advantages over animal models. Cells are low-maintenance, high throughput, and allow underlying cellular functions and molecular mechanisms to be studied with relative ease compared to animal models. In vitro research also provides the ability to work with human cells to assay nanoparticle toxicity, something that is not possible with in vivo studies. In vitro nanotoxicity research is not necessarily straightforward though. Interpretation of nanoparticle toxicity data is often quite difficult because proper assessment relies on many factors, such as particle parameters, cell line selection, and biomarker detection. Work with cell lines has other limitations as well, particularly when studying processes at the tissue, organ, or whole body level. Due to the unique properties related to size, shape, and composition it is difficult to use in vitro studies to predict how engineered nanomaterials will interact with complex biological systems (Fischer & Chan 2007). In specific regard to the Drosophila nanotox model, it presents many of the same advantages promised by in vitro research, including the tools
to directly assay specific molecular pathways and mechanisms of nanoparticle toxicity. Ultimately, when it comes to understanding the complex interactions between nanoparticles with a biological system which lead to whole organism changes, such as in behavior or development, there is no replacement for animal models.

Overview of significance of fly toxicity model

With the need for a fast through-put in vivo system to assay the toxicity of nanomaterials, it is clear that there is a place for the Drosophila model in the field of nanotoxicology. This research serves to emphasize the great leverage provided by the suite of developmental, reproductive, genomic, and genetic approaches available in flies for decoding details of the biological interactions of nanomaterials. Establishing the Drosophila model will allow for great amounts of nanoparticle toxicity information to be collected quickly and easily and serve as a triage for development of studies in more complex mammalian organisms.
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Table 1.1. Description of nanoparticle characterization methods.

<table>
<thead>
<tr>
<th>Physiochemical properties</th>
<th>Characterization methods</th>
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<tr>
<td>Shape</td>
<td>TEM, UV-vis</td>
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<tr>
<td>Agglomeration or aggregation state</td>
<td>DLS, UV-vis, TEM</td>
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<tr>
<td>Surface chemistry/charge/area</td>
<td>ζ-potential, XPS</td>
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<tr>
<td>Dosing metric</td>
<td>Weight, surface area, concentration</td>
</tr>
<tr>
<td>Stability over time/dissolution</td>
<td>DLS, UV-vis, ICP-MS, colorimetric assays</td>
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<td>Uptake</td>
<td>ICP-MS, TEM, fluorescence</td>
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Abbreviations: TEM, transmission electron microscopy; DLS, dynamic light scattering; UV-vis, UV-visible spectroscopy; XPS, X-ray photoelectron spectroscopy; ICP-MS, inductively coupled plasma mass spectrometry
CHAPTER 2

INHALATION METHOD FOR SYSTEMIC DELIVERY OF NANOPARTICLES TO THE \textit{DROSOPHILA} RESPIRATORY SYSTEM FOR TOXICITY TESTING

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Keywords: Nanoparticle, \textit{Drosophila}, Inhalation model, Nebulizer, Nanoparticle toxicity
Abstract

The growth of the nanotechnology industry and subsequent proliferation of nanoparticle types present the need to rapidly assess nanoparticle toxicity. We present a novel, simple and cost-effective nebulizer-based method to deliver nanoparticles to the *Drosophila melanogaster* respiratory system, for the purpose of toxicity testing. FluoSpheres®, silver, and CdSe/ZnS nanoparticles of different sizes were effectively aerosolized, showing the system is capable of functioning with a wide range of nanoparticle types and sizes. Red fluorescent CdSe/ZnS nanoparticles were successfully delivered to the fly respiratory system, as visualized by fluorescent microscopy. Silver coated and uncoated nanoparticles were delivered in a toxicity test, and induced Hsp70 expression in flies, confirming the utility of this model in toxicity testing. This is the first method developed capable of such delivery, provides the advantage of the *Drosophila* health model, and can serve as a link between tissue culture and more expensive mammalian models in a tiered toxicity testing strategy.
Introduction

There is a need to rapidly and cost-effectively assess the toxicity of nanoparticles (US Environmental Protection Agency 2007, National Nanotechnology Initiative 2006, Oberdörster 2005, Service RF 2007), defined as particles with one dimension in the nanoscale range. There are more than 800 nanoparticle-containing consumer products on the market (Woodrow Wilson International Center for Scholars). In addition, nanoparticles released from man made combustion sources have been reported to constitute up to 36% of urban atmosphere particulate matter (Shi et al 2001). The estimated cost for comprehensive testing of all existing nanoparticles for the U.S. is over $1.1 billion (Choi 2009) and this amount will increase as new nanomaterials are developed. The present rate of industrial production and use of novel nanomaterials far outpaces the capability of health and safety regulators to assess their potential danger. This knowledge gap is anticipated to widen further as worldwide nanotechnology spending approaches an estimated $2.5 trillion by 2014 (Lux Research). Nanoparticles have properties different from bulk materials due to their increased surface area, affecting their ability to penetrate the skin, pass through cell membranes, cross the blood-brain barrier, be absorbed into lymphatic channels, and reach bone marrow, lymph nodes, the heart and lungs, and the central nervous system (Oberdörster et al 2004, Oberdörster et al 2005). Thus, there is a compelling need to rapidly assess the toxicity of nanoparticles.
The ability of nanoparticles to generate both significant local and systemic toxic responses via respiratory system exposure makes this pathway of introduction of particular interest (US Environmental Protection Agency 2004). As a result, respiratory system research has been one of the primary contributors to the field of nanotoxicity (Oberdörster et al 1996, Donaldson et al 2002, Kreyling et al 2002). However, this base of knowledge is severely limited in comparison to the number of existing nanoparticles. Current pulmonary nanotoxicity methods using mammalian models are powerful, but involve delivery apparatus that are too cost prohibitive for widespread use, and thus insufficient to evaluate the vast number of nanoparticle types in production. There is a need for an inexpensive nanoparticle toxicity model, using a delivery apparatus simple enough for benchtop use yet capable of delivering a wide range of nanoparticles, and an organismal model that is inexpensive, experimentally powerful, and relevant to human health (Schneider 2000). Consequently, we have developed a method capable of delivering nanoparticles to the *Drosophila melanogaster* respiratory system, based on a simple nebulizer delivery.
Materials and methods

Nebulizer design

A nebulizer based system has been previously used to deliver soluble drugs (Broderick et al 2006), but has not been used with nanoparticles, which are relatively large and insoluble, nor have nanoparticles been delivered to the fly respiratory system. Here, the mouthpiece of a medical nebulizer (Minimate compressor, model #PM5, Precision Medical, Inc. Northampton, PA) is attached to a 8 cm long piece of ¾ inch rubber tubing subdivided by a single layer of cheesecloth into two chambers, a smaller (3 cm) section (damper chamber) and a larger (5 cm) section (exposure chamber, Fig. 2.1). The damper chamber reduces the air sheer and turbulence before aerosolized nanoparticles enter the exposure chamber containing the flies. The mouthpiece and the distal end of the exposure chamber are also covered with a layer of cheesecloth, to reduce the rate of air flow and prevent escape of the flies; this mesh does not accumulate nanoparticles but does absorb a small amount of dose (~80 μl of a 9 ml dose), and once saturated does not influence the administered dose. Nanoparticles are suspended in water at known concentrations, and 9 ml of this suspension is loaded into the nebulizer reservoir. The mouthpiece of the nebulizer is then connected to the damper chamber, and the flies (up to 20) are placed into the exposure chamber where they are exposed to the aerosolized nanoparticles. The nebulizer delivers a flow rate of 7 liters/minute at a driving pressure of 13.5 psi, this results in a delivery rate of aerosolized nanoparticles of 0.6 ml/min, with droplet sizes on the order of 0.5 μm.
diameter—much smaller than the diameter of the spiracle openings (25 μm~60 μm) through which air enters the fly respiratory system. The resulting whole body exposure chamber design is comparable in effect to the versatile but cost prohibitive inhalation chambers which have been used for mice and rat nanoparticle inhalation studies (Grassian et al. 2007, Yu et al. 2007, Sung et al. 2008).

Nanoparticles

A variety of nanoparticles were used to demonstrate the utility and flexibility of this inhalation method. Green fluorescent polystyrene FluoSpheres® 24, 100, and 210 nm in diameter were purchased from Invitrogen Corporation™. These are supplied as aqueous suspensions containing 2% solids and 2 mM of sodium azide. FluoSpheres® do not aggregate in solution, and all sizes fall within a narrow range as determined by TEM (Molecular Probes Handbook, 2009). Red fluorescent, 5.7±0.5 nm CdSe/ZnS core-shell nanoparticles were used to facilitate visualization of nanoparticles in the fly tracheal system. These are well-characterized through TEM, and UV and PL spectra, and following sonification have a minimal agglomeration state in water (NN-Labs, LLC). Silver uncoated nanoparticles (20 nm) were purchased from NovaCentrix. These were characterized in our laboratory using TEM and tend to agglomerate under our study conditions (Fig. 2.2D). Polysaccharide-coated 10-nm silver particles were generously provided by Dr. Dan Goia (Clarkson University, Center for Advanced Materials Processing, Potsdam, NY). These were previously characterized in our laboratory by
TEM (Ahamed et al 2010); the polysaccharide coating keeps the particles from agglomerating, resulting in uniform dispersion under our experimental conditions.

*Administered dosage to fly respiratory system*

Nanoparticles have not been previously introduced to the fly respiratory system, which differs in morphology from mammalian respiratory systems. Air enters the trachea of *D. melanogaster* through spiracles — pores located along the length of the body. Trachea branch into smaller and smaller diameters, called tracheoles, until they reach cell size diameters (~1 μm) and deliver O2 to cells. Spiracles are normally closed at rest, but open under anoxic conditions. Small insects rely almost exclusively on passive diffusion for the movement of gasses within the tracheal system; the major sites to air resistance lie in the spiracles, not in the trachea (Buck & Keister 1958), such that spiracle opening and closing regulates air intake into the tracheal system. To facilitate spiracle opening during nanoparticle exposure, flies are agitated during exposure to increase O2 demand. Given the relative diameters of the nebulized water droplets (0.5 μm diameter) and the spiracle openings (25 μm-60 μm) particles can easily enter the tracheal system, such that after a short exposure the concentration of particles in the tracheal system is equivalent to that in the chamber.

The rate of particle loading into the tracheal system is much more rapid than that of the rate of clearance (via diffusion and/or endocytosis) to the tissues (Snyder et al., 1995). Thus, over the short exposure times used here (up to 10 min), clearance to
the soma is negligible and dosage is equivalent to the number of particles in the tracheal system. This is equal to the volume of the tracheal system multiplied by the airborne concentration of nanoparticles in the exposure chamber. The airborne concentration of nanoparticles is equal to the saturated vapor density of water (23 g/m³) multiplied by the volume of the chamber (25 ml), resulting in $5.75 \times 10^{-4}$ g (or ml) water in the chamber during nebulization, and a chamber concentration of nanoparticles = $5.75 \times 10^{-4}$ ml water $\times 100$ μg NPs/ml = $5.75 \times 10^{-2}$ μg NPs/chamber, or $2.3 \times 10^{-3}$ μg/ml chamber volume. The volume of the fly tracheal system is $\sim 1.8 \times 10^{-3}$ μl (extrapolated from the volume in the ground beetle *Platynus decentis*, a slightly larger insect, Westneat et al 2003), resulting in a dose of $4.14 \times 10^{-9}$ μg/fly, or $3.45 \times 10^{-12}$ μg NPs/μg fly tissue — the equivalent of a 0.3 μg exposure to a 100 kg human. Once exposure ceases flies are allowed to rest, the spiracles close and the dose is absorbed by the soma. Dose can be manipulated through nanoparticle concentration in water, or through repeated exposures.

*Toxicity test of silver nanoparticles*

Heat shock protein 70 (Hsp70) expression is a useful marker of physiological stress (Mukhopadhyay et al 2003), and is used in a silver nanoparticle toxicity assay. Silver nanoparticle dosing solutions were prepared in 10 ml water at a concentration of 50 μg/ml. Twenty flies were exposed to coated and uncoated silver nanoparticles for 10 min, 2 times/day for five days. Flies were then sacrificed, and total protein isolated by
homogenization in RIPA lysis buffer [1X TBS (0.5 MTris–HCl and 1.5 MNaCl) pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide] in the presence of a protease inhibitor. Estimation of total protein was done using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). A negative control consists of a water-only exposure, a positive control for Hsp70 expression was comprised of adults that were temperature shocked for 1 h at 37±1 °C (Krebs & Feder 1997).

Thirty micrograms of protein extract from the control and Ag nanoparticle-treated flies were analyzed by SDS-PAGE and transferred to a PVDF transfer membrane. The membrane was then probed with anti-Hsp70 (Heat shock protein 70) antibody (Santa Cruz Biotech, CA) to determine relative expression level.

Fly strains and culture

Wild-type Drosophila melanogaster (Oregon RS, stock number 4269) and a tracheal-specific (breathless) GFP reporter strain (w[*]; P{w[+mC]=GAL4-btl.S}2, P{w[+mC]=UASp-Act5C.T:GFP}2, stock 8807) were obtained from the Bloomington Stock Center. Flies were reared on standard Bloomington stock center cornmeal/malt media.
Results

FluoSphere® and silver nanoparticle nebulization

To assess the system's capability, the ability of the nebulizer to aerosolize nanoparticles of differing size and physical composition was first tested. FluoSpheres® were suspended in 3 mL water (50 μg/mL deionized water), and hole punch size pieces of filter paper (3 mm diameter) were placed into the chamber and exposed for five minutes/treatment. After exposure, the filter paper was examined for fluorescence. FluoSpheres® were successfully nebulized, as revealed by their deposition on filter paper (Fig. 2.2 A,B,C). A qualitative decrease in intensity of green fluorescence occurred with the 210 nm particles, an indication that relatively fewer particles were aerosolized and a potential upper limit to the nebulizer's capacities. Next, metallic nanoparticles (20-nm uncoated Ag, 50 μg/ml deionized water) were nebulized, captured on micromesh grids, and visualized by transmission electron microscopy (Fig. 2.2D). The tendency of silver nanoparticles to agglomerate at higher concentrations is responsible for the observed size range of particles deposited.

Delivery of CdSe nanoparticles to fly respiratory system

To verify that this method can deliver nanoparticles to the fly respiratory system, we used a tracheal-specific (breathless) GFP reporter strain of flies, allowing
easy and definitive identification of the tracheal system. Fig. 2.3 presents a cross-section through the abdomen and thorax of (A) wild-type Ore RS, (B) breathless GFP strains of *D. melanogaster*, showing GFP expression in the tracheal system, and (C) breathless GFP flies exposed to nebulized red fluorescent CdSe/ZnS (100 μg/ml deionized water). The red-fluorescing CdSe/ZnS nanoparticles are clearly visible against the green of the tracheal tissue, confirming successful delivery of nanoparticles to the tracheal system. Note that individual nanoparticles are not resolved at this magnification.

*Hsp70 protein expression in silver nanoparticle exposed flies*

To confirm the utility of this methodology in toxicity assays, flies were exposed to coated and uncoated silver nanoparticles in a chronic toxicity assay, and tested for Hsp70 protein expression. Western blots probed with anti-Hsp70 antibody demonstrated that Hsp70 protein expression was higher in silver nanoparticle exposed flies as compared to the negative control (Fig. 2.4).
Discussion

A fundamental merit of this delivery method in toxicity testing is that it takes advantage of the power of *Drosophila* as a health model system. Over 60% of human disease genes have fly homologs, indicating the fly response to physiological insult is comparable to humans (Schneider 2000, Koh et al 2006, Marsh & Thompson 2006). In addition, chronic, generational, and genotoxic effects can be assessed *in vivo*, studies impractical in mammalian systems, providing both a rapid and comprehensive means to assess nanoparticle toxicity, and a triage to identify candidates for further testing in mammalian systems as part of a tiered toxicity testing strategy, for example as outlined by the European Union's Registration, Evaluation, Authorization and restriction of CHemicals (REACH) program.
Acknowledgements

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Fig. 2.1. Diagram of the nebulizer nanoparticle delivery system.
Fig. 2.2. Deposition of nanoparticles of different size and composition. Aerosolized FluoSpheres® A (24 nm), B (100 nm), C (210 nm), and 20-nm silver uncoated nanoparticles (D).
Fig. 2.3. Tracheal GFP expression and CdSe/ZnS particles within trachea. Wild-type (A) and Gal4-UAS /breathless GFP reporter (B) strains of *D. melanogaster*, and CdSe/ZnS particles (C) within the *Drosophila* tracheal system.
Fig. 2.4. Western blot analysis of Hsp70 protein expression in silver nanoparticle treated flies. Negative control (water only, lane 1), Ag coated (lane 2), Ag uncoated (lane 3), and positive control (lane 4).
CHAPTER 3

DIFFERENTIAL TOXICITY OF SILVER AND TITANIUM DIOXIDE NANOPARTICLES ON DROSOPHILA MELANOGASTER DEVELOPMENT, REPRODUCTIVE EFFORT, AND VIABILITY: SIZE, COATINGS, AND ANTIOXIDANTS MATTER

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Keywords: silver nanoparticles; titanium dioxide nanoparticles; nanoparticle size, nanoparticle coating; oxidative stress; Drosophila; vitamin C
Abstract

Silver and titanium dioxide nanoparticles are known to induce oxidative stress in \textit{vitro} and \textit{in vivo}. Here we test if they impact development, mating success, and survivorship in \textit{Drosophila melanogaster}, and if so, if these effects are reversible by antioxidants. Ingestion of nano-titanium dioxide during the larval stage of the life cycle showed no effects on development or survivorship, up to doses of 200µg/mL. Conversely, ingestion of nano-silver had major dose, size, and coating-dependent effects on each of these aspects of life history. Each of these effects was partially or fully reversible by vitamin C. Larvae growing on nano-silver supplemented with vitamin C showed a greater than twofold increase in survivorship compared to flies reared on nano-silver alone, and a three-fold increase in mating success. Vitamin C also rescued cuticular and pigmentation defects in nano-silver-fed flies. Biochemical assays of superoxide dismutase and glutathione show these markers respond to nano-titanium dioxide and nano-silver induced oxidative stress, and this response is reduced by vitamin C. These results indicate that life history effects of nano-silver ingestion result from oxidative stress, and suggest antioxidants as a potential remediation for nano-silver toxicity. Conversely, the lack of nano-titanium dioxide life history toxicity shows that oxidative stress does not necessarily result in life history effects, and argues that nanoparticle toxicity needs to be examined at different levels of biological organization.
Introduction

Nanoparticle-based technologies are a rapidly growing facet of our industrial, medical and environmental economies. Their toxicological properties, different from bulk form, have been subject to intensive research (Oberdörster et al 2005, Nel 2006, Rivera et al 2010). Silver and titanium dioxide nanoparticles, used in clothing, food industry, paints, cosmetics, electronics, coatings and medical products, are of particular concern because they are two of the fastest growing product categories in the nanotechnology industry (Cheng et al 2004, Cohen et al 2007, Lee et al 2007, Vigneshwaran et al 2007).

Oxidative stress is an emerging, general mechanism underlying nanoparticle toxicity (Nel et al 2006, Mocan et al 2010). Previous work in our lab (Ahmed et al 2010) finds that nano-silver ingestion in Drosophila larvae activates oxidative stress pathways, including superoxide dismutase (SOD), catalase, caspase-3 and caspase-9, glutathione and malondialdehyde (MDA), a product of lipid peroxidation. Titanium dioxide is best known for reactive oxygen species (ROS) production via photoactivation (Hirakawa 2006), however, studies find ROS production from both rutile and anatase in the absence of photoactivation (Gurr et al 2005, Braydich-Stolle et al 2009).

Yet to be determined is whether oxidative stress has effects on the whole organism, in terms of development, reproduction, and survival. The lowest level of cellular response to oxidative stress entails the induction of antioxidant molecules and detoxification enzymes via Nrf-2 activation (Xiao et al 2003). Thus, acute exposures may
have only transient cellular effects that do not impact the health of the organism.

Conversely, sustained ROS production can lead to inflammation, circulatory problems, and DNA damage that could ultimately impact the life history of the organism through effects on development, viability, and/or reproductive effort.

There are few in vivo studies on life history effects of chronic titanium dioxide and silver nanoparticle exposure, almost all in aquatic organisms (reviewed in Menard et al 2010). Such studies are needed, both to determine the potential for life history toxicity in mammalian models, and the potential for environmental damage through their extensive commercial production and consumer use. D. melanogaster provides a relevant model for investigating human health, as counterparts of genes responsible for more than 700 different human genetic diseases including neurological, immunological, cardiovascular, auditory, visual, developmental and metabolic disorders are found in Drosophila (Rieter et al 2001, Koh et al 2006, Wolf et al 2006, Khurana et al 2006).

Moreover, the Nrf-2 gene and its function are conserved in Drosophila, making it specifically relevant to model oxidative stress responses (Sykiotis & Bohmann 2008). The cost effectiveness, experimental flexibility, and short generation time of Drosophila permit rapid in vivo assessment of the vast number of nanoparticles being produced, including life history effects of chronic exposure.

We find that nano-silver ingestion had negative, dose-dependent effects on survivorship, mating success and development, with smaller, uncoated particles showing the greatest toxicity. Conversely, titanium dioxide had no effect on fly life history. Vitamin C ingestion partially or fully reversed each of these life history effects.
of nano-silver ingestion, and reduced the response of oxidative stress markers SOD and glutathione (GSH). While antioxidant reversal of nanoparticle induced stress has been observed in vitro (Kim et al 2009, Sharma et al 2010, Foldbjerg et al 2010, Akhtar et al 2010), this is the first in vivo study that shows nanoparticle toxicity can be alleviated by antioxidants. The in vivo reversal of each facet of nano-silver toxicity indicates that the primary mechanism of nano-silver toxicity is oxidative stress. The lack of nano-titanium dioxide life history toxicity provides a caution that biochemical toxicity does not necessarily extrapolate to whole organism toxicity.
Experimental Procedures

Nanoparticles used in this study

Uncoated and polysaccharide-coated 10nm and 60nm silver particles were generously provided by Dr. Dan Goia (Clarkson University Center for Advanced Materials Processing, Potsdam, NY). The coated silver nanoparticles were synthesized by the reduction of silver ions in a solution of a polysaccharide (acacia gum), which leads to surface coating. Nanoparticles were diluted from bulk concentrations to 1mg/mL working stock concentrations and sonicated for 1 min at 35–40 W to aid in mixing and forming a homogeneous dispersion.

Nanoparticle characterization

Transmission electron microscopy (TEM) characterization was performed to determine nanoparticle size and morphology using a Hitachi H-7600 tungsten-tip instrument at an accelerating voltage of 100 kV. Nanoparticles were examined after deposition of nanoparticles suspensions onto carbon film-coated Cu TEM grids. The AMT software for the digital TEM camera was calibrated for size measurements of the nanoparticles. Information on mean size and SD was calculated using point to point method (Murdock et al., 2008).

Dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) were used for characterization of hydrodynamic size and zeta potential (ζ) of nanoparticles suspended
in H₂O, performed on a Malvern Instruments Zetasizer Nano-ZS instrument as described by Murdock et al. (2008).

Fly Husbandry

OreRS flies were obtained from the Bloomington Stock Center, Bloomington, IN and reared on standard cornmeal-malt-yeast medium (Bloomington Stock Center recipe) at 25°C. Nanoparticles and vitamin C were added to the media while cooling and decanted into 60mm x 15mm petri plates for larval feeding experiments. Control plates consisted of 20mL standard Drosophila cornmeal media. In treatment lines, standard medium was supplemented with nanoparticles resulting in final suspensions from 5µg to 200µg nanoparticles per mL of medium, depending on the experiment. In antioxidant experiments, medium was supplemented with ascorbic acid (vitamin C) or vitamin C palmitate (a lipid-soluble vitamin C) to a final concentration of 50mM.

Survivorship assay

Drosophila embryos were laid over a two hour time period on control medium, fifty of which were collected and moved to control or treatment plates for the assay. Seven plates of fifty embryos each were used for each treatment. Percent survivorship was calculated as the number of embryos that pupated divided by the total number of embryos. The time from first larval instar hatch to pupation was also recorded. Adult phenotypes were scored following eclosion for cuticle maturation and melanization, two features found to vary with nano-silver dose.
Mating success assay

Virgin male and female flies that survived ingestion of 60 nm uncoated silver during the larval stage were mated in pairs. Twenty pairs were used for each treatment. The number of successful matings (those resulting in offspring) and number of progeny produced were recorded and compared to control lines (mated pairs not exposed to nano-silver during larval development).

Biochemical assays

Reagents.

Reduced nicotinamide adenine dinucleotide (NADH), reduced glutathione (GSH), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), nitrobluetetrazolium (NBT), and phenazine methosulphate (PMS) were purchased from Sigma-Aldrich, MO. All other chemicals used were of the highest purity available from commercial sources.

Preparation of crude extracts. For glutathione and superoxide dismutase assays, the control, Ag NP, and vitamin C treated larvae were homogenized in 0.1M sodium phosphate buffer (pH 7.4) containing 0.15M KCl. Following centrifugation (2300×g for 15 min at 4 °C), total protein of the supernatant was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). The supernatant (crude extract) was maintained on ice for use in biochemical assays.
Glutathione.

GSH level was quantified using Ellman's reagent (Ellman, 1959). Briefly, a mixture of 0.25mL of 1mg/mL crude extract and 0.9mL of 5% TCA was centrifuged (2300×g for 15 min at 37 °C). Then 0.5mL of supernatant added into 1.5mL of 0.01% DTBN and the reaction was monitored at 412nm. The amount of GSH was expressed in terms of nmol/mg protein.

Superoxide dismutase.

SOD activity was determined employing a modified version (Kakkar et al 1984) of the method described earlier (Nishikimi et al 1972). Briefly, at room temperature the reaction was initiated by adding 0.2mL of a 780μM NADH solution into a reaction mixture containing 1.25mL of 0.052M sodium pyrophosphate buffer (pH 8.0), 0.1mL of 186μM PMS, 0.3mL of 300μM NBT, 0.1 ml of distilled water and 50μl of 1mg/mL crude extract (total volume of 2.0mL). The reaction was stopped after 1.5 min at room temperature by adding 1.0mL acetic acid and then 4.0mL of n-butanol. Following centrifugation (2300×g for 15 min at room temperature) the color intensity of chromogen in butanol was measured at 560nm. A reaction mixture devoid of enzyme served as a control. One unit of enzyme activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in 1 min at room temperature and specific activity expressed as units/min/mg protein.
Statistical analysis

The mean and standard deviation of percent survivorship, percent mating
success, and time to pupation were calculated and statistically analyzed using an exact
binomial test (Goldstein 1954). Best-fit lines for each treatment were generated using
linear regression, and used to determine LD$_{50}$ (survivorship) or ED$_{50}$ (mating success).
LD$_{50}$ and ED$_{50}$ values and biochemical assays were compared to controls using Student’s
t-test ($\alpha = 0.05$).
Results

Nanoparticle characterization

Silver 10nm coated particles were previously characterized by DLS and TEM (Ahmed et al., 2009) and show good dispersion and uniform size. TEM analyses show that all nanoparticles tested are uniform in size (Figs. 3.1-3.4). DLS and zeta potential analyses indicate the particle suspensions are relatively stable and form agglomerates in water.

Survivorship is reduced and developmental time extended by nano-silver ingestion

Nano-silver ingestion had major, dose-dependent effects on survivorship to pupal stage (Fig. 3.5). Uncoated particles were more toxic than coated particles, and smaller particles were more toxic than larger particles. Time to pupation was slowed by nano-silver ingestion in a dose-dependent manner (Fig. 3.6). This effect plateaus around 180 hours, apparently extension of the pre-adult lifecycle is not viable beyond this time under our experimental conditions. Neither survivorship nor time to pupation was affected by titanium dioxide (rutile) ingestion at doses up to 200µg/mL.

Mating success is reduced by nano-silver ingestion

Nano-silver ingestion during the larval stage reduced adult mating success (Fig. 3.7). Lower doses of nano-silver were sufficient to disrupt reproduction compared to viability; ED_{50} (a dose that results in 50% viable matings compared to control) for
uncoated 60nm silver was 19.6 µg/mL ($R^2=0.90$), about 2/3 that of LD$_{50}$ for 60nm uncoated Ag. Many nano-silver-treated flies did not produce offspring, and those that did averaged fewer than half the progeny of control lines.

**Nano-silver ingestion impairs adult cuticle development and melanization**

Nano-silver ingestion during the larval stage resulted in cuticular and melanization defects in adults (Fig. 3.8). Flies that survived nano-silver ingestion had a soft, unpigmented cuticle. No such effect was observed in nano-titanium dioxide-fed flies. As epidermal pigments are secreted by the cuticle (Wright 1987), the cuticle defect is likely the root cause of these phenotypes.

**Vitamin C ingestion reverses toxic effects of silver ingestion**

Previous research finds that nano-silver ingestion causes oxidative stress (Ahmed et al., 2010). This suggests the potential for toxicity reversal through antioxidant treatment, which we verify here.

Larvae growing on 30µg/mL 60nm uncoated silver supplemented with 50mM vitamin C or vitamin C palmitate showed greater than twofold survivorship and threefold mating success compared to flies reared on nano-silver alone (Fig. 3.9). Time to pupation was reduced significantly (t-test), from 158.3+/-14.2 for nano-silver alone, to 151.0+/-18.7 ($H_0$: vitamin C < Ag; $p=0.003$) and 150.0+/-14.53 ($H_0$: vit C palmitate < Ag; $p<0.001$). Nano-silver induced cuticular and melanization defects were also reversed by co-ingestion of vitamin C (Fig. 3.8c,d).
Larvae growing on 30µg/mL 60nm uncoated silver supplemented with 50mM vitamin C showed reduced levels of superoxide dismutase (SOD) and increased, though not statistically significant, levels of glutathione (GSH) compared to flies reared on nano-silver alone (Table 3.1). This provides a biochemical mechanism whereby vitamin C ingestion can alleviate the effects on nano-silver on the life history traits examined here. Nano-titanium dioxide had comparable effects to nano-silver on SOD activity; but unlike nano-silver, had no effect on GSH levels.
Discussion

We show that nanoparticle induced oxidative stress can result in severe life history effects. However, as in the case of TiO$_2$, oxidative stress does not necessarily affect life history. Both biochemical and life history studies are needed to fully characterize nanoparticle toxicity.

Both nano-titanium dioxide and nano-silver have been shown to activate oxidative stress, DNA, and mitochondrial damage biochemical pathways (Hirakawa et al 2004, Hussain et al 2005, Jemec et al 2008, Wang et al 2008, Ahamed et al 2008, Sharma 2009, Ahamed et al 2010, Jin et al 2010). We find that these activations are not necessarily toxic to the whole organism. Nano-silver has major, negative effects on each facet of fly life history assayed in this study. Conversely, nano-titanium dioxide had no effect on survivorship, developmental rate, or adult cuticle phenotype. This shows that nanoparticles must be assayed at different levels of biological organization to determine their full toxicity profile.

Differences in silver and titanium dioxide life history toxicity may simply result from nano-silver showing greater biochemical toxicity. Although the ranges of particle variables relevant to toxicity in the aforementioned studies (different crystal structures, sizes, and shapes) complicate comparisons between nano-silver and nano-titanium dioxide, nano-silver has been shown to induce greater levels of oxidative stress than nano-titanium dioxide, including in a head-to-head study of silver and titanium dioxide.
nanoparticles comparable to those used in this study (Hussain et al 2005). While nano-titanium dioxide and nano-silver had comparable effects on SOD levels, nano-silver had a much greater impact on GSH levels than nano-titanium dioxide. GSH is the first line of defense against oxidative stress, its depletion in nano-silver-fed flies could underlie its greater life history toxicity.

Each facet of nano-silver toxicity is partially or wholly remediated by vitamin C. This indicates that oxidative stress is the major contributor to nano-silver toxicity at each level of organization. In *Drosophila*, oxidative stress pathways have dual function; they remediate oxidative stress but are also targets of the hormone ecdysone, which directs progression through larval stages of molting and pupal metamorphosis. Their activation in response to oxidative stress could disrupt the timing of these lifecycle events, a possible mechanism underlying the developmental phenotypes observed in treated flies. Such dual roles must be considered in evaluating biochemical toxicity in mammalian models. Moreover, oxidative stress may not be the only mechanism underlying nanoparticle toxicity, it is also possible that other mechanisms additional to oxidative stress are important to nanoparticle life history toxicity and underlie the different toxicity of nano-titanium vs. nano-silver.

Both aqueous and lipid-soluble forms of vitamin C reversed the effects of nano-silver ingestion. Two mechanisms through which vitamin C can act to reverse nano-silver toxicity are: 1) directly through reduction of reactive oxygen species (superoxide ion O$_2^{•}$, hydroxyl radical OH$^{•}$, and/or hydrogen peroxide H$_2$O$_2$) and/or 2) through the chelation of silver ions released by silver nanoparticles. Foldbjerg et al (2010) show in
vitro reduction of ROS in human lung cancer cell lines exposed to nano-silver when cells are pretreated with the antioxidant, N-acetyl-cysteine, however, this could result from antioxidant effects on either the nano-silver particle or the ion. Preliminary results in our lab find that the polysaccharide-coated nano-silver tested here release silver ions at a greater rate than uncoated nano-silver. Yet uncoated silver particles had greater toxicity than the same size coated particles, indicating silver ion release at most makes a small contribution to toxicity. A direct comparison of the efficacy of vitamin C on coated vs. uncoated nano-silver, with a silver ion control would help determine the mechanism of vitamin C reversal of nano-silver toxicity, and more generally nano-silver toxicity.

In addition to coating, size and agglomeration state are also relevant to toxicity. Though coated particles show less agglomeration in our DLS measurements, resulting in a relatively higher reactive surface area for the generation of ROS, they are less toxic than the same sized uncoated particle. The nature of the surface is clearly more relevant to toxicity than the amount of surface area available. Although the smaller 10nm silver particles showed somewhat greater toxicity than equivalently coated 60nm particles, the smaller particles provide six times more reactive surface area per unit mass, a much greater difference than found in their toxicity, in particular as smaller particles show less agglomeration in DLS measurements. The interaction between coating, size and agglomeration state in toxicity is clearly complex and in need of further study.

The lack of 1:1 correspondence between biochemical and life history aspects of nanoparticle toxicity strongly indicates a need to research the impact of biochemical
stress on the whole organism, in chronic toxicity models capable of testing for life history effects. Studies that focus on the relationship between *in vitro* and *in vivo* effects of oxidative stress may determine threshold values of biochemical ROS generation that result in life history toxicity, which would be relevant to setting exposure standards in industrial and commercial use.
Acknowledgments

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Figure 3.1. Characterization of silver 10nm uncoated nanoparticles. A. and B. represents the TEM characterization of 10nm uncoated Ag NPs. A total of 190 silver nanoparticles (Ag NPs) were measured by TEM for size distribution. A. depicts the morphology of silver nanoparticles and B. represents frequency of size distribution of Ag NPs. TEM mean ± SD of Ag NPs was 11.92±3.86 nm. C. and D. depicts DLS and LDV characterization of 10 nm uncoated Ag NPs. C. represents the Ag NPs diameter in water suspension (19.39 nm) and D. depicts the zeta potential (−37.1 mV).
Figure 3.2. Characterization of silver 60nm uncoated nanoparticles. A. and B. represents the TEM characterization of 60 nm uncoated Ag NPs. A total of 130 silver nanoparticles were measured by TEM for size distribution. A. depicts the morphology of silver nanoparticles and B. represents frequency of size distribution of Ag NPs. TEM mean ± SD of Ag NPs was 60.27±11.74 nm. C. and D. depicts DLS and LDV characterization of 60 nm uncoated Ag NPs. C. represents the Ag NPs diameter in water suspension (192 nm) and D. depicts the zeta potential (~29.4 mV).
Figure 3.3. Characterization of silver 60 nm poly-saccharide coated nanoparticles. A. and B. represents the TEM characterization of 60 nm poly-saccharide coated Ag NPs. A total of 139 silver nanoparticles were measured by TEM for size distribution. A. depicts the morphology of silver nanoparticles and B. represents frequency of size distribution of Ag NPs. TEM mean ± SD of Ag NPs was 66.54±15.7 nm. C. and D. depicts DLS and LDV characterization of 60 nm poly-saccharide coated Ag NPs. C. represents the Ag NPs diameter in water suspension (93 nm) and D. depicts the zeta potential (-32.7 mV).
Figure 3.4. Characterization of titanium dioxide nanoparticles. A. and B. represents the TEM characterization of titanium oxide NPs. A total of 200 titanium dioxide NPs were measured by TEM for size distribution. A. depicts the morphology of titanium dioxide NPs and B. represents frequency of size distribution of titanium oxide NPs. TEM mean ± SD of titanium dioxide NPs length was 38.22±12.14 nm and width was 13.53±3.54. C. and D. depicts DLS and LDV characterization of titanium dioxide NPs. C. represents the titanium oxide NPs diameter in water suspension (357 nm) and D. depicts the zeta potential (−25.5 mV).
Figure 3.5. Mean and SD percent survivorship for flies fed different concentrations of nanotitanium dioxide and nanosilver during larval development. Treatment line survivorship normalized to control lines (100%). LD$_{50}$ determined from best-fit line generated by linear regression (LD$_{50}$ µg/mL, $R^2$): 10nm Ag coated (32.4, 0.96), 10nm Ag uncoated (31.0, 0.95), 60nm Ag coated (47.4, 0.98), 60nm Ag uncoated (31.2, 0.96). Nanosilver ingestion had dose-, size-, and coating-dependent effects on survivorship. Conversely, nanotitanium dioxide had no effect on survivorship (LD$_{50}$ 763 µg/mL, $R^2$=0.99) at doses up to 200µg/mL (data point not shown). N = 350 embryos/treatment.
Figure 3.6. Survivorship of nanosilver and nanotitanium exposed larvae. Mean and SD time from larval hatch to pupation, for flies surviving different concentrations of nanotitanium dioxide and nanosilver during larval development.
Figure 3.7. Mating success is reduced by larval nanosilver ingestion. Treatment line mating success normalized to control lines (= 100%). ED$_{50}$ = 19.6 μg/mL, as determined from best fit line generated by linear regression ($R^2$ = .90). N = 20 mated pairs/treatment.
Figure 3.8. Larval nanosilver ingestion shows dose-dependent effects on adult melanization and cuticular development. The fly in Panel B was fed 30µg/mL Ag uncoated during larval development, and shows a soft adult cuticle lacking pigmentation compared to the control in Panel A. The cuticular and melanization phenotype are rescued by co-ingestion with vitamin C (Panel C, 30µg/mL Ag uncoated + vitamin C) and vitamin C palmitate (Panel D, 30µg/mL Ag uncoated + vitamin C palmitate).
Figure 3.9. Antioxidant reversal of nanosilver toxicity. The toxic effects of nanosilver ingestion on survivorship and reproductive effort are reversed by vitamin C. Mean and SD percent survivorship and mating pair success are presented for larvae reared on standard medium (negative control), 30µg/mL 60nm uncoated nanosilver, 30µg/mL 60nm uncoated nanosilver + 50mM vitamin C, and 30µg/mL 60nm uncoated nanosilver + 50mM vitamin C palmitate. Survivorship comparisons between control and each treatment line are significantly different (t-test, p<0.01). Survivorship comparisons between nanosilver and nanosilver+vitamin C treatments are significantly different (t-test, p<0.001). Mating success comparisons between control and each treatment line are significantly different (t-test, p<0.01), with the exception of vitamin C (p=0.34). Mating success comparisons between nanosilver and nanosilver+vitamin C treatments are significantly different (t-test, p<0.001).
<table>
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<th>GSH assay</th>
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CHAPTER 4

NANOSILVER INGESTION REDUCES DIVERSITY AND ABUNDANCE OF COMMENSAL GUT MICROBIAL COMMUNITY IN DROSOPHILA MELANOGASTER

Ryan T. Posgai, Tracy Collins, Kyle Murphy, John J. Rowe, and Mark G. Nielsen

(portion to be submitted to a peer-reviewed research journal)
ABSTRACT

Nanosilver ingestion causes oxidative stress *in vitro* and *in vivo*, due to their high surface/volume ratio. In *Drosophila*, nanosilver ingestion activates oxidative stress pathways, is toxic to development, longevity, and reproductive effort, and these effects are partially reversed by anti-oxidants (Posgai et al 2011). Here we examine if the antibacterial properties of nanosilver contribute to its toxicity through alterations of the normal commensal gut microbial community, which has been shown to influence immune function, longevity and mating success in *Drosophila*. We find that nanosilver ingestion does affect the gut microbial community. Using +/- screening, we have found one microbial species that is absent in nanosilver treated flies, and are working to identify it. Plating gut microbial contents on standard media under aerobic and anaerobic conditions finds a ten-fold colony reduction in nanosilver-fed flies: Aerobic: control = 3.5 x 10^5 cfu(colonies)/ml, nanosilver = 4.95 x 10^4 cfu/ml; Anaerobic: control = 3.85 x 10^5 cfu/ml, nanosilver = 3.95 x 10^4 cfu/ml. Using phylotypic bacterial PCR primers to 16S rDNA to characterize non-culturable microbes shows a 2-fold reduction in microbial abundance in nanosilver-fed flies. This difference (2 vs. 10-fold) indicates differential effects of nanosilver on the gut microbial community. Biolog plate screening finds alterations in gut microbial biochemical function, with the nanosilver-fed community lacking the ability to utilize a number of sole-carbon substrates, including: N-Acetyl-D-Glucosamine, Phenylethyl-amine, and α-D-Lactose. These results argue for further studies of chronic nanosilver ingestion, given the importance of gut microflora to
basic animal function (alterations in microflora underlie ulcers, obesity, cancers, type 2 diabetes, and autoimmune diseases in humans, and mating, longevity, immune system function in flies) and the widespread use of nanosilver in consumer products, including dietary supplements.
Introduction

When analyzing the make-up of the human genome, only the genes that are present within the 23 human chromosome pairs are typically considered for investigation. However, there are a large set of non-anthropomorphic genes that play an important functional role in humans. The non-human genes of reference belong to the copious amounts of commensal bacteria and various other microbes that humans host on as well as within our bodies. Collectively called the “human microbiome”, the 100 trillion commensal microorganisms constitute up to 3% of total body mass in the average individual and contain more than 8 million protein coding genes, nearly 360 times the number of human protein coding genes (Ley et al 2006, Human Microbiome Project Consortium 2012). Considering the overall size and close relationship with so many parts of the body, it is understandable why the microbiome is often referred to as the “forgotten organ.”

The human microbiome is made up of a number of separate micro-ecosystems. For example, the mouth, digestive tract, and nasal passage all have distinct and diverse microbial populations. Just as the relationships between organisms are important in shaping the dynamics and health of a macro-ecosystem (e.g. rainforest, barrier reef, etc.), the same applies to organisms at the micro-level. Also similar to macro-ecosystems, the ecology of micro-ecosystems of the human microbiome are terrifically complex and sensitive to disturbances (Konopka 2009, Steele et al 2011). Recently,
there has been a growing recognition of how the relative well-being of each of these micro-ecosystems can have a direct impact on human health.

There is mounting scientific evidence linking changes in the microbiome with the development of a large and diverse number of diseases. The freshly completed Human Microbiome Project is the result of a seven year National Institute of Health funded collaborative study with the explicit objective to “demonstrate that there are opportunities to improve human health through monitoring or manipulation of the human microbiome” (Human Microbiome Project Consortium 2012). There are a number of recent studies spurring the interest of such ambitious endeavors as the Human Microbiome Project. Turnbaugh and associates (2006) compared the gut microbiomes of lean and obese mice and humans and found a distinct difference in the microbial gut populations in obese individuals. These differences affect the metabolic potential of the gut microflora community in mice. This “obese microbiota” is transmissible to germ-free mice causing a greater increase in total body fat compared to mice cultured with “lean microbiota.” Gut microflora also play a critical role in the proper development of the immune system as indicated by the fact that germ-free mice have underdeveloped lymphoid tissues, spleens with few germinal centers, and poorly formed T and B cell zones, among other issues (Macpherson & Harris 2004). Another example of the importance of commensal microbiota to health is the well-established connection between gut microbial populations and irritable bowel diseases, such as Crohn’s disease and ulcerative colitis (Young et al 2011).
It is clear that the complex and delicate human-microbiome relationship is vitally important to our overall well-being. Therefore, concern about the potential effects of nanoparticle exposure on the human-microbiome is well merited. Due to the booming growth in the field of nanotechnology over the past decade, the incidence of human nanoparticle exposure has greatly increased. The most common type of nanoparticles found in consumer and commercial products are antimicrobial nanoparticles, especially silver nanoparticles (AgNPs). The antimicrobial properties of AgNPs are utilized in towels, socks, beverage containers, appliances, air filters, wound dressings, and even dietary supplements (The Woodrow Wilson’s Center Project on Emerging Nanotechnologies Consumer Products Inventory, http://nanotechproject.org/inventories/consumer/, accessed July 2012). However, with the increasing presence of AgNPs in manufactured and consumer products there is greater potential for consumption of AgNPs due to leaching of AgNPs into food products or the water supply. Once in the digestive tract, AgNPs could be toxic to normal gut microflora. The potential effect of antimicrobial NPs, such as AgNPs, on the microbiome has not been investigated in any living system.

*Drosophila melanogaster* is a relevant model to research the role of nanoparticle toxicity and gut microbial communities. *D. melanogaster* serves well as this model by taking advantage of the ability to inexpensively and rapidly assess chronic, generational, developmental, and genotoxic effects *in vivo*, studies impractical in mammalian systems. In terms of biology, the fly gut shares genetic, morphological and microbiological features with the human gut, and is growing as a model organism to understand the
relationship of the gut microbial community and the immune system on overall health (Ryu et al 2010, Jarchum et al 2011). Fly and human intestines share similar tissue, anatomy, and physiological functions (Ohlstein & Spradling 2006, Pitsouli et al 2008, Takashima et al 2008). Developmentally, both the mammalian gut and Drosophila midgut are of endothelial origin (Skaer 1993, Tepass & Hartenstein 1994). These similarities, along with the logistical advantages of using the fly model, make Drosophila a powerful tool for studying the effects of nanosilver on gut microbiota.

Through our previous research using Drosophila, we’ve shown that nanosilver ingestion activates oxidative stress pathways, is toxic to development, longevity, and reproductive effort, and these effects are partially reversed by anti-oxidants (Ahamed et al. 2010, Posgai et al 2011). AgNPs toxicity is primarily associated with oxidative damage to cellular components as a result of the particle induced generation of ROS. Oxidative stress proteins play an important role in regulating fly gut microbiota, by specifically removing harmful flora and maintaining those needed for fly health. Nano-silver has the potential to specifically disrupt this process through its impact of oxidative stress pathways, in addition to directly killing beneficial gut microbes through its antimicrobial effects. However, the mechanism of toxicity of AgNPs ingestion in Drosophila is not entirely clear. Because disruption of normal gut microflora function is known to have broad ranging negative health implications, the potential of AgNPs to harm individuals through toxic antimicrobial effects on important gut microflora is a compelling alternative mechanism of toxicity. Here we examine if the antibacterial properties of nanosilver contribute to its toxicity through alterations of the normal
commensal gut microbial community, which has been shown to influence immune function, longevity and mating success in *Drosophila*. We also explore whether effects on gut microbiota due to AgNPs ingestion can be mitigated by dietary vitamin C supplementation.
Methods

Fly strain and culture

OreRS flies were obtained from the Bloomington Stock Center, Bloomington, IN and reared on standard Drosophila food containing agar-agar, maize powder, sugar, yeast, nepagin, soy flour and propionic acid at 25 °C.

Media treatment preparation

Controls consisted of 20 ml of standard Drosophila media in 60 mm x 15 mm petri dish. For treatment lines, standard medium was supplemented with 10 nm AgNPs resulting in final nanoparticle suspension of 15 μg/ml of medium. The 15 μg/ml 10 nm AgNPs concentration was chosen as sub-lethal exposure dosage based on our previously published study (Posgai et al., 2011). In antioxidant experiments, AgNP treatment medium was supplemented with ascorbic acid (vitamin C) to a final concentration of 50 mM.

Ag nanoparticle exposure

Fifty embryos were collected on 60 mm x 15 mm petri plates containing 20 mL standard Drosophila media. After three days, larvae were transferred either to control, 15 μg/ml 10 nm AgNPs, 15 μg/ml 10nm AgNPs + vitamin C, or 15 μg/ml TiO2 treatment plates. Larvae were maintained on treatment plates throughout the remainder of their development, through pupation and eclosion. Three days post-eclosion, adult flies were
collected and the surface of the flies were sterilized with 70% ethanol such that any bacteria that remains associated with the fly are internal. The flies were then homogenized in PBS buffer at a concentration of twenty flies per milliliter buffer.

**Quantification of gut bacteria from *D. melanogaster***

*Micro-flora metabolic profile assay*

Fifty milliliters of LB broth was inoculated with 2 milliliters of fly homogenate and incubated with 200 rpm shaking at room temperature for 48 hours. After 48 hours, the LB broth inoculate was pelleted and washed 3X with 40 milliters of PBS buffer. After the third wash, the pellet was re-suspended in 12 milliters of PBS buffer. Resuspension was plated onto a Biolog EcoPlate™, 100 μl per well. After 5 days, absorbance of the product was measured at 590 nm using a microplate reader according to manufacturer's instruction.

**Assay of culturable microbial isolates**

For enumeration of cultivable flora, homogenates were serial diluted in PBS and plated on LB (1.5% agar) plates. Plates were incubated in aerobic and anaerobic conditions at 37°C for 48 hrs. Following incubation, colony morphologies were noted and the c.f.u./ml was determined.
**Assay of culture-independent bacteria**

For culture-independent quantification of bacterial population, quantitative PCR was performed using Syber Green technologies. Genomic DNA was isolated using UltraClean® Microbial DNA Isolation Kit and total 16s DNA was amplified using the universal primers Eub338F (5’-ACTCCTACGGAGGAGCAGCAG-3’) and Eub518R (5’-ATTACCGCGGTGCTGG-3’) (Fierer et al. 2005). The following phylum and class specific 16s primers were used to characterize the microbial community structure: Firmicutes LgcF (5’-GCAGTAGGGAATCTTTCCG-3’) and Eub518R, Actinobacteria Actino235F (5’-CGCGGCCTATCAGCTGG-3’) and Eub518R, Alphaproteobacteria Eub338F and Alf685R(5’-TCTACGRATTTCACCYCTAC-3’), Bacteriodes Cfb319F (5’-GTACTGACTGCAGC-3’) and Eub518R, and Gammaproteobacteria Gamma395F (5’-CMATGCCGCGTGTGTGAA-3’) and Gamma871R(5’-ACTCCCCAGGCGGTCDACTTA-3’) (Fierer et al 2005, Mühling et al 2008). Quantitative PCR reactions were run in a Cepheid SmartCycler® System. 16s rDNA copy numbers were determined using standards curves of TOPO-TA pCR2.1 vectors containing a single copy of the gene of interest.
Results

Micro-flora metabolic profile assay

The metabolic assay (Biolog) is a dedicated chemical triage of microbes’ ability to grow on thirty-one different carbon sources. The Biolog EcoPlate™ contains 31 carbon substrates (and a control well without a substrate) in three replicates. Growth is assayed by spectrophotometric measurement of the formation of violet formazan that occurs as respiring cells reduce a colorless tetrazolium dye which is included in each well along with the single carbon source. The utilization of different substrates by different groups of microorganisms varies. Therefore, the functional diversity of a microbial community can be assayed by observing variation of the pattern and intensity of Biolog plate color development, called the “metabolic fingerprint.” The community-level physiological profile is usually assessed for three key characteristics: color pattern development (similarity), rate of color change in each well, and richness of well response (diversity). This analysis does not include any rate data due to numerous issues with contamination as a result of opening and closing the Biolog plate for multiple readings at different time points.

Functional diversity can be examined from a variety of perspectives using Biolog data. The simplest aspect is substrate richness (S), which is the number of different substrates that are used by the microbial community. Richness analyses are based on the assumption of a relationship between S and n, the total number of individuals (Table
4.1) (Kennedy & Smith 1995). TiO$_2$ and negative control treatments had equal measurements of community richness. Nano-Ag fed flies had less than one-sixth the level of species richness of control and TiO$_2$ (Table 4.2).

Another component of species diversity indices is species evenness ($E_{var}$), which indicates the distribution of the individuals within species designations (Table 4.1) (Kennedy & Smith 1995). This value approaches the value of one as a single substrate dominates. AgNPs fed flies had the lowest evenness value of 1. Negative control and TiO$_2$ plates had the most even distribution of substrate utilization of the four treatments (Table 4.2).

The Shannon-Wiener Index (H) encompasses both substrate richness and substrate evenness (Zak et al 1994), where $p_i$ is the ratio of the activity on a particular substrate to the sum of activities on all substrates (Table 4.1). Control treatments had an H value nearly 5X higher than that of AgNP samples. Ag+vitamin C supplemented treatments had an H value equidistant between AgNPs alone and the control.

Overall similarity between samples can be compared using the average well color development (AWCD) for each plate (Garland and Mills 1991). The AWCD is the mean of the absorbance values for a whole plate. Control flies had the highest AWCD, and nano-silver fed flies had the lowest (Table 4.2). Neither Ag+vitamin C nor TiO$_2$ flies had significantly different AWCD values from the control. Flies fed AgNPs alone had a significantly lower (p<0.05) AWCD value compared to the control.
Assay of culturable microbial isolates

Homogenates of flies for all treatment types were plated and grown on LB media under both aerobic and anaerobic conditions. Under aerobic conditions there was a ten-fold colony reduction in nanosilver-fed flies: Aerobic: control = $2.5 \times 10^4$ cfu/ml, nanosilver = $8.85 \times 10^2$ cfu/ml; Anaerobic: control = $2.5 \times 10^6$ cfu/ml, nanosilver = $9.1 \times 10^4$ cfu/ml (Fig. 4.1). TiO$_2$ fed flies had equivalent colony growth $2.4 \times 10^4$ cfu/ml compared to controls and Ag+vitamin C flies had more colony growth $2.1 \times 10^3$ cfu/ml than nano-silver only fed flies (Fig. 4.1).

Using +/- screening, we have found one microbial species that is absent in nanosilver treated flies. There was a morphologically distinct large colony type present on negative control fly plates and absent on nano-silver fed fly culture plates. Nano-silver plates only had small colonies. Large colony and smaller colony bacteria were isolated and cultured with a range of AgNPs concentrations to test for differential nano-silver tolerance. Large colonies were less tolerant to AgNPs than small colonies (Fig. 4.2 a and b).

Assay of culture-independent bacteria

Phylotypic bacterial PCR primers to 16S rDNA were used to characterize non-culturable microbes. Multiple exposure trials were performed to determine the level of variation that occurs in the bacterial community composition. There was a 2-fold reduction in microbial abundance in nanosilver-fed flies in trial #1 and no appreciable
difference in trial #2 (Fig. 4.3 a and b). Levels of bacteria in TiO$_2$ and Ag+vit C fed flies was generally equivalent to the control in both trials.

To determine if nano-silver ingestion affects the ecological diversity of the fly gut microbiome, class specific 16S DNA primers were used to characterize the microbial community structure. Primers were designed for a number of different classes, but only two, the firmicutes and acidobacteria, have been investigated thus far. There was a two-fold decrease in acidobacteria and a 5-fold increase in firmicutes for nano-fed flies (Fig. 4.4).
Discussion and Conclusions

Taken together, the results of the metabolic profile, culturable bacteria, and culture-independent bacteria paint an interesting picture of the effect of nano-silver ingestion on the fly microbiome. Shannon-Wiener, evenness, and richness analyses of the microbial metabolic profile, or the “metabolome”, demonstrate a clear decrease in the diversity of substrates capable of being utilized by nano-silver flies. Further evidence of a change in the ecology of the gut microbial community in nano-silver fed flies is indicated by the disappearance of the large phenotype bacteria colonies. AWCD data indicate that there is not only less diversity, but perhaps a decrease in total number of bacteria. Cfu/ml counts of nano-silver fly cultures also show a decrease in the total number of both anaerobic and aerobic bacteria. However, culture independent analyses seem to tell a different story. The total amount of bacterial DNA is similar between nano-silver and negative control flies. This suggests that there is differential killing of gut bacteria by AgNPs and that the gaps in the microbial community created by the disappearance of the affected bacteria are filled in by the more nano-silver tolerant bacteria type or types.

These results have potentially far reaching implications. While the direct consequences of decreased microbiome ecological diversity on the health of the fly are yet unclear, there is increasingly abundant evidence of the importance of a healthy gut
microbiome to human well-being. The gut commensal microbial community is an emerging research target for understanding autoimmune disorders, obesity, type 1 diabetes, autism, multiple sclerosis, and schizophrenia (Parracho et al. 2005, Chervonsky 2010, Berer et al. 2011, Wen et al. 2008).

There is a complex relationship between commensal gut bacteria and the host immune system. Bacteria produce peptidoglycan among other small molecules, which typically trigger activation of the immune deficiency (IMD) pathway of the host immune system (De Gregorio et al 2002). However, the gut cell lining has a host mediated decreased sensitivity to the presence of commensal microorganisms. Communication between the microbiota and the host establishes and maintains immune homeostasis, enabling protective immune responses against pathogens while preventing adverse inflammatory responses to innocuous commensal bacteria. One possible explanation for the change in microbial community seen in nano-silver fed Drosophila is that the ingested nanoparticles irritate the cells of the fly’s digestive tract causing a robust activation of the IMD pathway and thus a disruption of the delicate bacteria/host relationship. Activation of the IMD pathway in the Drosophila gut epithelium has been shown to have an effect on the ecology of the microflora community and a significant impact on fly health. For example, overexpression of anti-microbial peptides caused activation of the IMD pathway and resulted in dominance of a typically minor pathogenic bacterium, G707 Gluconoacetobacter (Ryu et al. 2008).

It is worth noting that in our previous work we found that ingestion of nanosilver, but not nano-titanium dioxide, resulted in cuticle and melanization defects,
activation of oxidative stress pathways, reduced mating success, and reduced survivorship, all of which were reversible by dietary supplementation with antioxidants (Posgai et al. 2011). Anti-oxidant supplementation partially protected the gut microbial community from the deleterious effects of nanosilver, but it does not appear to have the restorative equivalence of antioxidants on fly life history traits. This result does little to clarify roles of oxidative stress and/or loss of gut microbes as mechanism/s of nanosilver toxicity.

To further elucidate the answer to the question of what mechanism is responsible for nanosilver toxicity, future research will use fecal transplants, antibiotics to mimic potential antimicrobial effects of nanosilver, and chemical based reproduction of potential nanosilver induced oxidative stress using H₂O₂. Drosophila larvae fed nanosilver will receive a dietary supplementation of feces from control line flies. If fecal transplants are successful at reversing nano-silver mediated toxicity effects on fly life history traits it would indicate a direct nano-silver toxicity mechanism via an impact on the gut microbial community. For the antibiotic and chemical oxidant studies, they can be compared to nano-silver to determine if either recapitulates the effects induced by AgNPs. If H₂O₂ fed flies show a similar response to nano-silver fed flies it would be evidence of oxidative stress as the primary mechanism of toxicity, and if antibiotics show a deleterious effect it would again point towards the microbial community as the important factor.

Drosophila represents a new and robust model for studying how nanoparticle exposure may impact the delicate, but important, balance that exists in the relationship
between a host and its commensal microbiota. Clearly, further study of the effects of nanoparticle ingestion will enhance our understanding of the mechanism/s of nanoparticle toxicity and the role gut microbial community ecology on human health.
References


Table 4.1. Microbial community functional diversity equations.

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<td>Richness</td>
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<tr>
<td>Shannon-Wiener Index (H)</td>
<td>( H' = - \sum_i (n_i / N) \times \log(n_i / N) )</td>
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<tr>
<td>Evenness (Evar)</td>
<td>( Evar = 1 - 2 / (\pi \times \arctan(\sum_i { \log(n_i) - \sum_j { \log(n_j) } }^2 / S)) )</td>
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\( n_i \) and \( n_j \) are the number of individuals in species \( i \) and \( j \) respectively, \( S \) is the total number of species, and \( N \) is total number of individuals.
Table 4.2. Measure of microbial community functional diversity.

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<th>Evenness (E\textsubscript{var})</th>
<th>Average Well Color Development (AWCD)</th>
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<td>Negative Control</td>
<td>1.615</td>
<td>1.077</td>
<td>0.795</td>
<td>0.11</td>
</tr>
<tr>
<td>Ag</td>
<td>0.351</td>
<td>0.179</td>
<td>1</td>
<td>0.02*</td>
</tr>
<tr>
<td>Ag + vitamin C</td>
<td>1.065</td>
<td>0.538</td>
<td>0.973</td>
<td>0.06</td>
</tr>
<tr>
<td>TiO\textsubscript{2}</td>
<td>1.479</td>
<td>1.077</td>
<td>0.788</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*significantly different from control (p<.05)
Figure 4.1. Quantification of culturable bacteria from negative control, AgNPs, AgNPs+vitamin C, and TiO$_2$ fed flies. Fly homogenate was cultured on LB agar plates under aerobic and anaerobic conditions. Total cfu/mL was calculated 48 hours after inoculation.

*Ag+vit C and TiO$_2$ treatments were not cultured under anaerobic conditions.*
Figure 4.2. Toxicity of AgNPs to small and large colonies of cultured aerobic bacteria of the fly microbiome. Differential toxicity of AgNPs to cultured isolates of (a) small colonies and (b) large colonies of bacteria collected from flies exposed to AgNPs.
Figure 4.3. Quantification of 16s DNA from *D. melanogaster*. Quantitative PCR analysis of microbial 16s DNA from two separate sets of exposure treatments (a) and (b).
Figure 4.4. Effect of Ag ingestion on acidobacteria and firmicutes populations of *D. melanogaster*. Quantitative PCR analysis using class specific 16s DNA primers for acidobacteria and firmicutes.
CHAPTER 5

DISCUSSION AND CONCLUSIONS

When this project was originally conceptualized the Drosophila model had not been used in any capacity to investigate the toxicity of nanoparticles. In fact, despite the storied history of the contributions of Drosophila to the advancement of scientific knowledge there were relatively few examples in the literature of the fly model being used to study toxicity of any type. Historically, the fly has been used in a limited capacity to study small molecule toxicity, drug toxicity, and in herbicides (David et al 1976, Duckworth et al 1994, David et al 1996). It was used fairly commonly in the 1970’s but seems to have lost favor shortly after. Recently, though it has seemed to regain popularity. This resurgence has been driven through a combination of opportunity and necessity. The relatively recent genome sequencing information has highlighted many new similarities between human and fly genetic genes providing an opportunity to exploit these relationships in the fly to better understand human gene function. Also, over the last half-decade or so global economic hardship has led to a general decrease in the availability of funding for basic scientific research. In the field of toxicology, this has created a need for more cost efficient in vivo alternatives to the
traditional mammalian models. The need for quick and inexpensive basic toxicity data is even more heightened in the relatively new discipline of nanotoxicology.

The field of nanotechnology is a booming industry and looks to continue to grow well into the foreseeable future. It has long been known that materials take on unique properties as they approach the nanoscale, but handling materials of such a small size is not easy to do. However, new advances in modern synthetic chemistry have allowed for more precise control of nanomaterial production and manipulation. This has created a recent flood of essentially novel particles, many of which have properties that hold tremendous potential for use in biomedicine, manufacturing, and electronics. New nanomaterials are being created and finding applications in consumer products at a faster rate than toxicologists can learn how these materials move within, interact with, and are transformed by environmental and biological systems.

Recognizing this shortcoming, we decided to begin developing the *Drosophila* model for the study of nanotoxicity. The first goal was to establish the fly as a model for studying nanoparticle inhalation. The primary target of potential nano-exposure is the respiratory system *via* inhalation of air-borne nanoparticles. The majority of *in vivo* nanoparticle toxicity researched has focused on the delivery of nanoparticles to the respiratory system (Warheit et al 2004, Oberdörster et al. 2005). Using mammals, there are basically two ways of delivering nanoparticles to the respiratory system, direct installation or aerosolization. Installation is not a practical option for the fly model, and while aerosolized exposure is the preferred nanoparticle delivery method, it typically involves large, complex, and expensive exposure chambers. Since we did not have
access to a traditional large aerosol exposure chamber, we developed a smaller, simpler, and less expensive chamber using a modified nebulizer. As proof of concept that the nebulizer was an effective method of delivering nanoparticles to the *Drosophila* respiratory system we had to prove three things: 1) that the nebulizer was capable of aerosolizing nanoparticles, 2) that the aerosolized nanoparticles were delivered to the respiratory system of the fly and, 3) that the flies were affected by exposure to the nanoparticles using AgNPs as a positive control. We were able to show that nanoparticles were aerosolized by the nebulizer by placing filter paper in the exposure chamber to capture FluoSpheres®, which are green fluorescent polystyrene NPs, and visualizing the NPs on the paper using fluorescent microscopy. Validation of NPs within the respiratory system of the fly was not as easy. The respiratory system of *Drosophila* differs in morphology from mammalian respiratory systems. Air enters the trachea of *D. melanogaster* through spiracles — pores located along the length of the body. Trachea branch into smaller and smaller diameters, called tracheoles, until they reach cell size diameters (~1 μm) and deliver O$_2$ to cells throughout the entire body. When NPs exposed flies were dissected, we could see fluorescent NPs within the fly tissue but we could not specifically distinguish the trachea. To overcome this, we obtained a tracheal-specific (*breathless*) GFP reporter strain of flies (kindly provided by Dr. Mark Krasnow at Stanford Univ.). We then used a red fluorescent CdSe/ZnS nanoparticle to contrast the GFP-labeled tracheal tissue in order to confirm NP delivery to the respiratory system. Finally, we showed that flies exposed to AgNPs had increased expression of heat shock protein 70 (Hsp70), a known physiological stress marker. Thus, we were the first
researchers to introduce NPs to the fly respiratory system and demonstrate a physiological response.

After validating the fly inhalation delivery method, we switched to a more holistic approach to study the toxicity of NPs using *Drosophila*. Feeding fly larvae AgNPs had a deleterious effect on a broad spectrum of life history traits. Flies fed nano-silver had decreased survivorship, extended length of development, lower fecundity, and a distinct pigment-lacking adult phenotype. To determine whether these effects were due to the specific physico-chemical properties of AgNPs or whether they were part of a generic “nano-sized” toxicity effect, the flies were also fed titanium oxide (TiO$_2$) nanoparticles. TiO$_2$ NPs had no significant effect on fly life history traits, even at doses 10x higher than the effective dose of AgNPs. This indicated that nano-silver has unique toxicity causing properties.

The primary cause of AgNPs toxicity is believed to be oxidative stress resulting from an increase in ROS production (Asharani et al 2009). This suggested the potential to reverse or protect flies from AgNPs toxicity through the use of antioxidants. Supplementation of AgNP-laced fly food with the antioxidant ascorbic acid (vitamin C) partially reversed all of the deleterious effects, including the physiological oxidative stress response markers. These results support the paradigm of nanosilver mediated oxidative stress as primary cause of AgNPs toxicity.

Antioxidants did not fully protect flies from nano-silver, suggesting another potential mechanism of toxicity other than oxidative stress may be responsible for AgNPs toxicity in *Drosophila*. It has not yet been examined in any model whether the
antimicrobial properties of nano-silver alter the commensal gut microbial community when ingested and, if so, how do antimicrobial effects contribute to its toxicity profile? Given the important role of gut microflora in basic animal function and the widespread use of nano-silver in consumer goods, these questions need to be addressed.

Through a combination of plate colony counts, metabolism profile comparisons, and qPCR 16s universal primer analyses we have shown that nanosilver ingestion changes the composition of the gut microbial community. Loss of certain species of gut microbes has been shown to lead to problems in reproduction and survivorship, some of the same issues which we find in our *Drosophila* life history studies, suggesting loss of microflora is a plausible component of nano-silver toxicity. Studying the details of how the relationships of the gut microbial community are changed and how these changes affect the whole fly will offer important insight into the mechanisms of nanosilver toxicity and the potential implications of increased NP exposure to human health.

**Future Directions**

Now that we have established the fly as a nanotoxicity model, it can begin to be used for the quick and inexpensive systematic toxicity assessment of a battery of nanoparticle types. For example, one of the more pressing areas of concern in the field of nanotoxicity is exposure to micro-plastics and nano-plastics. When plastic materials degrade they eventually break down into micrometer and nanometer sized particles. As greater amounts of plastic pollutants enter our waterways, there is concern about their potential toxicity to the environment and to human health. The *Drosophila* model could
be used to test the effects of micro- and nano-pollutants on life history, oxidative stress, etc.

One important area of information that remains unknown in regards to nanotoxicity in the fly is that of nanoparticle fate within organism. It is difficult to track and observe fluorescent nanoparticles within the fly due to the natural autofluorescence of the fly tissue. This problem could be resolved using inductively coupled plasma-mass spectrometry (ICP-MS). Fly organs can be dissected and separated from each other and comparisons of relative amounts NPs in each tissue could be measured using ICP-MS.

The numerous genetically modified strains of *D. melanogaster* also provide much potential for better understanding the mechanisms nanoparticle toxicity. For example, oxidative stress resistance is a hallmark of long lived strains of flies. It would be interesting to see if these flies are resistant to nano-silver. Also, there are flies that have genotypes for over expression or under expression of oxidative stress related genes, or strains of flies that are arsenic resistant. The responses of these mutant fly strains to nanoparticle exposure would provide vital nanotoxicity data.

Perhaps the most exciting area of future fly nano-research is studying how nanoparticle ingestion affects the fly microbiome and learning how these microbial community changes affect the health of the whole fly. The gut commensal microbial community is an emerging target in human disease research. Using qPCR 16s DNA analysis to continue to identify the specific changes in microbial species composition and abundance will be important for understanding which microbes are resistant to
nanoparticle exposure as well as which ones are most susceptible. Using germ free flies could help distinguish the direct toxic effects of nanoparticles from the indirect effects resulting from changes in the gut microbial community. In germ free flies fed nanoparticles, the indirect effects will be reduced or eliminated but the direct toxic effects will remain. The microbial community composition is also known to be important to mate selection in *Drosophila*. The mating preference of nano-silver fed flies may be affected due to changes in their microbiome.

These are just a few examples of how the vast pool of research tools available using *Drosophila*, in combination with the constantly expanding list of novel nanomaterials, provides a tremendous opportunity to study nanoparticle toxicity with the fly model system.
REFERENCES


