Characterization and Interactions of Nanoparticles in Biological Systems

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Amber Nagy

Graduate Program in Integrated Biomedical Science Program

The Ohio State University

2010

Dissertation Committee:
Professor W. James Waldman, Advisor
Professor Prabir K. Dutta
Professor Susheela Tridandapani
Professor Marshall V. Williams
Abstract

Nanoparticles are particles with at least 1 dimension less than 100 nm in size. Many consumer products already contain nanoparticles; however the risks and consequences of acute and chronic nanoparticle exposure have not yet been adequately evaluated. Additionally, nanoparticle manufacturing plants are becoming more prevalent around the world. As such, there is cause for concern regarding the effects of nanoparticle related occupational hazards and also incidental nanoparticle exposure to the general public.

This communication sought to further investigate nanoparticle/cell interactions, ensuing toxicity and cellular responses within biological systems. Three model nanoparticles were synthesized: quantum dots (QDs), modified carbon nanoparticles (CNPs) and a zeolite substrate containing silver nanoparticles.

QDs were chosen to model mechanisms of nanoparticle internalization and compartmentalization. It was found that QDs interact with scavenger receptors, and enter cells via a clathrin coated pit mediated pathway. The kinetics of QD internalization was established; QDs were found to associate with macrophage cell membranes within 2.5 minutes, and are confined to lysosomes 9 minutes after exposure. QDs were found to be approximately 9 nm in size and were found to aggregate when subjected to acidic conditions. Cadmium ions were found to leach from the core at low pH. Macrophages
exposed to quantities 20 times greater than needed for imaging were found to induce TNF-α secretion and cytotoxicity, via apoptosis.

To understand how the surface functional groups on nanoparticles drives inflammation and cytotoxicity, CNPs were modified with iron species, benzo(a)pyrene or ozone. Experiments utilizing primary human monocyte-derived macrophages revealed large variability in individual cell responses, ranging from increases in cytokines including TNF-α, to upregulation of complement factors. Carbon nanoparticles were added to cultures of murine alveolar macrophages and those modified with iron or B(a)P had little proinflammatory response. However, treating CNPs with O₃ immediately prior to exposing macrophages resulted in a significant decrease in TNF-α secretion that was found to be a result of changes in the oxidative state of modified CNP surfaces. Additionally, free radical content was sustained after ozonated CNPs were suspended in cell culture media, indicating that mechanisms other than oxidative stress may drive CNP mediated cell responses.

Finally, a novel antimicrobial zeolite support containing silver nanoparticles was created. These supports were found to have superior antimicrobial activity against *E. coli*. Zeolite micropatterning was not found to be a significant factor in bacterial killing. In addition, antibacterial activity was not found to be contact dependent. The upregulation of genes involved with metal transport, ATPase efflux pumps and multiple antibiotic resistance was revealed using gene microarrays. Increased antioxidant gene expression, including superoxide dismutase, glutaredoxin and thioredoxin was also noted, indicating that oxidative stress may be driving the antimicrobial activity of zeolite silver.
nanoparticle supports. Lastly, these supports were also found to be significantly cytotoxic to macrophages, and research is ongoing to determine if the mechanism of silver nanoparticle toxicity is similar to bacteria.

Physicochemical properties of nanoparticles, including charge and surface functional groups were found to play a role in nanoparticle-cell interactions. However, more definitive studies regarding specific pathways that are involved with nanoparticle internalization, inflammatory responses and toxicity are warranted before proper guidelines regarding nanoparticle exposure are established.
Dedication

This thesis is dedicated to my parents, who have supported all of my endeavors and encouraged me every step of the way.
Acknowledgments

First and foremost, I thank my advisor and mentor, Dr. W. James Waldman for allowing me complete freedom to pursue this project using my own ideas, and molding me into the scientist I am today. I am grateful to my mentor, Dr. Prabir Dutta, whose door was always open. His success, expertise and thoughtfulness have inspired me to set goals high and to never give up until I reach the top, and then to begin climbing another mountain. I am thankful for the scientific knowledge and personal advice I received from my committee members Dr. Marshall Williams and Dr. Susheela Tridandapani.

I am indebted to my labmate and friend, Dr. Mindy Dunn, who always had open ears and offered a helping hand. I am grateful to Dr. Adriana Estrada Bernal for her scientific expertise and patience. I also thank all of the chemistry graduate students including Dr. Brian Peebles, Dr. Bill Schumacher, Dr. Supriya Sabbani, Andrew Zane and Mike Severance, each of whom had instrumental contributions to this project. This work was completed using financial support provided by Ohio State University’s Alumni Grant for Graduate Research and Scholarship and NIOSH grant number.

I am thankful for the great conversations and technical support provided by Dr. Jim Van Brocklyn and Dr. Joanne Trgovcich. Ohio State’s Campus Microscopy and Imaging Facility staff, especially Dr. Sara Cole, also provided essential technical support. I would like to thank all of my friends and family members for their smiles and well
wishes; they kept me going during the hardest of times. Last, but surely not least, I am grateful for the support, friendship and encouragement from my partner and love, Cosby Lindquist.
Vita

June 1997 .................................................Elyria High School

2000 ..........................................................B.S. Biology, Youngstown State University

2003 ..........................................................M.S. Microbiology, Texas Tech University

2006 to present .........................................Graduate Research Associate, Department

of Integrated Biomedical Sciences, The

Ohio State University

Publications


Studies of Iron-Loaded Carbon Particles.” Environmental Science and

Technology. 2010, 44 (17), 6887-6892.


“Synthesis of Micropatterned Silver-Zeolite Films and Its Application as an

Antimicrobial Substrate.” Microporous and Mesoporous Materials. 2010, 135,

131–136.

Schumacher, W., Nagy, A., Waldman, W.J., and Dutta, P.K. “Direct Synthesis of

Aqueous CdSe/ZnS-based Quantum Dots using Microwave Irradiation.” Journal


Fields of Study

Major Field: Integrated Biomedical Science Program
Table of Contents

Abstract .............................................................................................................................................. ii

Dedication ........................................................................................................................................ v

Acknowledgments ............................................................................................................................. vi

Vita .................................................................................................................................................. viii

List of Tables .................................................................................................................................... xiv

List of Figures ................................................................................................................................... xv

Chapter 1: Overview .......................................................................................................................... 1

Rationale ......................................................................................................................................... 1

Hypothesis and Approach ............................................................................................................... 2

Chapter 2: Literature Review .......................................................................................................... 7

Quantum Dots ................................................................................................................................. 8

Synthesis, Properties and Applications of Quantum Dots ............................................................ 9

Mechanisms of Quantum Dot Cellular Internalization and Compartmentalization .................. 20

Quantum Dot Induced Inflammation and Toxicity ....................................................................... 23

Carbon Nanoparticles .................................................................................................................... 26

Carbon Nanoparticle Synthesis, Properties and Applications ..................................................... 27
<table>
<thead>
<tr>
<th>Internalization and Intracellular Localization of QDs</th>
<th>81</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxicity and Proinflammatory Response</td>
<td>83</td>
</tr>
<tr>
<td>Discussion</td>
<td>85</td>
</tr>
<tr>
<td>Chapter 4: Murine Alveolar Macrophage Responses to Modified Carbon Nanoparticle Exposure</td>
<td>99</td>
</tr>
<tr>
<td>Introduction</td>
<td>99</td>
</tr>
<tr>
<td>Experimental Methods</td>
<td>102</td>
</tr>
<tr>
<td>Carbon Nanoparticle Modification:</td>
<td>102</td>
</tr>
<tr>
<td>Carbon Nanoparticle Chemical Characterization</td>
<td>104</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>106</td>
</tr>
<tr>
<td>Luminol Assay</td>
<td>107</td>
</tr>
<tr>
<td>Cytotoxicity and ELISAs experiments</td>
<td>108</td>
</tr>
<tr>
<td>Gene Expression Microarrays</td>
<td>110</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>112</td>
</tr>
<tr>
<td>Results</td>
<td>113</td>
</tr>
<tr>
<td>Chemical Characterization of Carbon Black Nanoparticles</td>
<td>113</td>
</tr>
<tr>
<td>Human Macrophage Responses to Modified Carbon Nanoparticles</td>
<td>115</td>
</tr>
<tr>
<td>Murine Alveolar Macrophage Responses to Modified Carbon Nanoparticles</td>
<td>119</td>
</tr>
<tr>
<td>Discussion</td>
<td>120</td>
</tr>
</tbody>
</table>
Chapter 5: Antimicrobial Mechanism and Toxicity of Silver Zeolite Platforms........ 151

Introduction .................................................................................................................................................. 151

Experimental Methods ............................................................................................................................... 154

Materials ...................................................................................................................................................... 154

Submicron Synthesis of Zeolite AgNPs ........................................................................................................ 155

Zeolite AgNP Platform Chemical Characterization ................................................................................. 157

Biological Characterization ....................................................................................................................... 158

Results ......................................................................................................................................................... 163

Physicochemical Characterization of Zeolite AgNPs ............................................................................. 163

Antimicrobial Activity and Mechanism of Zeolite AgNPs .................................................................. 164

Discussion .................................................................................................................................................... 168

Chapter 6: Concluding Statements and Future Directions................................................................. 195

Literature Cited ............................................................................................................................................. 202
List of Tables

Table 2.1 Carbon black manufacturing processes. ................................................................. 66

Table 4.1 Quantitative deconvolution of spectra for Flammruss 101. Changes after ozonolysis and exposure to ambient conditions................................................................. 132

Table 5.1 Comparison of upregulated E. coli genes: Zeolite supports versus negative controls............................................................ 188

Table 5.2 Comparison of upregulated E. coli genes: Ag$^+$ versus negative controls...... 189

Table 5.3 Comparison of upregulated E. coli genes: Zeolite + Ag$^0$ nanoparticles versus negative controls................................................................. 190

Table 5.4 Comparison of upregulated E. coli genes: Zeolite versus Zeolite + Ag$^0$ nanoparticles. ................................................................. 191

Table 5.5 Comparison of upregulated E. coli genes: Ag$^+$ versus Zeolite + Ag$^0$ nanoparticles. ................................................................. 192
List of Figures

Figure 1.1 Comparisons of objects that are millimeters to angstroms in size. .................. 5
Figure 1.2 Fate of nanoparticles in the body........................................................................... 6
Figure 2.1 Schematic of a quantum dot. .................................................................................. 64
Figure 2.2 Nanoparticle intracellular compartmentalization................................................... 65
Figure 2.3 Cellular interactions of nanoparticles..................................................................... 67
Figure 2.4 Silver resistance genes, transcripts and protein products. ..................................... 68
Figure 3.1 Chemical characterization of QDs........................................................................... 91
Figure 3.2 QDs associate with scavenger receptors................................................................. 92
Figure 3.3 Clathrin-coated pits serve as one mechanism of QD internalization. ................. 93
Figure 3.4 QDs are quickly compartmentalized within lysosomes. ........................................ 94
Figure 3.5 QD induced cytotoxicity in murine alveolar macrophages. ................................. 95
Figure 3.6 QDs induce apoptosis in macrophages within 24 hours of exposure. ............... 96
Figure 3.7 QDs stimulate TNF-α secretion from macrophages after 24 hours of exposure. .......................... 97
Figure 3.8 Model of QD/macrophage interactions. ................................................................. 98
Figure 4.1 X-ray photoelectron spectrum of Flammruss 101 impregnated with 10% iron (II) as iron (II) acetate, in the Fe 2p region................................................................. 130
Figure 4.2 Quantitative deconvolution of spectra for Flammruss 101, showing changes that occurred on ozonolysis and exposure to ambient conditions................................. 131
Figure 4.3 The changes in the FTIR spectrum of ozonated Flammruss 101, purged of gas content and then left to sit overnight in ambient conditions. ........................................ 133

Figure 4.4 EPR signal from Flammruss. ............................................................................. 134

Figure 4.5 Stability of free radicals on Flammruss over time. ........................................ 135

Figure 4.6 Stability of Flammruss + ozone over time. .................................................... 136

Figure 4.7 Luminol assay of reactive oxygen species generated from human monocyte-derived macrophages after exposure to iron containing Flammruss. ............................ 137

Figure 4.8 Luminol assay of reactive oxygen species generated from human monocyte-derived macrophages after exposure to iron containing Flammruss exposed to ascorbate. ........................................ 138

Figure 4.9 Luminol assay of reactive oxygen species generated from human monocyte-derived macrophages. ...................................................................................... 139

Figure 4.10 Luminol assay of reactive oxygen species generated from human monocyte-derived macrophages. ...................................................................................... 140

Figure 4.11 Average TNF-α production from monocyte-derived after 24 hours exposure. ......................................................................................................................... 141

Figure 4.12 Monocyte-derived macrophages collected from 2 individual donors were incubated with several modified cNPs for 6 hours. ................................................. 142

Figure 4.13 Temporal and treatment-dependent changes in TNF-α secretion in human monocyte derived macrophages................................................................. 143

Figure 4.14 Gene expression arrays of human-monocyte derived macrophages after treatment with modified carbon nanoparticles......................................................... 144

Figure 4.15 Expression of MARCO on murine alveolar macrophages. ......................... 145

Figure 4.16 Murine alveolar macrophages exhibit decreased MARCO expression after incubation with carbon nanoparticles irrespective of LPS stimulation. ................. 146

Figure 4.17 Murine alveolar macrophages internalize modified Flammruss and treatment effects aggregation of particles. ................................................................. 147

Figure 4.18 Murine macrophage TNF-α secretion. ............................................................. 148
Figure 4.19 Murine TNF-α does not adsorb to modified carbon nanoparticles. .......... 149

Figure 4.20 Cytotoxicity of modified carbon nanoparticles in murine alveolar macrophages. .................................................................................................................................................. 150

Figure 5.1 Cartoon of zeolite structure and morphology........................................... 175

Figure 5.2 Schematic of zeolite AgNP fabrication.......................................................... 176

Figure 5.3 Representation *E. coli* exposure to zeolite supports and subsequent assessment of growth and viability .................................................................................................................................................. 177

Figure 5.4 Scanning electron micrograph of zeolite supports............................................. 178

Figure 5.5 Scanning electron micrographs of molds used to create zeolite + AgNP supports.................................................................................................................................................. 179

Figure 5.6 Viability of *E. coli* after exposure to zeolite supports................................. 180

Figure 5.7 Viability of *E. coli* after exposure to zeolite supports that were separated from bacteria using transwell plates.................................................................................................................................................. 181

Figure 5.8 Viability of *E. coli* after exposure to supernatants collected from zeolite supports that were soaked in LB media for 3 hours.................................................................................................................................................. 182

Figure 5.9 Efficacy of zeolite AgNP supports................................................................. 183

Figure 5.10 Dose-dependent and temporal degradation of *E. coli* RNA exposed to Ag⁺. .................................................................................................................................................. 184

Figure 5.11 *E. coli* RNA integrity of samples tested in gene expression array analyses. .................................................................................................................................................. 185

Figure 5.12 Quality assessment of *E. coli* gene expression arrays............................... 186

Figure 5.13 Viability of *E. coli* after exposure to Ag⁺ or zeolite supports....................... 187

Figure 5.14 Murine macrophage viability is significantly reduced in a dose-dependent manner.................................................................................................................................................. 193

Figure 5.15 Antimicrobial mechanism of zeolite substrates containing AgNPs.......... 194

Figure 6.1 Scavenger receptor ligand Poly I does not prohibit positive QDs from associating with cells .......................................................... 200
Figure 6.2 Large doses of positively-charged QDs induces apoptosis in less than 2 hours.
Chapter 1: Overview

Rationale

The past several decades have ushered in an era where nanotechnology is common in every facet of life. Nanoparticles are used in many manufacturing endeavors including the production of electronics, automobiles, paints and coatings, appliances, cosmetics and fabrics. In addition, nanoparticles are on the cutting edge of biomedical research, and are used for therapeutic delivery, cellular imaging, tracking, and as biological sensors, among other applications.

What are nanoparticles? The excepted definition is that nanoparticles are particles with a mean diameter less than 100 nm [1, 2], see Figure 1.1. However, there is movement to update this definition to specify that nanoparticles less than 30 nm have unique intrinsic properties, including changes in morphology and electronic and atomic structure, leading to increased surface reactivity [3] compared to bulk samples. Studies had been conducted that investigate the dangers of nanoparticles exposure [4-13], however this research and the discipline of nanotoxicology are still in their infancy. In addition, the manufacturing of nanoparticles in large quantities often precedes occupational exposure risks and assessments. There are data that confirm the ability of nanoparticles to translocate within the body, and their fate can have detrimental effects on human health ([14], Figure 1.2), although this factor can also be exploited to use
nanoparticles for disease treatment. Overall, there is a lack of research that investigates the correlation between the physico-chemical components of nanoparticles and their biological effects.

Hypothesis and Approach

To further define these relationships, the research outlined here was conducted using three different types of nanoparticles as models to identify and clarify nanoparticle interactions during in vitro cellular exposure. The three models that have been selected for this research are quantum dots (QDs), carbon-based nanoparticles (CNPs) and silver nanoparticles (AgNPs). Each model is very different in chemical composition and each has unique applications. All are nanoparticles that are becoming increasingly prevalent in everyday life, and the likelihood of exposure is growing. The purpose for this research is to test the following underlying hypothesis:

**The physical and chemical attributes of nanoparticles affect their interaction and toxicity within relevant biological systems**

To test this hypothesis, the following approach was taken:

*Identify cellular responses resulting from exposure to different model nanoparticles.*

**Model Nanoparticle 1: Quantum Dots**

Murine alveolar macrophages (MH-S) cells were incubated with QDs that were synthesized using an efficient, inexpensive method and chemically characterized using transmission electron microscopy and dynamic light scattering to determine size. XPS
was used to determine the surface elemental composition while UV spectroscopy and photoluminescence were used to determine the absorbance and emissions spectra, respectively. To assess the functionality and toxicity of these QDs, monolayers of MH-S cells were plated and subjected to increasing concentrations of QDs. Confocal microscopy was used to establish internalization and intracellular compartmentalization while flow cytometry and LDH release were used to quantify toxicity and mechanism of cell death.

Model Nanoparticle 2: Carbon-based Nanoparticles

Air pollution is a threat in every metropolitan city, and has been associated with reduced quality of living and shorter lifespan. Because of the complex nature of air pollution particulate matter (PM), we chose to model air pollution by modifying CNPs with iron (Fe$^{2+}$ and Fe$^{3+}$) and benzo(a)pyrene (B(a)P), a polycyclic aromatic hydrocarbon and expose the particles to primary human monocyte derived macrophages. Since individual responses to air pollution are quite variable, we also exposed select modified CNPs to a murine alveolar macrophage cell line (MH-S). A common manufactured CNP was chosen: Flammruss 101 (Lamp Black), which is ~90 nm in diameter. Human macrophage and murine alveolar macrophages cultures were subjected to a panel of modified CNPs for various time intervals. Cells were allowed to incubate with the CNPs before supernatants were harvested and assayed for cytotoxicity in the form of trypan blue staining and lactate dehydrogenase release and for the proinflammatory cytokine, tumor necrosis factor- alpha (TNF-α), using enzyme-linked immunosorbent assays (ELISAs). Human macrophages were also analyzed for changes in proinflammatory
markers using gene expression array analyses. In addition, CNPs underwent chemical characterization using electron paramagnetic resonance (EPR) and infra-red spectrometry (IR) and X-ray photoelectron spectroscopy (XPS) to identify free radical content and conduct surface elemental analyses, respectively.

**Model Nanoparticle 3: Immobilized Silver Nanoparticles**

We established a procedure to immobilize silver nanoparticles (AgNPs) onto a zeolite platform. In addition, we were able to further increase the surface area of this nanobiomaterial by micropatterning its surface. These AgNP platforms were characterized using scanning electron microscopy to confirm patterning and addition of AgNPs onto the zeolite template. Because silver is known to have antimicrobial properties, we tested the antibacterial efficacy of our nanobiomaterial by determining the viability of *Escherichia coli* after incubation with these AgNPs and found that they demonstrate potent bactericidal action in a very short time period. The mechanism of antibacterial action was identified using gene expression microarrays. The toxicity of AgNPs to MH-S cells was also investigated using flow cytometry.

Collectively, this work assesses the effects of nanoparticle and cellular interactions and not only provides insight into consequences of nanoparticle exposure, but also demonstrates that nanoparticles, despite their size, have complex relationships in biological settings. These relationships include cell membrane engagement, receptor association and endocytosis as well as intracellular compartmentalization and the ensuing cellular responses which include, but are not limited to toxicity and inflammation.
Figure 1.1 Comparisons of objects that are millimeters to angstroms in size.
Figure 1.2 Fate of nanoparticles in the body.

(a) Phagocytosis in lung resulting in reactive oxygen species
(b) Translocation to the brain via nasal cells and trachea-bronchial epithelia.
(c) Translocation and evasion of immune cells in the circulatory system; translocation to heart, liver, kidney and digestive tract
(d) Overall systemic disruption, cardiovascular, lung and liver disease. Adapted from [14].
Chapter 2: Literature Review

Nanoparticle Background

Nanoparticles are particles with at least one dimension less than 100 nm. Recently, much attention has been given to these particles because of their unique, interesting properties. As the diameter of nanoparticles decrease, their surface area increases dramatically. This property alone is particularly attractive and has stimulated biomedical research in areas such as drug delivery, imaging, disease modeling, cell tracking and antimicrobial agents. The primary advantage of increasing surface area with decreasing size is that one can theoretically prolong circulation by escaping immune cell detection and deliver therapeutics to a specific site by homing nanoparticles, thus effectively concentrating a drug to a target of interest, i.e. tumors. This principle applies not only to therapeutic delivery, but also for nanoparticles such as quantum dots and others. Nanoparticles have been proven to be effective chemotherapeutics, and they are getting incorporated into many other components of biomedical sciences. There are many types of nanoparticles including dendrimers, liposomes, micelles, nanogels and as well inorganic nanoparticles. Dendrimers are highly branched molecules that have the ability of engaging many targets simultaneously. They can be designed to carry several drugs of varying hydrophobicities and range in their degree of complexity. Liposomes are comprised of two phospholipid bilayers that can be constructed to be carriers of either
hydrophobic or hydrophilic materials. Micelles are made of one phospholipid bilayers that can be synthesized to transport both types of materials as well. Nanogels are typically composites of materials such as silica or chitosans and also can be created to carry a variety of materials. All of the aforementioned nanoparticles are the subject of intense research. However, the focus here is on three different manufactured nanoparticles and their interactions within biological systems.

In this section, the current state of research regarding synthesis, mechanism of action, cellular interactions and cytotoxicity of quantum dots, silver nanoparticles and carbon nanoparticles will be reviewed.

Quantum Dots

Quantum dots (QDs) are fluorescent semiconductor nanocrystals that have unique properties which can be exploited making them superior to traditional organic fluorophores. Because they are resistant to photobleaching, size tunable and have the ability to be multiplexed, they make excellent candidates for use in both clinical and basic sciences as tools for bioimaging, drug delivery and cell tracking. Although QDs have promise for use in the medical field, they are still costly to purchase. As such, many approaches have been investigated to synthesize QDs. For the purpose of brevity, individual types of semiconductor QDs will not be reviewed, but rather examples of successful QD use in the field of nanomedicine will be investigated. The principle behind QD function and mechanisms of synthesis will be discussed. Then, applications and
current literature focusing on the use of QDs in disease prevention, diagnosis and therapy will be outlined. Finally, the mechanism of QD uptake and toxicity will be reviewed.

Synthesis, Properties and Applications of Quantum Dots

Russian scientists in the early 1980’s were among the first to describe the size-dependent absorbance spectra of semiconductor nanocrystals [15, 16]. Several years later, the effective mass model was proposed by Brus [17] to corellate semiconductor energy band gaps with size. When semiconductors are photo-excited, a hole, or positive charge, is left in the valence band and the electron is raised to the conductance band. This electron/hole pair is collectively termed an exciton. For nanocrystal semiconductors, the minimum amount of energy that is needed for an electron to be raised from the valence band to the conduction band must be greater than the energy gap for the bulk material. Since a nanocrystal exciton resembles a hydrogen atom, we can apply electrostatic binding principles to understanding electron excitement and their transition between energy levels.

QDs are unique from their bulk counterparts because they are small enough that the distance between the electron and hole approaches that of the material’s exciton Bohr radius. In nanocrystal semiconductors, the size of the radius of the semiconductor’s core is less than the Bohr radius, and is finite and discrete, a property known as quantum confinement. In bulk, a semiconductor’s Bohr radius is continuous. Thus, researchers can take advantage of these properties, and by changing the size of the core, they also change
the size of the band gap, and, as a result, the emission wavelength. Since the energy of the bandgap is always larger for QDs compared to bulk, the energy that the electron loses to reemerge in the valence band has higher energy and a shorter radiation wavelength. The phenomenon is aptly termed “blue shift.” The emission wavelength is longer than the absorption wavelength of the lowest exciton energy peak, and energy difference between these peaks is known as Stoke’s shift.

In addition to having emission properties that are size tuneable, the absorption properties of QDs are also discrete. QDs have a series of absorption peaks that correspond to the energy transitions between discrete exciton energy levels. So, the absorption of light will not occur at wavelengths longer than the first exciton peak. This feature can be exploited in that QDs that emit at different wavelengths can be excited using the same wavelength, provided that the wavelength used has higher energy than that of the first exciton peak. The intensity of the photons that are emitted when QDs are stimulated is determined by the percent of absorbed photons that result in emitted photons, and is termed quantum yield. Quantum yield is controlled by the surface chemistry of the semiconductors and can often be improved by adding shells to the QD core. These shells consist of another semiconductor material provided that it has a wider band gap than the semiconductor core. Finally, capping agents such as polyethylene glycol (PEG) and mercaptoapropanoic acid (MPA) are commonly added to the QDs. These capping agents serve dual function: solubilize QDs in aqueous solutions and prevent leaching of toxic core elements such as cadmium.
As previously discussed, core composition and size control the optical properties of QDs. Modifications such as surfactants, linkers and capping agents encase the core and are used to prevent ion leakage, improve dispersion and, in some cases, improve the quantum yield of the material, a schematic of a QD is shown in Figure 2.1. Post-synthesis, QDs are commonly characterized via X-ray powder diffraction, transmission electron microscopy, zeta potential, dynamic light scattering, and X-ray photoelectron spectroscopy to determine the materials crystallinity, charge, size, and surface elemental composition, respectively. This section will first summarize the synthesis of QDs using aqueous or organic solvents, followed by a discussion on the technology of core capping.

Aqueous QDs are attractive because they have great potential in labeling proteins, cells, peptides and therapeutics due to their biocompatibility. As such, much time and effort has been spent by researchers to develop superior, non-toxic aqueous QDs. Initially, aqueous QDs were prepared in the presence of phosphate [18] and thiol stabilizers [19, 20] using wet chemical methods. Many others have since created aqueous QDs using thiol stabilizers, which allows for synthetic control. Although these QDs were found to be stable and soluble, their photoemissions were not ideal. It was found that inorganic passivation of the core can overcome this problem. Zeng et al. [21] achieved aqueous Type II CdTe QDs using successive layer by layer ion absorption/reaction methods, whose quantum yields were effectively improved by 5x when a CdS shell was incorporated. While it is noted that aqueous QDs do not approach the same degree of crystallinity as organic QDs because of the separation of nucleation and growth that occurs during organic synthesis, aqueous preparation results in reproducible QDs that can
be prepared in large, stable batches. The freedom to control surface functional groups which is granted by using thiols as stabilizers drives their solubility and biocompatibility and also has great promise for the field of nanomedicine.

Initial efforts using organic solvents with ionic precursors resulted in semiconductors with surface defects, which were also unstable and prone to aggregation. However, colloidal semiconductor nanocrystals prepared with atomic precursors in organic solvents using wet chemical synthesis were found to be defect-free and stable, in addition to maintaining mono-dispersion in aqueous solutions. Murray et al. [22] revolutionized QD synthesis by controlling nucleation via injection of metallic precursors into a hot organic solvent (“hot injection method”). This technique effectively separated nucleation and core growth due to the quick cooling of the mixture after the injection. The use of Cd(CH$_3$)$_2$ and the surfactants TOP (tri-n-octylphosphine) and TOPO (tri-n-octylphosphine oxide) during QD synthesis was a contributing factor to this approach. Modern QD synthesis employs the use of CdO or Cd salts as a precursor, as they were found to be more stable and less toxic [23]. Current investigation is ongoing to better understand the chemical reactions that occur during hot injection QD synthesis. In addition to the aforementioned synthesis procedure, there is also procedure in which nucleation and growth are concomitant (“heating-up method”) and initial growth can be supplemented by a second injection, in a process called Ostwald ripening [24], where larger crystals are formed at the expense of small crystals. The heating up method is better suited for large scale production of QDs and therefore is the subject of extensive research. Both methods have been substantially modified with regard to precursor
solvent, including the replacement of TOPO with more inexpensive, environmentally friendly solvents, although organic QD dispersion remains to be problematic.

Core-shell systems have been developed to increase quantum yield and stability. Most core-shell synthesis protocols are two steps and have the advantage of removing unreacted precursor material. The size of the shell is controlled by slowly adding shell precursors at a temperature lower than that by which the core was nucleated. Shell thickness can be controlled using the successive ion layer adsorption and reaction methods, where cationic and anionic precursors are added alternately and have been shown to increase photoluminescence [25]. Much focus has been given to the development of capping agents to improve QD stability and dispersion. Silica [26-28] and other polymeric [29-31] modifications and many other approaches have been successful at increasing QD biocompatibility. While progress has been made in QD production, new advancements in core/shell and capping systems and the transition from cadmium chalcogenides to doped QDs are crucial to develop high quality, efficient and safe QDs that are biocompatible.

The era of personalized medicine has emerged. Nanotechnology in the medical field has significantly impacted the notion of personalized medicine. Individual treatment for genetic disorders, cancer and neurological diseases is ongoing and the use of nanotechnology is a driving force. The use of QDs in basic science is already underway, and research is progressing toward their use from bench to bedside. While the unique properties of QDs have great potential for applications in vitro, their long term effects and toxicity are still being questioned, preventing their use in human trials. The
following sections will outline the progression of QD related research in the field of nanomedicine regarding the following aspects of medicine: disease prevention, diagnostics and therapy. In addition, cellular toxicity mechanisms will be addressed.

The use of nanoparticles in disease prevention is an active area of research. Such examples include the use of anti-inflammatory, anti-proliferative flavonoid compounds, which serve to reduce risk of cardiovascular disease and brain disorders such as Parkinson’s and Alzheimer’s disease. Recently, flavonoids have been incorporated into liposomes or polymeric nanoparticles to initiate better delivery, and recently reviewed [32]. Nanoparticle carriers doped with metal chelators, used in the treatment and prevention of neurodegenerative Alzheimer’s disease, show great promise in preventing and reversing the progression of amyloid Beta tangle formation [33]. Nanoparticle based delivery systems have also shown great promise in HIV prevention [34]. Current research using nanoparticles in vaccine development, delivery and efficacy also shows great promise in the realm of disease prevention [35-37].

Although there is good evidence that nanotechnology will positively impact preventative medicine, there are few studies being conducted using QDs for disease prevention. One study sought to prevent blood brain barrier breakdown by silencing matrix metalloproteinase-9 (MMP-9) using siRNA conjugated to quantum dots. In this study, Bonoiu et al. [38] hypothesized that reduction in MMP-9 would protect the brain from invading infected and inflammatory cells and prevent neurodegenerative disease exacerbation by reducing endothelial cell permeability. Using QDs as an efficient
nanocarrier for siRNA, MMP-9 was inhibited by 80%, and the study confirmed that QDs can play an integral role in the delivery of siRNA.

The ability of QDs to be multiplexed allows for them to be used to detect several molecules simultaneously. These properties were exploited and used to quantify cytokines at physiological levels (pM), which can be used to sensitively monitor cytokine production [39], correlate levels with disease conditions and potentially prevent disease progression, and possibly predict cancer metastasis. There have been others [40, 41] who have successfully used microarray and multiplexing technology to measure cancer biomarkers and research is ongoing in this field to develop multiplexing methods that can detect even lower levels of molecules in experimental samples.

There are many studies that are investigating the use of QDs in disease diagnostics. Because of their unique photoemitting properties, QDs invite researchers to utilize them to visualize protein expression (or lack thereof), track cells and image anything from individual molecules, to entire cells, tumors and even organs. QDs are also used as biosensors, which is often used to measure and validate intracellular processes in response to diseases or disease therapy. As stated above, the versatility and optical properties allow for detection of biomarkers in very low quantities. Microfluidic biochips have been designed into a platform that used QDs to detect cancer biomarkers at even femtomolar levels, which is 4 orders of magnitude more sensitive than using organic dyes [42]. Other “lab on a chip” approaches have been successful as well, including the use of QDs for the simultaneous detection and interaction of two proteins using fluorescent emission and light scattering as quantitative output measures [43]. In another example of
multiplexing, researchers have used microtubules that were functionalized with TNF-α and IL-2 antibodies to capture cytokines in experimental samples and detected them using QDs conjugated to antibodies against these molecules [44].

In pioneering work by Xu, et al. [45], genotyping of single nucleotide polymorphisms (SNPS) was demonstrated by conjugating QDs to microspheres along with allele specific nucleotides. Amplicons of genomic DNA were created by multiplex PCR and then hybridized to the microsphere-QDs containing SNPs before analysis via flow cytometry where each SNP can be identifying through its unique fluorescence. This technology can be used to identify types of mutations that are responsible for cancer development and be correlated with cancer progression and outcome.

In addition to their usefulness for detecting biomarkers related to cancer, QDs have the propensity to be used for bacterial detection at titers > $10^2$ CFU/mL [46] in a variety of environments including juice, water and urine. QDs have also been used to detect and identify pathogenic bacteria and viruses as well. *Bacillus anthracis* and *Bacillus cereus* were effectively bound when QDs were conjugated to synthetic peptides from gamma-phage lysin protein [47]. Detection of viruses such as hepatitis B [48], avian influenza virus [49] and respiratory syncytial virus has been successful [50]. QDs as biosensors for molecules such as biothiols [51], which is linked to diseases such as Alzheimer’s and Parkinson’s as well as liver and cardiovascular disease, have also been successfully created and used in vitro using biological samples.

Imaging is one of the areas that has benefitted the most from the development and characterization of QDs. There are many problems that have been resolved by using QDs
as fluorescent tags. For one, it allows for imaging of thicker tissue with the introduction of QDs emitting in the near IR spectra. In addition, photobleaching is far reduced compared to traditional organic dyes, allowing for longer laser exposure time. As mentioned above, the ability of QDs to be multiplexed (several QDs excited with one laser wavelength) and their discrete emission spectrum significantly reduces bleedover. Many applications utilizing QDs exist and continue to evolve over time. Bacterial strains have been identified via extracellular QD labeling. In particular, Gram + bacteria were identified using wheat germ agglutinin. Pathogenic bacteria were identifying by exploiting their host dependency of iron, which is harvested through transferrin [52]. QD labeled plasmid DNA for transfection have been achieved [53] as was detecting specific genetic sequences using fluorescent in situ hybridization of DNA [54] and mRNA [55] using QD based tracking. In fact, QDs have been used to track single proteins [56] including motor proteins (kinesins) and the energetics of intracellular cargo transport [57]. Cancerous cell lines were among the first live cells that were able to be successfully imaged [58, 59], and have been used to visualize receptor interactions, specifically erb2/HER receptor mediated signal transduction [60] and neuronal glycine receptors [61]. Processes such as apoptosis has been detected using QDs mediated Annexin V labeling [62]. QDs have been used to track various stages of development; one example includes an early study by Dubertret et al. [63], where QDs were coated with phospholipid micelles and then injected into Xenopus embryos. The QDs were tracked into the tadpole stage of development and were not found to be toxic or defective. Zebrafish embryos have also been imaged using QDs and that axon tracts and vasculature may develop
under the control of the same signals in some body parts but under the control of different guidance in other body parts [64].

Several groups have successfully imaged the vasculature. In 2003, Larson et al. [65] injected mice with QDs and was able to see vasculature through skin several hundred millimeters thick using multiphoton microscopy. More recently, chick vasculature was imaged using QDs emitting at 655 nm, a wavelength that was able to distinguish between tissue autofluorescence and QD emission [66]. Artherosclerosis using a mouse model was visualized and cellular interactions were observed using multiplexed QDs, providing insight into leukocyte recruitment and plaque maturation [67]. Others have built upon the in vivo use of QDs by imaging the lymphatic system [68-70]. Solid tumors have been imaged as well by conjugating QDs to tumor specific peptides [71, 72]; however, it had been found that the QD capping agent can interfere with QD localization, causing them to be internalized by circulating phagocytes, preventing their arrival at the appropriate target [73]. Bone marrow cells have been labeled with QDs and efficiently tracked to solid tumors allowing the investigators to better understand tumor vasculature and the tumor microenvironment [74]. Although use of QDs in biological and clinical imaging is a relatively new practice, studies indicate that they show great promise for use in disease diagnostics and detection. A great deal of research still needs to be conducted in order better understand risks and consequences associated with long term exposure and degradation products of QDs in humans.

Much can be learned from tracking QDs in both in vitro and in vivo systems. Technology has allowed researchers to conjugate small molecules, inhibitors,
therapeutics, proteins, nucleic acids, peptides and much more to QDs. As such, we can now track the course of a molecule from its point of inception all the way to its target. In addition, we can look at specific cell to cell interactions and we can even track individual particles using QDs. Tada et al. elegantly showed that they could track QDs to breast cancer cells using monoclonal antibodies toward HER2 receptors in a mouse model [75], providing proof of principle that indicates that QDs can be engineered to not only target specific cells, but also deliver cargo to intracellular compartments with good precision. Another group exploited the overexpression of glucose regulated protein 78 (GRP78) in cancer cells by conjugating the GRP78 peptide ligand Pep42 to QDs and unraveling their interaction and clathrin mediated internalization mechanism in a xenograph mouse model [76]. This study also provided evidence that QDs conjugated to cell specific ligands can be used to deliver therapies with good efficiency while simultaneously providing means to image cargo dissociation. Derfus et al. [77] conjugated homing peptides and enhanced green fluorescent protein (EGFP) siRNA to PEGylated QDs and determined that GFP transfected HeLa cells fluorescence was successfully knocked down, indicating that this technology can be applied toward tracking drug delivery to metastatic cancers. Indeed, proof of principle was confirmed in an in vitro cancer model when chitosan nanoparticles encapsulated with QDs were decorated with HER2 targeting antibody and HER2/neu siRNA and gene silencing in SKBR3, a HER overexpressing cancer cell line, was observed [78]. Complimentary to these findings, Weng et al. [79] created liposomes impregnated with doxorubicin conjugated with targeting peptides and QDs that resulted in efficient tumor cell homing, and thus drug delivery. Another group was able to
concurrently target, image and deliver cancer therapeutics by synthesizing a QD with an aptamer that recognizes prostate specific antigen and doxorubicin, which has fluorescent properties. This technology is unique in that there is bi-fluorescence resonance energy transfer where doxorubicin is quenched due to the intercalation of the aptamer and doxorubicin, and doxorubicin quenches the QD. When the drug dissociates the fluorescence of the QD and the drug are relinquished allowing for imaging of both [80].

It is interesting to note that tumor cell targeting and drug delivery can be achieved, yet groups have yet to report on tumor reduction and drug induced cytotoxicity. Interestingly, Holden et al. [81] were able to conjugate QDs to macrophage cell membranes in efforts to target hypoxic areas commonly found within tumors [81]. This novel concept provides a refreshing perspective toward indirect drug delivery in a variety of maladies, considering macrophages are among the first responders toward homeostatic disruption which often occurs during the onset of disease. While there are not many examples documenting the use of QDs in drug delivery, their use has been established and will surely be expanded upon in the future.

Mechanisms of Quantum Dot Cellular Internalization and Compartmentalization

Few studies have been conducted that research the mechanism of nonspecifically targeted QDs. One group extensively studied the mechanism of endocytosis of neutral, or positively or negatively charged QDs. It was found that negatively charged QDs were most efficient at entering keratinocytes, and mainly did so via a scavenger receptor, lipid
raft pathway [82]. However, Anas et al. have evidence that peptide conjugated QDs utilize clathrin coated pits to gain entry into 3T3 fibroblasts and an epidermal ovarian cancer cell line [83]. Our group has shown that negatively charged QDs quickly engage macrophages and are excluded from the nucleus and appear to be vesicle bound, while commercially purchased PEGylated QDs were not internalized as rapidly and appear more diffuse [84]. Nabiev et al. demonstrated that nonfunctionalized QD size and charge dictates intracellular localization, and that QDs are moved from compartments via active cytoplasmic transport mechanisms [85]. It is likely that QDs that have not been conjugated to a homing peptide or protein have redundant mechanisms for entering cells, and that these mechanisms are charge and size dependent as well as cell and species specific.

Although the fate of nonfunctionalized QDs has not been well researched, QDs have been created to be useful in biological settings by modifying their surfaces. The linker polyethylene glycol is often used to functionalize QDs. Universal methods have been proposed [86] which allow for construction of QDs with varying lengths of PEGs, which can be modified to suit individual experimental purposes. Many research groups have taken advantage of other biocompatible linker groups, particularly thiols to complex QDs to homing peptides, antibodies and other proteins. Chen et al. [87] designed chimeric phage vectors containing tumor targeting integrins, researchers were able to visualize receptor/ligand interactions with QDs, and avoided using chemical conjugation methods. The streptavidin/biotin system has shown promise for targeting QDs to receptors [88]; the strong affinity of avidin to biotin allows for stable imaging. The
process of receptor-mediated endocytosis involved ligand/receptor binding, followed by complex internalization via several pathways, including clathrin coated pits, caveolae and lipid rafts.

Receptor mediated delivery of QDs has been demonstrated numerous times, including targeting QDs and cargo using folate receptors [89-91], GABA receptors in the CNS [92, 93] and mannose receptors [94], most of which result in an intracellular localization to the endosomal/lysosomal pathway. For example, it was shown that negatively charged dots associate with scavenger receptors [82]. The reason for this association is still being investigated. However, the scavenger receptor MARCO (macrophage receptor with collagenous structure) was found to have a positively charged basic cluster containing several arginines [95]. They further show that both this cluster as well as an acidic cluster are essential for ligand binding. Researchers have shown that negatively charged environmental particles such as TiO$_2$ and SiO$_2$ [96-98] bind scavenger receptors. Therefore, it is understood that the charge and electrostatic properties of QDs may play a role in their internalization mechanism. It is known that some biological molecules dissociate from their receptors during the endosomal acidification process, allowing the receptor to recycle back to the plasma membrane and for cargo to escape the endosome. Figure 2.2 illustrates that charge dependent internalization of nanoparticles may influence their intracellular delivery and compartmentalization, as well as their exocytosis [99]. It is plausible that this same pH dependent mechanism can be exploited for QD/drug dissociation and delivery, although this research is ongoing.
Investigators must be clever in their nanoparticle design as early studies showed the propensity of QD clearance through macrophages in the reticuloendothelial system thus preventing them from reaching their target, although PEG capping agents have been shown to improve in vivo target homing [100]. It has been suggested that increasing the length of PEG extends QD circulation time, but size is increased, which can prevent receptor/ligand interactions due to steric properties [101]. The clearance of QDs in vivo remains a topic of interest because of conflicting literature. Lin et al. [102] found QDs to be sequestered in the kidneys of mice that had been injected 28 days previously, and found severe kidney damage, while Chen et al. [103] found excretion of water soluble silica coated QDs to occur within 5 days of injection in mice. Clearly, many studies regarding the excretion and fate of QDs need to be conducted before QDs can be considered for use involving humans.

Quantum Dot Induced Inflammation and Toxicity

One of the main setbacks of QD use in humans is the fact that most of them contain materials, such as cadmium and selenium, which have been proven to be toxic, at least in their bulk form. Cadmium can be toxic in low doses and does not have specific function in the human body. Cadmium has the tendency to bioaccumulate and is difficult to remove from the body; chronic exposure to cadmium can result in kidney and liver dysfunction and potentially death. There is question as to whether nano-sized cadmium exhibits the same effect as bulk cadmium. Studies have been conducted that investigate
the cytotoxicity of QDs with particular focus on QDs that are comprised of CdSe cores. One early study showed differences in mercapto-undecanoic acid functionalized QD toxicity were size and time dependent [104]. Size dependent cytotoxicity was confirmed in studies by Lovric et al. [105] using CdTe quantum dots. This same group also determined the mechanism of QD cytotoxicity could be attributed to the formation of reactive oxygen species [106] and that this phenomena may be able to be controlled by carefully constructing QDs with efficient core capping agents. Chan et al. [107] built upon this concept by further establishing that ROS generated in neuroblastoma cells exposed to CdSe core QDs induced apoptosis via activation of JNK mediated signaling, and also that by capping CdSe QDs with ZnS, cell death could be prevented. Groups have investigated the long term effects of QD exposure, and it was determined that core type plays a vital role in toxicity. QDs made with CdSe cores were found to be less cytotoxic than CdTe QDs, and that cytotoxicity of CdTe QDs could not be prevented by capping [108]. The mechanisms of cytotoxicity in this case were attributed to not only the formation of ROS but also Cd$^{2+}$ leaching. Others corroborate this finding, in particular one group found that CdS QD cytotoxicity is dependent on size and the mechanism of toxicity shifts from ROS driven to Cd$^{2+}$ dependent as concentration is increased [109].

Most studies focus directly on ROS dependent toxicity, and a study using neurons exposed to unmodified CdSe QDs showed that QD internalization resulted in calcium deregulation and sodium channel disruption, both of which can affect the role of ROS mediated protective responses [110]. In vivo experiments were performed on mice that were injected with charged QDs and it was found that negatively charged QDs induced
pronounced vascular thrombosis that could be prevented by the pre-administration of heparin [111], indicating that QD charge and surface functionality can affect the degree of QD cytotoxicity. Zebrafish have also been used as model organisms to understand the cytotoxicity of QDs and developmental dysfunction. In this study, surface charge was again found to be a strong contributing factor although free metal ion concentration only weakly correlated to cytotoxicity levels; in this study PEGylated QDs with a neutral terminal group were found to be the least toxic [112]. Recently, researchers found that CdSe core QDs severely impacted the fertilization and maturation of mouse oocytes and also slowed embryo development. However, when QDs were capped with ZnS, these effects were not seen [113], indicating once again that the QD shell type and capping agent are important factors that need serious consideration when using QDs in living systems. Short and long term toxicity studies in rats also suggests that surface properties, size and composition are important factors that modulate toxicological outcomes; in these experiments, they found that CdSe core ZnS shell QDs are not notably toxic over time.

Although most studies focus on the ability of QDs to result in toxicity, one group investigated the magnitude of a QD induced proinflammatory cytokine response. Lee et al. [114] determined that human primary monocytes upregulated TNF-α and CXCL-8 in response to ROS generated from QD exposure. This important study is among the first to investigate other cell responses and has long term implications regarding the chemical recruitment and trafficking of other immune cells to areas where QDs localize.

In conclusion, QDs have the propensity to provide a major breakthrough for the advancement of personalized medicine. Although this technology is quite young, many
‘proof of principle’ applications have been proposed and adequately demonstrated their feasibility and worth. Most studies toward the use of QDs seem to focus mainly on tumor targeting and imaging; yet they also show great promise for use in other diseases including imaging and targeting amyloid beta proteins which have been shown to modulate the pathology of Alzheimer’s disease. In addition, QDs can be used to track cells in atherosclerotic lesions, or trace bacteria and viruses upon the onset of infection. The possible uses for QDs in nanomedicine are numerous. However, researchers must use caution and carefully establish dosing regimens as well as understand the possible toxicological outcomes associated with QDs as chronic and acute toxicity data collection and interpretation is not yet complete.

Carbon Nanoparticles

Carbon-based nanoparticles are derived from a variety of sources, however a large portion of these nanoparticles come from the combustion of fossil fuels, including coal and oil. Several types of carbon-based nanoparticles exist: diesel exhaust particles (DEP), welding fumes, coal fly ash and carbon black and all are considered to be occupational hazards, while DEP is also a health concern for the public. Carbon black is relatively low in toxicity and is intentionally made for uses in inks, pigments and paint; use of carbon black in inks dates back to the ancient Chinese and Egyptian times. However, carbon black is of particular interest because it is known to have high surface

26
area per unit of mass and can have primary particle sizes as small as 10 nm, and the toxicity of nano-sized carbon black is still under debate.

Air pollution standards are not globally controlled. In the United States, the 2010 National Ambient Air Quality Standards for PM$_{2.5}$, (particulates with a diameter less than 2.5 microns) is 15 μg/m$^3$ on average annually, and 35 μg/m$^3$ for a 24 hour period [115]. The level of ozone for an 8 hour period is .075 parts per million (ppm) [115]. While the United States and European Commission both have standards to regulate air quality, highly populated countries such as China and India do not have such stringent standards. Therefore, it is of great importance to understand the effects that carbon-based nanoparticles have on cellular responses. In this section, the properties and synthesis of carbon nanoparticles, specifically DEP and carbon black nanoparticles (CBNPs), will be examined. In addition, the applications and potential for human exposure will be discussed. Finally, the cellular responses including uptake, inflammation and toxicity will be reviewed.

**Carbon Nanoparticle Synthesis, Properties and Applications**

Carbon black nanoparticles are formed through the incomplete combustion, or ‘thermal oxidative decomposition’ of hydrocarbons in an environment with limited air supply. A second process that is used to form carbon black is termed ‘thermal decomposition’, in which the carbon black is formed in an anaerobic environment, however the former process is by far more prevalent. There are several subtypes of
thermal oxidative decomposition and thermal decomposition (Error! Reference source not found., adapted from [116]). Furnace black is produced in large scale and the process is performed in controlled environments. The size of carbon blacks is dictated by the temperature of the reactor, with higher temperatures producing finer particles. Organic compounds that decorate the surface of carbon blacks can mirror those found on DEP and can be removed via methods such as soxhlet extraction for a purer product. Efforts have recently been focused on increasing the quality and purity of ultrafine carbon black for uses in fuel cells, UV absorbers and fillers among others. As such, several new synthetic approaches for the manufacturing of carbon black have been detailed. Researchers have attempted to utilize scrap tires as hydrocarbon sources for the production of carbon black [117, 118], however, the quality of carbon black produced was somewhat variable. Guo and Kim established a method using polyethylene [119] and polystyrene and high density polyethylene [120] as carbon sources, and a direct current thermal plasma system to decompose the polymers into carbon black. This method proved efficient and yielded high quality carbon black while recycling plastics rather than consuming fossil fuel during production.

Carbon black is arranged in grape like clusters and is 97% pure elemental carbon [121]. Carbon black’s most important physical properties are particle and aggregate size, morphology and microstructure while important surface characteristics are structure, porosity, surface area and chemical composition, including surface functional groups, all of which influence the material’s performance. Carbon black is unique in that with decreasing aggregate size there is an increase in tensile strength and improvement in
reinforcement performance. Additionally, with increasing porosity, there is improved electrical conductivity. During the manufacturing and emission process, carbon black encounters other compounds including transition metals, such as Fe$^{2+}$ or Fe$^{3+}$ and ozone. These compounds can easily adsorb to the surface, and change the chemical properties and reactivity of the particles. This is of special concern for CBNPs, with the increased surface area allowing for more binding sites thereby increasing the prospect of redox reactions, which can ultimately lead to the formation of reactive oxygen species (ROS). Carbon black incorporated into plastics can reduce surface resistance by serving as an electrostatic dissipater by forming conductive bridges for energy transfer. In order for electrical conductivity to occur in plastics, the percolation threshold of carbon black must be met. The percolation threshold is the distinct range by which the conducting capabilities of plastic are increased in direct correlation with the loading level of carbon black. Carbon black also serves as an excellent Ultra Violet (UV) light absorber and is used to stabilize plastics and dissipate UV energy as heat while maintaining excellent tensile strength. As such, there is great interest in creating CBNPs to increase the efficiency and quality of carbon black for these purposes.

Diesel exhaust particles are complex emissions that result from the incomplete combustion of diesel fuel, however the exact composition is affected by factors such as vehicle load, speed, type of fuel and engine, as well as additives and oil. The particle size distribution of DEPs is bimodal and can range between 7.5 to 42 nm in diameter for nuclei mode and 42 nm to 1 μm for accumulation mode [122]. The levels of DEPs emitted have been found to increase with speed [123]. The amount of DEPs and their
organic fraction is also dependent on the vehicle load size, with small loads resulting in fewer DEPs with higher organic content, including polycyclic aromatic hydrocarbons (PAHs), while large loads result in more DEPs with less organic compounds [124]. Because DEPs have large surface area, they have the propensity to carry organic compounds, like PAHs, which are carcinogenic. In addition, water soluble organic compounds can be formed and subsequently oxidized after exposure to ozone, thereby creating carboxylates on the surface, and improving water solubility [125].

Epidemiological studies have correlated increased particulate matter exposures with increased mortality risks, and that the length of exposure and exposure concentrations are primary factors associated with this risk [8]. The health effects of DEP exposure has been reviewed in [126], and highlights the relationship between air pollution and the onset of allergies, asthma and cardiovascular diseases. There is a body of evidence that supports the hypothesis that recurring exposure to air pollution causes DNA damage, which can ultimately lead to the onset of cancer [127]. Accordingly, DEPs are classified as ‘reasonably anticipated to be a human carcinogen’ [124].

There are no known applications for DEPs and they are viewed as health hazards, to be reviewed later in this document. The most common application for carbon black is in tires for automobiles. However, novel applications of carbon black are steadily emerging, including utilizing their conductive properties for the engineering of artificial noses [128], reviewed in [129], and sensing the presence of chemical threats including organophosphate nerve agents [130]. Carbon black nanoparticles are often used to improve fillers for tires and provides better dexterity [131, 132].
Mechanisms of Carbon Nanoparticle Cellular Internalization and Compartmentalization

Most of the research investigating environmental particle uptake revolves around inert particles such as TiO$_2$ and amorphous SiO$_2$. Little research has been conducted regarding the uptake mechanisms of carbonaceous particles, and even fewer studies have been conducted on internalization of nanoparticles. A review of potential pathways and cellular responses after nanoparticle and cell interactions are discussed in Figure 2.3, adapted from Oberdörster et. al [133]. Kobzik was among the first to investigate mechanistic entry of environmental particulates such as TiO$_2$, Fe$_2$O$_3$ and SiO$_2$ into cells [134]. This work identified macrophages scavenger receptors as a mode for particle internalization. Interestingly, DEP internalization was not inhibited from uptake when the scavenger receptor ligand poly inosinic acid (Poly I) was applied, indicating that they have a distinct entry pathway. Further, this group identified the Class A scavenger receptor: macrophage receptor with collagenous structure (MARCO) as a novel receptor involved with the binding of unopsonized environmental particles in mice [97] and humans [96]. Molecular assays identified amino acid residues located in specific cytoplasmic domains are necessary for scavenger receptor functions such as adhesion, internalization and intracellular trafficking [135]. Chao et al. [136] found that scavenger receptors regulate the apoptotic effect of silica particles via a caspase dependent pathway in murine alveolar macrophages. Hamilton et al. [137] supported these findings with
evidence that mouse alveolar macrophage uptake and toxicity of SiO$_2$ was significantly reduced in the presence of MARCO antibody. The scavenger receptor cysteine rich domain plays a significant role in particle binding, although the collagenous domain has also been shown to bind environmental particles [98]. Interestingly, scavenger receptor SRA-I have been found to negatively regulate interleukin 12 (IL-12), while MARCO, was shown to positively regulate IL-12 production and MARCO expression on macrophages was dependent on Th-1 or Th-2 polarizing factors [138]. Further, MARCO deficient mice were studied and shown to have limited ability to remove *Streptococcus pneumonia* and TiO$_2$ particles. In addition, the mice had significantly increased numbers of polymorphonuclear leukocytes (PMNs) and levels of proinflammatory cytokines TNF-\(\alpha\) and macrophage inflammatory protein-2 (MIP-2) after 4 hours of bacteria and particle exposure [139], which supports the importance of scavenger receptors in mounting appropriate lung innate immune responses after exposure to pathogens and environmental particles. Murine alveolar macrophages and lung epithelial cells were found to internalize carbon black and DEP, and macrophages were found to internalize more carbon black. Cytochalasin D was found to block 80% of DEP internalization in both cell types over a 24 hour exposure [140], indicating that particle uptake is dependent on actin polymerization.

MARCO and SRA-I and –II scavenger receptors were found to play an important role in regulating the immune responses against inhaled ozone. In this study, Dahl et al. [141] found that MARCO deficient mice had greater lung injury, specifically increased influx of neutrophils and increased levels of protein and 8-isoproastane, an indicator of
oxidative stress, compared to wild type mice. This group also provides evidence that MARCO protects against oxidized lipid surfactants after ozone exposure and plays an important role in protecting the lungs against inflammation. It is thought that due to its reactivity, ozone does not contact lung cell membranes directly, but rather reacts with reducing molecules such as glutathione and ascorbate, which leads to the formation of secondary ROS and oxidized lipids, the latter which are scavenged by SRA-I/II and MARCO [142].

The literature is divided with regard to intracellular compartmentalization. Research dating back to 1989 has shown that carbon black can translocate from the lung into the lymphatic system [143]. Several researchers found that the type and size of nanoparticle played a role on whether the particles are free in the cytoplasm or membrane bound [9, 144].

It is important to note that most of the data collected that investigates environmental particle uptake does not focus exclusively on nanoparticles, and there are other mechanisms that carbon nanoparticles may utilize to enter cells. For example, Geiser et al. [145] demonstrated that TiO$_2$ nanoparticles were internalized by red blood cells via non-phagocytic mechanisms, possibly diffusion or through adhesive interactions. They also found that the nanoparticles translocated from lung compartments, and were even found in heart connective tissue, indicating that inhaled nanoparticles do not remain confined in the lung. Others support this finding, and have evidence of particles translocating to the liver, kidneys and even the brain [146-149]. However, one study investigating the impact of acid functionalized CBNPs and single walled carbon
nanotubes (SWCNTs) in mice found that mice treated with SWCNTs had lower cardiac function and the onset of cardiac damage, although there was no evidence of nanoparticle translocation to the heart tissue [150]. There is ongoing research that focuses on the consequences of nanoparticle translocation to the brain including inflammation and damage due to the generation of free radicals [151-153]. Donaldson et al. [154] provides an inclusive review of CBNP toxicology after inhalation.

Carbon Nanoparticle Induced Inflammation and Toxicity

The interest in exploring the possible repercussions of nanoparticle exposure has ushered in an explosion of research dedicated to investigating the toxicity of carbon nanoparticles. Carbon black has been found to be non-toxic [155], and most of the exposure related illness has been linked to lung overload after years of chronic exposure. The role that size dependence has on toxicity has been investigated. In the mid 1990’s Oberdörster et al. found significant differences in lung persistence, injury and inflammation when they treated rats with two different sized TiO$_2$ particles [156]. Brown et al. [5] concluded that increases in proinflammatory factors were a consequence of increased surface area, after comparing different sized polystyrene particles ranging from 64 to 535 nm and revealing a significant increase in protein and lactate dehydrogenase in the bronchoalveolar lavage from animals instilled with the smallest particles compared to the largest. In addition, IL-8 gene expression was also increased in A549 lung epithelial cells treated with 64 nm particles, but not larger particles. A CBNP size dependent
increase in heme-oxygenase levels in both rat alveolar epithelial cells and alveolar macrophages was found, and could be attenuated by the addition of antioxidants, indicating that particle surface area plays a factor in CBNP derived oxidative stress [157]. Lung epithelial cells were found to be multinucleated and undergo apoptosis that was dependent on zinc transport after treatment with DEP from petroleum sources when compared to cells treated with particulate matter derived from biodiesel [158]. Others found that the increased surface area of small particles correlated with higher redox activity of larger particles [159]. Still others hypothesize that the nanoparticle composition also plays a role in the biological effects in response to particle exposure. For example, Schwarze et al. [160] found that surface reactivity may drive biological responses including toxicity and inflammation. Research in our lab corroborates this finding by illustrating that synthesized CBNPs containing iron significantly increased the release of proinflammatory factors compared to non-iron containing CBNPs [161, 162]. On the other hand, macrophages incubated with non-iron containing CBNPs were found to increase tumor necrosis factor-alpha (TNF-α) production in a dose dependent manner, but the addition of iron did not increase this response [13]. The authors of this study concluded that the contents of the particles may stimulate different types of cells (CBNPs stimulate macrophages, while transition metals stimulate epithelial cells) and that iron is sequestered within macrophages in an inactive form, which prevents potentiation between the iron and CBNPs. When mouse macrophages were incubated with CBNPs or CBNPs + FeCl₃, an increase in reactive oxygen species was observed. However, incubation with CBNPs, but not CBNPs + FeCl₃, resulted in TNF-α induction, but were
not toxic [163]. Studies were completed to understand the inflammogenicity of bioavailable iron found on the surface of coal fly ash. These researchers found that iron induced IL-8 induction from exposed human bronchial cells and that the amount of bioavailable iron increased with decreasing particle size [164]. Macrophages collected from rats that were treated with CBNPs had decreased phagocytic capabilities and had enhanced chemotaxis toward complement factor C5a [165]. Lundborg et al. [166-168] has shown that alveolar macrophages exposed to CBNPs had impaired phagocytosis of environmental particles and bacteria. DEP was found to dampen the response of exposed cells to LPS, while carbon black did not. Similarly, when human alveolar macrophages were exposed to DEP and then stimulated with LPS or lipoteichoic acid, a Gram + bacterial product, a reduction in proinflammatory cytokine production was noted, indicating that exposure to air pollution can impair the normal lung immune functions [169]. Toll like receptors (TLR) 2 and 4 were induced by particulate matter and thought to play a role in air pollution related inflammation [170, 171]. Toll-like receptor-4 was further investigated to determine if it plays a role in DEP induced inflammation. Mice with point mutations in TLR-4 had less inflammation which was pinpointed by the corresponding reduction of MIP-1 levels, which signals for an influx of inflammatory cells, indicating that TLR-4 functions, in part, as a receptor that mediates inflammation in response to DEP exposure [172].

Inflammatory cytokines produced by alveolar macrophages were increased when cells were treated with particles that had all soluble components removed [173], and the complexity of air pollution should be considered when attempting to identify
proinflammatory mechanisms. Organic extracts from DEP were used to compare the inflammatory responses of pulmonary epithelial cells and macrophages. Interestingly, these DEP fractions induced oxidative stress, c-Jun-N terminal kinase (JNK) activation, IL-8 production and apoptosis due to disruption in mitochondrial function in exposed pulmonary cells [174]; the same study showed that macrophages were less susceptible to oxidative damage, and could be protected in the presence of a thiol antioxidant, N acetylcysteine (NAC). Using electron paramagnetic resonance (EPR), large quantities of free radicals have been identified on PM2.5, including semiquinone radicals, which were found to damage DNA in human cells; this damage was reversed when oxidant scavengers such as superoxide dismutase, catalase and desferoxamine were added to the culture [175]. Xia et al. [176] noted that the quinone enriched polar fraction of DEP induced mitochondrial dysfunction and apoptosis in murine macrophages and concluded that changes in membrane potential after environmental particle exposure may be due to the adsorbed chemical content of the particle. DEPs have chemical properties that allow for the catalysis of reactive oxygen species, in part due to redox potentials of compounds contained in DEP extracts and the particle itself [177]. Studies on bronchial epithelial cells showed that environmentally persistent free radical formation due to the physicochemical composition of nanoparticles resulted in decreases cellular antioxidants, which triggered a state of oxidative stress, eventually leading to cell death [178]. Free radicals production was positively correlated with CBNP induced cytotoxicity in human lung epithelial cells [179]. However, ultrafine particulate matter, but not carbon black
nanoparticles induced heme oxygenase, TNF-α production, phospho-jun-N terminal kinase (pJNK) in murine macrophages.

The upregulation of proinflammatory transcription factors such as nuclear factor-kappa beta (NF-κβ) and activator protein-1 (AP-1) have been documented in vivo and in vitro after exposure to CBNPs. Stone et al. were among the first to note that exposure to CBNPs induced an influx of intracellular calcium through monocyte’s plasma membrane, which was attenuated by a calcium channel blocker verapamil [180]. Rat alveolar macrophages treated with CBNPs were found to have a dose dependent increase in intracellular Ca^{2+} levels that could be regulated by the addition of a calcium channel blocker. In addition, in the presence of the verapamil, the activator protein-1 (AP-1) transcription factor was significantly lower, as were TNF-α mRNA and protein levels [181]; these authors conclude that CBNPs may induce their proinflammatory effects by influencing intracellular calcium levels, which in turn may modulate ROS production and control within the cell.

Modifications of CBNPs often occurs during manufacturing process and in the atmosphere, and epidemiological evidence exists that suggests that carbon nanoparticles and ozone can interact and increase the generation of free radicals, specifically the hydroxyl radical, in a synergistic fashion. EPR identified that ozonated carbon black has a more intense and stable signal compared to non-ozonated carbon black. The difference in signals was attributed to the formation of surface functional groups, including epoxides and ozonides which react with carbon black [182]. When graphite was ozonated, a shift in EPR spectrum was noted and the formation of carbonyls, ether and ozonides were
noted by infra-red spectroscopy [183]. Repine et al. [184] found that rat macrophages exposed to carbon particles containing higher levels of free radicals, as characterized by EPR, exhibited greater particle uptake, mitochondrial damage and higher leukotriene B₄ release compared to cells exposed to particles with low unpaired electron spin densities. In other research, spin trapping and EPR was used to identify free radicals in the presence of particulate matter 2.5 (PM₂.₅), particulate matter 10 (PM₁₀), DEP and gasoline exhaust particles (GEP) alone or plus ozone [185]. Indeed, a more intense EPR signal was observed, and the authors conclude that this synergistic increase in free radicals may be a factor in ROS damaged proteins and nucleic acids. Other research supports this finding, and a study of humans that were exposed to DEP followed by ozone for several hours had a significant influx of macrophages and neutrophils in bronchial lavages compared to clean air followed by ozone exposure [186, 187]. In human pulmonary epithelial cells, IL-8 gene expression was found to be up regulated via activation of NF-Kβ, after treatment with DEP and a subsequent treatment of ozone [188]. It is interesting to note, however, that the organic content of carbon particles played a role in inflammatory responses. In a study by Madden et al. [189], rats were exposed to DEP with or without ozone treatment or carbon black with or without ozone treatment, and inflammatory markers were observed. In rats treated with DEP + 0.1 ppm ozone, the levels of lavage protein and lactate dehydrogenase (LDH) were significantly increased. However, this effect was not seen in rats treated with carbon black + 0.1 ppm ozone. In vivo studies using mice that were preexpoed to carbon nanotubes and then low levels of ozone (0.5 ppm) were found to have reduced PMN influx into the lung compared to mice treated
with carbon nanotubes and air, after 24 hours. In addition, carbon nanotube + ozone mice exhibited increased LDH levels 5 hours after challenge, but reduced to baseline after 24 hours, whereas carbon nanotubes alone had significantly higher LDH for both 5 and 24 hours post exposure, along with significantly increased secretion of TNF-α and IL-1β [190]. These data did not reveal a proinflammatory synergism between carbon nanotube inhalation and ozone, and the authors conclude that the mice developed a cross-tolerance in response to dual treatment [190].

While ozone is thought to be a pro-inflammatory oxidant, studies have tested its use as an anti-inflammatory, antiapoptotic treatment. In fact, ozone oxidative preconditioning, has been used to prevent inflammation during ischemia/reperfusion events. In work by Chen et al. [191], a rat model of renal damage caused by ischemia and reperfusion was tested using ozone oxidative preconditioning. These studies indicate that ozone treatment significantly reduced gene expression of TNF-α, IL-1β and intracellular adhesion molecule (ICAM) and also significantly reduced apoptotic mRNA levels of caspase 3 [191]. Mice pretreated with ozone administered intraperitoneally then challenged with lipopolysaccharide (LPS) were found to have reduced TNF-α serum levels as well as significantly increased activity of antioxidant enzymes, including glutathione peroxidase, catalase and glutathione-S transferase [192]. Rats treated with ozone and carbon tetrachloride, a known free radical inducer, were found to have reduced hepatic cell damage by increasing the level of antioxidants defenses prior to challenge [193]. Aqueous ozone has also been found to inhibit the activation of NF-κβ, a transcription factor that regulates proinflammatory cytokine expression in oral cells, and
that the signal transduction pathway for NF-κβ was inhibited at Iκβ or upstream. Further, NF-κβ inhibition was not found to be due to ozone itself, but rather due to the ozonated sulfhydryl groups in the amino acid cysteine and the aromatic amino acid tryptophan. Cells from a human monocyctic/macrophage cell line were exposed to 0.1, 0.2 or 0.5 ppm of ozone for one hour, and cytotoxicity and TNF-α production were not changed compared to control cells, and the authors conclude that lung epithelial cells, rather than macrophages may drive the inflammatory phenotype in response to ozone exposure [194]. There are data that support this finding, where nasal epithelial cells exposed to increasing doses of ozone exhibited increased NF-κβ activation and increased TNF-α levels [195]. On the contrary, Bosson et al. [196] found that airway epithelial cells exhibited suppressed NF-κβ nuclear expression, phosphorylated c-Jun and reduced IL-8 secretion after cells were exposed to ozone. While these studies show that ozone can create an anti-inflammatory, anti-apoptotic environment, direct ozone exposure has been shown to be a health hazard, cause reduced lung function, increased susceptibility to bacterial infections and exacerbate asthma and allergic reactions.

Human bronchial epithelial cells were exposed to DEPs or CBNPs for 24-72 hours, and proliferation was decreased in cell treated with DEP but not CBNPs. In addition, the proinflammatory marker granulocyte-macrophage colony stimulating factor (GM-CSF) was increased in a dose dependent manner for DEP treated cells, while cell treated with the same amount of CBNPs did not secrete GM-CSF [197]. Tao et al. [198] demonstrated that inflammatory responses to pollutants are affected by intracellular interactions between several pulmonary cell types, and that co-culture model systems
may provide better insight to inflammatory mechanisms compared to single cell models. Other researchers have found that supernatants from alveolar macrophages treated with particulate matter had potent abilities to activate bronchial epithelial cells; in addition, this response was attenuated by incubating cells with antibodies to IL-1β and TNF-α, indicating that these cytokines serve to initiate the inflammatory response in the pulmonary microenvironment [7]. These findings were also observed in a rabbit model, where animals were instilled with supernatants from a co-culture of human alveolar macrophages and bronchial epithelial cells that had been exposed to PM$_{10}$ contained factors such as granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-6 (IL-6) which act on the bone marrow to release PMNs destined for the activated site [199]. GM-CSF was found to be induced when human bronchial epithelial cells were incubated with CBNPs collected from an urban setting. Interestingly, the data suggest that the particle chemical composition regulates the production of GM-CSF, and the release of GM-CSF was dependent on the activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) signaling cascades [200].

While the literature detailing the dangers of CBNPs is growing, there is still contradictory data with regards to defining the causal factors that result in cell and system damage. Further, modifications to CBNPs that occur during manufacturing or in ambient air can have a profound effect on their toxicity and inflammatory responses. The literature reviewed above cites examples of enhanced inflammation and toxicity, and also
implications of these outcomes. Future studies, and those recorded in this thesis, will aim to discern what drives cellular responses to CBNP exposure.

Silver Nanoparticles

The use of silver for preservation of food and water and as a use for disease prevention and treatment has been documented for centuries. Ancient Chinese, Egyptian and Roman artifacts and writings reveal the use of silver vessels for food, water and wine storage. In fact, wealthy British people evaded the harshest symptoms from the Plague because they used silver utensils and cookware, preventing spread of the bacteria, \textit{Yersinia pestis}. The use of silver was prominent in photograph development, although this has declined with the advent and increased popularity of digital photography. In contrast, there has been a renewed interest in silver as antimicrobials with the onset of antibiotic resistance. As such, the demand for and development of silver products has tremendously increased in the past 5 years. Silver nanoparticles (AgNPs) have become the subject of intense research due to the inherent increased surface area, as well for their optical properties. This section will examine the properties, synthesis and applications of AgNPs; the cellular internalization mechanisms of AgNPs will also be summarized. Finally, the toxicity mechanisms of silver ions (Ag\textsuperscript{+}) and AgNPs and other associated cellular responses will be compared and reviewed.
Silver nanoparticles can be synthesized through a myriad of processes. One such method entails the use of normal or reverse micelles which are based on sodium dodecylsulfate or diethyl sulfosuccinate, respectively. This methodology uses silver ions as the metal nanoparticle precursor, with hydrazine as a reducing agent, and relies on the micelle aggregate to dictate the size and shape of the nanoparticle. [201]. Size selection of the AgNP occurs via size-selective precipitation where two solvents are used to promote aggregation of larger particles while smaller nanoparticles remain in the solution supernatant after a centrifugation step; this process can be repeated to get suspensions of nanoparticles that are uniform in size. There are many variations of this procedure, and new protocols are frequently published. Here, several methods will be reviewed, but by no means represent an exhaustive list of AgNP synthesis. Sun et al. [202], developed a method where silver nanoparticle shape and size could be easily controlled. In these experiments, various amounts of silver nitrate were reduced using polyethylene glycol followed by the addition of poly(vinyl pyrrolidone) (PVP), a capping agent, at different concentrations. It was found that smaller cubic AgNPs could be created by using 0.125 M AgNO₃ and shorter growth times. Other work has also utilized PVP, but relied on a rotating platinum electrode as the cathode to directly electroreduce bulk silver ions, which facilitated the movement of AgNPs away from the cathode to the solution, while PVP as a stabilizer prevented the deposition of Ag⁺ at the cathode region [203]. The type of capping agent is important for AgNPs to remain dispersed when put into buffered
media. AgNPs containing PVP were not found to aggregate when put in acidic or highly buffered media, while citrate and uncoated AgNPs were found to aggregate in these conditions [204]. Silver nanoparticles have also been chemically synthesized with the aid of dendrimers. One successful example involved using photoreduction through UV irradiation and polyamido amine (PAMAM) dendrimers, where AgNP size was controlled by the size and surface functionality (amine or carboxylated) of the dendrimer. The resultant AgNPs were larger when prepared with carboxylate terminated dendrimers, possibly due to aggregate formation [205]. AgNP synthesis requires multiple steps and organic solvents, which can pose various environmental threats, therefore, researchers are attempting to create AgNPs via green methods. For example, saccharides including glucose, galactose, lactose and maltose have been successfully implemented as reducing agents for the production of AgNPs [206]. In this study, disaccharide based reduction resulted in the smallest AgNPs (< 25 nm), and also had the highest antibacterial performance [206]. Another green approach for the formation of AgNPs from AgNO₃ utilizes H₂O as the solvent, glucose as the reducing agent and starch, specifically amylase, as the designated capping molecule [207]; this approach yielded AgNPs that were ~5.3 nm in size. In keeping with the green theme, scientists have examined the use of biological proteins and investigated the possibilities of mimicking nature for the purposes of creating AgNPs. One example is the use of nitrate reductase, an enzyme found in many species, as a reducing agent of AgNO₃ to form silver nanoparticles [208].

Indeed, other researchers have borrowed from the biological realm in their quest to synthesize AgNPs. In fact, bacterial strains, including *Pseudomonas stutzeri*, a silver
resistant strain isolated from a silver mine, have developed mechanisms to convert Ag\(^+\) into AgNPs of various shapes and sizes [209], although the mechanism has yet to be elucidated, though it is hypothesized that the bacteria has developed strategies to pump silver beyond the membrane or bind silver to prevent toxicity. Work to further understand the mechanism by which bacteria can precipitate silver from AgNO\(_3\) was performed by Naik et al. [210]. This research identified three bacterial peptides that had a selective affinity for silver. Among these peptides, two were able to precipitate silver, and were found to be more acidic which may facilitate the growth of AgNPs at different rates, and account for their size and shape distributions. Fungi have also been shown to reduce AgNO\(_3\) to form AgNPs in the range of 5-25 nm, however the fungal mechanisms differ from bacterial mechanisms in that the reduction can occur extracellularly, which is likely mediated by protein exudates [211]. Other green AgNP synthetic methods have been thoroughly reviewed by Sharma et al. [212].

While there are many avenues to create AgNP suspensions, others have taken a nano-hybrid approach, which utilizes other materials as a substrate or carrier during the synthetic process. For example, zeolites which are porous aluminosilicates, have the propensity to accommodate cations within their supercages via ion exchange. Several researchers have demonstrated that silver can be incorporated into zeolites as both ions and nanoparticles [213-216]. These materials are unique in that they can gradually release silver ions and have great potential for uses in food preservation, medicine air duct work and water filtration. Several prototypes of catheters containing AgNPs in their coatings have been efficient and effective in fighting bacteria associated with urinary
tract infections [217, 218]. Indeed, AgNPs have been incorporated into films that may be used to coat surgical tools. In these studies, the antimicrobial properties of films containing AgNPs was shown; additionally, mouse fibroblasts were found to be unaffected by AgNPs, and were even shown to have increased attachment, and factor that is important in wound healing [219]. Coatings on surgical blades consisting of AgNPs were found to efficiently and effectively lyse various strains of bacteria including *Bacillus anthracis* and *Klebsiella pneumomiae* after 1.5 hours, although the amount of time needed to kill *Staphylococcus aureus* was 3 hours [220]. Vegetable oil based paints have also had AgNPs incorporated into their formulation, and exhibited good antibacterial activity against both *S. aureus* and *E. coli* [221]. Zeolites impregnated with AgNPs used for coating air ducts in air conditioning systems have successfully reduced air borne bacterial loads [222], and show further promise for preventing nosocomial infections. Dedrimers have also been synthesized with AgNPs incorporated into their structure. These composites have potential for simultaneous imaging, labeling and therapeutic delivery as they were shown to have exceptional optical properties and are biologically compatible [223].

Like other nanoparticles, silver nanoparticles have greater surface area than their bulk counterparts. In addition, silver has excellent optical properties that have been exploited. Some of these properties include surface resonance enhancement and resonance wavelength tunability. In turn, AgNPs have been used to increase light absorption in solar cells and can also be used to detect changes in proteins and DNA [224, 225].
Many other applications of AgNPs exist. They have been implemented in water purification [226] and also incorporated in water filtration schemes [227]. Because of their antimicrobial properties, AgNPs have been added to cosmetics and deodorants. For example, one study investigated zeolites containing AgNPs as a spray for the control of axillary odor. This prototype significantly reduced the number of odor causing bacteria in human subjects without producing obvious side effects at the application area [228]. Along these lines, AgNPs have been incorporated into fabrics to prevent the growth of odor causing bacteria that can colonize clothing [229, 230]. Other uses for AgNPs as a preservative in cosmetics have been demonstrated. Kokura et al. [231] tested the effects of AgNPs on skin cells and found that they were non-toxic and remained stable for over a year, while maintaining antimicrobial efficiency, although it should be noted that there are very few studies dedicated toward examining the properties of AgNPs contained within these such products, including skin cream, lotions and moisturizers. In dentistry, zeolites containing AgNPs have been incorporated into filling material for root canals [232, 233], where they were found to inhibit bacterial growth of pathogenic bacteria associated with endodontic infections. However, it should be noted that the antimicrobial effects of such materials have the same impact on normal oral flora which are essential for healthy teeth and gums. Studies by Thom et al. [233] established that the addition of silver to root canal filling material had increased evidence of hemolysis, indicating that these materials may be capable of damaging and killing oral cells. More studies investigating the risks associated with such materials are needed to discern the potential hazardous outcomes before their use is approved in patients.
Several applications for the use of AgNPs as bioimaging agents or enhancers have been reported. Silica cores coated with AgNPs were shown to produce photoacoustic signals that were detected in a dose dependent manner, which would allow for in vivo imaging of whole organs [234]. Meanwhile, Hu et al. [235] used AgNPs as localized surface plasmon resonance enhanced scattering probes to image pancreatic cancer cells that can be used for imaging in both dark field and transmission electron microscopy. Sensing parameters from humidity [236] to biological pH [237, 238] have been created using AgNPs. Surface enhanced Raman spectroscopy using AgNPs has been used to image live yeast cell walls [239] and HeLa cells [240].

The applications of silver as antimicrobials in earlier times have been well documented, see [241] for a review. The use of AgNPs as antimicrobials has been demonstrated numerous times [218, 242-247]. For example, *Streptococcus mutans*, a Gram + bacteria was exposed to AgNPs, gold and zinc nanoparticles, and the minimum bactericidal concentration for AgNPs were 30 and 90 times more effective than zinc and gold nanoparticles, respectively [243]. Suresh et al. [248] cleverly compared the antimicrobial activity of AgNPs that were created through biosynthesis (AgNPs were created when *Shewanella oneidensis* was incubated with AgNO$_3$) or through chemical synthesis. Their data revealed that the biosynthetic AgNPs were more effective antimicrobial than chemically synthesized AgNPs; interestingly, the Gram + bacteria *Bacillus subtilis* was more susceptible than the Gram – bacteria, *Escherichia coli* [248]. While other researchers corroborate this finding [249, 250], other studies have found that Gram – bacteria are far more sensitive to the bactericidal effects of AgNPs. Specifically,
a suspension of AgNPs that was 10 times more concentrated was needed to kill *S. aureus* compared to *E. coli* [244].

While the role of AgNPs as an antibacterial agent has been fairly well documented, the efficiency of AgNPs as antivirals has been less studied. There are a few studies that indicate that AgNPs can disable viruses; one such study indicated that biogenic AgNPs are more potent than their chemically synthesized counterparts [251]. In other studies, AgNPs have been shown to prevent plaque formation after Vero cells were infected with Monkeypox virus that had been incubated with AgNPs [252], while AgNPs were shown to prevent HIV infection [253]. Additionally, AgNPs have prevented hepatitis B replication by preventing the formation of RNA and extracellular virions [254].

**Mechanisms of Silver Nanoparticle Cellular Internalization and Compartmentalization**

The mechanisms of AgNP cellular internalization have yet to be fully characterized. Since AgNPs are noble metals, like AuNPs, it is possible that non-targeted nanoparticles may enter mammalian cells through the same mechanisms. Studies by Geiser et al. [145], have demonstrated that AuNPs can enter red blood cells via diffusive mechanisms since they were found in the cytosol without membranes encapsulating them. In contrast, other studies indicate that AuNPs enter peripheral blood macrophages via macropinocytosis [255], a transport mechanism confirmed by others [256]. Yen et al. studied the interactions of AuNPs and AgNPs in macrophages, and found both to be
confined in cytoplasmic vesicles, although these authors hypothesize that the AuNPs may bind serum proteins prior to internalization due to their charge, entering cell through a more complicated process compared to AgNPs [257]. Macropinocytosis and clathrin mediated AgNP uptake has been proposed in fibroblasts, with AgNPs appearing in the cytoplasm and nucleus [258]. When AgNPs of different sizes were coated with antibodies, they were found to alter basic cell processes such as proliferation and apoptosis. Specifically, NPs that were 40 to 50 nm and coated with herceptin had greater apoptotic effects on breast cancer cells [259]; additionally, the uptake of antibody coated nanoparticles was improved with 40 nm particles compared to 10 nm particles.

Several researchers have provided information regarding the intracellular compartmentalization of AgNPs. Arora et al. found AgNPs in the cytoplasm and mitochondria of primary liver cells and in the mitochondria of fibroblasts [260]. Others have found AgNPs in the mitochondria and nucleus in fibroblasts [261]. Liposomes containing silver were found to localize to lysosomes in human keratinocytes [262], while keratinocytes exposed to Lung cancer cells exposed to AgNPs with different sugar coatings were found in the cytoplasm; it was noted that the coating type dictated the rate of uptake, with lactose increasing the rate of internalization in fibroblasts [263]. Also in fibroblasts, Lesniak et al. [223] found silver/dendrimers within endocytic vesicles, likely endosomes. Macrophages exposed to AgNPs also easily internalized AgNPs, which were found to localize to vacuoles within the cells, but note that the mechanism of internalization is unknown [264].
In vivo studies, mice that had silver grids implanted had silver sulfide nanocrystals found in many organs including the brain, spleen, kidney, liver and lymph nodes as well as in collagen fibrils; the authors hypothesize that macrophages facilitate the release, or dissolution, of silver ions from the grids, allowing silver sulfide particles to circulate throughout the body [265]. Studies investigating the ability of nanoparticles to cross the blood brain barrier (BBB) found that AgNPS and copper nanoparticles introduced to rats intravenously resulted in edema localized to the proximal frontal cortex and the ventral surface of the brain, 24 hours after exposure [266]. Similar findings were found in mice that were exposed to AgNPs via entry through systemic circulation or directly into the brain ventricular space [267]. In a porcine skin model, AgNPs were found in the upper stratum corneum layers of the skin, and caused local focal points of inflammation after 14 days of AgNP application, while exposure to a culture of keratinocytes were found to have AgNPs enclosed in cytoplasmic vacuoles [268]. When AgNPs were applied to human skin that was damaged or not, silver penetration was noticed. Transmission electron microscopy revealed the presence of AgNPs in deep stratum corneum and the outermost layer of the epidermis after 24 hours [269]. Rats exposed to AgNPs via oral dosing over a four week period were found to have slight liver damage, 28 days post exposure, and silver was found to be distributed to organs such as liver, lungs and brain [270].

While the mechanism of AgNP cellular internalization has yet to be established, it is evident that AgNPs are not confined to specific locations in the body. They have the ability to translocate, through the blood and lymphatic system and deposit in organs such
as the brain, lungs, liver and kidneys. In the early 1980’s it was demonstrated that silver ion reduction can occur, resulting in insoluble silver phosphates and silver chlorides, which may be transformed into silver sulfide, which have the propensity travel throughout the body, and accumulate in the brain, and even cross the placental barrier [271]. Liver toxicity and damage have been reported and the primary route of AgNP excretion in animals, including humans was found to be in the feces [272]. While the most notable side effect of silver exposure is argyria, or skin discoloration, the cellular responses and toxicity of AgNPs are not well documented and a review of the current literature is reported below.

Silver Nanoparticle Induced Inflammation and Toxicity

The mechanism of AgNP toxicity can result in several ways. First, AgNP can release Ag$^+$ ions, which have been well documented to cause toxicity in bacteria and mammalian cells. Several studies have examined the kinetics of Ag$^+$ release from AgNPs. One such study revealed that Ag$^+$ release was dependent on both proton and dissolved oxygen content, and that release was pH and temperature dependent. These authors argue that AgNPs will not persist as particles but will undergo dissolution when in biological conditions [273]. This research exemplifies the necessity to conduct research pertaining to the toxicity of AgNPs under the correct experimental conditions and controls. If, in fact, the dissolution of AgNPs is inevitable, the interactions of Ag$^+$ in the biological systems should also be investigated in great detail. On the other hand,
studies performed in soil bacteria indicate that interactions AgNPs factors such as soil humic acid content, not the dissolution of AgNPs to Ag\(^+\) mitigate cell death [274]. Aggregation of AgNPs also may influence the toxicity, and factors such as pH, electrolyte content and ionic strength can change aggregation state. Studies on AgNPs capped with several types molecules had different aggregation states, and the addition of chloride and calcium resulted in the formation of silver colloids and large aggregates, regardless of capping type, except for PVP capped AgNPs, which remained suspended regardless of the pH, electrolyte content and ionic strength [204]. Second, AgNPs can be internalized and cause physical disruption of electron transport in the mitochondria. Indeed, the activity in all four mitochondrial complexes in brain, liver, heart and skeletal muscle were found to be significantly reduced, indicating that AgNP exposure can impair cellular metabolism [275]. It is hypothesized that AgNPs interact with sulfhydryl groups, and oxidation of these groups prevent or reduce electron transport. Mitochondrial damage was exhibited in germ line stem cells, which were more sensitive to AgNPs than liver cells [276]. They can induce DNA damage [277] and prevent RNA formation [254]. Lastly, it is hypothesized that AgNP exposure can result in the formation of ROS, leading to oxidative stress and depletion of antioxidant proteins, thereby inducing apoptosis. Researchers show that size of AgNPs is correlated to ROS driven cell death in alveolar macrophages that had been exposed to AgNPs ranging from 15 nm to 55 nm for 24 hours [264]. Arora et al. [278] demonstrated that exposure to AgNPs resulted in significant decreases in superoxide dismutase and glutathione, while catalase and glutathione peroxidase levels were not changed; lipid peroxidation was significantly increased
compared to untreated cells for both human fibrosarcoma and skin carcinoma cell lines. These authors concluded that cytotoxicity was clearly caused by AgNP induced oxidative stress, and that the mechanism of cell death shifts from apoptosis to necrosis after higher doses of AgNPs, indicating that the effective concentrations of AgNPs needed for antimicrobial activity should not interfere with coinciding wound healing. When fibroblasts were exposed to Ag⁺, increases in ROS, specifically superoxide, was detected and the expression of antioxidant genes was downregulated; the expression of metal protective proteins such as metallothionein was increased, indicating fibroblasts use several mechanisms to cope with influxes of Ag⁺ [279]. Dermal fibroblasts were found to have decreased DNA synthesis, which was designated as the primary factor controlling Ag⁺ induced cytotoxicity [280].

The formation of ROS has been implicated in the genotoxicity caused by exposure to AgNPs. Studies in human cells (fibroblasts and glioblastoma cells) revealed that DNA damage was dose dependent, and more prevalent in the glioblastoma cells. This work goes on to identify that AgNPs were able to enter the nucleus, thus directly interacting with DNA. The mechanism of DNA damage was found to be dose dependent, and occurred in the Gap2/mitotic phase, with more damage occurring in glioblastoma cells [261]. In work with hepatoma cells, researchers showed that AgNPs induced expression of genes associated with cell cycle checkpoints before the mitotic phase, and also increased the expression of metallothionein genes [281]. Further, they showed that the addition of cysteine reduced cell death and the expression of genes associated with stress indicating that Ag⁺ ions released from the AgNPs play a role in toxicity. Ahamed
et al. [277] observed that AgNPs upregulated p53 expression, which is associated with cell cycle arrest; the DNA repair enzymes Rad51 and phosphor-H2AX were also increased in murine embryonic stem cells and embryonic fibroblasts, and that coatings on AgNPs may modulate damage further.

Proliferation of germ line stem cells has been shown to be reduced after exposure to AgNPs, via signals downstream of glial cell line-derived neurotrophic factor/fyn signaling [282]. Further, the antiproliferative activity of AgNPs has been demonstrated in human glioblastoma cells and fibroblasts that was found to be mediated by Ca$^{2+}$ flux, which in turn may have delayed apoptosis and also prevented proliferation [258]. When fibroblasts were exposed to increasing concentrations of Ag$^+$, cytotoxicity, as measured by ATP levels, DNA synthesis and total protein levels was increased. This effect was reversed by the addition of fetal bovine serum (FBS), likely due to the binding and sequestration of Ag$^+$ by serum proteins containing thiol groups, which bind metal ions with great affinity [283]. Proliferation was found to be reduced in mouse macrophages that had been exposed to 10 ppm of AgNPs for up to 72 hours, and the smallest AgNPs were found to be the most cytotoxic [257], while cells exposed to 1 ppm showed no cytotoxicity. Fibroblasts were found to undergo apoptosis via a mechanism that driven by ROS and JNK signaling. In this study, Bax, an apoptotic factor, and cytochrome c release were found in the mitochondria, which indicated that AgNP exposure triggered mitochondria dependent apoptosis [284]. These effects were attenuated and apoptosis was prevented when cells were treated with NAC, indicating that ROS was a primary factor in AgNP induced toxicity.
Several studies have investigated the proinflammatory profiles from cells that have been exposed to silver and AgNPs. It should be cautioned that silver ions and AgNPs can bind sulfur groups located on amino acids and proteins. This can interfere with protein function, and therefore may result in false negative results (personal observation). However, Carson et al. [264] observed significant increases in the release of TNF-α, MIP-2 and IL-1β from alveolar macrophages. Others did not find increases in IL-1, IL-6 or TNF-α in murine macrophages that had been exposed to AgNPs, regardless of particle size [257]. Proinflammatory cytokine induction was measured in human mesenchymal stem cells exposed to both AgNPs and Ag⁺, and increases of IL-8 but not IL-6 were found, indicating that subtoxic levels of AgNPs (2.5 μg/mL) can result in cell activation, and recruit other cells to the site of inflammation [283]. Interestingly, Wong et al. demonstrated that AgNPs exert an anti-inflammatory response [285], while another group also found that macrophages from human donors had a dose-dependent decrease in IL-5, IFN-γ and TNF-α after they were activated with phytohaemagglutinin, suggesting that AgNPs may be of use to prevent infiltrating inflammatory immune cells and promote wound healing [286].

While toxicity of AgNPs has began to be explored in mammalian cells, it is also important to understand the mechanism of toxicity in bacteria, particularly because of the escalating interest in their use as topical therapies. Levels of Ag⁺ and AgNPs that were found to be toxic to bacteria were also found to be toxic to keratinocytes and fibroblasts [287], which brings into question the safety of silver-based therapeutics. With the evolution of antibiotic resistant strains of bacteria, researchers are exploring the
possibilities that bacteria can also develop resistance to silver. Several studies have demonstrated the ability of bacteria to combat the toxic effects of AgNPs and Ag⁺. The origin of silver resistance has been traced back to nine genes which code for proteins that form a type of efflux pump [288], Figure 2.4. In Salmonella, silver resistance was found to be encoded by three RNAs: one that was a silver specific binding protein, and two efflux pumps [289]. Therefore, the use of silver for therapies must be closely monitored as to not repeat the current drug resistance dilemma.

Antimicrobial activity of AgNPs has been found to be size dependent. Panacek et al. [206] demonstrated that AgNPs of 25 nm had greater antibacterial activity than larger ones, and that these particles were capable of killing both Gram + and Gram – bacteria, although the concentration needed for biocidal action was varied. This work was supported in another study, where AgNPs the smallest (5 nm) particles had the best potential to be toxic, and this correlated well with the amount of intracellular ROS produced [290]. Interestingly, Gram - bacteria were found to be more sensitive to AgNPs compared to Gram + bacteria, and a time-dependent bactericidal effect was also revealed [291]. This was confirmed by Shrivastava et al. [292], who found that a dose dependent increase in cell death for Gram – bacteria that was antibiotic resistant or not, while the same trend was not observed for Gram + bacteria. The mechanism of death was found to be penetration of bacterial cell walls and stimulating the dephosphorylation of tyrosine residues on bacterial proteins essential for survival. Other studies confirmed this finding; S. aureus growth was not found to be significantly different from the controls, even at doses as high as 33 nM [244], however for E. coli and yeast a significant difference was
observed. The mechanism of toxicity was hypothesized to be due to the formation of ROS, since the addition of NAC prevented cell death. It should be noted that the difference in efficacy of AgNPs as antimicrobials may be attributed to the thickness of the cell wall, as Gram + bacteria have a thick peptidoglycan layer that can protect the organism from AgNP penetration. Additionally, AgNPs were shown to bind bacteria and damage the cell membrane. Studies in *E. coli* indicated that AgNPs adhere to, bind and penetrate cells and kill bacteria within 24 hours by degrading the membrane, and therefore compromising cellular equilibrium and membrane potential [293].

Research investigating the shape of AgNPs and their antibacterial activity found that triangular AgNPs had better antimicrobial activity than Ag⁺, spherical AgNPs or AgNP rods [294], and that this activity may be related to the percent of active facets on the particle.

The concurrent use of AgNPs and antibiotics were found to increase bactericidal efficiency. In studies by Shahverdi et al. [295], the presence of the antibiotics penicillin G, amoxicillin, erythromycin, clindamycin and vancomycin and AgNPs, the antibacterial activity was increased against *E. coli* and *S. aureus*, although the effect was more notable in *S. aureus*, likely due to the mechanism of action on peptidoglycan.

The mechanism of how AgNPs are toxic was analyzed via a proteomic approach. The mechanism of death was found to be consistent with cell membrane destabilization, as indicated by the increase in membrane protein precursors. Additionally, the levels of intracellular potassium and ATP significantly decreased, which indicated membrane deenergization, decreased proton motive force and destabilization. Other studies by this
group investigated how the oxidation status of AgNP surfaces affects their antibacterial activity [296, 297]. It was found that the bactericidal tendency was dependent on the chemisorptions of Ag\(^+\) on the surface; without oxidation of the AgNP surface, no antibacterial effects were observed [297]. Antibacterial activity is also found to decrease when AgNPs are exposed to high salt concentration, presumably due to the decrease in bioavailable Ag\(^+\), while stabilizing agents like BSA can prevent aggregation and preserve bactericidal activity.

The antibacterial activity of Ag\(^+\) has been hypothesized to be through ROS. Research showed that superoxide was generated by \textit{E. coli} strains containing reporter genes for the superoxide-sensor protein, under aerobic condition. This was confirmed by the lack of superoxide production in anaerobic conditions, confirming the role of ROS in cell death [298]. These investigators propose that ROS are generated via respiratory chain or antioxidant enzymes dysfunction caused by thiol/Ag\(^+\) interactions. Similarly, bacteria treated with Ag\(^+\) released from a washing machine were found to have reduced viability; however \textit{E. coli} was more susceptible than \textit{S. aureus}. The mechanism of cell death was found to be a result of the cell membrane perturbation and separation from the cell wall leading to the release of intracellular contents [299].

As noted previously, zeolites can be impregnated with silver with good antibacterial activity. The mechanisms by which the bactericidal effects are initiated are similar to those of AgNPs and Ag\(^+\). The benefits of using zeolites as silver carriers is that they can be designed to have time release capabilities and have the potential to be reused [214], which is beneficial for application such as drug delivery. Inoue et al. [300]
determined that the amount of dissolved oxygen available to zeolites containing silver was an important factor that affected bactericidal activity, as cell viability was reduced only under aerobic conditions. The mechanism of cell death was thought to be a result of the formation of ROS, since antibacterial activity was abolished in the presence of antioxidant proteins such as catalase, and other scavengers. A detailed study compared the mechanism of action of silver zeolite and AgNO₃. In this work, the inhibitory effects of several substances were tested to determine if bacterial cell death could be prevented during exposure to either silver zeolite or AgNO₃. The addition of cysteine, methionine, histidine, tryptophan, BSA and yeast extract all prevented bacterial death during exposure to silver zeolites, while the activity of AgNO₃ was inhibited by cysteine and histidine; manganese, magnesium and ferrous ions also prevented the toxic effects of AgNO₃ [301]. These studies also provide evidence that ROS induces cell death since catalase deficient bacterial strains were more susceptible the bactericidal effects of both silver zeolite and AgNO₃, supporting the possibility that both ions and ROS facilitate the antibacterial activity of silver zeolites.

The environmental and ecological data regarding the impact of AgNPs in aquatic and terrestrial systems is severely lacking. There have been several recent studies investigating the consequences of AgNP exposure to indicator organisms. An algal species, Chlamydomonas reinhardtii was exposed to AgNPs and AgNO₃ to determine whether the mechanism of toxicity was due to the release of Ag⁺ or direct interactions with AgNPs using photosynthesis as an endpoint. It was determined that free Ag⁺ was not the sole component of toxicity, and that algae may actually facilitate the dissolution of
AgNPs to Ag$^+$ under oxidative conditions [302]. In Daphnia magna, AgNPs were found to be far more toxic than AuNPs, and the investigators suggest that bimetallic formulation of AgNPs may reduce toxicity to freshwater organisms [303]. A study of zebrafish indicated that colloidal AgNPs resulted in significantly greater toxicity and increased malformation of embryos, compared to AuNPs of the same size, indicating that surface chemistry and reactivity is important in assessing the potential of ecotoxicity caused by AgNPs exposure [304]. This work is in agreement with another group who determined that zebrafish malformations occurred in a AgNP dose dependent manner [305]. Gaiser et al. [306] demonstrated the feasibility of AgNPs to be transferred between species by testing the ability of AgNPs to be taken up by fish gastrointestinal tracts and also via human intestinal epithelial cells. In addition, particle size was determined to be a factor affecting cell viability as AgNPs were more toxic than bulk silver. Fish cells exposed to increasing concentrations of silver nanospheres were found to have dose dependent cytotoxicity and genotoxicity, as was evident by significant increases in the formation of multinucleated cells and chromosomal aberrations [307]. Studies of uptake in Rainbow trout indicate the AgNPs can accumulate in fish gills and liver, as well as increase oxidative metabolism in these tissues [308]. Meanwhile, studies in trout hepatocytes indicate that AgNPs reduce membrane integrity and metabolic activity in a dose dependent manner [309]. Plants have also been shown to internalize AgNPs, and detrimental effects such as impaired cell division and chromosomal breaks [310], although the literature on this topic is lacking. It appears as though there are assay dependent differences with regard to the toxicity of AgNPs in plants [311].
In light of the literature reviewed in the section, it is evident that there is a serious need for additional research regarding the effects of AgNPs in not only mammalian systems, but also prokaryotic, aquatic, terrestrial and botanic models. The original research that is presented in the subsequent chapters of this thesis will attempt to build upon the data that has already been established, with specific emphasis on the mechanisms of silver zeolite mediated toxicity in *E. coli* and murine macrophages. This research will reveal similarities and differences in the susceptibility of organisms to AgNP exposure and their cellular responses.
Figure 2.1 Schematic of a quantum dot.
Figure 2.2 Nanoparticle intracellular compartmentalization and fate impacted by surface charge. Adapted from [99].
<table>
<thead>
<tr>
<th>TYPE</th>
<th>PRODUCTION PROCESS</th>
<th>CARBON SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thermal-oxidative decomposition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Open System-diffusion flames</td>
<td>Degussa gas black</td>
<td>Coal tar distillates</td>
</tr>
<tr>
<td>Closed System-turbulent flow</td>
<td>Furnace Black</td>
<td>Aromatic oil from coal tar, crude oil or natural gas</td>
</tr>
<tr>
<td></td>
<td>Lamp Black</td>
<td>Aromatic oil from coal tar or crude oil</td>
</tr>
<tr>
<td><strong>Thermal decomposition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous</td>
<td>Acetylene black</td>
<td>Acetylene</td>
</tr>
<tr>
<td>Discontinuous</td>
<td>Thermal black</td>
<td>Natural gas and oils</td>
</tr>
</tbody>
</table>

Table 2.1 Carbon black manufacturing processes.
Figure 2.3 Cellular interactions of nanoparticles.

A. Nanoparticle interacts with cell surface. B. Particle is internalized. C. Cell surface receptor activation. D. Upregulation of signaling proteins and activation of signaling pathways. E. Free particles (non-membrane bound) in cytosol. F. Nanoparticle interacts with mitochondria, respiration is compromised resulting in apoptotic or necrotic cell death. G. Free nanoparticle enters nucleus interacts with DNA resulting in genotoxicity and mutations, also increases expression of proinflammatory factors. Adapted from [133].
Figure 2.4 Silver resistance genes, transcripts and protein products.
Chapter 3: Biological Characterization and Internalization Mechanisms of Microwave Synthesized CdSe/ZnS Quantum Dots in Murine Macrophages

Introduction

The emergence of nanotechnology has ushered in many new and exciting applications that can be used to prevent, diagnose, and treat disease. The use of nanoparticles has already proved to be quite useful as demonstrated by the increase of FDA-approved treatments that have incorporated the use of nano-sized drug carriers. Quantum dots (QDs) are fluorescent semiconductor nanoparticles that have several advantages over traditional organic fluorophores. Because they are resistant to photobleaching, size tunable and have the ability to be multiplexed, they are already being used in both clinical and basic sciences as tools for microbial detection [46, 48-50], bioimaging [59, 79, 312], drug delivery [76, 80, 313] and tracking [53, 57, 61, 75]. Nanoparticles have been used to quantify cytokines at physiological levels [39], correlate levels with disease conditions and have promising potential in prevention of disease progression and predicting cancer metastasis [40, 41]. One of the most interesting and effective applications of QDs is their use in imaging. Because of their size and robust fluorescence, they can be used in single molecule tracking [56, 57, 61, 75, 314]. Cancer cell lines were among the first live cells that were successfully imaged [58, 59], and QDs have been used to visualize receptor interactions, specifically erb2/HER receptor mediated signal transduction [60] and neuronal glycine receptors [61].
Although QDs have great promise for use in the medical field, they are costly to produce and there can be variance between batches. As a result, many approaches have been investigated to improve QD synthesis. Improvement in aqueous QD solubility was demonstrated by the incorporation of phosphate [18] and thiol stabilizers [19, 20], that also allows for conjugation to proteins and other molecules. Recently, we described an efficient and reproducible protocol for QD synthesis from aqueous solution that yielded highly fluorescent, biocompatible QDs [84].

One of the main obstacles of QD use in human subjects is that most contain materials, such as cadmium (Cd) and selenium (Se) that can be toxic in large quantities, at least in bulk form. Cadmium has the tendency to bioaccumulate and is difficult to remove from the body; chronic exposure to cadmium can result in kidney and liver dysfunction and potentially death. Studies have been conducted that investigate the cytotoxicity of QDs with particular focus on QDs with Cd cores. In 2004, Shiohara et al. showed differences in CdSe QD toxicity in Vero cells, HeLa cells and human primary hepatocytes was concentration and time dependent [104]. Size dependent cytotoxicity in rat pheochromocytoma cells and murine microglial cells was confirmed by Lovric et al. [105] using CdTe quantum dots. The mechanism of QD cytotoxicity in MCF-7 breast cancer cells has been attributed to the formation of reactive oxygen species (ROS) [106]. Others [110] showed that QD internalization by hippocampal neurons resulted in calcium deregulation and sodium channel disruption, both of which can affect the role of ROS mediated protective responses. QDs containing shells and caps are less toxic because they prevent core material from leaching into the cell, but further investigation is needed to
resolve the mechanism of QD induced cytotoxicity. QDs have been shown to have a decrease in fluorescence and can aggregate when placed in acidic conditions [315] suggestive of reduced stability. Few studies investigate proinflammatory responses related to QD exposure, however, one study determined that human primary monocytes upregulated TNF-α and CXCL-8 in response to ROS generated from QD exposure [114].

The mechanism by which non-targeted nanoparticles enter cells is not well understood and there is evidence that multiple pathways may be used. One recent study reported that commercially available QDs can use scavenger receptors and lipid raft-mediated endocytosis for cellular entry [82]. Modified LDL and negatively-charged inorganic nanoparticles have the propensity to bind scavenger receptors which supports the role of these receptors in QD uptake. The intracellular destination of QDs has been shown to range from the cytoplasm and nucleus [85, 316] to lysosomes [82].

Because the chemistry of QD synthesis is continually changing, it is difficult to understand and assess the practicality and risks associated with using QDs in biomedical sciences. In order to thoroughly understand the consequence of nanoparticle exposure, it is essential to properly characterize nanomaterials intended for applications in biological systems. Cytotoxicity, inflammatory properties and other cellular responses caused by nanoparticles should be analyzed prior to widespread biomedical application of these nanomaterials (i.e. conjugation to drugs or biomolecules). In this communication, we characterize nanoparticle and cellular interactions using QDs as a model. Here, we tested aqueous CdSe/ZnS QDs prepared using a rapid, microwave-based protocol to determine cytotoxicity and induction of proinflammatory cytokines by macrophages following
exposure. We also investigated cellular interactions with QDs, including association with cell surface scavenger receptors, mechanism of internalization and intracellular fate.

Experimental Methods
Quantum dot synthesis and chemical characterization (zeta potential, atomic absorbance and UV/Vis analyses) were performed by Andrew Zane and Michael Severance at Ohio State University Analytical Chemistry labs.

Chemicals for QD Synthesis
Cadmium chloride hemipentahydrate, CdCl₂ · 2.5 H₂O (≥ 98%), and sodium borohydride, NaBH₄ (99%), were purchased from Aldrich. Zinc chloride, ZnCl₂ (99.99%), mercaptopropanoic acid (MPA), and Se powder (>99.5%, 200 mesh) were obtained from Acros. Sodium hydroxide, NaOH, and ammonium hydroxide, NH₄OH (28-30%), were purchased from Mallinckrodt Chemicals. All chemicals were used without further purification. The H₂O used for QD synthesis was purified by a Barnstead NANOpure Infinity ultrapure water system.

Quantum Dot Preparation
Negatively charged COO-QDs were prepared using our previously published protocol [84]. Briefly, fresh solutions of NaHSe were prepared for each QD batch by mixing NaBH₄ with H₂O followed by the addition of Se powder under an inert environment created by constant flushing of N₂ gas through the sample. The components
reacted for ~3.5 hours and then supernatants containing NaHSe were diluted to 20 mM using N$_2$-saturated H$_2$O. A solution of Cd-MPA was created by mixing MPA in H$_2$O with 5.0 mM CdCl$_2$ · 2.5 H$_2$O stock solution and adjusting the pH to 9.5 using 1 M NaOH and held at room temperature until needed. Lastly, a 60 mM Zn(NH$_3$)$_4^{2+}$ stock solution was prepared by dissolving ZnCl$_2$ in H$_2$O and titrating with NH$_4$OH, and was stored at 4°C. Nucleation of CdSe dots occurred when NaHSe stock was injected into Cd-MPA solution. This mixture was stirred for one hour prior to adding Zn(NH$_3$)$_4^{2+}$ stock solution. These contents were then microwave irradiated (MARS5 microwave system, CEM Corp.) for 90 minutes at 150 °C.

Chemical Characterization

*Spectroscopic Measurements*

All absorption measurements were obtained using a Shimadzu UV-2501PC UV-visible spectrometer. All emission measurements were obtained using a Horiba Jobin Yvon Fluorolog 3 fluorimeter.

The as-prepared quantum dot solution (930 nM, concentration calculations were done using extinction coefficient of $2.9 \times 10^6$ cm$^{-1}$M$^{-1}$ at 488 nm) was diluted 10:1 with purified water and placed in a quartz cuvette to measure the optical spectra. The excitation wavelength used for the emission spectrum was 375 nm, and all slits were set for a 2.5 nm band-pass.

To obtain a quantum yield measurement, the quantum dot solution was compared to a solution of rhodamine 6G. Both solutions were diluted to have an absorbance of
0.020 at a wavelength of 480 nm. At this wavelength and concentration, rhodamine 6G is known to have a quantum yield of 95% [84]. The peak emissions of both samples were measured with 480 nm excitation, and the quantum yield calculated.

To determine possible time-dependent leaching of cadmium at a pH of 4.5, solutions of quantum dots (final concentration 466 nM) were adjusted to this pH using 1 M hydrochloric acid, and centrifuged at different times (0.5 hours, 3.5 hours, 11.5 hours, 24 hours) to stop further dissolution. The samples were centrifuged using a Thermo-Fisher Scientific MX-150 ultracentrifuge at 150,000 RPM for 1 hour, and the emission of the supernatant indicated that there were no QDs in solution. The cadmium concentration was determined using a Perkin Elmer 3100 atomic absorption spectrometer. A linear calibration curve was made using standard solutions of 0.05, 0.30, 0.50, 1.00, and 2.00 ppm Cd\textsuperscript{2+}. The samples were diluted 10:1 using purified water to place them within the calibration curve.

Size and Charge Measurements

Titrations of zeta potential and size vs. pH were obtained using a Malvern Zetasizer Nano, and an attached Malvern MPT-2 auto-titrator. Zeta potential measurements were made using the method of phase analysis light scattering. Measurements were conducted at 25°C at a forward scattering angle of 15°. Zeta potentials were calculated from the measured electrophoretic mobility using the Henry equation with a value of 1.5 for Henry’s constant according to the Smoluchowski approximation. This approximation is consistent with the high dielectric constant of water.
Measurements were conducted at 25°C with a backscattering angle of 273°. Dynamic light scattering results were analyzed using several algorithms. The data presented were analyzed using the constrained regularization method, CONTIN [317]. A quartic weighting scheme was used for multimodal analysis. CONTIN analysis provided the distribution of particle radii based on scattering intensity. The relative number distribution was then obtained by transformation of the results according to Mie theory and the known properties of the scattering material.

Cell Culture

The murine alveolar macrophage cell line (MH-S) was purchased from the American Type Culture Collection (Manassas, Va), and propagated using RPMI cell culture media (Gibco) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 0.1% penicillin streptomycin (Gibco), herein referred to as complete media. Cells were passaged every 3-4 days and incubated at 37°C with 5.0% CO₂. Cells were acclimated to these conditions for at least 12 hours prior to QD exposure.

Biological Characterization and Internalization Mechanistic Assays:

Cytotoxicity and ELISAs experiments

Cells were plated in 24 well plates at a density of 1 x 10⁵ cells/well. QDs were diluted to concentrations of 4, 25, 100 or 500 nM in complete cell culture media and added to wells for 24 hours after old media was aspirated. Cells treated with 1% Triton-100 (Sigma) served as a positive control for lactate dehydrogenase (LDH) release assays.
while cells treated with *E. coli* lipopolysaccharide (Sigma) served as the positive control for tumor necrosis factor-alpha (TNF-α) and Interleukin-1Beta (IL-1β) ELISAs. Supernatants were collected from each well and clarified by centrifugation at 16,000 RCF for 2 minutes to pellet QD aggregates and cell debris. Supernatants were transferred to clean microfuge tubes and stored at -80°C until analysis for cytokine secretion or LDH release. All experiments were performed at least 3 times.

**LDH cell viability assays**

Lactate dehydrogenase is a stable enzyme confined to the cytoplasm in healthy cells. The release of LDH into surrounding media indicates a breach in the plasma membrane, and therefore can be used as an indicator of cell viability. Supernatants were pipetted in duplicate into flat bottom 96-well plates and assayed for LDH activity in duplicate using kits from Clontech per manufacturer’s instructions. Optical densities (ODs) representing enzyme activity was measured colorimetrically using a BMG LabTech Omega plate reader at an absorbance wavelength of 490 nm and a reference wavelength of 690 nm. Cell viability was calculated using the following equation:

\[
\% \text{ Cell Death} = \frac{\text{mean OD experimental wells} - \text{mean OD negative control}}{\text{mean OD positive control} - \text{mean OD negative control}} \times 100
\]

**ELISAs**

TNF-α and IL-1β secretion was measured via ELISAs using DuoSets purchased from R&D Systems according to the manufacturer’s recommendations. Supernatants were assayed in duplicate and plates were read using a BMG LabTech Omega plate.
reader at an absorbance wavelength of 450 nm with a correction wavelength of 570 nm. Experimental sample concentrations were calculated with Omega software using a four parameter logistic standard curve.

Flow Cytometry

All flow cytometry experiments were performed in triplicate. Cells were seeded in 6-well plates (Corning) at a density of \(1.5 \times 10^6\) cells/well and allowed to adhere overnight prior to QD exposure. Flow cytometry staining procedures are described below.

Sytox Red Staining

Media was aspirated and macrophages were then incubated with increasing concentrations of QDs suspended in complete cell culture media (ranging from 62.5 nM to 500 nM) for 24 hours after which they were washed 3 times with sterile phosphate buffered saline (PBS) and harvested with trypsin. Cell concentration was adjusted to \(1 \times 10^6\) cells/tube and suspended in 1 mL of Hank’s Buffered Saline Solution (HBSS, Gibco) prior to adding 1 µL (5 nM) of Sytox Red (Invitrogen). Cells were incubated at room temperature for 15 minutes and analyzed using flow cytometry (FACScalibur, BD Biosciences) with an excitation wavelength of 488 nm for QD detection and 635 nm for Sytox Red detection.

Annexin V Staining

Cells were exposed to no QDs or concentrations of 62.5 to 500 nM in complete culture media for 24 hours. Cells were then washed with PBS three times prior to being harvested by trypsinization. Cell concentration was adjusted to \(1 \times 10^6\) cells per tube, and
were resuspended in 100 μL Annexin binding buffer (Invitrogen); 5 μL of Annexin V-APC (BD Biosciences) was then added to each tube. Tubes were incubated in the dark at room temperature for 15 minutes before an additional 400 μL of binding buffer was added. Cells were analyzed for fluorescence using a FACScalibur flow cytometer at an excitation wavelength of 488 nm for detection of QDs and 635 nm for Annexin-APC.

**Scavenger Receptor Competition Assay**

Media was removed and replaced with fresh media containing 100 μg/mL of Poly-inosinic acid (Poly I), a non-specific scavenger receptor ligand, for 30 minutes. Fresh media containing 100 μg/mL Poly-I containing 62.5 nM to 500 nM QDs was then added to cells. Untreated cells served as controls. Cells were incubated for 6 hours then harvested and analyzed for fluorescence by flow cytometry using a FACScalibur flow cytometer at an excitation wavelength of 488 nm.

**Endocytosis Inhibition**

Cells were pretreated for 30 minutes with fresh media containing 50 μM of chlorpromazine, a clathrin inhibitor, or 50 μM nystatin, a caveolae inhibitor. All inhibitors were purchased from Sigma and diluted to working concentration using PBS. Untreated cells served as controls. After pretreatment, media was removed and replaced with media containing the appropriate inhibitor with no QDs, 25 nM, 75 nM or 150 nM QDs. Cells were incubated with or without inhibitor and with or without QDs for 2 hours, then washed 3 times with PBS. Cells were then harvested by trypsinization, suspended in HBSS and analyzed by flow cytometry using a FACS calibur flow cytometer at an excitation wavelength of 488 nm.
Live Cell Imaging

Cells were plated on glass coverslips situated in 45mm petri dishes at a density of 5 x 10^4 cells/cover slip and incubated overnight. Prior to live cell imaging and QD addition, cells were incubated with serum-free X-VIVO media (Lonza) containing 25 nM of LysoTracker Red (Invitrogen) for at least 15 minutes. QDs (final concentration of 75 nM) were added following 3 minutes of image collection. Images were acquired sequentially at an excitation wavelength of 442 nm for QD detection and 561 nm for lysotracker detection every 6 seconds for a total of 20 minutes. In additional experiments, cells were incubated with LysoTracker Red and QDs for 45 minutes prior to image collection. Images were acquired using a Hamamatsu C9100 EM-13 camera connected to a Visitec Infinity3 2D array scanning multibeam confocal scanner and an IX81 microscope equipped with a 100x, 1.40 N.A. UPlanApo objective lens. Movies were constructed using MetaMorph software (Molecular Devices) using image stacks that were background subtracted with the statistical correction option of the “Background and Shading Correction” application. Out of focus images were removed prior to production of movies.

Colocalization analysis of raw data image stacks was performed using MetaMorph (Correlation Plot plugin). The correlation coefficient (r) measures the correlation between intensities of corresponding pixels in two images (the red and green channels). The correlation coefficient calculation can return a range of values from -1.0 to +1.0 where a value of +1.0 shows the data are perfectly correlated and a value of -1.0 shows an inverse relationship of the pixel values between the two images.
Statistical Analysis

Comparisons between QD treatments were analyzed for statistical significance using One-way ANOVA performed with SigmaPlot software.

Results

QD Characteristics

The microwave-based synthesis procedure for the QDs used in this study has been reported previously [84]. These QDs can be best described as containing a CdSe core with CdS intermediate shell followed by a ZnS outer shell. 3-Mercapatopropionic acid (3-MPA) capped off the QDs, as well as being the source of sulfur for the QDs. Transmission electron microscopy indicated a particle diameter of ~5 nm.

Figure 3.1a shows the characteristic absorption and emission spectra of QDs (93 nM) used in this study. The emission maximum is at ~560 nm, and quantum yields of optimized preparations ranged from 13-20%.

Figure 3.1b shows the particle sizes (931 nM) and zeta potential (186 nM) of the QDs as a function of pH. The zeta potential sample was diluted using supernatant from centrifuged QD solutions suspended in PBS buffer. The size was obtained from dynamic light scattering using the CONTIN fit followed by a number size distribution, as described in the literature [318]. The hydrodynamic diameter is relatively constant between pH 7-11 and averages out to ~9.4 nm. This size shows a marked increase below
pH~6, indicating aggregation. 3-MPA has a pK\textsubscript{a} of 4.38 [319], and as protonation of the carboxylate groups proceeds, aggregation of the QDs occurs [320]. The zeta potential is negative reflecting the surface MPA groups on the QD. There is a gradual increase in the zeta potential between pH 11 to 6 (-40 to -35 mV) followed by a more pronounced increase (-35 to -29 mV) between pH 6 to 5, consistent with protonation of the MPA.

The QDs were incubated at a pH of 4.5 in water (466 nM) under ambient conditions, and the concentration of free Cd\textsuperscript{2+} in solution was determined by atomic absorption spectrometry. Concentration of Cd\textsuperscript{2+} increased from 98 µM after one hour of incubation to 133 µM after 24 hours at pH 4.5.

Internalization and Intracellular Localization of QDs

We tested whether QDs associate with scavenger receptors by performing a competition assay using Poly-I, a non specific scavenger receptor ligand. To determine if QDs were taken up by cells in the presence or absence of Poly-I, flow cytometry analysis was performed. When cells were incubated with 100 µg/mL Poly I, a clear reduction in fluorescence is seen (Figure 3.2). Because association with cells was not completely inhibited following the addition of Poly-I (Figure 3.2), we hypothesized that QDs are associating with additional cell surface receptors. There are several mechanisms that facilitate the endocytosis of receptors after they have been engaged, including internalization through caveolae and clathrin coated pits. To determine which of these serves as a mechanism of QD internalization, we used an inhibitor approach to block internalization of QDs. Prior to the addition of QDs to cell cultures, macrophages were
pretreated with cytochalasin D (inhibits actin polymerization and phagocytosis), nystatin (binds cholesterol and disrupts caveolae coat structure) or chlorpromazine (prevents clathrin recycling). Neither nystatin treated cells nor cytochalasin D treated cells displayed a reduction in fluorescence after exposure of cells to QDs suspended in the corresponding inhibitor. However, in cells treated with chlorpromazine, fluorescence is significantly reduced, indicating that these QDs enter cells primarily using clathrin coated pits (Figure 3.3). To determine the location of QDs and further confirm the clathrin pathway following entry, we used live cell confocal imaging. Indeed, we observed QDs localizing to the cell membrane within minutes (time (t) = 2:47) after QDs were introduced to the culture, followed by their appearance in intracellular vesicles (t = 8:54) and mature vesicles at t = 11:57 and t = 17:26 (Figure 3.4a-e). We stained acidic organelles using Lysotracker Red to visualize the late endosomal pathway and were able to localize the QDs to the lysosomes within 20 minutes of their introduction into live cultures. The image presented in Figure 3.4f is representative of QD/lysosomal colocalization after 45 minutes of incubation. We quantified the amount of colocalization in this cell over time and QDs were found to have a correlation coefficient (r value) of 0.576521697 +/- 0.065927, indicating that the majority of QDs are retained in acidic compartments. Colocalization of QDs and lysotracker is consistent with transport through the endosomal pathway to the lysosomes.
Cytotoxicity and Proinflammatory Response

To determine if our QDs induce cytotoxicity or apoptosis in murine alveolar macrophages, we incubated cells with increasing concentrations. Here, we used concentrations above the necessary loading level needed to illuminate the cells to determine if QD-mediated cytotoxicity could be detected. Cells analyzed by flow cytometry after 24 hours of QD exposure show a non dose-dependent increase in membrane permeability, as indicated by Sytox Dead Red nuclear staining. An increase of approximately 10% labeled cells is observed compared to the untreated cell population. (Figure 3.5a). Membrane damage after 24 hours of exposure was examined further by enzymatic LDH analysis of media-derived supernatants to measure LDH release. Minimal LDH activity was observed after exposure to 250 nM QDs, as demonstrated by where less than 5% of cells demonstrated increased membrane permeability compared to unexposed macrophages. However, cells treated with 500 nM QDs exhibited significantly more (p < 0.001) membrane damage compared to the other three doses, with 25% cell death (Figure 3.5b). We demonstrate that flow cytometry is a more sensitive test for determining membrane permeability at lower exposure concentrations, perhaps because individual cells are analyzed whereas LDH is an indirect and collective measurement of membrane permeability.

To determine if our QDs induced an apoptotic response in these cells, Annexin V staining was performed after a 24 hour exposure. Macrophages treated with 250 nM QDs or less were found to have between a 5 to 15% increase in the number of apoptotic cells compared to the unexposed QDs, while cells treated with 500 nM were found to have
over 3.5 times (41.7%) as many apoptotic cells, indicating that the mechanism of cytotoxicity is apoptosis (Figure 3.6). It should be noted that the QD concentrations required to visualize a cytotoxic response were 12.5 to 25 fold higher than concentrations necessary for cellular visualization [84].

To assess the potential of QDs to elicit inflammatory responses, levels of cytokines TNF-α and IL-1β in supernatants of QD-exposed macrophages were measured by ELISAs. TNF-α and IL-1β were chosen for these experiments because they are common indicators of inflammation and are produced by macrophages. Supernatants derived from cells incubated with QDs for short periods (2 hours) did not result in proinflammatory cytokine induction (data not shown). However, a slight, yet significant dose dependent increase in TNF-α secretion was observed after cells were exposed for 24 hours (Figure 3.7). TNF-α levels range from undetectable for untreated cells and cells treated with 62.5 and 125 nM QDs to 23.75 ± 3.84 pg/mL for supernatants from cells treated with 250 nM. Supernatants from cells treated with 500 nM show an increase of 33.19 ± 8.03 pg/mL of TNF-α. These same supernatants were also analyzed for IL-1β, which was undetectable (data not shown). Macrophages treated with 1 μg/mL of lipopolysaccharide alone resulted in TNF-α levels greater than 500 pg/mL indicating that these cells are capable of activation. These results demonstrate that QDs do not induce a significant proinflammatory response (less than 6.125 pg/mL), except after very large doses of QDs.
Discussion

QDs have been synthesized using a number of capping agents including polyethylene glycol (PEG) [321], dihydrolipoic acid [322], mercaptoacetic acid [323] and MPA [84, 106, 108] in order to improve biocompatibility. Several groups have investigated the role of scavenger receptors and their affinity to negatively charged molecules [98, 137, 324, 325], and although QDs have been successfully bioconjugated [83, 326, 327], their non-specific uptake mechanism in macrophages has not been thoroughly investigated. Small, thioglycolic acid capped CdTe QDs (2.1 nm) were found to be internalized by macrophages within 10 minutes, and localized to the nucleus and perinuclear space, while larger CdTe QDs (3.4 nm) localized within cytoplasmic compartments after 30 minutes [85]. Murine macrophages treated with carboxylated QDs were found to internalize QDs within 30 minutes, while cells treated with NH$_2$-PEG coated QDs internalized QDs more slowly; organic QDs were not able to be detected within the cells [315].

To our knowledge, we are the first to identify the mechanism of uptake of carboxylated QDs via scavenger receptor mediated endocytosis in murine macrophages and visualize their compartmentalization in real time. In the presence of poly-inosinic acid, a non-specific scavenger receptor ligand, we found fluorescence was reduced, indicating that QDs associate with scavenger receptors (Figure 3.2). However, there are clearly other receptors that participate in QD binding, since cell fluorescence reduced by a factor of ten, and not eliminated altogether in the presence of this inhibitor. Researchers have shown that negatively charged inorganic materials associate with scavenger
receptors [97, 98]. Zhang et al. [82] reported that scavenger receptors are involved with QD internalization in keratinocytes. The scavenger receptor MARCO (macrophage receptor with collagenous structure) was found to have a positively charged basic cluster containing several arginines [95]. The negative zeta potential at pH ~7.4 (-37 mV) would make the QDs attracted to positively charged clusters on the receptor molecules. Another possibility is that since the QDs were suspended in media containing FBS, serum proteins can bind QDs, and this association can influence cell entry, and receptor interaction. These studies suggest that the charge and electrostatic properties of QDs may play a role in their internalization mechanism.

It is clear that in our system, QDs primarily enter macrophages via clathrin coated pits since incubation with a clathrin inhibitor significantly decreased QD uptake (Figure 3.3). The fact that QD internalization was prevented by blocking the clathrin mediated endocytic pathway indicates that receptor-mediated endocytosis is the primary route of entry for QDs. However, we cannot determine if the QDs are directly associating with the receptors or are entering the cells through protein adsorption and subsequent receptor engagement. Work investigating non-specific QD uptake in tumor cell lines revealed that carboxylated QDs were internalized via receptor-mediated endocytosis and clathrin coated pits [328]. In other studies, negatively charged QDs were found to enter keratinocytes via lipid rafts and little interaction with clathrin was observed [82]. These differences may be attributed to the fact that macrophages have a large number of clathrin coated pits and other cells, such as fibroblasts and endothelial cells have been found to
have a greater number of caveolae [329]. Therefore, we conclude that the mechanism of QD endocytosis may be cell specific.

For the first time, we capture macrophage internalization and compartmentalization of QDs in real time. Carboxylated QDs interact very quickly with cell membranes and are internalized in minutes. Other research has also indicated that negatively charged QDs enter cells rapidly [315, 328]. Clift et al. [315] reported that macrophages internalized QDs within minutes and Zhang et al. [82] demonstrated that upon internalization, the QDs are both vesicle bound and localize to lysosomes in keratinocytes. We simulated the change in QD properties as it moves from a neutral to acidic environment of the endosome/lysosome by titrating QDs (Figure 3.1b). As the carboxyl groups become protonated, QDs aggregate and their zeta potential becomes less negative, consistent with previous studies [82]. Thus, the QDs in the lysosomes (pH ~4.5) are definitely aggregates. We were also able to colocalize QDs and lysosomes trafficking throughout the cell in real time (Figure 3.4).

Since we have confirmed and established a mechanism for QD internalization in macrophages, we also sought to determine the QD loading levels that macrophages could withstand. Data from our group and others [82, 84] indicate that QD concentrations as low as 20 nM are easily detected within cells. In this report, we tested doses up to ~25x that (500 nM) to determine the concentration at which our QDs initiate cell death. When macrophages were incubated with large concentrations of QDs (500 nM), greater than half of the cells were found to be apoptotic. We conclude that, for this macrophage cell line, QD exposure becomes toxic between 250 and 500 nM and the mechanism of cell
death is consistent with apoptosis due to positive Annexin V staining. While the exact
pathway that triggered apoptosis was not specifically addressed in this report, Lovríc et
al. [106] determined the mechanism of MPA-coated QD cytotoxicity (1-10 μg/mL) in
MCF-5 breast cancer cells was due to the formation of reactive oxygen species (ROS),
perhaps caused by the disintegration of the QD coating. Chan et al. [107] further
established that ROS generated in neuroblastoma cells following exposure to QDs with a
CdSe core (150 to 300 nM) induced apoptosis via activation of JNK mediated signaling.
These investigators also showed that cell death could be prevented by capping CdSe QDs
with a ZnS shell. QDs made with CdSe cores (10 μg/mL) were found to be less cytotoxic
than CdTe QDs, although cytotoxicity of CdTe QDs could not be prevented by capping
[108]; however the addition of thiol-capped shells has been shown to stabilize Cd core
QDs [330]. It has been proposed that mechanisms of QD cytotoxicity (187.5 nM to 3
μM) are due to both the formation of ROS and Cd²⁺ leaching, but the addition of a CdS
shell reduces toxicity which can be further abrogated by the addition of a ZnS shell [331].
Others corroborate this finding, in particular one group found that CdS QD cytotoxicity
(10 – 80 μg/mL) is dependent on size and the mechanism of toxicity shifts from ROS
driven to Cd²⁺ dependent as concentration is increased [109]. It is established that
cadmium related ROS generation can induce apoptosis at 10 to 50 μM [332]. The
dissolution of QDs at acidic pHs within the lysosome is thus a source of Cd²⁺. Elemental
analysis indicates concentrations of 133 μM Cd²⁺ from a 466 nm QD solution at pH 4.5
within a 24-hour period, well above the level required to induce apoptosis.
We next sought to determine if our QDs initiated a proinflammatory cytokine response. We determined that although there is a dose dependent increase in TNF-α release in cells treated with very large doses of QDs (250 or 500 nM), these levels were minimal compared to other proinflammatory molecules. Little research has examined the upregulation of proinflammatory cytokine in response to QD exposure. One study by Lee et al. [114] found that QDs (0.2 – 25 nM) induced the upregulation of TNF-α and CXCL-8 via ROS formation, while another study demonstrated that cadmium alone (50 μM) resulted in upregulation of IL-8 in intestinal cells, however, TNF-α and Interferon-γ levels remained unchanged [333]. It must be stressed that the variability in cell responses, including cytokine secretion, can be cell type and species dependent. However, in this cell line, we conclude that exposure to carboxylated QDs results in a small, but significant, proinflammatory response.

A model of macrophage responses to aqueous CdSe/ZnS QDs is presented in Figure 3.8. We have defined a route of QD internalization beginning with cell surface association with scavenger receptors, followed by internalization via a clathrin mediated pathway, and ending with rapid compartmentalization within acidic organelles (Figure 3.8). We have also reported that our QDs are not cytotoxic at relevant doses and do not illicit a pronounced proinflammatory cytokine response in macrophages. Advancements in the field of QD synthesis has yielded a protocol that is efficient, simple and inexpensive to synthesize. However, defining the mechanism by which nanoparticles enter cells is also important because it can not only provide insight into the material’s toxicity and cellular responses, but also can be used to tailor their synthesis for targeting.
intracellular compartments. As such, these findings can be used towards the overall biological characterization of nanoparticles and their interactions in mammalian systems.
Figure 3.1 Chemical Characterization of QDs.

A.) Absorption and emission spectrum of QDs (QD concentration = 93 nM.) Excitation wavelength for emission spectrum was 375 nm. B.) Plots of QD radius and zeta potential as a function of pH. Average diameter between pH 7 and 10.8 is 9.4 ± 4.4 nm. QD concentration for size titration was 931 nM, and for zeta potential 186 nM.
Figure 3.2 QDs associate with scavenger receptors.

A reduction in the number and intensity of fluorescent cells is observed when co-treated with scavenger receptor ligand Poly-I (100 μg/mL) after 6 hours, indicating that Poly I can effectively compete with QDs for these receptors.
Figure 3.3 Clathrin-coated pits serve as one mechanism of QD internalization.

Cells treated with chlorpromazine (50 μM) have a significant reduction in QD uptake compared to untreated cells, indicating that clathrin coated pits participate in the internalization mechanism of our QDs.
Figure 3.4 QDs are quickly compartmentalized within lysosomes.

A.) Macrophages stained with Lysotracker red just prior to addition of QDs. B.) QDs form a halo around the outside of the cell within 2 minutes. C.) Yellow vesicles indicative of colocalization are apparent after ~ 9 minutes. D.) QDs are clearly in lysosomes by 12 minutes. E.) Vesicles begin to track in the cell by 17 minutes post exposure. In Panel F, macrophages were incubated with Lysotracker followed by the addition of 150 nM QDs (final concentration =75 nM) for 45 minutes prior to imaging. The majority of QDs are found within lysosomes. R is the correlation between QDs and Lysotracker.
Figure 3.5 QD induced cytotoxicity in murine alveolar macrophages.

A.) Macrophages were exposed to QDs for 24 hours and membrane permeability was assessed via flow cytometry. A slight non-dose dependent increase in permeability was observed among cells treated with the QDs compared to the negative control. Figure is representative of three independent experiments. B.) Macrophage membrane permeability was measured by quantifying LDH release into supernatants after 24 hours of exposure. LDH levels for cells treated with 500 nM was significantly higher compared to the other treatments (p < 0.001).
Figure 3.6 QDs induce apoptosis in macrophages within 24 hours of exposure.

Macrophages were incubated with increasing concentrations of QDs and apoptosis was measured via flow cytometry. The number of apoptotic cells nearly doubled for concentrations up to 250 nM, while the number of apoptotic cells increased more than 3.5 times after incubation with 500 nM QDs. Figure is representative of three independent experiments.
Figure 3.7 QDs stimulate TNF-α secretion from macrophages after 24 hours of exposure.

Macrophages were incubated with increasing concentrations of QDs and TNF-α levels in supernatants were analyzed via ELISA. A significant increase in TNF-α levels (denoted by *) was found in cells treated with 250 or 500 nM QDs compared to the control (p < 0.001), although these amounts are negligible compared to known proinflammatory stimuli.
Figure 3.8 Model of QD/macrophage interactions.

1.) QDs associate with cell surface receptors (scavenger receptors) within 2 minutes
2.) QDs begin to be internalized via clathrin coated pits around 9 minutes
3.) QDs found within acidic compartments at 12 minutes
4.) Endosomes and lysosomes fuse with QDs at ~18 minutes
5.) Majority of QDs within lysosomes by 45 minutes
Chapter 4: Murine Alveolar Macrophage Responses to Modified Carbon Nanoparticle Exposure

Introduction

Manufactured carbon nanoparticles are used for many applications including additives in rubber, and as pigments for inks and paint. They possess increasing tensile strength and surface area with decreasing size, which makes them attractive candidates for plastic reinforcement. The manufacture of carbon nanoparticles has increased in recent years and there is concern for occupational exposure to engineered nanoparticles [334]. In addition, the combustion of diesel leads to the formation of complex particulate matter that ranges in size depending on vehicle use [122, 123] and exposure can occur in an occupational setting and to the general public. Poor air quality has been linked to increased risk for cardiopulmonary diseases and mortality [8, 335-337]. The most common route of carbon nanoparticle exposure is through inhalation, and carbon nanoparticles can be found deep in the respiratory track, including the alveoli [338]. The degree of cytotoxicity and the pulmonary inflammatory potential of carbon nanoparticles have been correlated to its surface chemistry. The emission of carbon nanoparticles into the atmosphere provides an opportunity for interaction with other elements including transition metals and gases which adds to the complexity of understanding the mechanism of pulmonary toxicity and inflammation after exposure. The adsorption of polycyclic aromatic hydrocarbons (PAH) and metals on particulate matter during the
combustion process can further enhance associated cellular responses including granulocyte macrophage colony-stimulating factor (GM-CSF) release, NF-κβ activation and TNF-α production in macrophages and bronchial epithelial cells [161, 162, 174, 339]. Human monocyte-derived macrophages were found to undergo an oxidative burst upon treatment with carbon nanoparticles containing iron, but not carbon alone [340]. Macrophages were found to undergo ROS dependent apoptosis after exposure to diesel emission particles (100 μg/mL), and cell death was reduced when macrophages were incubated with particles that had their organic content extracted [341]. Nano-sized carbon based air pollutants were found to have increased PAH content compared to PM$_{2.5}$, and PAH levels were positively correlated with levels of iron [342]. Human alveolar epithelial cells (A549s) were found to have more multinucleated and apoptotic cells when treated with a 80:20 mix of petroleum diesel emission particles:biodiesel emissions particles at 25 μg/mL, compared to a 20:80 mixture [158]. Accordingly, supernatants derived from type II alveolar epithelial cells treated with 125 μg/mL carbon black nanoparticles were found to increase the mobilization of macrophages, indicating that carbon nanoparticles can promote a proinflammatory environment and recruit granulocytes to the afflicted area to aid in particle clearance [343]. On the other hand cytotoxicity was correlated with the ability of nanoparticles to generate reactive oxygen species rather than their PAH content, when A549 cells were treated with 5 μg/mL of carbon black [179].

Atmospheric ozone has been found to have confounding effects on the inflammatory potential and toxicity of carbon nanoparticles. A recent study correlated
the mortality of individuals from cardiovascular causes and PM$_{2.5}$ and the mortality of individuals from respiratory causes and ozone levels; however there was not an effect of ozone on the risk of death from cardiovascular causes when considering the concentration of PM$_{2.5}$ [344]. Murine models in vivo indicate that concurrent exposure to carbon nanotubes and ozone results in an attenuated pulmonary inflammatory response [190]. However, others have found that humans exposed to 300 μg/m$^3$ ambient PM$_{10}$ followed by ozone exposure (200 ppb) 5 hours later exhibited a significant influx of neutrophils and macrophages after 24 hours [186]. Human lung epithelial cells were found to secrete significantly more IL-8 in response to DEP (100 μg/mL) after pre treating the cells with ozone (500 ppb) [188]. This work was supported by Madden et al. [345] where DEP particles were treated with ozone (100 ppb) and then instilled into rats (1 to 500 μg/mL) were found to illicit significant influxes of neutrophils and increased lactate dehydrogenase (LDH) into bronchioalveolar lavage fluid. Interestingly, this study also examined the effects of ozonation on carbon black and found that there was not a significant change in LDH activity when particles were exposed to ozone prior to instillation. In contrast, DEP, which has greater organic content, was found to induce increased inflammation and LDH levels indicating that the organic content may not be important in driving the toxic responses to carbon black [345].

Different species and cell types can elicit variable responses to nanoparticle exposure. Therefore, in these experiments, we exposed both human monocyte-derived macrophages (MDMs) and murine alveolar macrophages (MH-S) to several types of modified carbon nanoparticles. As such, the aim of these studies was to determine if
modified carbon nanoparticles induce cytotoxicity and proinflammatory cytokine secretion in murine alveolar macrophages, and, in some cases, human monocyte-derived macrophages.

Experimental Methods
Carbon Nanoparticle Modification, Fourier Transmission Infrared spectrometry (FTIR) and X-Ray Photoelectron Spectroscopy (XPS) was performed by Brian Peebles at Ohio State University Analytical Chemistry labs.

Carbon Nanoparticle Modification:

*Carbon nanoparticles impregnated with iron*

Flammruss 101 (DeGussa) was wet impregnated with 0.1 to 10% iron by weight as iron (II) acetate (>95%, Acros Organics) by weighing 100 mg of carbon and the proper weight of iron salt in sparged water under nitrogen and adding them to a glass tube in a glove bag. The sealed tube and vacuum line apparatus was vortexed and sonicated for 10 minutes, still under nitrogen, and the water was allowed to evaporate under vacuum under room temperature.

*Carbon nanoparticles modified with benzo(a)pyrene*

Flammruss 101 was modified with 97% pure benzo(a)pyrene (B(a)P, Sigma) by first purifying B(a)P through a silica gel column. B(a)P was dissolved at a concentration of 4 mg/10 mL in reagent grade dichloromethane (Sigma). Flammruss was activated under vacuum at 600°C, and a suspension of B(a)P was added under N₂ such that the
final particles contained 1% B(a)P by weight. Excess dichloromethane was evaporated under vacuum at room temperature.

*Diesel emission particles (DEP)*

DEP was recovered from the exhaust pipe of a diesel-powered truck.

*Ozonated carbon*

To ozonate carbon nanoparticles (CNP + O₃), approximately 0.5 grams of Flammruss 101 were loaded into a burette plugged at the bottom and at the top with glass wool. The bottom of the burette was connected to the outlet of an Enaly EOZ-300Y corona discharge ozone generator (Ozone Solutions, Hull, Iowa). The generator was supplied with a flow of approximately 100 mL/minute compressed air, which was just enough to start suspending the particles in the gas flow. Ozonation was performed for 4 hours at ambient temperature.

*Carbon nanoparticle ozone treatment in cell culture plates*

Flammruss 101 was suspended at a concentration of 2 mg/mL in anhydrous methanol (99.9%, Alfa Aesar) by vortexing and sonication. Nanoparticles were then loaded in triplicated into individual wells of a sterile 24-well tissue culture at concentrations of 2.5, 5 or 10 μg/cm². Residual methanol was allowed to evaporate prior to ozonation. Three plates were prepared; one plate was not ozonated prior to macrophage addition, one plate was ozonated for 4 hours followed by ageing overnight prior to macrophage addition, and the third plate was ozonated for four hours and macrophages were immediately added. Ozone exposures were performed using an Enaly EOZ-300Y corona discharge ozone generator at a flow of approximately 100 mL/minute.
compressed air that had been passed through a hydrocarbon trap and a desiccant. Macrophages were suspended in X-VIVO 15 serum-free media (Lonza) and added to each well at a cell density of 2.5 x 10^5 cells/mL. Negative control particle-free wells were treated with 20 μL of methanol alone.

Carbon Nanoparticle Chemical Characterization

*Electron Paramagnetic Resonance (EPR)*

Flammruss alone or exposed to O_3 as solids or suspended in either deionized water, Dulbecco’s PBS (Invitrogen), complete cell culture media or X-VIVO 15 phenol red-free media (Lonza) were analyzed for free radical content using EPR. Samples were added to quartz capillary tubes and free radical content was measured on a Bruker EMX X-band spectrometer. Flammruss was treated with ozone for 1 hour and stored in a nitrogen atmosphere prior to EPR analyses. The EPR spectrometer was operated at the X-band at 2 mW, with a receiver gain of 2 x 10^1 for all samples.

*Fourier Transmission Infrared spectrometry (FTIR)*

Flammruss alone, Flammruss that was exposed to ozone and allowed to aerate over time as well as freshly ozonated Flammruss was evaluated for changes in surface functionality using FTIR. A Perkin-Elmer Spectrum 400 FTIR spectrometer was fitted with a Pike Technologies 100 mm gas cell with calcium fluoride windows. Flammruss samples were deposited onto the windows after they were suspended in acetone to achieve a thin layer of particles that would not obstruct the infrared laser. Ozone was introduced to flammruss by sealing the cells and connecting the inlet of the gas cell to the
outlet of the ozone generator. Infrared spectra were collected before ozonation, after ozonation, after purging the cell for 15 minutes with air, and after leaving the window with the carbon film overnight in ambient conditions.

Since the carbon films absorbed strongly across the infrared spectrum, the blank spectrum taken using the cell filled with air, was multiplied by an attenuation factor before subtraction, so that bands that appeared in the spectrum of the empty cell were completely eliminated from the carbon spectrum. Attenuation factors ranged from 0.7 to 0.8. Blank-subtracted spectra of the untreated carbon samples were smoothed by 37-point boxcar averaging, and then the baselines were subtracted using multipoint linear baseline correction. Difference spectra of carbon samples that have been exposed to ozone were taken by first subtracting the blank-subtracted spectra of untreated carbon (without any mathematical manipulation of the raw data other than attenuation factor) and then 37-point averaging and correcting the baseline using multipoint linear correction.

**X-Ray Photoelectron Spectroscopy (XPS)**

X-ray photoelectron spectroscopy was performed on Flammruss after impregnation with iron (II) acetate and before and after ozonation using a Kratos Ultra Axis spectrometer with a monochromatic Al kα source using Kratos Vision 2 software. The CasaXPS software was used for deconvolution and data analyses. The manufacturer’s recommended setting of the binding elemental C 1s peak to 284.8 eV to calibrate the energy positions. The Fe 2p regions of carbon particles impregnated with iron were examined to speciate the iron on the surface. High resolution carbon spectral
measurements were made by performing a rough fit manually, and then performing five fitting iterations using the Marquardt algorithm.

Cell Culture

Human Peripheral Blood

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque (Histopaque, Sigma) density gradient centrifugation from buffy coats purchased from the American Red Cross as previously described [346]. Because complete donor anonymity is a strict condition of this arrangement, no Institutional Review Board human subjects protocol is required, as specified by the NIH and Ohio State University IRB guidelines. To promote monocyte differentiation into the macrophage phenotype, PBMCs were aspirated from the plasma/Histopaque interface, washed three times in phosphate buffered saline solution (PBS, buffered to pH 7.4), suspended in RPMI 1640 (GIBCO) supplemented with 10\% pooled normal human serum and 0.1\% penicillin/streptomycin. Suspended cells were transferred to Teflon plates where they were incubated for 5-7 days at 37°C in a humidified atmosphere of 5\% CO₂/95\% air [347]. The cells were then transferred to Optilux 96-well microtiter plates (Falcon) for luminol assay or 24-well or 6-well plates for particle exposures prior to cytokine measurements or gene arrays, and incubated 24 h prior to removal of nonadherent cells as previously described [162]. Monocyte-derived macrophages (MDM) prepared in this manner routinely marked 90-95\% positive for CD14 with undetectable levels of CD3⁺ T cell contamination as determined by immunofluorescence flow cytometry.
**Murine Macrophages**

The murine alveolar macrophage cell line (MH-S) was purchased from the American Type Culture Collection (Manassas, Va), and propagated using RPMI cell culture media (Gibco) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 0.1% penicillin streptomycin (Gibco), herein referred to as complete media. Cells were passaged every 3-4 days and incubated at 37°C with 5.0% CO₂. Cells were acclimated to these conditions for at least 12 hours prior to QD exposure.

**Luminol Assay**

The luminol assay was used to measure oxidative burst kinetics in response to modified carbon nanoparticles. The following components were added to each of triplicate wells containing human monocyte-derived macrophages: 100 μL serum free culture medium, 100 μL luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, sodium salt, Sigma) for a final concentration of 0.5 mM and carbon modified nanoparticles at concentrations ranging from 5 to 20 μg/cm². For experiments using ascorbate as a reducing agent, a 10 mM stock solution of ascorbic acid (Sigma) dissolved in ddH₂O and diluted to make 0.1 mM and 1 mM solutions. Particulate suspensions were prepared by adding 10 mg of particulates to 5 mL ascorbate solution for a stock concentration of 2 mg/mL, which was further diluted to 0.25 mg/mL using PBS. Cells were exposed to particulate suspensions at a concentration of 10 μg/cm² for 30 minutes with gentle shaking at room temperature. Plates were centrifuged at 750 x g for 20 minutes and supernatants were replaced with PBS prior to analysis. For one experimental group, supernatants were replaced with 1 mM ascorbate solution (no particles). Zymosan
(78.125 \( \mu g/cm^2 \)) was added to triplicate wells containing 100 \( \mu l \) each of media and luminol, and served as a positive control for oxidative burst. A set of triplicate control wells containing MDMs, culture medium, and luminol, but no stimulant, were also included in each experiment. For experiments containing ascorbate treatment as a reducing agent, all reagents were maintained at 4\(^\circ\)C and plates were kept on ice during addition of reagents. Luminescence was measured immediately on a Top Count Scintillation and Luminescence counter (Packard). The plates were then incubated for 5 to 10 minute intervals at 37\(^\circ\)C in a humidified atmosphere of 5% CO\(_2\)/95% air, and recounted. This process was repeated for up to 120 minutes. Luminescence indices for each time point were calculated by dividing the mean luminescence counts per minute (cpm) of 3 replicate treated wells by the mean cpm of 3 negative control wells.

**Cytotoxicity and ELISAs experiments**

Cells were plated in 24 well plates at a density of 1 x 10\(^5\) cells/well. Modified carbon nanoparticles were suspended in PBS and added to confluent macrophage murine macrophage monolayers at a concentration ranging from 2.5 to 20 \( \mu g/cm^2 \). Cells treated with 1% Triton-100 (Sigma) served as a positive control for lactate dehydrogenase (LDH) release assays, while cells treated with *E. coli* lipopolysaccharide (Sigma) served as the positive control for tumor necrosis factor-alpha (TNF-\(\alpha\)). Supernatants were collected from each well and clarified by centrifugation at 16,000 RCF for 2 minutes to pellet uninternalized carbon nanoparticles. Supernatants were transferred to clean microfuge tubes and stored at -80\(^\circ\)C until analysis for cytokine secretion or LDH release. All experiments were performed at least 3 times.
**LDH cell viability assays**

Lactate dehydrogenase is a stable enzyme confined to the cytoplasm in healthy cells. The release of LDH into surrounding media indicates a breach in the plasma membrane, and therefore can be used as an indicator of cell viability. Supernatants were pipetted in duplicate into flat bottom 96-well plates and assayed for LDH activity in duplicate using kits from Clontech per manufacturer’s instructions. Optical densities (ODs) representing enzyme activity was measured colorimetrically using a BMG LabTech Omega plate reader at an absorbance wavelength of 490 nm and a reference wavelength of 690 nm. Cell viability was calculated using the following equation:

\[
\text{% Cell Death} = \frac{\text{mean OD experimental wells} - \text{mean OD negative control}}{\text{mean OD positive control} - \text{mean OD negative control}} \times 100
\]

**ELISA**

TNF-α secretion was measured via ELISAs using DuoSets purchased from R&D Systems according to the manufacturer’s recommendations. Supernatants were assayed in duplicate and plates were read using a BMG LabTech Omega plate reader at an absorbance wavelength of 450 nm with a correction wavelength of 570 nm. Experimental sample concentrations were calculated with Omega software using a four parameter logistic standard curve.
Gene Expression Microarrays

Human monocyte derived macrophages were cultured as stated above. Cells were seeded in 6-well plates (Corning) and allowed to adhere overnight, washed to remove nonadherent cells. Prior to carbon nanoparticle exposure, fresh RPMI containing 10% pooled human serum and 0.1% penicillin streptomycin was added to each well. Cells were exposed to modified carbon nanoparticles for 6 hours. Uninternalized particles were washed way using PBS. Ribonucleic acid (RNA) was harvested using the Trizol method [348]. Prior to gene expression hybridization, cDNA was synthesized by creating an annealing mixture containing 1-3 μg total RNA and a proprietary buffer and adding it to a cDNA mastermix containing RNase free H₂O, cDNA synthesis buffer, RNase Inhibitor, and cDNA enzyme mix (all from SuperArray) and incubating for 50 minutes at 42°C followed by 75°C for 5 minutes and cooling to 37°C. A mixture of RNA polymerase buffer (SuperArray), biotinylated UTP (Sigma) and RNA polymerase enzyme (SuperArray) was prepared to synthesize, label and amplify cRNA from the cDNA. This mix was added to the tube containing cDNA and incubated for 4 hours at 37°C. Prior to use in the gene expression arrays, RNA was purified using the SuperArray cRNA cleanup kit, where cRNA was transferred to a microfuge tube and combined with 315 μL lysis and binding buffer (SuperArray) and 315 μL reagent grade ethanol (Sigma). Samples were loaded onto individual spin columns and centrifuged at 8,000 x g for 30 seconds. Flow through was removed and 600 μl washing buffer was applied to each column and centrifuged at 8,000 x g for 30 seconds. Flow through was again removed and an additional 200 μl of wash buffer was added and samples were centrifuged at 11,000 x g
for 1 minute. Flow through was removed and samples were spun at 180° of their previous orientation for 2 minutes at 11,000 x g. cRNA was eluted from the column by collecting samples in fresh tubes after incubating the column with 25 ul of 10 mM Tris buffer (pH 8) for 2 minutes and centrifuging the column for 1 minute at 8,000 x g. RNA was quantified using 1 μL on a NanoDrop (Thermo Scientific). Gene array membranes were prehybridized in tubes by adding 5 mL deionized water for 5 minutes. Hybridization solution (Superarray) was heated to 60°C, water was removed from the tubes and replaced with 2 mL of hybridization solution. The tubes were then placed in clean glass hybridization tubes and prehybridized in a hybridization oven for 1 hour at 60°C with continuous agitation. Old hybridization solution was decanted and 0.75 mL of hybridization solution containing labeled cRNA (1-3 μg/tube) was added to the membranes and allowed to hybridize overnight at 60°C with continuous agitation. A wash solution (Solution 1) containing 2X sodium citrate/sodium chloride buffer (SSC) and 1% sodium dodecyl sulfate (SDS) and a wash solution (Solution 2) containing 0.1x SSC and 0.5% SDS were prepared. After hybridization was completed, the target hybridization mix was removed and 5 mL of Solution 1 was added to each tube and membranes were agitated in the hybridization oven at 60°C for 15 minutes. The solution was discarded and replaced with 5 mL of Solution 2, and tubes were incubated at 60°C for 15 minutes. The solution was removed and gene array tubes and membranes were cooled to room temperature. Blocking solution (2 mL) was added to each tube and agitated at room temperature for 40 minutes. Diluted alkaline phosphatase-conjugated streptavidin was prepared in Buffer F (SuperArray) at a dilution of 1:8000 and 2 mL was
added to each membrane and agitated for 10 minutes. The membranes were then washed 4 times for 5 minutes each with 4 mL of Buffer F. The membranes were then rinsed 2 times with Buffer G (SuperArray), and 1 mL of CDP-Star chemiluminescent substrate was added to each tube. Membranes were exposed to film (Kodak) to acquire binding activity. Data was analyzed using GEArray Expression Analysis Suite.

Flow Cytometry

All flow cytometry experiments were performed in triplicate. Cells were seeded in 6-well plates (Corning) at a density of 1.5 x 10^6 cells/well and allowed to adhere overnight prior to carbon nanoparticle exposure at 20 μg/cm^2. Flow cytometry staining procedures are described below.

**MARCO Fite staining**

To determine if interaction with Flammruss 101 alone or modified with benzo(a)pyrene (B(a)P) resulted in upregulation of the scavenger receptor MARCO, murine macrophages were either pretreated with lipopolysaccharide or not for 3 hours prior to exposure to particles at 20 μg/cm^2 for 6 hours. Treated cells were washed 3 times with PBS and gently harvested with cell scrapers. Cells were suspended at a concentration of 1 x 10^6 cells/tubes in 100 μL. Cells were blocked using 0.5 mg/mL mouse Fc block (BD Pharmingen) for 5 minutes prior to adding 10 μg/mL of Fite-conjugated mouse anti-MARCO antibody (Affinity Bioreagents) for 20 minutes on ice, in the dark. Cells were washed 2 times with PBS and resuspended in 500 μl of PBS prior to flow cytometric analyses using a FacsCalibur (BD Biosciences) at excitation wavelength
of 488 nm. Control cells were prepared exactly the same, except that the isotype control 
Fitc-conjugated rat-IgG 1 (BD Pharmingen) was used in place of MARCO antibody.

Statistical Analysis

Comparisons between carbon nanoparticles and TNF-α levels and cytotoxicity 
were analyzed for statistical significance using One-way ANOVA performed with 
SigmaPlot software.

Results

Chemical Characterization of Carbon Black Nanoparticles

Carbon was modified with iron (II) acetate and the surface elemental composition 
was investigated using XPS; the spectrum and peak assignment table was adapted from 
[349] and is shown in Figure 4.1. The amount of iron (II) and iron (III) on the surface at 
the Fe 2p spectral region was found to be 42% (711.4 eV binding energy) and 58% 
(713.0 eV binding energy), respectively. Several satellite peaks were fitted but not 
quantified due to lack of sensitivity factors and applicable iron species.

To identify changes in surface functionality after exposure to ozone, the C 1s and 
O 1s spectral region was examined using XPS. The spectra from Flammruss, ozonated 
and aerated Flammruss and freshly ozonated Flammruss and their deconvolution peak 
data (Figure 4.2, Table 4.1) represent C-C and C-H (284.8 eV), hydroxyl/ether C-O 
(285.4 eV), carbonyl C=O (287.6 eV), carboxyl O-C=O (288.6 eV) and π → π* (290.9 
eV) [349]. The O:C ratio increased 5.7- fold (0.009 to 0.051) when comparing 
Flammruss and freshly ozonated Flammruss. The surface O:C ratio between Flammruss
and ozonated, aerated Flammruss increased 6.0-fold (0.009 to 0.054). Notably, the relative area of the $\pi \rightarrow \pi^*$ transition decreased from 14.5% to 5.66% immediately after ozone exposure compared to Flammruss. The ozonated, aerated Flammruss $\pi \rightarrow \pi^*$ transition decrease was slightly less (5.81%). The carbonyl and carboxyl relative area was increased from 1.76% to 2.95% and 1.64% to 2.39%, respectively when comparing Flammruss to freshly ozonated Flammruss, while aerating ozonated Flammruss results in a slightly higher relative area of carboxylated carbon (3.71%) and return of carbonyl relative area back to baseline measurements (1.66%) [349].

Changes in the FTIR spectrum were examined after Flammruss was freshly ozonated and compared to spectrum obtained after Flammruss was ozonated and aerated with compressed air in ambient conditions overnight are shown in Figure 4.3. Changes in infrared spectral branches found between 1700 and 1800 cm$^{-1}$ were associated with a gas phase species were detected between aerated and freshly ozonated Flammruss. An acid anhydride band found at 1770 cm$^{-1}$ was still evident after aeration, indicated surface functionality changes that may have been masked by gas phase interference. Accordingly, a band found at 1734 cm$^{-1}$ associated with carboxylic acid was also present after aeration, indicated surface functionality changes that may have been masked by gas phase interference. A change in a band found at 1592 cm$^{-1}$, which corresponds with structural carbon, was evident. In accordance with XPS spectra, there was an increase in the band found at 1280-1285 cm$^{-1}$, which is associated with carbonyl/ether functionality.

The surface free radical content of Flammruss and ozonated Flammruss was investigated using electron paramagnetic resonance. In Figure 4.4A, Flammruss alone
and after ozonation give strong peaks, with ozone causing the peak to shift to a slightly higher g factor [349]. However, when these samples were suspended in water, the ozonated Flammruss had an attenuated EPR signal (Figure 4.4B). To determine the persistence of free radical content of ozonated Flammruss in biological media, particles were suspended in PBS, RPMI + FBS or X-Vivo serum-free media. All three conditions supported the detection of free radicals on ozonated Flammruss (Figure 4.4C). The persistence of free radical content in Flammruss and freshly ozonated Flammruss suspended in serum-free media was investigated over time. Free radicals were stable and persistent over a period of 75 minutes for both conditions, as seen in Figure 4.5 and 4.6, respectively. The concentrations were different between the two particles so signal intensity cannot be compared.

Human Macrophage Responses to Modified Carbon Nanoparticles

Modified carbon nanoparticles were incubated with primary human macrophages and the luminol assay was conducted to determine if the particles could induce an oxidative burst within a biological systems. The top graph in figure 4.7 revealed that macrophages exposed to 20 μg/cm² carbon nanoparticles impregnated with either 10% weight Fe (II), or 10 μg/cm² of carbon nanoparticles impregnated with less iron (0.1, 0.5, or 1%) had little impact on luminescence (luminol index ranging from ~1 to 2.5 ) compared to zymosan stimulated cells (luminol index peaking at ~60). The bottom graph in Figure 4.7 revealed that cells treated with 20 μg/cm² carbon nanoparticles impregnated with 10% iron (II) had a luminol index 1.5 times greater than cells treated with 10
μg/cm² carbon nanoparticles impregnated with 0.1 to 1% iron (II), although this trend was not statistically significant. Macrophages exposed to 10 μg/cm² carbon nanoparticles impregnated with 10% iron (II) suspended in increasing concentrations of ascorbate that was washed before oxidative burst was measured also had low measurements of oxidative burst compared to zymosan stimulated cells (Figure 4.8, top). Cells that were treated with particles that did not have the particles washed had luminol indices that remained at 2.5 for 100 minutes before a decline was observed (Figure 4.8, bottom). Luminol indices ranging from ~1.5 to 2.5 and peaked after 10 minutes and returned to baseline by 110 minutes post exposure (Figure 4.8, bottom). Comparisons in luminescence were made between 2 macrophage donors exposed to 5 μg/cm² of Flammruss, Flammruss impregnated with 10% iron (II), Flammruss treated with B(a)P and DEP. Both donors exhibited an oxidative burst (luminol index ~60) when stimulated with zymosan, but there was little activity upon treatment with particles (Figure 4.9A and B, top panels). Donor A had little change in luminol index when treated with 5 μg/cm² carbon nanoparticles (Figure 4.9A, bottom); Donor B had a small increase in luminol index to ~2.5 when treated with 5 μg/cm² Flammruss + B(a)P, although this increase was not statistically significant (Figure 4.9B, bottom panel). When cells from these same 2 macrophage donors were treated with 20 μg/cm² carbon nanoparticles, Donor A again had little change in luminol index (Figure 4.10A); after 10 minutes of exposure, Donor B had a slight increase in luminol index to ~3 when treated with 20 μg/cm² Flammruss + B(a)P (Figure 4.10B).
Four individual macrophage donors were exposed to 0 to 10 μg/cm² Flammruss impregnated with iron (II) for 24 hours. ELISA analysis for the proinflammatory cytokine TNF-α revealed no significant difference in protein secretion; error bars represent standard deviation (Figure 4.11). Previously, Waldman et al. [162] determined that TNF-α does not adsorb to the surface of carbon, so the levels of cytokine present in supernatants of exposed cells appears to be an accurate measurement for proinflammatory cellular responses. Donor variations appear to play a significant role in cell responses to modified carbon nanoparticle exposure. The error bars in Figure 4.11 are large, so an experiment was conducted to determine levels of TNF-α secretion from 2 donors after exposure to 20 μg/cm² Flammruss, Flammruss + B(a)P and Flammruss + Fe (as iron II). A clear difference between the donors’ resting TNF-α secretion level was noted as donor A had TNF-α levels at ~100 pg/mL, while donor B was greater than 300 pg/mL. Donor B exhibited a decrease in TNF-α secretion after treatment with modified carbon nanoparticles, and treatment with Flammruss + Fe reduced TNF-α levels to nearly 0 pg/mL (Figure 4.12). The opposite effect was observed for donor A, where Flammruss and Flammruss + B(a)P exposed cells displayed little increase in TNF-α secretion above baseline, but cells treated with Flammruss + Fe secreted greater than two times the amount of TNF-α (over 200 pg/mL) after 6 hours (Figure 4.12).

To simulate a model where individuals are exposed to modified nanoparticles like those found in air pollution during a respiratory illness, human macrophages from 3 individual donors were pretreated with lipopolysaccharide (LPS, 1 mg/mL) prior to exposure to 80 μg/cm² LPS + Flammruss for 8 or 24 hours. Unstimulated macrophages
and cells stimulated with LPS alone or Flammruss alone served as controls. In Figure 4.13, donor A macrophages had TNF-α levels that were greater than 1000 pg/mL when resting, or treated with LPS or both LPS and Flammruss. Treatment with Flammruss only attenuated the amount of TNF-α secreted into supernatants after 8 and 24 hours by approximately 200 pg/mL. Donor B had resting TNF-α levels of 400 pg/mL, and LPS stimulation increased TNF-α by ~100 pg/mL. Adding Flammruss alone did not change TNF-α levels, however, for cells concurrently treated with LPS and Flammruss, nearly double the amount of TNF-α was found in supernatants after 8 hours of exposure. Similar results were observed for donor B after 24 hours of exposure except that cells treated with LPS only released similar levels compared to cells treated with both LPS and Flammruss (~700 pg/mL). Treatment with Flammruss alone did not increase TNF-α above untreated cells (Figure 4.13). Donor C exhibited a yet another different response after exposure. After 8 hours of treatment, resting macrophages released 800 pg/mL of TNF-α. LPS stimulation, treatment with Flammruss alone or in conjunction with LPS actually decreased TNF-α release by approximately 100 pg/mL, 650 pg/mL and 350 pg/mL, respectively (Figure 4.13). After 24 hours, macrophages from donor C that were treated with LPS had ~900 pg/mL TNF-α compared to untreated cells (~650 pg/mL). Cells treated with Flammruss alone exhibited a reduction in TNF-α secretion by 550 pg/mL, and cells treated with both LPS and Flammruss were also found to have less (~250 pg/mL) TNF-α in supernatants (Figure 4.13).

Macrophages from 3 individual donors were subjected to proinflammatory cytokine transcription analyses after treatment with 20 μg/cm² Flammruss or Flammruss
+ B(a)P. Only 1 factor, complement component 3 (C3) was found to be consistently upregulated when comparing gene expression between the three donors. C3 was upregulated 2.5-fold in donor A, 3.3-fold in donor B and 1.8-fold in donor C (Figure 4.14). Upregulation in TNF-α transcription was variable and donors B and C exhibited an increase in TNF-α gene expression.

Murine Alveolar Macrophage Responses to Modified Carbon Nanoparticles

Due to the large variability in cell responses in human macrophage experiments, a murine alveolar cell line was used to understand interactions and responses of cells exposed to modified carbon nanoparticles.

Inorganic particles have been shown to associate with scavenger receptors, specifically macrophage receptor with collagenous structure (MARCO). Flow cytometry was performed to determine the basal expression of MARCO on mouse macrophages. Figure 4.15 shows that serum components compete with MARCO antibody for receptor binding sites. However, to ensure that this was not an artifact due to Fc receptor binding, cells were blocked prior to antibody addition. Figure 4.16 revealed macrophages stimulated with LPS exhibited similar amounts of MARCO expression compared to control cells. However, it appears that MARCO expression may be slightly decreased when cells are exposed to 20 μg/cm² Flammruss or Flammruss + B(a)P for 6 hours compared to untreated cells and LPS stimulated cells (Figure 4.16).
Freshly ozonated Flammruss suspended in PBS prior to introduction to macrophages cultured in serum-free X-Vivo media appeared to be better dispersed compared to unmodified Flammruss and Flammruss that was ozonated and aerated for 24 hours (Figure 4.17); cells internalize irrespective of treatment. Untreated cells had resting levels of approximately 40 pg/mL of TNF-α in supernatants after 24 hours. TNF-α levels were increased 2 to 2.5 fold when cells were exposed to Flammruss alone and Flammruss that was ozonated and aerated and this response trended toward dose dependence. Cells exposed to freshly ozonated Flammruss secreted significantly less (p < 0.01) TNF-α compared to unmodified Flammruss and aerated Flammruss (Figure 4.18B). Macrophages exposed to supernatants that were conditioned with Flammruss alone or ozone modified Flammruss did not secrete significantly higher levels of TNF-α, indicating that surface modification on freshly Flammruss may drive this reduction (Figure 4.18A). Figure 4.3.19 demonstrated that freshly ozonated Flammruss did not adsorb TNF-α. These experiments were performed at sub-toxic concentrations of carbon nanoparticles, and freshly ozonated Flammruss decreased cytotoxicity in macrophages, although this decrease was not statistically significant (Figure 4.3.20).

Discussion

Modified carbon nanoparticles are formed during manufacturing and natural processes. Flammruss 101, a 90 nm carbon black nanoparticle formed through incomplete combustion of oil, is commonly used in pigments and as rubber additives. Carbon black has low solubility and toxicity. We confirmed that toxicity of unmodified
Flammruss is low and that modification with ozone did not increase toxicity, but rather appeared to decrease the level of cytotoxicity in murine alveolar macrophages. Most carbon black nanoparticle toxicity is associated with lung overload.

The very process of Flammruss manufacture results in persistent free radicals on the surface of carbonaceous nanoparticles. The source of free radicals on carbon nanoparticles has been identified as unpaired electrons that are the result of hydrogen subtraction from carbon. In the current study, solid Flammruss alone and freshly ozonated Flammruss were examined for free radical content. A shift in unpaired electron spin was noted for Flammruss particles that were treated with ozone compared to unmodified Flammruss. Peña et al. reported that free radical content of carbon black decreased with increasing oxygen content and when particles were heat treated [350].

When carbon samples were suspended in water, ozonated Flammruss EPR signal was attenuated compared to unmodified Flammruss (Figure 4.4B). Persistent free radicals were observed after ozonated Flammruss was suspended in different biological media. The inherent nature of free radicals found on the surface on Flammruss may contribute to the release of proinflammatory cytokines in response to carbon nanoparticle exposure in this murine alveolar macrophage cell system. Macrophages incubated with Flammruss alone and Flammruss exposed to ozone and aerated responded similarly. A two-fold increase in TNF-α secretion was observed for both particles, while freshly ozonated Flammruss particles did not elicit an increase in TNF-α secretion above baseline. Because macrophages exposed to preconditioned supernatants collected from each type of particle did not change transient TNF-α levels and particles were not found to adsorb TNF-α
protein (Figure 4.18A and 4.19), particles themselves must be stimulating the cells to secrete TNF-α. The difference in inflammatory response among the particles is hypothesized to be due to changes in the oxidative state of modified Flammruss particle surfaces. Chemical analysis of unmodified and modified Flammruss revealed an increase in carboxyl groups present on the surface of freshly ozonated Flammruss (3.71%) compared to aerated Flammuss (2.39%). It appears that this change is due to the formation of carbonyl groups after exposure to ambient air. Carbonyl species are associated with oxidative stress in cells, and protein carbonylation is an initial change that occurs under oxidative stress that targets proteins for degradation and limits enzymatic function [351]. It is unknown whether the increase in carboxyate groups found on freshly ozonated Flammruss prevents cytokine secretion, however carboxylation may be the driving factor that improved ozonated Flammruss dispersion in cell culture.

Ozone alone is a powerful oxidizing agent and causes pulmonary inflammation and toxicity via oxidation of proteins and lipids. Studies investigating the concomitant exposure of carbon nanoparticles and ozone together are limited. One study found that ozonating carbon black did not result in increased proinflammatory factors in instilled rats [189]. The ozone level that Flammruss was exposed to in the current study may have reduced any biological potency of reactive byproducts, and it is possible that Flammruss regained it inflammatory potential after such surface groups equilibrated. However, there are several studies that suggest ozonation can create a phenomenon known as oxidative preconditioning, and actually be anti-inflammatory. Studies in rats undergoing ischemia/reperfusion events in kidneys were found to have significantly reduced levels of
TNF-α, IL-1β and ICAM expression and fewer apoptotic cells if preconditioned with ozone 15 days prior to ischemia/reperfusion [191]. Exposure to ozone can boost endogenous antioxidants and regulate reactive oxygen species, which results in a protective environment during trauma. It has been well established the respiratory tract cannot tolerate ozone and exposure results in pulmonary hypersensitivity [352]; however the blood is better equipped with antioxidants (glutathione, ascorbate, etc.) [353], and has been successfully used as a therapeutic for conditions such as gangrene, bed sores and abscesses. It is unclear why murine alveolar macrophages exposed to freshly ozonated Flammruss particles did not respond with an increase in proinflammatory cytokine secretion, although cell culture conditions can contain components that augment that ozonated particle surfaces. Aside from a possible change in antioxidant expression, ozonated particles seemed to be better dispersed. The chemical functionality was slightly different among the particles and this may have impacted the mechanism of particle internalization and, therefore the cellular response.

The expression of MARCO, a scavenger receptor that has been shown to interact with environmental particles [96-98, 139], was evaluated on murine alveolar macrophages. Cells treated with LPS were found to express MARCO, however the media components were found to play a role in the detection of MARCO expression. Cells cultured in normal cell culture media did not express MARCO after LPS treatment, while cells cultured with serum-free X-Vivo media were stained positive for MARCO. One explanation for this phenomenon is that serum proteins bind MARCO and compete with antibody for binding sites. On the other hand, serum proteins may interact with LPS
and may have prevented cell interaction and the expression of MARCO. Murine alveolar macrophages were found to constitutively express MARCO, and treatment with 20 μg/cm² Flammruss or Flammruss + B(a)P in the presence and absence of LPS did not increase MARCO, but rather slightly decreased expression. From these experiments, it was concluded that exposure to both unmodified and modified Flammruss may decrease scavenger receptor expression. This decline may result in a decline in particle uptake and susceptibility to bacterial infections. Other researchers have found that cigarette smoke (which contains particulates and B(a)P) decreased MARCO expression in a human monocyte cell line and also significantly impaired *Mycoplasma pneumoniae* clearance [354]. In the current study, it appears that carbon nanoparticles decreased MARCO expression and this may ultimately result in decreased clearance in pulmonary pathogens or other particulate clearance. As such, the interaction of carbon nanoparticles with specific cell surface receptors is an area that warrants further investigation. Lastly, it has been found that there is variability between mouse strain MARCO expression and understanding differences between strains can potentially be used to extrapolate reasons for pollution sensitivity in different human populations.

Large degrees of variation were discovered in monocyte-derived macrophages derived from individual donors. Gene expression analysis revealed differential gene expression of proinflammatory cytokines including IL-12, TNF-α and complement protein 3. Several donors’ macrophages had little response to exposure to unmodified Flammruss, while others had more substantial cytokine expression. One protein that was consistently upregulated when exposed to Flammruss + B(a)P was C3. Researchers have
shown that complement mediates the TH2 response which is associated with allergies as reviewed in [355]. Air pollution has also been correlated with allergen sensitization and provocation [356]. Interestingly, scavenger receptors (including MARCO) are upregulated after allergen sensitization and challenge in mice; further, pulmonary dendritic cells from SR-AI/II deficient mice exhibited more migration and induced transferred OVA-specific T cell proliferation in lymph nodes [357]. This indicates that scavenger receptors may modulate innate immune response and function after an allergen exposure. In the current study, carbon nanoparticle exposures down regulate MARCO expression, and it is tempting to speculate that MARCO expression after nanoparticle exposure may result in exacerbation of allergic responses after challenge, although this hypothesis has not yet been tested.

The fact that cells from different individuals also varied widely in the secretion of TNF-α after particle exposure indicated that particle type and surface components, including the oxidation state of metals on the carbon surface and PAH content can drive innate immune responses. In fact, Goulaouic et al. [358] demonstrated that carbon nanoparticles coated with different PAH content actually decreased IL-1β, IL-12 and IL-8 secretion in a human monocyte cell line. Benzo(a)pyrene alone increased cytokine levels. The authors hypothesized that the sorption of PAHs onto carbon nanoparticles reduced its bioavailability, and that particle specific surface areas was reduced by the presence of PAHs and accountable for the attenuation of proinflammatory cytokines [358].

We had no history regarding the donors’ health and demographics, thus it is difficult to conclude if outstanding factors, previous exposure and possibly conditioning
modulated cellular responses. In a rat model, labeled polystyrene particles were found to translocate from the lungs into the circulatory system when animals were primed with LPS, indicating that individuals with existing pulmonary inflammation may be at greater risk for systemic infection [359]. Air pollution also can come into contact with bacteria, viruses [171, 360] and pathogen components while circulating in ambient air. In addition, populations experiencing preexisting conditions such as pneumonia have been shown to have severe lung dysfunction and an increase in pulmonary related hospitalizations was increased when ambient air quality is subpar [361-363]. Here, a series of experiments was performed on macrophages isolated from 3 different donors that were pretreated and simultaneously exposed to LPS along with 80 μg/cm² Flammruss to simulate preexisting lung infections and poor air quality (Figure 4.13). In all cases, LPS + Flammruss resulted in increased TNF-α secretion compared to Flammruss alone, although for 2 of the 3 donors, TNF-α levels were attenuated compared to untreated cells. It is possible that the donors were experiencing an infection during the time of blood draw and that these cells were already in an activated state. Therefore, it is not possible to make definitive conclusions regarding proinflammatory responses driven by carbon nanoparticle exposure.

When iron was wet impregnated onto Flammruss, chemical characterization using XPS revealed the presence of both Fe (II) and Fe (III) at 41.7% and 58.3% out of 100 atoms (Figure 4.1). There was no significant difference in TNF-α levels after 24 hours for human macrophages from 4 donors treated with 0-10 μg/cm² (Figure 4.11). These data opposed findings of Waldman et al. [162] where the addition of iron to synthesized
carbon nanoparticles induced a significant dose dependent increase in TNF-α, however one donor tested did have an increase in TNF-α levels above baseline and 2-fold greater than Flammruss (Figure 4.12).

To investigate the hypothesis that the ROS production may be a mechanism driving TNF-α production, several types of nanoparticles were tested for their ability to generate extracellular ROS. For human macrophages from 2 donors exposed to low (5 μg/cm²) concentrations of carbon nanoparticles, there was not a significant increase in luminescence. When cells from these same donors were exposed to higher concentrations (20 μg/cm²), donor A had no change in luminescence. Although a significant difference was not achieved, donor B appeared to have a slight increase in ROS when treated with Flammruss + B(a)P (Figure 4.10). Because nearly equal amounts of iron (II) and iron (III) was found on the surface of iron impregnated Flammruss, particles were exposed to ascorbate to determine if this iron was reducible and capable of generating ROS. While luminol indices were still ~6 times less than the positive control, when ascorbate was not washed off, there was an increase in luminol index. This indicated that there were reducible iron species found on the surface, but a reducing agent must be present. Particles that were suspended in ascorbate-containing media and washed may have contained iron species that had reoxidized and were no longer producing ROS. It does appear that the concentration of iron impregnated into Flammruss and the concentration of particles added to cell cultures influenced ROS measurements; cells exposed to particles with less iron (0.1, 0.5 or 1%) and at lower concentrations (10 μg/cm²) had very little changes in luminol indices compared to
Flammruss impregnated with more iron (10%) and added to the culture at a high concentration (20 μg/cm²). When mouse macrophage-like cells were exposed to carbon nanoparticle and FeCl₃, no increase in TNF-α was found [163]. In another study by Wilson et al. [13], carbon nanoparticles (15 μg/mL) incubated with large amounts of iron (100 μM) were found to increase ROS in macrophages and also decrease glutathione levels. It is interesting to note that mouse macrophages sequestered iron and that the presence of iron and carbon nanoparticles did not increase TNF-α levels in an additive manner. As stated above, in the current study, no increase in ROS (as measured by luminescence) was found. It is possible that the iron dissociated from Flammruss and was sequestered in a non-bioavailable form and thus not Fenton active. Welch et al. [364] performed extensive studies investigating the oxidation of Fe(II). Phosphate buffer (pH 7.0), was found to autoxidize Fe(II) in 5 minutes, and found to chelate iron. Iron is tightly regulated in macrophages, and in the current system, it is possible that Fe(II) that was found by XPS was quickly oxidized when introduced to phosphate containing biological media. The intracellular compartmentalization of modified carbon nanoparticles was not investigated here, so it is unknown whether iron on the surface directed the carbon nanoparticles to storage vesicles. The mobilization rate of iron increases with decreasing pH, thus if iron containing carbon nanoparticles are kept in vesicle that are not acidic, iron may not be of consequence to the cell. The mobilization of iron from carbonaceous particles is further reviewed in [164].

The present investigation is not an exhaustive examination of proinflammatory factors that can occur after nanoparticle exposure. Other cytokines (IL-12, IL-8, MIP,
etc.) should be measured at both the transcript and protein level to get a more accurate portrayal of macrophage mediated inflammatory responses. The mechanism of carbon nanoparticle cytotoxicity should be measured in more detail, included investigating apoptotic pathways. In addition, more experiments should be performed investigating oxidative stress at the cellular level and measurement of intracellular free radicals should be conducted. More sophisticated experiments also need to be conducted regarding the chemical characterization of carbon surface groups including iron oxidation state in conditions that are biologically relevant. For example, Peebles [349] found that Flammruss became highly aggregated when suspended in PBS. These aggregates would have less surface area than individual nanoparticles and the lack of uniform particle aggregate size may account for variations in inflammatory responses. Because carbon nanoparticles would likely encounter pulmonary surfactants, it would also be worthwhile to perform chemical characterizations in conditions mimicking the respiratory track so a more accurate correlation can be drawn between the physicochemical properties of modified carbon nanoparticles and the ensuing cellular responses. Lastly, because of the large variations in cellular responses among human macrophage donors, it is of great importance to have a sufficiently large sample size before conclusions are drawn. Age and preexisting pulmonary conditions (among other factors) can severely skew data and should be accounted for, if possible.
Figure 4.1 X-ray photoelectron spectrum of Flammruss 101 impregnated with 10% iron (II) as iron (II) acetate, in the Fe 2p region.

<table>
<thead>
<tr>
<th>Name</th>
<th>Position (binding energy)</th>
<th>Mole Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{2+}$ 2p$_{3/2}$</td>
<td>711.43</td>
<td>41.7</td>
</tr>
<tr>
<td>Fe$^{2+}$ 2p$_{1/2}$</td>
<td>713.03</td>
<td>58.3</td>
</tr>
</tbody>
</table>
Figure 4.2 Quantitative deconvolution of spectra for Flammruss 101, showing changes that occurred on ozonolysis and exposure to ambient conditions.
Table 4. Quantitative deconvolution of spectra for Flammruss 101. Changes after ozonolysis and exposure to ambient conditions.
Figure 4.3 The changes in the FTIR spectrum of ozonated Flammruss 101, purged of gas content and then left to sit overnight in ambient conditions.
Figure 4.4 EPR signal from Flammruss.

A.) Solid Flammruss +/- ozone exposure
B.) Flammruss +/- ozone suspended in H\textsubscript{2}O
C.) Flammruss + ozone in complete media, serum-free X-Vivo media and PBS
Figure 4.5 Stability of free radicals on Flammruss over time.
Figure 4.6 Stability of Flammruss + ozone over time.
Figure 4.7 Luminol assay of reactive oxygen species generated from human monocyte-derived macrophages after exposure to iron containing Flammruss.
Figure 4.8 Luminol assay of reactive oxygen species generated from human monocyte-derived macrophages after exposure to iron containing Flammruss exposed to ascorbate.
Figure 4.9 Luminol assay of reactive oxygen species generated from human monocyte-derived macrophages.

A.) Donor A exposed to low concentrations of 4 types of carbon particles
B.) Donor B exposed to low concentrations of 4 types of carbon particles
Figure 4.10 Luminol assay of reactive oxygen species generated from human monocyte-derived macrophages.

A.) Donor A exposed to high concentrations of 4 types of carbon particles
B.) Donor B exposed to high concentrations of 4 types of carbon particles
Figure 4.11 Average TNF-α production from monocyte-derived after 24 hours exposure.
Figure 4.12 Monocyte-derived macrophages collected from 2 individual donors were incubated with several modified cNPs for 6 hours.
Figure 4.13 Temporal and treatment-dependent changes in TNF-α secretion in human monocyte derived macrophages.
Figure 4.14 Gene expression arrays of human-monoocyte derived macrophages after treatment with modified carbon nanoparticles.
Figure 4.15 Expression of MARCO on murine alveolar macrophages.
Figure 4.16 Murine alveolar macrophages exhibit decreased MARCO expression after incubation with carbon nanoparticles irrespective of LPS stimulation.
Figure 4.17 Murine alveolar macrophages internalize modified Flammruss and treatment effects aggregation of particles.
Figure 4.18 Murine macrophage TNF-α secretion.

A.) Macrophages incubated with carbon nanoparticle conditioned supernatants

B.) Macrophages incubated directly with modified carbon nanoparticles.
Significance denoted by asterisk, n=3, p <0.01
Figure 4.19 Murine TNF-α does not adsorb to modified carbon nanoparticles.
Figure 4.20 Cytotoxicity of modified carbon nanoparticles in murine alveolar macrophages.

A.) Cells incubated with carbon nanoparticle conditioned supernatants
B.) Cells incubated directly with modified carbon nanoparticles
Chapter 5: Antimicrobial Mechanism and Toxicity of Silver Zeolite Platforms

Introduction

Because an era where antibiotic resistance is a growing concern has begun, there is a renewed interest in developing products containing silver for uses as antimicrobials. For thousands of years, silver has been used for food and beverage preservation [241]. In modern times, the successful use of silver for medical purposes was reported in 1852, when silver sutures were used to close fistulas in slaves after child birth [241]. Since then, silver has been used for hygienic purposes (prevent gingivitis and for dental repair), wound healing, and for treatment of newborn eye diseases, among other things. The use of silver as an antimicrobial agent declined with the discovery of antibiotics, yet the evolution of antibiotic resistant pathogens has brought about a revival in silver based applications. Silver is now an additive in consumer products including bandages, socks, shirts, water filters, antiperspirants, combs, paints and washing machines.

The environmental and mammalian toxicology of silver nanoparticles (AgNPs) is still debatable and is the subject of fervent investigation. The elution of AgNPs into wastewater is an environmental concern; researchers have found that populations of nitrifying bacteria found in sludge are reduced when exposed to a large treatment of AgNPs [365], which has severe implications on waste water treatment. The aquatic toxicology of AgNPs has been investigated in fish. Limited studies revealed that AgNPs are genotoxic and cytotoxic to fish cells at concentrations > 0.3 μg/cm² [307]; in trout
exposures, 10 nm AgNPs increased the expression of genes associated with oxidative stress [308]. Terrestrial toxicity was demonstrated in soil nematodes, which exhibited decreased fecundity that correlated with increased expression of superoxide dismutase 1 and 3 after treatment with 0.1 and 0.5 mg/L [366]. Studies in earthworms revealed increased apoptosis when soil contained greater than 4 mg/kg AgNPs [367].

The antimicrobial mechanism of AgNPs is also thought to be driven by surface area and the formation of ROS. Baker et al. [368] found that complete bacterial death could be achieved at 8 μg/cm² and smaller particles were more efficient antimicrobials. Others support this finding, and found that the amount of chemisorbed Ag⁺ and aggregation status influences antimicrobial efficacy [297]. The formation of ROS has been implicated in bacterial toxicity [290], and is thought to perturb cell membrane integrity [369].

In mammalian cells, AgNP toxicity has been found to correlate with particle size in some studies. Lui et al. [370] found that AgNPs (5 and 20 nm) increased ROS and that smaller particles enter cells easier than larger ones in four cells lines, including lung, liver and breast cancer cells. When murine peritoneal macrophages were exposed to 2 to 25 ppm AgNPs, cytotoxicity was evident after 24, 48 and 72 hours that was also accompanied by a decrease in nitric oxide production [371]. Foldbjerg et al. [372] found, both AgNPs (29 nm) and Ag⁺ temporally induced apoptosis that correlated with increased intracellular ROS In a human macrophage cell line. This same group demonstrated similar results in a lung epithelial cancer cell line. In these studies, cytotoxicity and apoptosis occurred in a dose-dependent manner; cells treated with > 10 μg/mL were
found to be rescued from death if they were treated with antioxidants indicating that the mechanism of AgNP cytotoxicity is ROS dependent [373]. When fibroblasts and glioblastoma cells were exposed to AgNPs (6-20 nm), a concentration dependent increase in mitochondrial dysfunction as measured by decreased ATP content, and genotoxicity, as measured by comet assay and increased micronuclei, was induced by oxidative stress [261].

Zeolites are crystalline aluminosilicates that have a microporous 3-dimensional structure. Zeolite has the capacity to undergo ion exchange, a property that can be easily exploited for many uses. Previously, the synthesis of a patterned zeolite template containing AgNPs and its antimicrobial efficiency was described [374]. Others have also characterized antimicrobial activities of silver-exchanged zeolites and found that Ag$^+$ ions migrate from the substrate and that they are also sufficient to cause bacterial death of both *E. coli* and *S. aureus* [213]. Kwakye-Awuah et al. [214] also demonstrated the antimicrobial power of silver zeolites: complete bacterial death was observed after 1 hour, even when zeolite was retrieved, washed and reused for up to three experiments.

There is growing concern surrounding the increasing use of AgNPs. The use of silver has already proved successful for many biomedical applications and AgNPs may expand these applications as an efficient antimicrobial for disease prevention and treatment. Yet, there are growing environmental concerns surrounding AgNPs. As such, we sought to develop a method to immobilize AgNPs onto a zeolite platform. In the current study, the synthesis, and antimicrobial capacity, efficacy and mechanism of zeolite loaded AgNP platforms was investigated using *E. coli*. In addition, because
inhaled is a relevant route of nanoparticle exposure, the toxicity of these platforms was also evaluated against murine alveolar macrophages.

Experimental Methods

The synthesis and chemical characterization of zeolite platforms containing AgNPs was performed by Dr. Supriya Sabbani at the Ohio State University Department of Chemistry laboratories.

Materials

Silver nitrate (99%), potassium nitrate, trypan blue, polyethyleneglycol, Ludox SM-30, poly(methyl methacrylate) (PMMA), hydrazine (Aldrich) were purchased from Aldrich. PEG-600 (Fluka), DARVAN (R. T. Vanderbilt company, Inc), aluminum hydroxide (Alfa Aesar, 80.5%), sodium hydroxide (Mallinckrodt, 98.8%), 25 wt.% tetramethyl ammonium (TMA) hydroxide aqueous solution (Sachem), AKP30 high-purity alumina powder (Sumitomo Chemical Co. Ltd., Tokyo, Japan), with an average particle size of 300 nm, Silastic T-2 polydimethylsiloxane (Dow Corning), 200 proof ethyl alcohol (Pharmco) and 1-octanol (puriss, Fluka, Buchs, Switzerland) were also purchased and used without further purification. LB broth powder was purchased from Agros Chemical. Agar, 100 mm sterile petri dishes and chloroform were obtained from Fisher Scientific and transwell plates and 6 and 24- well plates were obtained from Corning. Qiagen supplied the RNeasy RNA isolation kit. The E. coli strain XL-1 Blue
was provided by Dr. Joanne Trgovcich. Murine alveolar macrophages were purchased from American Type Tissue Culture. Fetal bovine serum was purchased from Atlanta Biologicals while RPMI was purchased from Gibco. Penicillin streptomycin, Trizol, Sytox Red and Hank’s buffered saline solution were purchased from Invitrogen.

Submicron Synthesis of Zeolite AgNPs

Alumina Supports

Macroporous alumina oxide supports served as a substrate for zeolite membrane preparation and their preparation is described in detail in [374].

Zeolite Y

Faujasite zeolite Y ranging from 80-200 nm in sized were prepared from a clear solution using ratio: 0.037 Na₂O:1.0 Al₂O₃:3.13(TMA)₂O:4.29 SiO₂:497H₂O. A precursor gel was prepared by mixing 152 g H₂O, 4.58 g Al (OH)₃, and 53.6 g TMAOH and stirred for 2 hours. Dowex proton-exchange resin was used to adjust the pH of 25 g Ludox SM-30 to 8.1. After the resin was removed from the silicate solution, 20 g was added to the TMA aluminate solution along with 30 mg NaOH. This mixture was stirred continuously for 3 hours before being transferred to a 250 mL Nalgene bottle and was then heated at 98°C for 4 days. Zeolite crystals were washed with dH₂O and dried at 60°C overnight prior to calcinations at 550°C in flowing air for 24 hours to remove template from zeolite pores. Figure 5.1 is a representation of zeolite Y and its structure.

Zeolite Y Seed Layers
Single dip coating cycles were used to deposit zeolite Y onto alumina platforms. Alumina substrates were submerged and withdrawn from a watch glass filled with 16 mL of zeolite suspension at a speed of 0.01 m/s, and seed layers were dried overnight at room temperature. Seeded supports were placed at 45° angle (face up for one day and face down for 3 days) in 250 mL Nalgene bottle containing the same zeolite precursor solution used for zeolite Y crystallite synthesis above. Secondary growth was performed 1 day face up and 3 days face down at 100°C. Prior to calcination, the supports were rinsed and thoroughly dried. Supports containing zeolite were calcined under the following conditions in a temperature controlled tube furnace: 1.) temperature was increased from room temperature to 350°C at a rate of 0.2°C/minute., 2.) maintained at 350C for 6 hours, 3.) increased temperature to 550°C at a rate of 0.2°C/minute and 4.) maintained at 550°C for 6 hours before they were 5.) cooled to room temperature at a rate of 0.5°C/minute.

**Patterned Zeolite Supports**

We previously reported a novel method for synthesizing micropatterned zeolite membranes [374]. Briefly, micropatterning on the alumina substrate by creating silica masters containing the photolithographically-generated patterns. A mold was fabricated by adding Silastic T-2 PDMS and its curing agent at a 10:1 ration. This mixture was poured over the micropatterned silica master and allowed to cure for 48 hours at room temperature. The resulting PDMS template was peeled and used as a mold for creating a PMMA template. Here, a 20 wt.% PMMA solution in anisole was drop cast on the PDMS mold and dried for 5–6 hours at room temperature. The PMMA template was peeled off and used to create patterned alumina substrates by drop casting the alumina slurry onto
the PMMA mold and allowing it to dry at room temperature. A ramp up rate of 0.2°C/minute was administered until the temperature reached 950°C and the alumina supports were released by pyrolysis. See Figure 5.2 for a schematic of this process.

**Impregnation of Zeolite Supports with AgNPS**

Patterned and non-patterned alumina supports containing zeolite Y were sodium exchanged with 1M NaCl for 12 hours followed by thorough washing with dH₂O. Films were treated with 0.005M AgNO₃ solution at 60 mL/support, then reduced by adding 100 μL hydrazine in a glove bag. Hydrazine was washed and films were ion exchanged with 1M NaCl to remove unreacted silver ions from the zeolite. Finally, supports were washed with dH₂O to remove residual sodium.

**Zeolite AgNP Platform Chemical Characterization**

**Scanning Electron Microscopy**

The morphology of alumina supports and zeolite membranes were obtained using a Philips XL-30 FEG scanning electron microscope.

**X-Ray Diffraction**

X-ray powder diffraction (XRD) patterns were recorded with a Rigaku Geigerflex diffractometer using Ni-filtered Cu Ka radiation (40 kV and 25 mA).

**Inductively Coupled Plasma Optical Emission Spectroscopy**

To quantify the amount of silver that was released from the supports with each use and bacterial exposure, supernatants were collected from exposed supports after 30 minutes and 48 hours. LB media and RPMI +FBS and pen strep media alone and spiked
with AgNO$_3$ at 25 ppm Ag was used for comparison. Silver content was measured using Inductively coupled plasma optical emission spectroscopy at Galbraith laboratory.

Biological Characterization

_E. coli Experiments_

Cultures of XL-1 blue _E. coli_ were incubated with the disks and assessed for viability using traditional colony counts. LB broth was prepared using a concentration of 25 g/L. LB agar plates were prepared with 1.5% agar. Individual clones were inoculated in 3 mL of LB broth and shaken at 225 rpm overnight at 37°C. Prior to exposing bacteria to the zeolite membranes, bacterial cultures were adjusted to obtain an initial optical density (OD) between 0.6 and 0.8 using 600 nm as an absorbance wavelength. Patterned-zeolite supports with or without silver nanoparticles were placed into 24 well tissue culture plates, and 2 mL of bacterial suspension was added to each well, including wells with bacteria only. Non-patterned zeolite supports with or without silver nanoparticles were placed into 6-well tissue culture plates, and 5 mL of bacterial suspension was added to each well, including wells with bacteria only. Experimental plates were incubated at 37°C and continuously shaken. Samples were removed, serially diluted and plated on LB agar at 0, 30, 60, 120 and 180 minutes. In addition, optical density was measured after exposure to supports using a Shimadzu spectrophotometer at an absorbance wavelength of 600 nm. A schematic of the experimental design is found in Figure 5.3. LB plates were incubated at 37°C overnight and colony forming units were counted to determine bacteria viability. All zeolite supports were reused after being decontaminated with 70% EtOH for 20 minutes prior to air drying.
RNA extraction

Bacterial RNA was extracted using standard procedures. *E. coli* was subjected to one of 4 conditions for times ranging between 5 minutes and 30 minutes:

1.) Bacteria only
2.) Bacteria treated with 25 ppm Ag⁺ from AgNO₃
3.) Bacteria exposed to zeolite supports
4.) Bacteria exposed to zeolite supports containing AgNPs

Bacteria was then collected from each well and pelleted at 4°C in 15 mL centrifuge tubes at 3250 x g for 15 minutes. Supernatants were discarded and the pellets were homogenized in 5 mL of Trizol for 5 minutes. Each tube was shaken vigorously for 30 seconds after the addition of 1 mL of chloroform. Tubes were incubated at room temperature for 3 minutes prior to centrifugation at 4°C and 3250 x g for 15 minutes. The organic layer was then removed and placed into clean, RNase free microfuge tubes. Equal amounts of 100% ethanol was added to each tube and mixed by pipetting. RNA purification was then performed using RNeasy Mini kits as per the manufacturer’s instructions. At the final elution step, RNA was resuspended in 20 µL of RNase free H₂O and stored at -80°C until further use in gene expression arrays and quantitative reverse transcription polymerase chain reaction experiments (qPCR). The quality of RNA was examined using 1.8% agarose gels using TBE buffer and also quantified and analyzed using the Agilent 2100 Bioanalyzer Lab-On-A-Chip Agilent 6000 Series II chip.
Gene expression microarrays

Sample labeling and hybridizations were performed by David Newsom and data analysis by Peter White. The concentration of the samples provided was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer. RNA samples were analyzed by the FGC using an Agilent 2100 Bioanalyzer Lab-On-A-Chip Agilent 6000 Series II chip to determine the integrity of the samples. The RNA was of high quality and all samples passed our QC cutoff. Sample labeling and hybridization was performed according to the manufacturer’s protocols. Samples were hybridized to the E.Coli 8x15K Microarray (AMADID 020097).

Microarray slides were hybridized overnight, washed and then scanned with Agilent G2505C Microarray Scanner. This high resolution scanner represents the very latest technology from Agilent for Arrays and features an industry-leading extended dynamic range of $10^6$ (20-bits) for high sensitivity scanning without saturation, low-level detection resulting from optimized precision optics, broad dynamic range, minimal spectral cross talk that enables detection of weak features. The information about each probe on the array was extracted from the image data using Agilent Feature Extraction 10.9 (FE). This data is stored in the FE “.txt” files. The raw intensity values from these files is imported into the mathematical software package “R”, which is used for all data input, diagnostic plots, normalization and quality checking steps of the analysis process using scripts developed in-house by Peter White specifically for this analysis.

All analysis was performed using analysis scripts that were developed in-house using the R software environment for statistical computing and graphics. These scripts call on
several Bioconductor (http://www.bioconductor.org/) packages. Bioconductor is an open source and open development software project to provide tools for the analysis and comprehension of genomic data [375]. Significance analyses of microarrays (SAM) is a powerful tool for analyzing microarray gene expression data useful for identifying differentially expressed genes between two conditions [376]. SAM calculates a test statistic for relative difference in gene expression based on permutation analysis of expression data and calculates a false discovery rate (FDR) using the q-value method presented in Storey [377]. In outline, SAM identifies statistically significant genes by carrying out gene specific t-tests and computes a statistic for each gene, which measures the strength of the relationship between gene expression and a 5 response variable. This analysis uses non-parametric statistics, since the data may not follow a normal distribution. The response variable describes and groups the data based on experimental conditions. In this method, repeated permutations of the data are used to determine if the expression of any gene is significant related to the response. The use of permutation-based analysis accounts for correlations in genes and avoids parametric assumptions about the distribution of individual genes. For this experiment, SAM analysis was implemented in R using the Bioconductor Siggenes package. Another quality assessment tool is Relative Log Expression (RLE) values. Specifically, these RLE values are computed for each probe set by comparing the expression value on each array against the median expression value for that probe set across all arrays. Gene expression arrays were analyzed using a 10% FDR to generate the list of significantly differentially expressed genes. The q-values (FDR) for each gene are provided in the results table – the lower the value the more significant the result.
Murine Macrophages

The murine alveolar macrophage cell line (MH-S) was purchased from the American Type Culture Collection (Manassas, Va), and propagated using RPMI cell culture media (Gibco) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 0.1% penicillin streptomycin (Gibco), herein referred to as complete media. Cells were passaged every 3-4 days and incubated at 37°C with 5.0% CO₂. Cells were harvested using enzymatic release (0.25% trypsin/EDTA), neutralized with cell culture media and either applied to 6-well plates containing zeolite supports. In experiments investigating toxicity of Ag⁺, cells were plated and allowed to adhere overnight prior to exposure.

Cytotoxicity

Trypan Blue staining was used to determine cell viability in macrophages that were exposed to supports by retrieving 100 µL of the cell suspension and making a 1:3 dilution with 0.4% trypan blue. Cells were then observed and counted on a hemocytometer using light microscopy.

Macrophages were also incubated with increasing concentrations of AgNO₃ or KNO₃ for 30 minutes and cytotoxicity was determined using flow cytometry. Cells were harvested and the concentration was adjusted to 1 x 10⁶ cells/tube. They were then suspended in 1 mL HBSS prior to adding 1 µL (5 nM) of Sytox Red. Cells were incubated at room temperature for 15 minutes and analyzed using flow cytometry with an excitation wavelength of 635 nm for Sytox Red detection.
Results

Physicochemical Characterization of Zeolite AgNPs

There is currently much interest in developing nanomaterials containing micropatterning as such designs effectively improve surface area. In this work, a novel method was developed for the synthesis of a micropatterned alumina substrate seeded with zeolite (Figure 5.4, [374]). By generating a series of molds, first a silica master mold, followed by a PMDS stamp, a micropatterned alumina substrate with parallel lines of 5 x 5 μm (width x height) separated by 45 μm gaps was successfully seeded with nanozeolite Y. In Figure 5.4a and b, the micropatterning is clearly visible using SEM, and the addition of nanocrystalline zeolite coating ~2 μm thick is noted in Figure 5.4c and d.

During the demolding the alumina slurry from the PDMS mold, surface defects were often observed. In order to prepare more sturdy supports and preserve micropatterning, a PMMA mold was developed. Figure 5.5a-c, top panels provides visual evidence and a schematic of the support synthesis using the silica master mold, and the subsequent molds derived from PDMS and PMMA. The resultant alumina support (Figure 5.5d) was seeded with zeolite (Figure 5.5e) and examined for elemental content using X-ray diffraction patterns (XRD). The peak in Figure 5.5f revealed at 6°2Θ is due to the zeolite and the peak at 25°2Θ is due to the alumina. Silver nanoparticles were incorporated into the zeolite supports by ion exchanging sodium with silver, followed by reduction using hydrazine. Using SEM, the size of zeolite particles and silver nano-
clusters were found to be ~250 nm and < 50 nm, respectively, and were not entirely uniform in size (Figure 5.5a and b, bottom panels).

Elemental analyses of supernatants collected from supports soaked in LB media for 48 hours revealed that 20 ppm of Ag\(^+\) was released from non-patterned zeolite AgNP supports. Supernatants retrieved after 30 and 60 minutes of exposure were assessed for the presence of aggregates using DLS, and a broad distribution of aggregate size was noted. Thus, we are unable to determine if the source of silver is from small AgNPs or a result of AgNP oxidation and the release of Ag\(^+\) into solution.

Antimicrobial Activity and Mechanism of Zeolite AgNPs

To determine if micropatterning zeolite AgNP supports enhanced antimicrobial activity versus non patterned supports, *E. coli* was incubated alone, with zeolite supports, or with supports containing AgNPs. Turbidity measured using spectrophotometry revealed that bacterial growth was completely inhibited over a three hour exposure when *E. coli* was exposed to zeolite AgNPs (OD <0.1), compared to bacteria only and zeolite supports, which entered log phase growth at OD ~0.5 (Figure 5.6 top). While *E. coli* incubated with both patterned and non-patterned zeolite AgNPs completely attenuated bacterial viability within 2 hours of exposure, no difference in killing efficiency was observed as a result of patterning (Figure 5.6 bottom). To determine if the antimicrobial activity of zeolite AgNP supports was contact dependent, bacteria was separated from the supports using transwell plates containing inserts with pores of 0.400 μm. Bacterial death was evident within 60 minutes, and controls grew normally (Figure 5.7). In
addition, media from the bottom chamber was sampled to ensure that bacteria were unable to transcend the membrane. To confirm that antimicrobial action was not contact dependent, supports were incubated with media for 3 hours and then removed and bacteria was added to the tubes; all bacteria were again non-viable within 60 minutes (Figure 5.8). To test the longevity of antimicrobial activity of zeolite AgNP supports, viability assays were repeated on the same support a total of 6 times. Supports were steam autoclaved for sterilization between each use. When beginning with an E. coli culture concentration of approximately $1 \times 10^6$ CFU/mL per support, all bacteria survival was reduced to 0 within 2 hours after 4 uses, however, full viability attenuation was achieved within 3 hours for uses 5 and 6 (Figure 5.9).

The antimicrobial mechanism of AgNPs is still debatable. To identify the mechanism driving AgNP bacterial toxicity, a molecular approach was taken. The stability of RNA is imperative for organism survival. E. coli was incubated with increasing amounts of Ag$^+$ to determine the kinetics of silver toxicity. In Figure 5.10, it was revealed that concentrations as low as 6.25 ppm can cause RNA degradation within 30 minutes. Further experimentation determined that E. coli could withstand as much as 50 ppm Ag$^+$ for very short periods (5 minutes). As such, a dose of 25 ppm for 5 minutes was chosen for gene expression microarrays. Figure 5.11 demonstrated the integrity of E. coli RNA extracted from each experimental condition and used for the gene expression microarrays was of suitable quality. No degradation was evident in the negative controls, zeolite support controls, and the zeolite AgNP samples, while minimal degradation was
observed in Ag\textsuperscript{+} treated bacteria. Therefore, the RNA was used for gene expression microarrays.

Assuming that most genes are not changing in expression across arrays means ideally most of these relative log expression (RLE) values will be near 0. Boxplots of these values, for each array, provides a quality assessment tool. When examining this plot focus should be on the shape and position of each of the boxes. Typically arrays with poorer quality show up with boxes that are not centered about 0 and/or are more spread out. For this particular dataset there was no such problem array; however, sample NC3 was identified as an outlier and was excluded from the final analysis Figure 5.12. These supports were also tested for antimicrobial activity. *E. coli* treated with 25 ppm Ag\textsuperscript{+} for 5 minutes had significantly less bacterial colonies compared to untreated bacteria. Bacteria exposed to zeolite supports containing AgNPs for 30 minutes also displayed significant antibacterial properties compared to zeolite controls (Figure 5.13). Gene expression arrays revealed a number of significant differences between treatments. Tables 5.2-5.6 contain a list of selected candidate genes that may be associated with the mechanism of bacterial death. Incubation with zeolite supports induced the upregulation of several genes involved in metal transport, including iron transporters (increased 13.5 and 7 fold) and a copper transporter (upregulated 7.2 fold). The fused predicted multidrug transporter subunits of ABC superfamily gene was upregulated 11 fold (Table 5.2). *E. coli* treated with Ag\textsuperscript{+} had nitrite reductase and nitrite transporter genes upregulated 27 and 11 fold, respectively. In addition, genes involved with DNA transcription regulation and repression were upregulated more than 8-fold, compared to the negative control (Table
Zeolite supports containing AgNPs also had several metal transporters upregulated compared to negative controls. Notably, the zinc transporter gene was upregulated 247 times. Copper transporter was upregulated 77 fold and magnesium transporter was upregulated 11 fold. Two multi-antibiotic resistance genes were also upregulated ~20 fold. Several genes involved in antioxidant defense were upregulated including glutaredoxin (10.5-fold), thioredoxin (7.6-fold) and superoxide dismutase (3.2-fold). Additionally, several genes involved in sulfur transport were upregulated between 6 and 18-fold (Table 5.4). E. coli exposed to zeolite + AgNPs, also resulted in upregulation of thiosulfate transporter subunit and the sulfate/thiosulfate transporter subunit (14.9 fold and 11.9 fold, respectively) compared to zeolite supports only. Additionally, both copper transporter and magnesium transporter were upregulated over 9 fold. Antioxidants thioredoxin and glutaredoxin were also upregulated ~7.5-fold compared to zeolite controls. Interestingly, iron transporter genes were downregulated between 7 and 13-fold, although ferrochelatase was actually upregulated 9.5-fold in E. coli exposed to zeolite + AgNPs compared to zeolite supports alone (Table 5.5). E. coli treated zeolite + AgNPs, Zn-binding periplasmic protein was upregulated over 200-fold compared to bacteria treated with Ag+. Thiosulfate transporters were also upregulated between 8 and 14-fold. Several genes involved with copper including copper transporter, multicopper oxidase and periplasmic copper-binding protein were upregulated 25.2, 16.3 and 7.6-fold, respectively. Glutaredoxin and thioredoxin were upregulated more than 5-fold after exposure to zeolite + AgNP supports. Lastly, DNA-binding transcriptional dual activator of multiple antibiotic resistance was upregulated 4.8-fold compared to Ag⁺ treated cells.
Discussion

Zeolites are microporous minerals that can serve as suitable carriers for antimicrobial agents. In this work, we successfully created novel micropatterned zeolite supports harboring AgNPs [374]. Because of initial setbacks using PMDS molds (cracking and defects in micropatterning) a tertiary mold was created using PMMA. The alumina template on which zeolite was seeded better preserved micropatterning when it was demolded from PMMA, likely due to the temperature controlled pyrolysis used during removal. While we previously proved the proficiency of these zeolite AgNP supports, their antimicrobial mechanism was not immediately apparent. Others have tested silver-exchanged zeolites, however the supports used in this study differ in that they have undergone a reduction step followed by sodium exchange to produce AgNPs without residual Ag$^+$. While the purpose of micropatterning was to further increase the surface area of zeolite AgNP supports, patterning did not increase the antimicrobial efficiency of the supports. This could be because, in the present investigation, antimicrobial activity was not found to be contact dependent. Additionally, when supernatants were tested for silver content, 20 ppm of Ag$^+$ was released into media after zeolite AgNP supports were submerged in LB media for 48 hours under gentle agitation. This is in contrast with Su et al. [369], who found that Ag/clay conditioned supernatants did not have appreciable antimicrobial activity. There are several explanations for silver found in supernatants: 1.) AgNPs are oxidized over time, as found by Lok et al. [297], who revealed that AgNPs
began releasing Ag\(^+\) in less than 30 minutes, 2.) Although unlikely, perhaps not all Ag\(^+\) was removed during the final sodium exchange, resulting in a heterogeneous mixture of silver species associated with zeolites 3.) AgNPs may have been released from zeolite directly. However, the source of silver (dissolution of AgNPs to Ag\(^+\), or AgNP escape from zeolite cages) remains undetermined because dynamic light scattering revealed a large size distribution of particle sizes which cannot be definitively identified as silver-containing particles.

Because the synthesis of zeolite layers in not exactly uniform, the size and amount of nanoparticles formed is variable. Smaller AgNPs may undergo silver dissolution faster than larger AgNPs. Since zeolite AgNPs appear to have a finite span of effectiveness (Figure 5.9) it is proposed that after each use, larger AgNPs remain, and decrease the antimicrobial activity of the supports, due to decreased Ag\(^+\) oxidation kinetics. However, since the supports in the aforementioned experiment were autoclaved, the effects of heat and pressure on AgNP stability cannot be overlooked. In fact, Landry et al. [378] demonstrated crystalline nanosilver bandages that were exposed to temperatures > 90\(^\circ\)C were less bactericidal. Additionally, the crystalline structure became decomposed, and the speciation of silver was changed rapidly with increasing temperature. If the crystalline structure of nanosilver influences the antimicrobial activity of the bandage, then the destruction of nanosilver crystalline integrity may explain reduction in antimicrobial effectiveness. It is possible that during autoclaving, AgNPs were physically modified, resulting in decreased efficacy with each use. The use of 70% EtOH to clean zeolite AgNP was also tested, and the same trend of decreased
antimicrobial efficacy was noted. It is thus hypothesized that components of LB media may interact with AgNPs, thereby preventing antimicrobial activity due to lack of bioavailable silver species.

Several research groups studying AgNPs propose that the antimicrobial activity is due to the formation of reactive oxygen species. Kim et al. [244] reported that the minimal inhibitory concentration of 13 nm AgNPs for *E. coli* was found to be between 3.3 to 6.6 nM. The mechanism of bacterial death was found to be a result of persistent surface free radicals found on AgNPs, and that the antimicrobial activity of both AgNPs and Ag⁺ could be reversed by n-acetylcysteine [244]. Hybridization of AgNPs onto clay resulted in immobilized AgNPs with antimicrobial activities. In that study, bacterial contact with AgNPs was sufficient to cause cell death. The mechanism of cell death was found to be cell membrane disruption caused through the generation of ROS, and when bacteria were incubated with glutathione, their viability was rescued [369]. When bacterial reporter strains specifically responding to superoxide radicals were incubated with 100-300 ppm Ag⁺, it was apparent that the mechanism of antimicrobial activity was by ROS, specifically superoxide, which formed after perturbation of the electron transport chain [298]. Accordingly, the upregulation of superoxide dismutase indicates that ROS may also play a role in the antimicrobial mechanism of these zeolite AgNP supports.

In the current study, the upregulation of thioredoxin and glutaredoxin, which are crucial to maintaining oxidative balance, were noted after *E. coli* was exposed to zeolite supports + AgNPs (Table 5.5). However, it appears that this increase in gene expression
was not sufficient to rescue all bacteria, as a significant difference in viability was still observed. It should be noted that several genes associated with sulfur species transport and reduction were upregulated, indicating that bacteria were adjusting sulfur pools to accommodate for the need of these sulfur-containing antioxidants. It is plausible that bacteria produce H$_2$S as a detoxification mechanism, where Ag$^+$ would react with H$_2$S to form silver sulfide. Therefore, it is concluded that thiol-silver interactions are a major contributor to the antibacterial activity of zeolite AgNPs by inactivating essential antioxidant proteins, which is in agreement with several groups [298, 379-381]. In addition, Ag$^+$ could react with chloride, forming stable AgCl, but also deplete intracellular chloride, resulting in toxicity [382]. However, under conditions of high Cl$^-$ content, AgCl$_2^-$ a water soluble bioavailable anion can form [288]. There is also evidence that intracellular silver utilizes several different metal transporters, most favorably the copper transporter, as indicated by the highly significant upregulation of that gene. Therefore, it is not unlikely that AgNP toxicity mechanisms are similar to copper toxicity. Several mechanisms of silver nanoparticle toxicity likely exist, although these data suggest that an imbalance of antioxidant defenses occur, resulting in accumulation of ROS, killing the bacterium.

It is proposed that AgNPs (and Ag$^+$) enters bacteria via several metal transporters, namely copper and magnesium transporters. Slawson et al. [382] reported that Ag$^+$ toxicity was reduced when Cu$^{2+}$ is also present, indicating that silver may compete with copper for cellular entry. It is also noted that for zeolite AgNPs, several ATPase efflux pumps were upregulated as well as copper oxidase, which may serve to detoxify Ag$^+$. 
When comparing gene microarray data between *E. coli* that was treated with Ag\(^+\) versus bacteria exposed to zeolite + AgNP, there are drastic differences in the expression of metal transporters. This disparity is explained by the difference in experimental conditions. Bacteria were exposed to 25 ppm Ag\(^+\) by the addition of one bolus and harvested after 5 minutes. Figure 5.10 profiled the degradative kinetics of Ag\(^+\), where bacterial RNA was degraded irrespective of dose within 30 minutes at the concentration of silver recovered from zeolite AgNP supports. The kinetics of silver release from zeolite AgNPs was only tested after 48 hours of incubation. Therefore, the gene expression profiles between bacteria treated with Ag\(^+\) and zeolite AgNP supports cannot accurately be compared. However, it is noted that maltose transporter and \(\alpha\)-galactosidase, which are involved with sugar transport and maltose cleavage and transition metal ion binding, respectively, were upregulated by *E. coli* treated with 25 ppm Ag\(^+\). Guffanti and Corpe [383] noted that \(\alpha\)-galactosidase activity in *Pseudomonas fluorescens* was reduced in the presence of Ag\(^+\). Thus bacteria may counter this dysfunction by increasing intracellular levels of maltose and maltose cleaving enzymes (including \(\alpha\)-galactosidase) in efforts to maintain energy stores.

The increase in gene transcription of multi-antibiotic resistance genes marR and marA is remarkable because the known genes encoding silver resistance in *E. coli* are ybdE, ylcD, ylcC, ylcB, ylcA and ybcZ. Genes encoding for silver resistance in *E. coli* are encoded on both plasmids and chromosomes [384]. Most silver resistant bacterial strains have developed tolerance by utilizing Ag\(^+\) ATPase efflux pumps and antiporters rather than chemical detoxification mechanisms [288]. However, in the current study,
only ylcB, the gene coding for copper silver efflux protein, was upregulated when bacteria were exposed to zeolite AgNP supports compared to unexposed E. coli. Increased expression of mar genes, which are known to be associated with antibiotic resistance (including tetracycline and ampicillin resistance) [385] was observed and the current study indicates that they may also play a role in the evolution of silver resistant E. coli. However, further studies are essential to confirm the role of mar genes and their role in silver resistant bacteria. The proposed mechanism of antimicrobial activity for zeolite AgNPs is found in Figure 5.15. Zeolite AgNPs release Ag⁺ ions into solution. Ions enter E. coli via passive diffusion or metal transporters, where they preferentially bind cysteine residues on proteins. Essential proteins containing sulfhydryl groups (including glutaredoxin and thioredoxin) become dysfunctional leading to oxidative imbalance. Free radicals overwhelm the antioxidant capabilities of the bacterium, and eventually disrupt electron transport, killing the cell. Bacteria combat these events by upregulating genes coding for efflux pumps and also increase expression of sulfur transporters to reestablish oxidative balance. Silver resistant strains of bacteria exist, and resistance is thought to be controlled by sil genes. However, it is proposed that mar genes may also be significantly involved in the evolution of silver resistant bacterial strains.

Because of the increasing prevalence AgNPs in consumer and medical products, there are ample opportunities for mammalian cells to contact these nanoparticles through various routes including absorption, inhalation and ingestion. When murine alveolar macrophages were incubated with zeolite AgNPs, viability of cells was significantly decreased within 30 minutes. Further, a dose dependent increase in cell necrosis was
observed when cells were incubated with Ag⁺. Others have found that AgNPs are cytotoxic to fibroblasts [261] and lung epithelial cells [373]; both groups found cell death to be ROS dependent. The mechanism of zeolite AgNP mediated cell death in mammalian cells is under investigation, although it is thought to be similar to that of bacteria.
Window size: 7.4Å  
Internal cage size (super cage): 13Å  

Figure 5. 1 Cartoon of zeolite structure and morphology.
Figure 5.2 Schematic of zeolite AgNP fabrication; from [374].
Inoculate flask with bacteria from a single clone grown overnight. Allow bacteria to grow in shaking incubator 2-3 hrs; read optical density on spectrophotometer. Add bacteria to plate containing zeolite platforms. Incubate plates overnight and count colonies. Shake plate and incubate. Sample at 30, 60, 120, and 180 minutes for viability (plates) and growth (spectrophotometry).

Serial dilutions plated to determine bacteria viability.

Figure 5.3 Representation E. coli exposure to zeolite supports and subsequent assessment of growth and viability.
Figure 5.4 Scanning electron micrograph of zeolite supports, from [374].
Figure 5.5 Scanning electron micrographs of molds used to create zeolite + AgNP supports, from [374].

Top: a.) The PDMS mold made from the silicon mold; the following micrographs focus on the black square shown in the figure, b.) the PDMS mold, c.) the PMMA replica d.) after alumina deposition, sintering and removal of the PMMA, e.) the zeolite film grown on the alumina and f.) X-ray diffraction pattern of the zeolite film (* indicates the (111) reflection from the zeolite, the other strong peak is from the alumina, weaker peaks at 2h 10, 12, 23 and 27° all arise from the zeolite).

Bottom: a.) The zeolite film after growth of silver particles and (b) a magnified image of the surface. (Note that the SEM is from the right of the black square in Top a.) with grooves above the PDMS plane).
Figure 5.6 Viability of E. coli after exposure to zeolite supports.

ZNPT-control = non-patterned zeolite support negative control, ZPT-control = patterned zeolite support control, NPT + Ag\textsuperscript{0} = non-patterned zeolite support + silver nanoparticles, and PT + Ag\textsuperscript{0} = patterned zeolite support + silver nanoparticles. Error bars are standard deviations from 3 replicates.
Figure 5.7 Viability of E. coli after exposure to zeolite supports that were separated from bacteria using transwell plates.

ZNPT = non-patterned zeolite support negative control, NPT + Ag⁰ = non-patterned zeolite support + silver nanoparticles.
Figure 5.8 Viability of E. coli after exposure to supernatants collected from zeolite supports that were soaked in LB media for 3 hours.

ZNPT = non-patterned zeolite support negative control, NPT + Ag$^0$ = non-patterned zeolite support + silver nanoparticles.
Figure 5.9 Efficacy of zeolite AgNP supports.

Exposure time needed to eliminate all E. coli viability increased with each zeolite AgNP use.
Figure 5.10 Dose-dependent and temporal degradation of E. coli RNA exposed to Ag⁺.
Figure 5.11 E. coli RNA integrity of samples tested in gene expression array analyses.
Figure 5.12 Quality assessment of E. coli gene expression arrays.
Figure 5. Viability of E. coli after exposure to Ag\(^+\) or zeolite supports.

Top: E. coli viability is significantly reduced after incubation with Ag\(^+\) for 5 minutes, 
n=4, \(p < 0.005\).

Bottom: E. coli viability is significantly reduced after incubation with zeolite AgNPs for 30 minutes n= 4, \(p < 0.02\).

ZNPT = non-patterned zeolite support negative control, NPT + Ag\(^0\) = non-patterned zeolite support + silver nanoparticles/
Table 5.1 Comparison of upregulated E. coli genes: Zeolite supports versus negative controls.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>FDR</th>
<th>Adjusted p value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>fiu</td>
<td>predicted iron outer membrane transporter</td>
<td>13.5</td>
<td>1.7</td>
<td>0</td>
<td>putative outer membrane receptor for iron transport</td>
</tr>
<tr>
<td>nrdF</td>
<td>ribonucleoside-diphosphate reductase 2, beta subunit, ferritin-like protein</td>
<td>12.9</td>
<td>1.7</td>
<td>0</td>
<td>ribonucleoside-diphosphate reductase 2, beta chain, frag</td>
</tr>
<tr>
<td>yddA</td>
<td>fused predicted multidrug transporter subunits of ABC superfamily</td>
<td>11.0</td>
<td>1.7</td>
<td>0</td>
<td>putative ATP-binding component of a transport system</td>
</tr>
<tr>
<td>entA</td>
<td>2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase</td>
<td>7.6</td>
<td>1.7</td>
<td>0</td>
<td>2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase</td>
</tr>
<tr>
<td>entB</td>
<td>isochorismatase</td>
<td>7.3</td>
<td>1.7</td>
<td>0.0001</td>
<td>2,3-dihydro-2,3-dihydroxybenzoate synthetase, isochorismatase</td>
</tr>
<tr>
<td>copA</td>
<td>copper transporter</td>
<td>7.2</td>
<td>1.7</td>
<td>0.0001</td>
<td>putative ATPase</td>
</tr>
<tr>
<td>cirA</td>
<td>ferric iron-catecholate outer membrane transporter</td>
<td>7.1</td>
<td>1.7</td>
<td>0</td>
<td>outer membrane receptor for iron-regulated colicin I receptor</td>
</tr>
<tr>
<td>ybbW</td>
<td>predicted allantoim transporter</td>
<td>-6.8</td>
<td>2.37</td>
<td>0.0007</td>
<td>putative transport protein</td>
</tr>
<tr>
<td>entF</td>
<td>enterobactin synthase multienzyme complex component, ATP-dependent</td>
<td>6.8</td>
<td>1.7</td>
<td>0.00003</td>
<td>ATP-dependent serine activating enzyme</td>
</tr>
<tr>
<td>fhuE</td>
<td>ferric-rhodotorulic acid outer membrane transporter</td>
<td>6.7</td>
<td>1.7</td>
<td>0.00002</td>
<td>outer membrane receptor for ferric iron uptake</td>
</tr>
<tr>
<td>exoD</td>
<td>pseudo</td>
<td>-6.5</td>
<td>2.18</td>
<td>0.00206</td>
<td>putative exonuclease</td>
</tr>
<tr>
<td>glxR</td>
<td>tartronate semialdehyde reductase, NADH-dependent</td>
<td>-6.5</td>
<td>2.17</td>
<td>0.00015</td>
<td>putative oxidoreductase</td>
</tr>
<tr>
<td>fes</td>
<td>enterobactin/ferric enterobactin esterase</td>
<td>6.4</td>
<td>1.81</td>
<td>0.00001</td>
<td>enterochelin esterase</td>
</tr>
<tr>
<td>fes</td>
<td>enterobactin/ferric enterobactin esterase</td>
<td>6.4</td>
<td>1.81</td>
<td>0.00001</td>
<td>enterochelin esterase</td>
</tr>
<tr>
<td>entC</td>
<td>isochorismatase synthase 1</td>
<td>6.4</td>
<td>1.84</td>
<td>0</td>
<td>isochorismatase hydroxymutase 2, enterochelin biosynthesis</td>
</tr>
<tr>
<td>fes</td>
<td>enterobactin/ferric enterobactin esterase</td>
<td>6.4</td>
<td>1.78</td>
<td>0.00001</td>
<td>enterochelin esterase</td>
</tr>
<tr>
<td>fes</td>
<td>enterobactin/ferric enterobactin esterase</td>
<td>6.3</td>
<td>1.98</td>
<td>0.00003</td>
<td>enterochelin esterase</td>
</tr>
<tr>
<td>aceB</td>
<td>malate synthase A</td>
<td>-5.5</td>
<td>1.7</td>
<td>0</td>
<td>malate synthase A</td>
</tr>
<tr>
<td>zntA</td>
<td>zinc, cobalt and lead efflux system</td>
<td>5.4</td>
<td>2.33</td>
<td>0.01438</td>
<td>zinc-transporting ATPase</td>
</tr>
<tr>
<td>feIA</td>
<td>iron-enterobactin outer membrane transporter</td>
<td>5.4</td>
<td>1.9</td>
<td>0</td>
<td>outer membrane receptor for ferric enterobactin</td>
</tr>
<tr>
<td>lhi</td>
<td>hydroxypropionate-dependent protein</td>
<td>-5.2</td>
<td>2.44</td>
<td>0.0011</td>
<td>hydroxypropionate-dependent protein</td>
</tr>
<tr>
<td>fecI</td>
<td>KpLE2 plage-like element; RNA polymerase, sigma 19 factor</td>
<td>4.5</td>
<td>2.41</td>
<td>0.00015</td>
<td>probable RNA polymerase sigma factor</td>
</tr>
<tr>
<td>fes</td>
<td>enterobactin/ferric enterobactin esterase</td>
<td>4.5</td>
<td>2.44</td>
<td>0.00022</td>
<td>enterochelin esterase</td>
</tr>
<tr>
<td>fdhE</td>
<td>formate dehydrogenase formation protein</td>
<td>-4.4</td>
<td>5.87</td>
<td>0.05351</td>
<td>affects formate dehydrogenase-N</td>
</tr>
<tr>
<td>yeaU</td>
<td>predicted dehydrogenase</td>
<td>-4.3</td>
<td>1.87</td>
<td>0.00002</td>
<td>putative tartrate dehydrogenase</td>
</tr>
<tr>
<td>mdtJ</td>
<td>multidrug efflux system transporter</td>
<td>4.3</td>
<td>1.7</td>
<td>0.001</td>
<td>possible chaperone</td>
</tr>
<tr>
<td>fecR</td>
<td>transmembrane signal transducer for ferric citrate transport</td>
<td>4.2</td>
<td>2.42</td>
<td>0.00018</td>
<td>regulator for fec operon, periplasmic</td>
</tr>
<tr>
<td>cueO</td>
<td>multicopper oxidase (laccase)</td>
<td>4.0</td>
<td>1.81</td>
<td>0.00002</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>cspE</td>
<td>DNA-binding transcriptional repressor</td>
<td>-4.0</td>
<td>2.14</td>
<td>0.00002</td>
<td>cold shock protein</td>
</tr>
<tr>
<td>hspB</td>
<td>heat shock chaperone</td>
<td>4.0</td>
<td>2.5</td>
<td>0.00512</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>mrdD</td>
<td>formate-dependent nitrate reductase, membrane subunit</td>
<td>4.0</td>
<td>2.18</td>
<td>0.00009</td>
<td>formate-dependent nitrate reductase complex</td>
</tr>
<tr>
<td>exhD</td>
<td>membrane spanning protein in TonB-ExbD-ExbD complex</td>
<td>4.0</td>
<td>2.14</td>
<td>0.00001</td>
<td>uptake of enterochelin; TonB-dependent uptake of B colicins</td>
</tr>
</tbody>
</table>

188
## Table 5.2 Comparison of upregulated E. coli genes: Ag⁺ versus negative controls.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold change</th>
<th>FDR</th>
<th>Adjusted P value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ynaE</td>
<td>Rac prophage; predicted DNA-binding transcriptional regulator</td>
<td>-58.8</td>
<td>1.93</td>
<td>0.00008</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>nirB</td>
<td>nitrite reductase, large subunit, NAD(P)H-binding</td>
<td>27.9</td>
<td>1.84</td>
<td>0</td>
<td>nitrite reductase</td>
</tr>
<tr>
<td>cspB</td>
<td>Qin prophage; cold shock protein</td>
<td>-20.6</td>
<td>1.84</td>
<td>0.00004</td>
<td>cold shock protein; may affect transcription</td>
</tr>
<tr>
<td>Z3931</td>
<td>unknown protein encoded by prophage CP-933Y</td>
<td>-15.9</td>
<td>4.1</td>
<td>0.0288</td>
<td>unknown protein encoded by prophage CP-933Y</td>
</tr>
<tr>
<td>glcD</td>
<td>glycolate oxidase subunit, FAD-linked</td>
<td>-14.7</td>
<td>1.93</td>
<td>0</td>
<td>glycolate oxidase subunit D</td>
</tr>
<tr>
<td>cspG</td>
<td>DNA-binding transcriptional regulator</td>
<td>-14.2</td>
<td>1.93</td>
<td>0.00008</td>
<td>homolog of Salmonella cold shock protein</td>
</tr>
<tr>
<td>ycgF</td>
<td>predicted FAD-binding phosphodiesterase</td>
<td>-11.8</td>
<td>1.93</td>
<td>0.0002</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>glcF</td>
<td>glycolate oxidase iron-sulfur subunit</td>
<td>-11.8</td>
<td>1.93</td>
<td>0</td>
<td>glycolate oxidase iron-sulfur subunit</td>
</tr>
<tr>
<td>nirC</td>
<td>nitrite transporter</td>
<td>11.4</td>
<td>1.93</td>
<td>0</td>
<td>nitrite reductase activity [b3367]</td>
</tr>
<tr>
<td>adhE</td>
<td>iron-dependent alcohol dehydrogenase</td>
<td>10.6</td>
<td>1.93</td>
<td>0</td>
<td>CoA-linked acetaldehyde dehydrogenase</td>
</tr>
<tr>
<td>ygiT</td>
<td>predicted DNA-binding transcriptional regulator</td>
<td>8.9</td>
<td>2.71</td>
<td>0</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>arsR</td>
<td>DNA-binding transcriptional repressor</td>
<td>8.4</td>
<td>3.52</td>
<td>0</td>
<td>transcriptional repressor of chromosomal ars operon</td>
</tr>
<tr>
<td>gpmM</td>
<td>phosphoglycerate mutase III, cofactor-independent</td>
<td>7.7</td>
<td>3.51</td>
<td>0</td>
<td>2,3-bisphosphoglycerate-independent phosphoglycerate mutase</td>
</tr>
<tr>
<td>putA</td>
<td>fused DNA-binding transcriptional regulator/proline dehydrogenase</td>
<td>-7.0</td>
<td>4.59</td>
<td>0.00002</td>
<td>proline dehydrogenase, PSC dehydrogenase</td>
</tr>
<tr>
<td>malK</td>
<td>ATP-binding component of ABC superfamily/regulatory protein</td>
<td>6.6</td>
<td>5.16</td>
<td>0.0002</td>
<td>ATP-binding component of transport system for maltose</td>
</tr>
<tr>
<td>dcuC</td>
<td>anaerobic C4-dicarboxylate transport</td>
<td>6.4</td>
<td>6.27</td>
<td>0</td>
<td>transport of dicarboxylates</td>
</tr>
<tr>
<td>fruK</td>
<td>fructose-1-phosphate kinase</td>
<td>6.3</td>
<td>5.98</td>
<td>0.00025</td>
<td>fructose-1-phosphate kinase</td>
</tr>
<tr>
<td>psaE</td>
<td>predicted phosphate starvation inducible protein</td>
<td>-6.2</td>
<td>5.89</td>
<td>0.00022</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>aaeX</td>
<td>membrane protein of efflux system</td>
<td>5.8</td>
<td>6.03</td>
<td>0.00002</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>fruB</td>
<td>fused fructose-specific PTS enzymes: IIA component/HPr component</td>
<td>5.7</td>
<td>8.1</td>
<td>0.00044</td>
<td>PTS system, fructose-specific IIA/fpr component</td>
</tr>
<tr>
<td>treC</td>
<td>trehalose-6-P hydrolase</td>
<td>5.5</td>
<td>8.1</td>
<td>0</td>
<td>trehalase 6-P hydrolase</td>
</tr>
</tbody>
</table>
## Table 5.3 Comparison of upregulated E. coli genes: Zeolite + Ag⁰ nanoparticles versus negative controls.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>FDR</th>
<th>Adjusted p value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>zraP</td>
<td>Zn-binding periplasmic protein</td>
<td>246.7</td>
<td>2.24</td>
<td>0</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>copA</td>
<td>copper transporter</td>
<td>77.3</td>
<td>1.68</td>
<td>0</td>
<td>putative ATPase</td>
</tr>
<tr>
<td>cueO</td>
<td>multicopper oxidase (laccase)</td>
<td>26.8</td>
<td>1.68</td>
<td>0</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>marR</td>
<td>DNA-binding transcriptional repressor of multiple antibiotic resistance</td>
<td>21.9</td>
<td>1.68</td>
<td>0</td>
<td>multiple antibiotic resistance protein; repressor of mar operon</td>
</tr>
<tr>
<td>marA</td>
<td>DNA-binding transcriptional dual activator of multiple antibiotic resistance</td>
<td>19.9</td>
<td>1.68</td>
<td>0</td>
<td>multiple antibiotic resistance; transcriptional activator of defense systems</td>
</tr>
<tr>
<td>sbp</td>
<td>sulfate transporter subunit</td>
<td>18.8</td>
<td>1.68</td>
<td>0.00001</td>
<td>periplasmic sulfate-binding protein</td>
</tr>
<tr>
<td>ygiI</td>
<td>predicted oxidoreductase with NAD(P)-binding Rossmann-fold domain</td>
<td>13.2</td>
<td>3.08</td>
<td>0.00074</td>
<td>putative oxidoreductase</td>
</tr>
<tr>
<td>mgtA</td>
<td>magnesium transporter</td>
<td>11.8</td>
<td>3.08</td>
<td>0.00090</td>
<td>Mg²⁺ transport ATPase, P-type 1</td>
</tr>
<tr>
<td>grxA</td>
<td>glutaredoxin 1, redox coenzyme for ribonucleotide reductase (RNR1a)</td>
<td>10.5</td>
<td>3.08</td>
<td>0</td>
<td>glutaredoxin1 redox coenzyme</td>
</tr>
<tr>
<td>zntA</td>
<td>zinc, cobalt and lead efflux system</td>
<td>9.9</td>
<td>3.08</td>
<td>0.00209</td>
<td>zinc-transporting ATPase</td>
</tr>
<tr>
<td>fnuK</td>
<td>fructose-1-phosphate kinase</td>
<td>9.7</td>
<td>2.91</td>
<td>0.00004</td>
<td>fructose-1-phosphate kinase</td>
</tr>
<tr>
<td>fruB</td>
<td>fused fructose-specific PTS enzymes</td>
<td>8.6</td>
<td>2.52</td>
<td>0.00001</td>
<td>PTS system, fructose-specific IIA/fpr component</td>
</tr>
<tr>
<td>yhdD</td>
<td>predicted Mg²⁺ transport ATPase inner membrane protein</td>
<td>8.1</td>
<td>5.2</td>
<td>0.01448</td>
<td>putative transport ATPase</td>
</tr>
<tr>
<td>frmA</td>
<td>glutathione-dependent formaldehyde dehydrogenase</td>
<td>8.0</td>
<td>2.47</td>
<td>0.00001</td>
<td>formaldehyde dehydrogenase, glutathione-dependent</td>
</tr>
<tr>
<td>cysP</td>
<td>thiosulfate transporter subunit</td>
<td>7.9</td>
<td>3.02</td>
<td>0.00099</td>
<td>thiosulfate binding protein</td>
</tr>
<tr>
<td>trxC</td>
<td>thioredoxin 2</td>
<td>7.6</td>
<td>2.28</td>
<td>0</td>
<td>putative thioredoxin-like protein</td>
</tr>
<tr>
<td>yjiH</td>
<td>conserved inner membrane protein</td>
<td>7.6</td>
<td>3.75</td>
<td>0.00209</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>ihpB</td>
<td>heat shock chaperone</td>
<td>7.5</td>
<td>3.08</td>
<td>0.0032</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>alx</td>
<td>predicted inner membrane protein, part of terminus</td>
<td>7.1</td>
<td>2.47</td>
<td>0</td>
<td>putative transport protein</td>
</tr>
<tr>
<td>glkD</td>
<td>glycolate oxidase subunit, FAD-linked</td>
<td>-6.8</td>
<td>2.4</td>
<td>0</td>
<td>glycolate oxidase subunit D</td>
</tr>
<tr>
<td>ycgF</td>
<td>predicted FAD-binding phosphodiesterase</td>
<td>-6.8</td>
<td>3.58</td>
<td>0.0157</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>cysA</td>
<td>sulfate/thiosulfate transporter subunit</td>
<td>6.7</td>
<td>3.08</td>
<td>0.0002</td>
<td>ATP-binding component of sulfate permease A protein</td>
</tr>
<tr>
<td>nirD</td>
<td>nitrite reductase, NAD(P)H-binding, small subunit</td>
<td>6.4</td>
<td>2.75</td>
<td>0.00003</td>
<td>nitrite reductase</td>
</tr>
<tr>
<td>cysU</td>
<td>sulfate/thiosulfate transporter subunit</td>
<td>6.4</td>
<td>2.71</td>
<td>0.00004</td>
<td>sulfate, thiosulfate transport system permease T protein</td>
</tr>
<tr>
<td>cysW</td>
<td>sulfate/thiosulfate transporter subunit</td>
<td>6.2</td>
<td>3.16</td>
<td>0.00029</td>
<td>sulfate transport system permease W protein</td>
</tr>
<tr>
<td>glcF</td>
<td>glycolate oxidase iron-sulfur subunit</td>
<td>-5.6</td>
<td>2.52</td>
<td>0.00001</td>
<td>glycolate oxidase iron-sulfur subunit</td>
</tr>
<tr>
<td>soxR</td>
<td>Fe-S center for redox-sensing</td>
<td>5.3</td>
<td>3.08</td>
<td>0.0004</td>
<td>redox-sensing activator of soxS</td>
</tr>
<tr>
<td>spy</td>
<td>envelope stress induced periplasmic protein</td>
<td>5.3</td>
<td>2.47</td>
<td>0</td>
<td>periplasmic protein related to spheroplast formation</td>
</tr>
<tr>
<td>hemH</td>
<td>ferrochelatase</td>
<td>5.3</td>
<td>3.08</td>
<td>0.00016</td>
<td>ferrochelatase; final enzyme of heme biosynthesis</td>
</tr>
<tr>
<td>ndh</td>
<td>respiratory NADH dehydrogenase 2/cupric reductase</td>
<td>4.9</td>
<td>3.52</td>
<td>0.00003</td>
<td>respiratory NADH dehydrogenase</td>
</tr>
<tr>
<td>gbsR</td>
<td>tartronate semialdehyde reductase, NADH-dependent</td>
<td>-4.0</td>
<td>3.02</td>
<td>0.00159</td>
<td>putative oxidoreductase</td>
</tr>
<tr>
<td>sodB</td>
<td>superoxide dismutase, Fe</td>
<td>3.2</td>
<td>3.77</td>
<td>0.00015</td>
<td>superoxide dismutase, iron</td>
</tr>
<tr>
<td>ykB</td>
<td>copper/silver efflux system outer membrane protein CusC</td>
<td>3.2</td>
<td>6.65</td>
<td>0.0383</td>
<td>putative resistance protein</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Gene Name</td>
<td>Fold Change</td>
<td>FDR</td>
<td>Adjusted p value</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------</td>
<td>-------------</td>
<td>------</td>
<td>------------------</td>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>cysP</td>
<td>thiosulfate transporter subunit</td>
<td>-14.9</td>
<td>1.67</td>
<td>0</td>
<td>thiosulfate binding protein</td>
</tr>
<tr>
<td>nrdF</td>
<td>ribonucleoside-diphosphate reductase 2, beta subunit, ferritin-like protein</td>
<td>13.9</td>
<td>1.55</td>
<td>0</td>
<td>ribonucleoside-diphosphate reductase 2, beta chain</td>
</tr>
<tr>
<td>fiu</td>
<td>predicted iron outer membrane transporter</td>
<td>13.7</td>
<td>1.46</td>
<td>0</td>
<td>putative outer membrane receptor for iron transport</td>
</tr>
<tr>
<td>fepG</td>
<td>iron-enterobactin transporter subunit</td>
<td>12.6</td>
<td>1.52</td>
<td>0</td>
<td>ferric enterobactin transport protein</td>
</tr>
<tr>
<td>cysW</td>
<td>sulfate/thiosulfate transporter subunit</td>
<td>-11.9</td>
<td>1.88</td>
<td>0.00001</td>
<td>sulfate transport system permease W protein</td>
</tr>
<tr>
<td>copA</td>
<td>copper transporter</td>
<td>-10.8</td>
<td>1.52</td>
<td>0</td>
<td>putative ATPase</td>
</tr>
<tr>
<td>nrdE</td>
<td>ribonucleoside-diphosphate reductase 2, alpha subunit</td>
<td>10.4</td>
<td>1.8</td>
<td>0.00001</td>
<td>ribonucleoside-diphosphate reductase 2, alpha subunit</td>
</tr>
<tr>
<td>cysA</td>
<td>sulfate/thiosulfate transporter subunit</td>
<td>-10.3</td>
<td>1.8</td>
<td>0.00002</td>
<td>ATP-binding component of sulfate permease A protein</td>
</tr>
<tr>
<td>hemH</td>
<td>ferrochelatase</td>
<td>-9.5</td>
<td>1.67</td>
<td>0.00001</td>
<td>ferrochelatase: final enzyme of heme biosynthesis</td>
</tr>
<tr>
<td>mgtA</td>
<td>magnesium transporter</td>
<td>-9.0</td>
<td>2.5</td>
<td>0.00012</td>
<td>Mg2+ transport ATPase, P-type 1</td>
</tr>
<tr>
<td>cysD</td>
<td>sulfate adenylytransferase, subunit 2</td>
<td>-9.0</td>
<td>1.84</td>
<td>0.00001</td>
<td>ATP-sulfurylase</td>
</tr>
<tr>
<td>cysU</td>
<td>sulfate/thiosulfate transporter subunit</td>
<td>-9.0</td>
<td>1.62</td>
<td>0</td>
<td>sulfate, thiosulfate transport system permease T protein</td>
</tr>
<tr>
<td>cysI</td>
<td>sulfite reductase, alpha subunit, flavoprotein</td>
<td>-8.7</td>
<td>1.8</td>
<td>0.00001</td>
<td>sulfite reductase</td>
</tr>
<tr>
<td>fecI</td>
<td>KpLE2 phage-like element; RNA polymerase, sigma 19 factor</td>
<td>8.5</td>
<td>1.63</td>
<td>0</td>
<td>probable RNA polymerase sigma factor</td>
</tr>
<tr>
<td>fes</td>
<td>enterobactin/ferric enterobactin esterase</td>
<td>8.4</td>
<td>1.59</td>
<td>0</td>
<td>enterochelin esterase</td>
</tr>
<tr>
<td>yeeE</td>
<td>predicted inner membrane protein</td>
<td>-8.1</td>
<td>1.94</td>
<td>0.00006</td>
<td>putative transport system permease protein</td>
</tr>
<tr>
<td>trxC</td>
<td>thioredoxin 2</td>
<td>-7.8</td>
<td>2.32</td>
<td>0</td>
<td>putative thioredoxin-like protein</td>
</tr>
<tr>
<td>grxA</td>
<td>glutaredoxin 1, redox coenzyme for ribonucleotide reductase (RNR1a)</td>
<td>-7.6</td>
<td>2.5</td>
<td>0.00013</td>
<td>glutaredoxin1, redox coenzyme</td>
</tr>
<tr>
<td>sbp</td>
<td>sulfate transporter subunit</td>
<td>-7.6</td>
<td>2.5</td>
<td>0.00013</td>
<td>periplasmic sulfate-binding protein</td>
</tr>
<tr>
<td>entE</td>
<td>enterobactin synthase multienzyme complex</td>
<td>7.4</td>
<td>1.75</td>
<td>0.00001</td>
<td>2,3-dihydroxybenzoate-AMP ligase</td>
</tr>
<tr>
<td>entA</td>
<td>2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase</td>
<td>7.2</td>
<td>1.52</td>
<td>0</td>
<td>2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase</td>
</tr>
<tr>
<td>yigI</td>
<td>predicted oxidoreductase with NAD(P)-binding Rossmann-fold domain</td>
<td>-7.2</td>
<td>4.98</td>
<td>0.00295</td>
<td>putative oxidoreductase</td>
</tr>
<tr>
<td>cirA</td>
<td>ferric iron-catecholate outer membrane transporter</td>
<td>7.1</td>
<td>1.46</td>
<td>0</td>
<td>outer membrane receptor for iron-regulated colicin I receptor</td>
</tr>
<tr>
<td>entF</td>
<td>enterobactin synthase multienzyme complex component</td>
<td>6.8</td>
<td>1.8</td>
<td>0.00001</td>
<td>ATP-dependent serine activating enzyme</td>
</tr>
<tr>
<td>marR</td>
<td>DNA-binding transcriptional repressor of multiple antibiotic resistance</td>
<td>-6.8</td>
<td>1.52</td>
<td>0</td>
<td>multiple antibiotic resistance protein</td>
</tr>
<tr>
<td>entS</td>
<td>predicted transporter</td>
<td>6.6</td>
<td>1.46</td>
<td>0</td>
<td>putative transport</td>
</tr>
<tr>
<td>cysI</td>
<td>sulfite reductase, beta subunit, NAD(P)-binding, heme-binding</td>
<td>-6.1</td>
<td>2.08</td>
<td>0.00004</td>
<td>sulfite reductase, alpha subunit</td>
</tr>
<tr>
<td>fepD</td>
<td>iron-enterobactin transporter subunit</td>
<td>4.7</td>
<td>1.57</td>
<td>0</td>
<td>ferric enterobactin</td>
</tr>
</tbody>
</table>

Table 5.4 Comparison of upregulated E. coli genes: Zeolite versus Zeolite + Ag⁰ nanoparticles.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold change</th>
<th>FDR</th>
<th>Adjusted p value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>zraP</td>
<td>Zn-binding periplasmic protein</td>
<td>-214.4</td>
<td>1.88</td>
<td>0</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>copA</td>
<td>copper transporter</td>
<td>-25.2</td>
<td>1.88</td>
<td>0</td>
<td>putative ATPase</td>
</tr>
<tr>
<td>ynaE</td>
<td>Rac prophage; predicted DNA-binding transcriptional regulator</td>
<td>-17.9</td>
<td>2.15</td>
<td>0.00026</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>cysP</td>
<td>thiosulfate transporter subunit</td>
<td>-16.9</td>
<td>1.88</td>
<td>0</td>
<td>thiosulfate binding protein</td>
</tr>
<tr>
<td>cueO</td>
<td>multicopper oxidase (laccase)</td>
<td>-16.3</td>
<td>1.88</td>
<td>0</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>malR</td>
<td>ATP-binding component of ABC superfamily/regulatory protein</td>
<td>12.8</td>
<td>1.88</td>
<td>0.00001</td>
<td>ATP-binding component of transport system for maltose</td>
</tr>
<tr>
<td>sbp</td>
<td>sulfate transporter subunit</td>
<td>-12.7</td>
<td>1.88</td>
<td>0</td>
<td>periplasmic sulfate-binding protein</td>
</tr>
<tr>
<td>mgtA</td>
<td>magnesium transporter</td>
<td>-10.1</td>
<td>2.15</td>
<td>0.00008</td>
<td>Mg2+ transport ATPase, P-type 1</td>
</tr>
<tr>
<td>grxA</td>
<td>glutaredoxin 1, redox coenzyme for ribonucleotide reductase (RNR1a)</td>
<td>-9.3</td>
<td>2.15</td>
<td>0</td>
<td>glutaredoxin 1 redox coenzyme</td>
</tr>
<tr>
<td>cysU</td>
<td>sulfate/thiosulfate transporter subunit</td>
<td>-8.4</td>
<td>1.88</td>
<td>0</td>
<td>sulfate, thiosulfate transport system permease T protein</td>
</tr>
<tr>
<td>cusF</td>
<td>periplasmic copper-binding protein</td>
<td>-7.6</td>
<td>2.36</td>
<td>0.00158</td>
<td>orf, hypothetical protein</td>
</tr>
<tr>
<td>dcuB</td>
<td>C4-dicarboxylate antipporter</td>
<td>7.6</td>
<td>1.9</td>
<td>0</td>
<td>anaerobic dicarboxylate transport</td>
</tr>
<tr>
<td>yhaD</td>
<td>predicted Mg(2+) transport ATPase inner membrane protein</td>
<td>-7.4</td>
<td>3.12</td>
<td>0.01217</td>
<td>putative transport ATPase</td>
</tr>
<tr>
<td>yeeE</td>
<td>predicted inner membrane protein</td>
<td>-7.4</td>
<td>2.08</td>
<td>0.00090</td>
<td>putative transport system permease protein</td>
</tr>
<tr>
<td>cysW</td>
<td>sulfate/thiosulfate transporter subunit</td>
<td>-6.7</td>
<td>2.15</td>
<td>0.00011</td>
<td>sulfate transport system permease W protein</td>
</tr>
<tr>
<td>yetT</td>
<td>predicted oxidoreductase</td>
<td>6.7</td>
<td>1.88</td>
<td>0</td>
<td>putative oxidoreductase</td>
</tr>
<tr>
<td>chaA</td>
<td>calcium/sodium:proton antipporter</td>
<td>-6.4</td>
<td>1.88</td>
<td>0</td>
<td>sodium-calcium:proton antipporter</td>
</tr>
<tr>
<td>zntA</td>
<td>zinc, cobalt and lead efflux system</td>
<td>-6.3</td>
<td>2.2</td>
<td>0.00549</td>
<td>zinc-transporting ATPase</td>
</tr>
<tr>
<td>cysA</td>
<td>sulfate/thiosulfate transporter subunit</td>
<td>-6.3</td>
<td>2.15</td>
<td>0.00014</td>
<td>ATP-binding component of sulfate permease A protein</td>
</tr>
<tr>
<td>hsbB</td>
<td>heat shock chaperone</td>
<td>5.8</td>
<td>2.15</td>
<td>0.00053</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>mglA</td>
<td>maltose transporter subunit</td>
<td>5.7</td>
<td>1.92</td>
<td>0</td>
<td>part of maltose permease, inner membrane</td>
</tr>
<tr>
<td>atoS</td>
<td>sensory histidine kinase in two-component regulatory system with AtoC</td>
<td>5.6</td>
<td>1.88</td>
<td>0</td>
<td>sensor protein AtoS for response regulator AtoC</td>
</tr>
<tr>
<td>cysD</td>
<td>sulfate adenylyltransferase, subunit 2</td>
<td>-5.5</td>
<td>2.15</td>
<td>0.00012</td>
<td>ATP:sulfurylase</td>
</tr>
<tr>
<td>tdcA</td>
<td>DNA-binding transcriptional activator</td>
<td>5.5</td>
<td>2.29</td>
<td>0.00071</td>
<td>transcriptional activator of tdc operon</td>
</tr>
<tr>
<td>melA</td>
<td>alpha-galactosidase, NAD(P)-binding</td>
<td>5.4</td>
<td>1.88</td>
<td>0</td>
<td>alpha-galactosidase</td>
</tr>
<tr>
<td>trxC</td>
<td>thioredoxin 2</td>
<td>-5.3</td>
<td>1.88</td>
<td>0</td>
<td>putative thioredoxin-like protein</td>
</tr>
<tr>
<td>nirB</td>
<td>nitrite reductase, large subunit, NAD(P)H-binding</td>
<td>4.9</td>
<td>1.9</td>
<td>0</td>
<td>nitrite reductase</td>
</tr>
<tr>
<td>furC</td>
<td>fumarate hydratase (fumarase C),aerobic Class II</td>
<td>-4.8</td>
<td>1.92</td>
<td>0.00001</td>
<td>fumarase C=fumarate hydratase Class II; isozyme</td>
</tr>
<tr>
<td>putD</td>
<td>gamma-Glu-GABA hydrolase</td>
<td>-4.8</td>
<td>2.2</td>
<td>0.00056</td>
<td>probable amidotransferase subunit</td>
</tr>
<tr>
<td>marA</td>
<td>DNA-binding transcriptional dual activator of multiple antibiotic resistance</td>
<td>-4.8</td>
<td>1.95</td>
<td>0</td>
<td>multiple antibiotic resistance</td>
</tr>
</tbody>
</table>

Table 5. 5 Comparison of upregulated E. coli genes: Ag\textsuperscript{+} versus Zeolite + Ag\textsuperscript{0} nanoparticles.
Figure 5.14 Murine macrophage viability is significantly reduced in a dose-dependent manner after incubation with Ag\(^+\) for 15 minutes (n = 3, p < 0.05).
Dissolution of Ag$^+$ from Zeolite supports containing AgNPs occurs over time. Silver ions enter the cell via passive diffusion or metal transport proteins. Antioxidant proteins are inactivated due to the affinity of Ag$^+$ to thiol groups, resulting in oxidative stress and free radical formation. Reactive oxygen species disrupt electron transport and causes cell death. Additionally, Ag$^+$ causes physical disturbance in the cell wall and membrane resulting in the deregulation of Na$^+$ and K$^+$ within the bacteria. Due to the increase in free radicals, antioxidants are upregulated by the bacterium as are Sil and Mar genes, which are associated with survival and code for silver efflux pumps and metal oxidation. Silver ions chelate Cl$^-$, reducing the bioavailability of Ag$^+$, but also reducing intracellular Cl$^-$ pools. In silver susceptible bacteria, antioxidant defenses are overwhelmed and cell death occurs. However, the evolution of silver resistant bacteria should not be discounted as several classes of genes associated with silver and antibiotic resistance were upregulated.

Figure 5.15 Antimicrobial mechanism of zeolite substrates containing AgNPs.
Chapter 6: Concluding Statements and Future Directions

In the current era, technological advances in sciences are made and communicated rapidly. There is often an air of excitement with regard to such breakthroughs, and the emergence of the field of nanotechnology was no different. The possibilities for improving the quality of life may likely rely on nanotechnology. As such, the incorporation of nanotechnology, and nanoparticles, has already infiltrated the average American consumer’s lifestyle. However, while populations are quick to take advantage of some of conveniences that nanotechnology provides, the investigation into the acute and long term consequences that may be associated with these advantages is a much slower and tedious process. For example, nanoparticles are currently used for water filtration, yet how these materials affect biological function and their safety is largely unknown. The human and environmental repercussions of nanoparticle manufacture and use is just being realized. It was the focus of this thesis to determine how different physicochemical parameters associated with nanoparticles influence cell responses.

The use of QDs for biological imaging and drug delivery is an area undergoing rigorous experimentation. Here, the overall negative surface charge of microwave irradiated CdSe/ZnS QDs was found to dictate a mechanism of cellular internalization.
Ongoing (but yet incomplete) studies indicate that positive charged QDs (+QDs) are more aggregated in biological media. In the presence of the same chemicals shown to prevent negatively charged QDs (-QD) from associating with macrophages, +QDs were still found to associate with cells (Figure 6.1). These preliminary data indicate that surface charge may dictate the mechanism of nanoparticle entry, and even their intracellular fate. This information can be exploited for a variety of functions and provide another layer of specificity to pharmaceutical delivery. However, it must also be noted that these +QDs began to induce apoptosis in cells that had been exposed for 2 hours, whereas apoptosis was not apparent for 24 hours when macrophages were exposed to –QDs (Figure 6.2). Further testing is also needed regarding the capability of these QDs to induce a proinflammatory response.

The global population is expected to exceed 10 billion people by 2055. Most of the global population is found in growing third world countries where air pollution guidelines are not as stringent. It also seems that the automobiles used in these developing areas are not as sophisticated in regards to emissions protection, since they are typically older model automobiles. In addition, the manufacturing industry, including nanoparticle manufacture is booming. Conditions are ripe for air quality to worsen in these locales. When combining the fact that the infrastructure was not developed to support such growth, there will likely be much more traffic, and, as a consequence, more idling cars and trucks further contributing to poor air quality. As reviewed above, many factors contribute to the toxicity and pulmonary inflammation caused by poor air quality. The association between particulate matter size and mortality due to pulmonary
dysfunction is clear. What remains unclear is the confounding affects that gases and nanoparticles have with regard to pulmonary function, inflammation and fibrosis. The current work suggests that ozonation may modify nanoparticle surfaces in such a way that proinflammatory cytokines secretion is attenuated. Modification of surface function groups on carbon nanoparticles is inevitable, and it is worthwhile to study cohorts that reside in areas with consistently high ozone levels and compare them to others with lower ozone levels, but higher particulate pollution. While the carbon nanoparticle studies performed in this work were designed to tease apart individual surface group impacts on alveolar cell responses, it is noted that much more complex nanoparticles exist in reality. It is important from a mechanistic point of view to determine the contribution of individual components including metal, organics and biological constituents. However, it would be practical and helpful to also chemically and biologically characterize air samples collected from different locations. It may also be worthwhile to modify these captured air particles by extracting organics or precipitating metals to design better indicators for nanoparticle components that drive a pulmonary response. Future experiments that delve into mechanisms driving the decrease in TNF-α secretion in response to freshly ozonated CNPs are ongoing.

Perhaps the most practical application that was investigated in this work was the efficacy of zeolite supports containing AgNPs. Silver has been known to have antibacterial properties for thousands of years. The interest in nanoparticles and their unique properties that appear with decreasing size has no doubt been behind the renewed interest in AgNPs. However, unlike iron oxide nanoparticles or QDs, which do display
distinctive properties with decreasing size (superparamagnetism and enhanced optical properties, respectively), silver does not. Therefore, the most important aspect of using AgNPs is their increased surface area compared to bulk. To investigate if increasing surface area could enhance the killing power of silver, we used both AgNPs and also micropatterned the surface of zeolites. A significant difference was not initially found when comparing supports that were patterned versus unpatterned. However, further experiments are recommended to optimize and compare the performance of micropatterned zeolite AgNPs. Quantitative reverse transcription polymerase chain reaction experiments are underway to quantify the expression of genes if interest regarding the antimicrobial activity of zeolite AgNPs in *E. coli*. It is also worthwhile to develop an impregnation method that deposits homogenously sized AgNPs onto the zeolite structure. The longevity of individual supports (and potentially coatings) may be improved if the size of AgNPs is uniform and smaller, which may allow for increased dissolution of Ag$^+$ from AgNPs.

The applications of AgNPs are seemingly popping up everywhere. The Project on Emerging Nanoparticle Technologies [386] revealed there are over 1,000 consumer products available containing nanoparticles. Silver was found to be the leading material that products contain nanoparticles with over 250 products, mostly related to personal care. On the surface, the addition of AgNPs to products, including clothes, containers and cosmetics seems beneficial. Food preservation saves money, and most people prefer to have good hygiene. The problem lies in tolerance. Bacteria can develop tolerance to just about anything, as evidenced by the evolution of antibiotic resistant bacteria. The fate is
just as likely for bacteria and silver. However, while it may seem plausible to just go back to fabric that is silver-free, ecological and environmental damage will likely not be as easily reversible. Additionally, there is contradicting data with regard to the safety of AgNPs and mammalian cells. By most accounts, the form of silver and its bioavailability seem to dictate the toxicity of the particle. However, this is an area that deserves much more attention. The doses and mechanism of action of any therapeutic must be carefully analyzed in a multitude of systems before it is approved to be used as a medical treatment, yet these guidelines are not the same for the contents of consumer goods, including textiles, food, cosmetics and lotions. All of these goods have the ability to shed AgNPs (as some are designed to do so), which means they can be inhaled, ingested and absorbed into the body. Hence, the consequences of prolonged nanoparticle exposure are nontrivial. Experiments investigating the role of free radicals and antioxidant balance are ongoing. Additionally, experiments investigating the kinetics of AgNP and Ag+ release from zeolite AgNPs are proposed, as are studies detailing the bioavailability silver in both physiologically and environmentally relevant conditions.

While nanotechnology applications are blossoming, the field of nanotoxicology is lagging. Research understanding the molecular mechanisms of nanotoxicology is underway; however caution should be yielded as long term studies will not be concluded for years. As stated, the physicochemical components of nanoparticles often contribute to adverse consequences in biological systems. It is reasonable and recommended that nanoparticles undergo thorough and precise chemical and biological characterization so that exposure and environmental risks can be adequately identified.
Figure 6.1 Scavenger receptor ligand Poly I does not prevent positive QDs from associating with cells.
Figure 6.2 Large doses of positively-charged QDs induces apoptosis in less than 2 hours.
Literature Cited


373. Foldbjerg, R., D. Dang, and H. Autrup, Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549. Archives of Toxicology, 2010.


