INVESTIGATION AND CHARACTERIZATION OF THE CELLULAR UPTAKE OF NANOPARTICLES

By

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To Danton Sherwood for always being there, ready to help
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>10</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>11</td>
</tr>
<tr>
<td>LIST OF OBJECTS</td>
<td>16</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>17</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>19</td>
</tr>
<tr>
<td>Motivation</td>
<td>19</td>
</tr>
<tr>
<td>Objective</td>
<td>21</td>
</tr>
<tr>
<td>Dissertation Organization</td>
<td>23</td>
</tr>
<tr>
<td>Background and Literature Review</td>
<td>24</td>
</tr>
<tr>
<td>Nanoparticles</td>
<td>24</td>
</tr>
<tr>
<td>Nanotechnology and Nanomaterials</td>
<td>24</td>
</tr>
<tr>
<td>Nanotoxicology</td>
<td>25</td>
</tr>
<tr>
<td>Human and Environmental Exposures</td>
<td>26</td>
</tr>
<tr>
<td>Characterization of Nanoparticles</td>
<td>27</td>
</tr>
<tr>
<td>In Vitro Testing</td>
<td>27</td>
</tr>
<tr>
<td>In Vivo Testing</td>
<td>28</td>
</tr>
<tr>
<td>Particle Interactions in Biological Systems</td>
<td>28</td>
</tr>
<tr>
<td>Cellular Take Up of Nanoparticles</td>
<td>28</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>29</td>
</tr>
<tr>
<td>Polymer Coatings</td>
<td>31</td>
</tr>
<tr>
<td>Correlative Microscopy for the Study of Nanotoxicology</td>
<td>32</td>
</tr>
<tr>
<td>Correlative Microscopy</td>
<td>32</td>
</tr>
<tr>
<td>Microscopy Techniques for Particle Characterization</td>
<td>33</td>
</tr>
<tr>
<td>Microscopy Techniques for Characterizing the Interactions of Nanoparticles and Cells</td>
<td>33</td>
</tr>
<tr>
<td>Summary</td>
<td>35</td>
</tr>
<tr>
<td>2 THE STUDY OF NANOPARTICLE UP TAKE BY HUMAN Alveolar Basal EPITHELIAL CELLS USING CORRELATIVE MICROSCOPY</td>
<td>36</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>37</td>
</tr>
<tr>
<td>Cell Line</td>
<td>37</td>
</tr>
<tr>
<td>Dye Doped Silica Nanoparticles</td>
<td>37</td>
</tr>
<tr>
<td>Characterization of LSI Ru(bpy)3 Doped Silica Particles</td>
<td>38</td>
</tr>
<tr>
<td>Sample Preparation Confocal Microscopy</td>
<td>38</td>
</tr>
</tbody>
</table>
Sample Preparation for TEM .......................................................... 39
Confocal Microscopy ........................................................................ 40
Optical Microscopy ........................................................................... 40
Transmission Electron Microscopy .................................................. 40

Results ............................................................................................. 41
LSI Ru(bpy)3 Doped Silica Nanoparticles ........................................ 41
Confocal Microscopy Results ............................................................ 41
Optical Microscopy Results ............................................................... 43
TEM Results ..................................................................................... 43

Discussion ....................................................................................... 46
Summary ........................................................................................... 46

3 INVESTIGATION OF THE EFFECT COATINGS ON THE PROPERTIES OF PLATINUM NANOPARTICLES AND THEIR UPTAKE INTO BEAS CELLS .... 56

Oxidants and ROS ............................................................................ 56
Heme Oxygenase ............................................................................. 57
Colloidal Pt Nanoparticles ............................................................... 57
Catalytic Activity of Colloidal Pt ..................................................... 58
Expression of HO-1 in BEAS Cells ................................................... 59
Description of Work ........................................................................ 61

Materials and Methods ................................................................. 61
Preparation of Platinum Nanoparticles ........................................... 61
Nanoparticle Characterization ......................................................... 62
BEAS Cell Cultures .......................................................................... 63
Nanoparticle Dosing Experiments .................................................. 64
Sample Preparation for TEM ............................................................ 64
TEM Analysis of BEAS Cells .............................................................. 65

Results ............................................................................................... 66
Characterization of Platinum Nanoparticles ..................................... 66
DLS results ....................................................................................... 66
TEM results ..................................................................................... 66
Zeta potential ................................................................................ 68

Particle Up Take by BEAS Cells ...................................................... 69

Discussion ....................................................................................... 71
Summary ........................................................................................... 73
Future Work ..................................................................................... 74

4 INVESTIGATION OF THE EFFECT OF SURFACE COATING ON THE COAGULATION, CIRCULATION TIME AND ACCUMULATION OF GOLD NANOPARTICLES IN THE LIVER OF MICE ............... 89

Background ..................................................................................... 89
The Liver Functions ........................................................................ 89
Hepatocytes and Sinusoidal Endothelial Cells ................................ 90
Kupffer Cells ................................................................................ 91

Particle Properties that Affect Cellular Up Take ................................ 93
6 METHODOLOGY FOR THE STUDY OF NANOPARTICLES AND THE CELLULAR UPTAKE OF NANOPARTICLES USING OPTICAL AND ELECTRON MICROSCOPY ................................................................. 161

Description of Techniques .......................................................................................................................... 162
  Optical Techniques ................................................................................................................................. 162
  Electron Microscopy ............................................................................................................................... 164

Description of Work .................................................................................................................................. 165

Particle Characterization by Electron Microscopy ..................................................................................... 165

Methodology for Characterizing Nanoparticles Using Electron Microscopy ............................................. 166

Sample Preparation .................................................................................................................................... 166

TEM/STEM and SEM analysis .................................................................................................................... 167

Characterization of the Cellular Uptake of Nanoparticles in Tissue and Cells ........................................... 167

Methodology for Characterizing the Cellular Uptake of Nanoparticles in Cells ...................................... 169

Confocal Microscopy of Cells ................................................................................................................... 169

Brightfield, Darkfield or DIC Microscopy .................................................................................................. 170

TEM/STEM/EDS ....................................................................................................................................... 171

Characterization of the Cellular Uptake of Nanoparticles after In Vivo Testing......................................... 172

Methodology for Characterizing the Cellular Uptake of Nanoparticles after In Vivo Testing Using Correlative Microscopy .............................................................................................................. 173

Confocal Microscopy of Tissue Samples .................................................................................................... 173

Optical Microscopy of Thin Sections ......................................................................................................... 174

TEM/STEM/EDS Analysis of Tissue Sections ............................................................................................ 175

Conclusions ................................................................................................................................................ 177

LIST OF REFERENCES .............................................................................................................................. 181

BIOGRAPHICAL SKETCH .......................................................................................................................... 192
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>The percentage of corner and edge sites in tetragonal, near-spherical and</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>cubic crystals.</td>
<td></td>
</tr>
<tr>
<td>3-2</td>
<td>Particles size, dispersion and zeta potential measurements for the platinum</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>nanoparticles.</td>
<td></td>
</tr>
<tr>
<td>4-1</td>
<td>Test matrix of mice experiments.</td>
<td>122</td>
</tr>
<tr>
<td>4-2</td>
<td>Liver histology sections and the percentage of gold recovered from each</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>lobe.</td>
<td></td>
</tr>
<tr>
<td>4-3</td>
<td>Number, size and area fraction of agglomerated gold nanoparticles.</td>
<td>131</td>
</tr>
<tr>
<td>4-4</td>
<td>Samples analyzed by TEM.</td>
<td>131</td>
</tr>
<tr>
<td>5-1</td>
<td>Particle size distribution.</td>
<td>152</td>
</tr>
<tr>
<td>5-2</td>
<td>Zeta potential distribution measurements.</td>
<td>152</td>
</tr>
<tr>
<td>5-3</td>
<td>Weight of QDs in the daphnia gut.</td>
<td>153</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Brightfield TEM image of the LSI Ru(bpy)3 doped silica nanoparticles.</td>
<td>48</td>
</tr>
<tr>
<td>2-2</td>
<td>Live cell confocal images of the control A549 cells stained with Hoechst 33258 and Oregon Green 488. The cells were healthy and alive.</td>
<td>48</td>
</tr>
<tr>
<td>2-3</td>
<td>High magnification confocal image of two cells with nuclei stained blue surrounded by the green cell membrane.</td>
<td>49</td>
</tr>
<tr>
<td>2-4</td>
<td>Live cell confocal images of the A549 cells dosed with LSI Ru(bpy)3 doped silica nanoparticles. The nanoparticles, which are red in the images, were taken up into the cells but were not taken into the nuclei.</td>
<td>49</td>
</tr>
<tr>
<td>2-5</td>
<td>Confocal image of the A549 cells dosed with 40 μg/ml Ru(bpy)3 doped silica nanoparticles for 24 hours.</td>
<td>50</td>
</tr>
<tr>
<td>2-6</td>
<td>Transmitted light images of the control and dosed A549 cells.</td>
<td>51</td>
</tr>
<tr>
<td>2-7</td>
<td>Brightfield TEM image of a cell from the control sample.</td>
<td>52</td>
</tr>
<tr>
<td>2-8</td>
<td>Brightfield TEM images of the A 549 cell from the control sample.</td>
<td>52</td>
</tr>
<tr>
<td>2-9</td>
<td>Brightfield TEM images of an A549 cell dosed with 20 mg/ml silica nanoparticles for 2 hours Nanoparticles were observed in endosomes and around the periphery of the cell.</td>
<td>53</td>
</tr>
<tr>
<td>2-10</td>
<td>Brightfield TEM images of an A549 cell dosed with 20 mg/ml silica nanoparticles for 2 hours Nanoparticles around the periphery of the cell and at various stages of invagination.</td>
<td>53</td>
</tr>
<tr>
<td>2-11</td>
<td>Brightfield TEM images of the 20 nm diameter silica nanoparticles inside a membrane bound vacuole and outside a cell.</td>
<td>54</td>
</tr>
<tr>
<td>2-12</td>
<td>Brightfield TEM images of an A 549 cell dosed with 40 μg/ml silica nanoparticles for 24 hours.</td>
<td>54</td>
</tr>
<tr>
<td>2-13</td>
<td>TEM images of the A549 cell dosed with 40 μg/ml silica nanoparticles for 24 hours. The 16 nm diameter silica nanoparticles are resolved in the images.</td>
<td>55</td>
</tr>
<tr>
<td>2-14</td>
<td>HAADF STEM image of the agglomerated silica nanoparticles inside an endosome and the corresponding EDS spectrum.</td>
<td>55</td>
</tr>
<tr>
<td>3-1</td>
<td>Diagram of the three types of Pt nanoparticles used in this study, Pt no agents, Pt-den and Pt-PVP.</td>
<td>75</td>
</tr>
</tbody>
</table>
3-2 Brightfield TEM images of the colloidal Pt nanoparticles made without protective agents. ................................................................. 76
3-3 Brightfield TEM images of the Pt nanoparticles plus dendrimer. .................. 77
3-4 Brightfield TEM images of the Pt-PVP 10k nanoparticles. .......................... 78
3-5 Brightfield TEM images of Pt-PVP 55k nanoparticles. ............................... 79
3-6 Brightfield TEM images of Pt-PVP 1.3 M nanoparticles............................... 80
3-7 BEAS cells ..................................................................................... 81
3-8 Brightfield TEM images of control BEAS cells. ........................................ 81
3-9 Brightfield TEM images of the control BEAS cell. ..................................... 82
3-10 Brightfield TEM images of BEAS cells exposed to Pt-no agents nanoparticles and the EDS spectrum from Pt nanoparticles................................. 83
3-11 Brightfield TEM images of a BEAS cell exposed to Pt-den nanoparticles for 6 hours and the corresponding EDS spectrum........................................ 84
3-12 Brightfield TEM images of the BEAS cells exposed to Pt-PVP 10k nanoparticles for 6 hours .............................................................. 85
3-13 Brightfield TEM images of BEAS cells exposed to Pt-PVP 55k nanoparticles for 6 hours ........................................................................ 86
3-14 Brightfield TEM images of the BEAS cells exposed Pt-PVP 1.3M for 6 hours and the corresponding EDS spectrum ........................................ 87
3-15 STEM images of the Pt-PVP 1.3M nanoparticles in a vesicle in a BEAS cell... 88
4-1 A) Diagram of a liver with an enlarged section of the showing the cells that surround the interlobular viens. B) Histology section of the liver showing a vien surrounded by endothelial cells, Kupffer cells and Kupffer cells further out. ...................................................................................... 121
4-2 Brightfield TEM images of the native and PEG coated 40 nm gold particles.... 123
4-3 Brightfield TEM images of native and PEG coated gold nanoparticles.......... 123
4-5 High resolution TEM image of a gold nanoparticle. ..................................... 124
4-6 A plot of the particle size distribution by mass for the native gold nanoparticles. ...................................................................................... 125
Plot of the percentage native and PEGylated gold nanoparticles found in the liver after different exposure times

Transmitted light images of a mouse liver that was exposed to native gold nanoparticles for 5 minutes.

Transmitted light images of a mouse liver that was exposed to native gold nanoparticles for 24 hours.

Transmitted light images of a mouse liver that was exposed to PEGylated gold nanoparticles for 2 hours.

Transmitted light images of a mouse liver that was exposed to PEGylated gold nanoparticles for 24 hours.

Transmitted light images of a mouse liver that was exposed to PEGylated gold nanoparticles for 2 hours and 24 hours.

Darkfield images of the Gold nanoparticles in distilled water.

Darkfield images of a liver that was exposed to native gold nanoparticles for 5 minutes and 24 hours. The gold nanoparticles show up orange and yellow, depending upon agglomerate size, in the darkfield images. There are gold nanoparticles throughout sample.

Darkfield images of a liver that was exposed to PEGylated gold nanoparticles for 2 hours and 24 hours.

Brightfield TEM images of the livers exposed to native gold nanoparticles for 15 minutes. Large clusters of nanoparticles were found throughout the sample.

HAADF STEM image and corresponding EDS spectrum from the mouse liver exposed to native gold nanoparticles for 15 minutes.

HAADF STEM images of the mouse liver exposed to native gold nanoparticles for 15 minutes and the corresponding EDS spectrum.

Brightfield TEM images of the livers exposed to native gold nanoparticles for 24 hours. These sections contained large tightly packed clusters of nanoparticles.
4-21 HAADF STEM image of a cluster of gold nanoparticles in the mouse liver exposed to native gold nanoparticles for 24 hours and the corresponding EDS spectra. ................................................................. 135

4-22 Brightfield TEM images a portion of a liver exposed to PEGylated gold nanoparticles for 15 minutes ................................................................. 136

4-23 Brightfield TEM image of gold nanoparticles in the space of Disse adjacent to a red blood cell. .................................................................................. 136

4-24 Brightfield TEM image of 40 nm gold nanoparticles in the endosome of a Kupffer cell in the liver exposed to PEGylated gold nanoparticles for 15 minutes and the corresponding EDS spectrum ................................................................. 137

4-25 Brightfield TEM images of the PEGylated gold nanoparticles in Kupffer cell on the edge of a sinusoid that was exposed to PEGylated gold nanoparticles for 24 hours .................................................................................. 137

4-26 Brightfield STEM image of the gold nanoparticles in the Kupffer cell of the mouse liver exposed to gold nanoparticles for 24 hours and the corresponding EDS spectrum .................................................................................. 138

5-1 Daphnia magna or water flea .......................................................................................................................................................................................... 152

5-2 Brightfield TEM image of the rod shaped Invitrogen QDs .................................................................................................................................... 153

5-3 Brightfield TEM image of the gut tissue from a Daphnia magna that was exposed to PEG coated QDs .......................................................................................................................... 154

5-4 Brightfield TEM image of the lumen inside the gut of a Daphnia magna exposed to PEG coated QDs .......................................................................................................................... 154

5-5 Brightfield and darkfield STEM images of the gut from Daphnia magna exposed to PEG coated QDs .................................................................................................................................... 155

5-6 HAADF STEM image of an electron dense region in the lumen of a Daphnia gut exposed to PEG coated Q dots and the corresponding EDS spectrum ...... 155

5-7 Brightfield TEM image of the gut tissue from a Daphnia magna exposed to amine functionalized, PEG coated QDs .................................................................................................................................... 156

5-8 HAADF STEM image of area A, it includes the nonsecretory endothelial cell, microvilli and lumen of the gut exposed to amine functionalized, PEG coated QDs .................................................................................................................................... 156

5-9 HAADF STEM images of QDs in area A the gut of the Daphnia exposed to amine functionalized, PEG coated QDs .................................................................................................................................... 157
5-10 A HAADF STEM image of amine functionalized, PEG coated QDs in the lumen area of the gut with the corresponding EDS spectrum.......................... 157

5-11 HAADF STEM image of area B of the gut tissue exposed to amine functionalized, PEG coated QDs ................................................................ 158

5-12 HAADF STEM images of the endothelial cells in the gut exposed to amine Functionalize, PEG coated QDs .................................................................................. 158

5-13 HAADF STEM image of the endothelial cell and the corresponding EDS spectrum ........................................................................................................... 159

5-14 Brightfield TEM images of the Daphnia gut exposed to carboxyl functionalized, PEG coated QDs ......................................................................... 159

5-15 HAADF STEM images of the lumen, area A in Figure 5-14B. .................. 160

5-16 HAADF STEM images of the carbboxyl functionalized, PEG coated QDs in the lysed cells lining the epithelium in area C from Figure 5-14B. .......... 160

6-1 Flow chart outlining the methodology for nanoparticle characterization using electron microscopy .......................................................................................................................... 179

6-2 Flow chart outlining the methodology for characterizing cellular uptake of nanoparticles using optical microscopy ........................................................................................................... 179

6-3 Flow chart outlining the methodology for characterizing cellular uptake of nanoparticles using electron microscopy ........................................................................................................... 180
<table>
<thead>
<tr>
<th>Object</th>
<th>Serial section movie of the liver exposed to PEGylated gold nanoparticles for 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>page</td>
<td>131</td>
</tr>
</tbody>
</table>

16
INVESTIGATION AND CHARACTERIZATION OF THE CELLULAR UPTAKE OF NANOPARTICLES

By

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Chair: Name Kevin Powers
Major: Materials Science and Engineering

The focus of this study was to determine the effect of surface coatings on the cellular uptake of nanoparticles and their fate inside cells and tissue using correlative microscopy. The nanoparticle properties and cellular uptake, including unique identification of the composition, locations and distribution of nanoparticles in cells, were determined using multiple microscopy techniques. The effect of coatings on the properties of platinum nanoparticles and their uptake by BEAS cells was undertaken to determine their relationship to the expression of heme oxygenease (HO-1) enzyme. The 1.3M PVP platinum nanoparticles produced very fine and well dispersed nanoparticles that were observed in the lysosomes of the BEAS cells and the other nanoparticles studied were present in large agglomerates.

The effect of polyethylene glycol (PEG) coating on the circulation time, agglomeration and accumulation of gold nanoparticles in the liver of mice was studied. A new approach to measuring the PEG coating thickness using high resolution TEM and negative staining techniques was introduced. The amount and distribution of gold in sections of the liver was determined using darkfield reflected light microscopy. Image analysis was used to determine the size, number and area fraction of agglomerates in
the sections. The three dimensional distribution of gold nanoparticles in a single cell of the liver was obtained using ion abrasion scanning electron microscopy. The uncoated gold nanoparticles were taken up almost immediately by the Kupffer cells while the PEG coated nanoparticles were taken up after 2 hours. The native gold was observed in large, tightly packed agglomerates in lysosomes inside the cells, while the PEG coated nanoparticles were observed lining the inner surfaces of the lysosomes. Differences in the agglomeration of the gold nanoparticles had not been previously observed.

The effect of surface charge on the fate of QDs ingested by daphnia magna (water fleas) was explored. PEGylated QDs were not taken up by the nonsecretory cells that line the gut, they were observed in the lumen and lysed cells. A methodology for analyzing the cellular uptake of nanoparticles after in vitro and in vivo testing using optical and electron microscopy techniques is included.
CHAPTER 1
INTRODUCTION

Motivation

Engineered nanoparticles are being developed for a wide range of applications from consumer products to medical applications. Along with the increased production and use of nanoparticles comes an increased risk of deliberate and accidental exposure to humans and the environment during their production, use and disposal. This is a complex problem because on one hand, nanoparticles have unique properties that make them useful in areas, like medical imaging, cosmetics and cancer therapies, where human exposure to nanoparticles is intentional. On the other hand, the possibilities exist for unwanted human and environmental exposures to nanoparticles where toxicity may be an issue.

The potential risks and dangers of nanoparticle exposures to humans and the environment are not well understood. (Colvin et al., 2003) The field of nanotoxicology was developed to study the potentially harmful effects of nanoparticles on humans and the environment, because the standard toxicological models and testing methods were considered inadequate. (Donaldson et al., 2004; Holsapple et al., 2005) The research on nanoparticle toxicity is progressing much slower than the commercialization of the technology. The toxicity testing of nanoparticles to date has involved specific types of nanoparticles and modes of exposure, with a large number of different testing techniques and models being used. (Marquis et al., 2009) To complicate matters, it is uncertain if the toxicity data for bulk materials and larger particles can be used to assess the toxicity of nanoparticles. The toxicity of nanoparticles cannot be predicted.
based on the limited available test data, lack of adequate materials characterization and inconsistencies in testing procedures.

Standardized nanotoxicity testing procedures are needed to unify the testing procedures, and assess the toxicity of nanoparticles and risks associated with exposures. (Marquis et al., 2009) This is complicated by the fact that nanotoxicity testing encompasses human and environmental exposures as well as accidental and therapeutic exposures. Nanotoxicity testing procedures consist of three parts, characterization of the nanoparticles, in vitro tests, which are done outside the organism consist of exposing cells to nanoparticles in a controlled laboratory environment, and in vivo tests, which are done inside the organism and consist of nanoparticle exposures to whole organisms. (Donaldson et al., 2004, Hayashi et al., 2005; Kroll et al., 2009) Standardization of the testing procedures is difficult because each testing category has numerous components which are application dependent.

Microscopy techniques are an important aspect of all the components of nanotoxicity testing and will be an integral part of any standardized testing procedures. Optical, electron, x-ray and associated microscopy techniques provide detailed information on the properties of nanoparticles and the cellular uptake and distribution of nanoparticles that cannot be obtained with any other way. The use of multiple microscopy techniques, called correlative microscopy, is required to analyze a sample when working on such complex problems. Complications arise because of the large size disparity between the nanoparticles and most biological features. The nanoparticles can be as small as a few nanometers while most cells are many microns in size. The advantage of using correlative microscopy techniques is the ability to examine the
nanoparticles, cells and tissue samples with different techniques that provide complementary information over a wide range of magnifications. (Jahn et al., 2007) The information obtained from the nano scale imaging can be used to interpret what is seen on the macro scale.

Transmission electron microscopy (TEM) is considered the gold standard for determining the properties of the nanoparticles prior to testing, particularly the primary particle size and particle shape. These measurements are essential to ensure reproducible toxicity test results. (Oberdorster, et al., 2005; Powers et al., 2006; Warheit et al., 2008) The microscopy techniques used to characterize the cellular uptake of nanoparticles after in vitro and in vivo testing depend on the type of particles and type of testing. Optical techniques are used for viewing the interaction of the nanoparticles with whole cells or thick sections of cells. Electron microscopy techniques are used to locate and uniquely identify the location of the nanoparticles inside the cells along with any morphological changes that occur to the ultrastructure of the cells. It can be challenging to find nanoparticles in cells and tissue samples because of their small size, something like finding a needle in a haystack. This makes doing any type of statistical analysis based on the TEM images extremely challenging. Although TEM is best technique to locate nanoparticles in the cells it is best used in conjunction with other techniques for area and volume distribution measurements of the nanoparticles in cells and tissue. The use of multiple microscopy techniques is necessary for thorough characterization of the cellular uptake of nanoparticles and their effect on the cells.

Objective

The goal of this research was to investigate the effect of surface coatings on the cellular uptake of nanoparticles, and determine their fate inside the cells and tissue
using optical and electron microscopy. Four different experimental testing scenarios were explored in this thesis. The cellular uptake of dye doped silica nanoparticles by A549 cells was studied to determine how the uncoated nanoparticles were taken up into the cells and determine the information obtained and resolution limits of optical and electron microscopy techniques. Confocal microscopy was used to determine if the fluorescent nanoparticles were taken up inside the cell membrane or nucleus. TEM was used to take a more in depth look at the exact the location and distribution of the nanoparticles inside the cells. The uptake of platinum (Pt) nanoparticles by bronchial epithelial airway (BEAS) cells was studied in an effort to explain the up regulation of HO-1 enzyme by the uptake of one type of polyvinylpyrrolidone (PVP) coated platinum (Pt) nanoparticles. The particle size and dispersion are related to their distribution in the cells. The effect of polyethylene glycol (PEG) coating on agglomeration, circulation time and accumulation of gold nanoparticles by the liver of mice after intravenous injection was characterized using correlative microscopy. The PEG coating thickness was characterized using high resolution TEM and negative staining. A method for determining the size, number and area fraction of gold nanoparticles in the liver was developed using darkfield microscopy. The distribution of gold nanoparticles in the Kupffer cells was investigated using TEM and ion abrasion scanning electron microscopy (IASEM). The effect of surface charge on the fate of quantum dots ingested by daphnia magna (water fleas) was explored using TEM and energy dispersive x-ray (EDS) analysis. This testing was done in an effort to understand the fate of nanoparticles in the environment. A methodology for analyzing the cellular uptake of
nanoparticles after in vitro and in vivo testing using optical and electron microscopy techniques is included.

**Dissertation Organization**

The contents of this work are organized into six chapters. This chapter, Chapter 1, outlines the motivation and objectives of this work as well as provides a literature review of pertinent topics. Chapter 2 discusses the cellular uptake of uncoated, fluorescent dye doped silica nanoparticles by cultured lung epithelial cells. The properties of the nanoparticles and the cellular uptake behavior were explored using confocal, optical and electron microscopy. Chapter 3 discusses the effect of different surface coatings on the properties of Pt nanoparticles and their cellular uptake by BEAS cells in an effort to explain the up regulation of a specific gene associated with one type of coated Pt nanoparticle. The properties and cellular distribution of the Pt nanoparticles are discussed in terms of their effect on the catalytic activity of the Pt nanoparticles. Chapter 4 discusses the effect of surface coating on the circulation time, cellular uptake and biodistribution of nanoparticles in the liver of mice after intravenous injection of gold nanoparticles. Electron microscopy techniques were used to characterize the nanoparticles and nanoparticle distribution in the cells. EDS analysis was used to identify the nanoparticles by composition in the tissue samples. Optical microscopy was used to determine the area fraction and distribution of gold nanoparticles in the tissue samples. The experiments in this chapter explore the differences in cellular uptake of uncoated and polymer coated nanoparticles. Chapter 5 explores the effect of surface charge on the uptake quantum dots by the cells that line the gut of the Daphnia magna after ingestion. Chapter 6 presents a methodology for the study of the properties of
nanoparticles and the cellular uptake of nanoparticles after in vitro and in vivo testing. The methodology is based on the research from this thesis.

**Background and Literature Review**

**Nanoparticles**

Nanoparticles are particles that range in size from 1 to 100 nm. (NNIN, 2005) The term nanoparticles includes many types of particles that are classified according to their origin, composition, crystallinity and application. Engineered, inorganic nanoparticles are used in this study. They are manufactured specifically to take advantage of their unique physiochemical properties. (Buzea et al.; Oberdorster, et al., 2005; Stearns et al., 2001) The enhanced properties of nanoparticles are attributed to their size and morphological substructure. Very small particles have a high specific surface area and a large fraction of atoms at the surface compared to bulk materials. (Nel, et al., 2006; Oberdorster et al., 2005; Roduner et al., 2006) The surface properties of nanoparticles are linked with increased chemical reactivity and lower melting temperatures than larger particles and bulk materials because of the lower stability of the surface atoms. (Buzea et al., 2007; Roduner et al., 2006)

**Nanotechnology and Nanomaterials**

Nanotechnology is defined by the National Nanotechnology Initiative as the understanding and control of matter at dimensions between 1 and 100 nm where unique phenomena enable novel applications. (NNIN, 2005) Nanotechnology is an interdisciplinary field that encompasses science, engineering and medicine to study and produce products that incorporate nanomaterials. Nanomaterials have a physiochemical structure that is larger than atomic and molecular scale but smaller than 100 nm and have properties that are dependent on the nanostructure. (Nel et al., 2006)
Nanoparticles are a type of nanomaterial, particles that are less than 100 nm, while nanomaterials can be larger than 100 nm, but have features that are less than 100 nm. Nanotechnology is a very broad field that includes applications such as medicine, electronics, solar energy applications and consumer products. There are thousands of products being manufactured today that incorporate engineered nanomaterials. A few examples of products that contain nanoparticles are sunscreens, self cleaning glass, antimicrobial bandages, make up, and stain resistant clothing. Human and environmental exposures to engineered nanoparticles can occur during the manufacturing, use and disposal of nanotechnology products. Nanoparticles are also being tested and used in many medical applications such as image contrast agents, drug targeting, and drug delivery. There are concerns that these materials and products present a potential hazard to humans and the environment over their life cycle.

**Nanotoxicology**

The field of Nanotoxicology was developed to study the possible adverse effects of nanomaterials and nanoparticles on living organisms and the environment. (Oberdorster et al., 2005) There is concern regarding the toxicity of engineered nanoparticles because their potential risks and dangers are not fully understood. Most of the research to date has been in the field of particle toxicology, which is focused on the toxic behavior of air borne particles from natural and anthropogenic sources. (Oberdorster et al., 2005) It is not known if the hazards associated with the use of engineered nanoparticles can be estimated from the previously obtained toxicity data on particles larger than 100nm or bulk materials. (Stern et al., 2008) Engineered nanoparticles have unique chemical and physical properties that may also contribute to the increased reactivity and other hazards associated with these materials. (Holsapple et al., 2005)
More research is required to determine the exposure risks to humans and the environment, and to standardize testing procedures for risk assessments and safety evaluations of nanoparticles and nanomaterials. (Oberdorster et al., 2005) The key elements in nanoparticle toxicity testing for humans and animals include determination of the possible exposures, thorough physiochemical characterization of the nanoparticles, in vitro assays performed in a controlled test environment, and in vivo studies performed in the living organisms. (Holsapple et al., 2005; Marquis et al., 2009, Oberdorster et al., 2005)

**Human and Environmental Exposures**

The probability of human and environmental exposure to engineered nanoparticles increases as the production and use of products containing nanoparticles increases. Human exposures occur via four routes, dermal or through the skin, inhalation, injection and ingestion. (Buzea et al., 2007; Oberdorster et al., 2005) Once taken up, the nanoparticles can translocate and accumulate within the body. After which, they will be either retained or excreted from the body. The mechanisms and rates of translocation, accumulation and excretion of nanoparticles are largely unknown, as well as the potential for adverse effects associated with nanoparticle take up.

The effect of nanoparticle exposures on the environment and the effects on plants, insects, fish and animals are being studied. The potential hazards associated with environmental exposures are enormous. (Colvin et al., 2003) Nanoparticles have the potential to get into the water, air and soil during their life cycle affecting the food supply and habitat of most living organisms.
Characterization of Nanoparticles

Characterization of the nanoparticles is essential to ensure that the toxicity test results are reproducible and provide the basis for understanding the properties that determine their biological effects. (Gaumet et al., 2008; Powers et al., 2006; Warheit et al., 2008) Nanoparticles should be characterized in the as received state or as synthesized state. (Oberdorster et al., 2005) The physiochemical properties that are important factors in determining the toxicity of nanoparticles include particle size, composition, surface area, surface chemistry, surface charge, crystal structure, shape, state of agglomeration and porosity. (Buzea et al., 2007; Nel et al., 2006; Oberdorster et al., 2005) The recommended nanoparticle characterization techniques are TEM, dynamic light scattering (DLS), centrifugal sedimentation particle sizing (CPS), gas adsorption (BET), zeta potential, and x-ray photo electron spectroscopy (XPS). (Marquis et al., 2009; Powers et al., 2006)

The properties of the nanoparticles are dependent on their local environment and can be altered when they enter a biological system. Therefore nanoparticles should also be characterized in the “as-dosed” condition because the suspension fluid can affect the surface related properties. The properties of the fluid, such as the ionic strength, pH, temperature and the presence of organic molecules and proteins in the fluid, affect the properties of nanoparticles in the fluid. These properties directly affect the particle size, dispersion, stability, hydration, charge, composition of the surface layer, and ultimately the fate of the nanoparticles in a biological environment. (Nel et al., 2009)

In Vitro Testing

In vitro assays provide information on cell viability, the ratio of living to dead cells, and toxicity mechanism. In vitro testing is faster and less expensive than in vivo testing.
It is also more reproducible than in vivo testing because it is done in a controlled laboratory environment. The downside is that the in vitro test conditions cannot fully replicate the in vivo environment. The specific in vitro tests and cells that are used will depend on the route of entry and the target organ for the nanoparticles. (Oberdorster et al., 2005) In vitro screening tests, with high throughput, may eventually be developed to evaluate the toxicity of nanoparticles once the interactions of nanoparticles in the body and environment are more fully understood. (Hayashi et al., 2005; Kroll et al., 2009)

**In Vivo Testing**

In vivo studies are needed to study the interactions of nanoparticles within a living body. They provide information on the fate, biodistribution and elimination of nanoparticles from a living body or environment that cannot be obtained through in vitro testing. (Fischer et al., 2007) The movement of nanoparticles from the portal of entry to specific organs can only be determined through in vivo testing.

**Particle Interactions in Biological Systems**

**Cellular Take Up of Nanoparticles**

In vitro and in vivo tests have shown that nanoparticles can accumulate inside the cells of various organs. The body has phagocytic cells, or macrophages, that take up substances, such as bacteria, viruses, abnormal cells and foreign particles, to dispose of them either through dissolution or transport out to the body. When the substances cannot be disposed of they are encapsulated in membrane bound vesicules, making them incapable of causing harm to the body. (Aderem et al., 1999) The macrophages are part of the reticuloendothelial system (RES) which is part of the immune system of the body. Phagocytosis is the cellular take up of particulate materials. It occurs readily on micron sized particles that enter the body. (Wasdo et al.,
The process of phagocytosis begins with attachment of the particle or substance to the cell membrane. Extensions of the cell membrane, called pseudopods, surround the food or particle and fuse together to form a vesicle, or lysosome, which transports the food or particles into the cell. (Karp et al., 1996) The vesicles contain enzymes that break down cellular waste and food. Cellular uptake of agglomerated nanoparticles occurs by phagocytosis.

Endocytosis is the process that fluid and dissolved or suspended molecules and small particles are taken up into cells. The fluid and small particles are surrounded by the cell membrane which is pinched off to form an endosome, or membrane bound vesicle, that is released into the cytoplasm of the cell. Receptor mediated endocytosis is the process by which specific molecules are taken up into cells following their binding to receptors in pits on the plasma membrane. Once the receptor pits are filled, a vesicle is formed and the material is transported into the cell. The size of the vesicles is determined by the type of receptor which in turn controls the size of particles that can be engulfed. (Lynch et al., 2006; Verma et al., 2008) The vesicles form endosomes which can merge with lysosomes in the interior of the cell.

**Protein Binding**

Cellular uptake of nanoparticles is affected by the binding of proteins to the nanoparticles in the biological environment. Most particles that come in contact with biological systems are immediately coated by proteins, resulting in a protein corona around the particles. (Aggarwal et al., 2009; Cedervall et al., 2007; Klein et al. 2007) The protein coronas are dependent on the chemistry, surface properties and size of the nanoparticles as well as the biological medium. Absorption of certain proteins called opsonins, make the nanoparticles susceptible to be taken up by the macrophages of the
Opsonins interact with a variety of cellular receptors that facilitate uptake of the nanoparticles. (Aderem et al., 1999; Buzea et al., 2007)

The interactions that occur at the interface between the nanoparticles and the biological system are in a state of constant flux. The controlling interfacial components are the nanoparticle surface, the solid liquid interface between the nanoparticles and the biological medium, and the contact zone between the solid liquid interface and cells. (Nel et al., 2009) The proteins that bind to the nanoparticles change with time, protein availability and concentration of nanoparticles. (Cedervall et al., 2007; Ehrenberg et al., 2009) The proteins that bind to the nanoparticles are studied using techniques such as polyacrylamide gel electrophoresis, chromatography, and mass spectrometry. (Wasdo et al., 2008) The protein coronas are complex and contain many proteins. More than 50 proteins were identified in the corona surrounding various nanoparticles suspended in blood serum. (Aggarwal et al., 2009; Wasdo et al., 2008)

The characteristics of the nanoparticles and the biological medium affect protein adsorption and phagocytosis. The surface properties of the nanoparticles change when the nanoparticle interacts with the surrounding medium. The chemical composition, surface functionalization, shape, angle of curvature, porosity, surface crystallinity and roughness, and hydrophobicity are important characteristics that determine the surface interactions of nanoparticles in a specific medium. (Nel et al., 2009) Studies on protein adsorption by nanoparticles in blood plasma and human serum have shown that the affinity of certain proteins to bind to the nanoparticles is influenced by chemistry, electrostatic interaction, hydrophobic interactions and surface topology. (Dutta et al., 2007; Gref et al., 2000; Wasdo et al., 2008) Agglomeration and protein binding play a
critical role in determining the biodistribution, clearance and inflammatory potential of nanoparticles. (Aggarwal et al., 2009; Fadeel et al., 2010; Wasdo et al., 2008) The cellular response to nanoparticles in a biological medium is dependent on the adsorbed biomolecules or proteins more than the nanoparticles themselves. (Cedervall et al., 2007; Lynch et al., 2008)

**Polymer Coatings**

Polymer coatings on nanoparticles are used to minimize adsorption of proteins and cellular uptake. Opsonins that bind to the surface of nanoparticles are recognized by the macrophages of the RES and stimulate phagocytosis. Neutral and bioresistant polymer coatings, which prevent or minimize protein adsorption, are used to prevent or decrease nanoparticle detection and uptake by the macrophages of the RES. The amount and type of proteins that bind to the polymer coated nanoparticles are different from the proteins that attach to uncoated nanoparticles. (Zahr et al., 2006) Without the adsorbed proteins on the surface, the polymer coated nanoparticles are not detected by the macrophages of the RES and stay in circulation for longer times. (Aggarwal et al., 2009) In other applications, the polymer coatings are functionalized so that specific proteins are adsorbed for site specific particle uptake. This technique is used in the field of drug targeting and delivery, so that drug particles are taken up or delivered to a specific region of the body. (Sheng et al., 2009)

Polyethylene glycol (PEG) is commonly used as a coating to mask nanoparticles from the RES. The PEG can be covalently linked or physically adsorbed on the surface of the nanoparticles. It makes the surface of the particles hydrophilic with a neutral to slightly negative charge. The protein adsorption resistance of the PEG surface molecules is attributed to steric repulsion which results from a loss of conformational
entropy of the bound PEG chains and low interfacial free energy in aqueous media. (Otsuka et al., 2003; Zahr et al., 2006) The amount of adsorbed plasma proteins on PEG coated particles was found to decrease as the molecular weight of the PEG increased from 2000 to 5000 Da, but remained constant for PEG molecular weights above 5000. (Gref et al., 2000) The molecular weight of the PEG, affects the polymer chain length, density of the coating and the protein binding. The higher protein adsorption for the lower molecular weight PEG coatings were attributed to discontinuous surface coverage of the PEG due to short polymer chains. In other words, opsonins were able to bind to the particle surface in regions that were not covered with PEG. The higher molecular weight PEG has longer polymer chains and forms a more continuous coating on the nanoparticles. (Gref et al., 2000)

The thickness of the PEG coatings has been calculated from measurements of the particle diameter with and without the polymer coating using particle size analysis techniques like dynamic light scattering (DLS) and centrifugal particle sizing (CPS). (Dobrovolskaia et al., 2009) The accuracy of these measurements is questionable because they are based on the assumption that the density of the particles, which consists of the particle and the coating, is known. The measurements also assume that the particles are spherical and the polymer forms a uniformly thick coating on the surface of the nanoparticles. The morphology and uniformity of polymer coatings is not known at this time.

**Correlative Microscopy for the Study of Nanotoxicology**

**Correlative Microscopy**

Correlative microscopy is the use of multiple microscopic techniques to characterize the same specimen. The advantage of using correlative microscopy is that
the samples are analyzed over a wide range of magnifications, from low magnification optical techniques to high resolution electron microscopy techniques in two, three and four dimensions. Microscopy techniques are used in all aspects of nanotoxicity testing, including particle characterization and studying the interactions of nanoparticles with cells and tissues in both in vitro assays and in vivo testing.

**Microscopy Techniques for Particle Characterization**

Microscopy is considered the gold standard for measuring the properties of nanoparticles. (Oberdorster *et al.*, 2005) Nanoparticles, due to their small size, are analyzed using Scanning Electron Microscopy (SEM) and TEM. It can be used to measure the particle size, shape, morphology, particle size range, crystallinity, agglomeration state, surface area and coating morphology. The chemical composition of the nanoparticles and coatings can be determined using EDS or Electron Energy Loss Spectroscopy (EELS).

**Microscopy Techniques for Characterizing the Interactions of Nanoparticles and Cells**

The optical techniques are used to image the cells, tissue and whole animals, while TEM is required to analyze the nanoparticles and the ultrastructure of cells and tissue. Morphological information is gained through comparison of images taken using multiple techniques over a wide range of magnifications. (Jahn *et al.*, 2007; Porter *et al.*, 2006) A better understanding of the interactions between cells and nanoparticles is obtained using multiple microscopy techniques. Correlative microscopy includes any available microscopic techniques, such as light, probe, laser and electron microscope techniques. A few of the microscopy techniques that are typically used in biological sciences and the cellular uptake of nanoparticles are transmitted light, fluorescence
microscopy, confocal microscopy and TEM with elemental analysis such as Dispersive EDS or EELS. (Kapp et al., 2007; Porter et al., 2006; Stearns et al., 2001; Yen et al., 2009) The analysis can be in 2 or 3 dimensions. Typically, an optical technique is used to screen samples and to locate areas of interest. TEM is needed for ultrastructure information obtained through high resolution imaging and elemental analysis. The preparation of samples for TEM is very expensive and labor intensive. In many cases, the TEM results are necessary to aid in the interpretation of the optical results. After which the optical technique can be used as a screening process.

The latest advances in TEM analysis of biological materials are in cryo preparation, cryo TEM and TEM tomography. (Robinson et al., 2001; Sartori et al., 2007; van der Wel et al., 2005) Cryo preparation procedures, such as high pressure freezing, eliminate artifacts from the conventional preparation procedures of fixation, dehydration and embedding. The possibility of washing away nanoparticles is also eliminated when high pressure freezing is used. The frozen samples can be imaged directly in the cryo TEM with no staining. TEM tomography is based on a tilt series of two dimensional projections of the object along different directions that is reconstructed into a three dimensional projection of the original object.

IA-SEM or Focused Ion Beam SEM (FIB-SEM) is being used to present three dimensional views of cells and tissue. (Heymann et al., 2006; Heymann et al., 2009; Matthijs de Winter et al., 2009) It is a relatively new technique to the world of biological sciences. It combines the removal of thin layers of samples with imaging the newly exposed face of the sample. The result is a three dimensional image cube, which in this
case would show the nanoparticle distribution in three dimensions where most of the other techniques present the data in two dimensions.

Summary

There are still many aspects of nanoparticle toxicity that are not known or understood. Most nanotoxicity studies include some microscopy as supporting evidence for the toxicity results. Correlative microscopy presents a more in depth picture of the nanoparticle-cellular interactions that is needed to understand the toxicity of nanoparticles from the characterization of the as synthesized particles to the in vivo test results. This research aims to develop a microscopy methodology that includes a suite of microscopy techniques to determine the properties of nanoparticles and investigate the cellular uptake of nanoparticles after in vitro and in vivo tests.
In vitro testing is generally used as a first approach to determine the toxicity of nanoparticles. It is fairly rapid and inexpensive compared to in vivo testing and provides valuable information on the way nanomaterials interact with cells. (Hayashi et al., 2005; Kroll et al., 2009) There are numerous assays available for the determining viability and mechanistic processes. Correlative microscopy techniques are used to monitor the nanoparticle interactions with the cells and monitor nanoparticle uptake. The microscopy techniques used to image nanoparticle interactions with cells in vitro experiments are confocal microscopy of the live cell cultures, optical microscopy (transmitted light), TEM and Scanning Transmission Electron Microscopy (STEM) with EDS for elemental composition. The combination of techniques enables the structure of the cells and the nanoparticles to be imaged at magnifications from 2x to 500,000x or more. Confocal microscopy can be used to analyze the cellular take up and localization of particles in and around the live or fixed cells, as well as sections of cells. The optical microscope was used to image the fixed, dehydrated and embedded sections of cells prior to TEM analysis to determine the overall condition of the cells and locate areas of interest for the TEM analysis. TEM and STEM with EDS analysis was used to image the ultrastructure of the cells, determine the location of nanoparticles in the cells and to confirm the location and composition of the nanoparticles by elemental analysis.

Nanoparticle uptake by cultured human lung alveolar basal epithelial (A549) cells is well documented in the literature. (Jin et al., 2007; Stern et al., 2008) This study focused on documenting the cellular uptake of 20 nm dye doped silica nanoparticles by A549
cells using the following four microscopy techniques, confocal microscopy, optical microscopy, TEM and STEM with EDS analysis.

**Materials and Methods**

**Cell Line**

The A549 human lung alveolar basal epithelial Type II cell line was used for the experiments. They are part of squamous, flat scale like cells, subdivision of epithelial cells. The A549 cell line was obtained from the American Type Culture Collection, Rockville, MD, and maintained in Cellgro RPMI-1640 medium containing 2mM L-Glutamine, and supplemented with 10% fetal bovine serum (heat inactivated), and 1% antibiotic-antimycotic mixture. Cells were maintained in humidified atmosphere of 5% CO₂ and 95% air at 37°C. The cells were subcultured using 0.25% Trypsin and 0.53 mM EDTA solution for detachment. The culture medium was replaced every two to three days and cells were split 1:10 before reaching total confluency.

**Dye Doped Silica Nanoparticles**

Ru(bpy)₃, tris(2,2'-bipyridy)l-dichlororuthenium(II) hexahydrate, doped Silica Nanoparticles, lot # NP01734007, were obtained from Life Sciences, Inc, St. Petersburg, FL. The mean particle diameter was 20 nm. The particles had an excitation wavelength at 453 nm and fluorescence emission at 590 nm. The surface of the particles was not modified. The as received particles came in a concentrated solution of 6.0 mg/ml particles in 32 nM potassium phosphate with a pH 7.4. The particles were ultrasonicated for 5 minutes and diluted in media for dosing the cells.
Characterization of LSI Ru(bpy)3 Doped Silica Particles

A JEOL 200CX TEM was used for imaging the particles. Brightfield TEM images were taken of the particles. The particle size of 500 particles was measured from the TEM images and the mean particle size was calculated.

Sample Preparation Confocal Microscopy

The A549 cells were cultured directly in MatTek glass bottom culture dishes for confocal microscopy. A control sample of A549 cells was incubated in the confocal dishes for 48 hours and stained with fluorescent stains for Laser Scanning Confocal Microscopy (LSCM). All other cells were incubated for 24 hours in the confocal dishes prior to particle dosing with nanoparticles. The cells were washed two times with Phosphate Buffer Solution and then dosed with nanoparticles. The particle concentration was 20 $\mu$g/ml and 40 $\mu$g/ml particles diluted in normal growth media. The solution was ultrasonicated in a Branson Table top ultrasonic Cleaner for 10 minutes. The particle media solution was added to the cell culture dishes and incubated for 2, 12 and 24 hours.

Two fluorescent stains were used for confocal microscopy, Invitrogen Oregon Green 488 and Invitrogen Hoechst 33258. Oregon Green 488 is a fluorescent lectin, which is a carbohydrate binding protein that, in this case, binds to specific configuration of sugar molecules on the surface of the cells. It was added to the cell cultures to visualize the cell membrane. The absorption and fluorescence emission maxima of Oregon Green 488 dye was 496 nm and 524 nm, respectively. The cells were washed two times with PBS and fresh media was added to the cells prior to staining. The stain was added to the cell cultures approximately 30 minutes prior to the microscopy session. Hoechst 33258 fluorescent stain was used for labeling DNA and stains the cell
nucleus and mitochondria. The absorption and fluorescence emission maxima of the Hoechst were 350 nm and 461 nm, respectively.

Prior to confocal imaging, the cells were washed two times with Phosphate Buffer Solution (PBS) and then 1 ml of fresh media was added to the culture dish. The cells were kept on ice before and after LSCM imaging to keep them alive.

**Sample Preparation for TEM**

After confocal imaging, the samples were prepared for optical microscopy and TEM using a dish culture cell embedding technique. The monolayer of cells was fixed and embedded in the culture dish so the same cells could be imaged in both the LSCM and TEM. The media was removed from the cell culture dishes and cells were washed two times in Tyrode’s buffer (8.0 g NaCl, 0.2 g KCl, 0.05 g NaH$_2$PO$_4$·H$_2$O, 1.0 g NaHCO$_3$, 1.0 g Glucose, 0.1 g MgCl$_2$·2H$_2$O and 0.33 g CaCl$_2$·2H$_2$O at pH 7.4) to remove excess proteins and the media from culture dishes. The cells were then fixed in 1% glutaraldehyde in Tyrode’s Buffer. The cells were rinsed with Tyrode’s Buffer for 15 minutes after fixation. The cells were post fixed in 2% osmic acid in 0.1 M phosphate buffer for 1 hour. The osmic acid was drained and the cells were rinsed two times with phosphate buffer. The cells were dehydrated in graded water/ethanol solutions (70%/30%, 50%/50%, 30%/70%, 90%/10%, and two times 100% ethanol) and then infiltrated with graded Shell EPON® Resin 828 /ethanol mixtures (1:3, 1:1 3:1), followed by 100% EPON®. After the 100% EPON® step, a thin layer of EPON® resin was added to cover the bottom of the cell culture dishes. Molded plastic embedding capsules, called BEEM capsules, were placed over the areas of interest in the bottom of the cell culture dishes. The capsules are 8 mm in diameter and 20 mm long. The EPON® was polymerized overnight in an oven at 68°F. The BEEM capsule tubes were filled with
EPON® and returned to the oven for 24 hours. The BEEM capsules were removed from the cell culture dishes with the layer of cells located on the block face. The BEEM capsules were removed and the block trimmed for microtoming. The cell blocks were sectioned using the Leica Ultra Cut S Ultramicrotome. Thick sections, 500nm, were cut for optical microscopy and thin sections, 70-100 nm, were cut for TEM.

Confocal Microscopy

The live A 549 cells were examined using the Leica TCS SP2 AOBS (Acoustical Optical Beam Splitter) Spectral Laser Scanning Confocal Microscope (LSCM). The UV laser was used for excitation at 350 nm for imaging the cell nuclei stained with Hoechst 33258, and the Argon laser was used for excitation 450 nm and 488 nm for imaging Ru(bpy)3 dyed Silica nanoparticles and Oregon green stained cell membranes. The live cells were imaged with the 20x and 63x objective lenses. The LSCM was used to view optical sections through the depth, z direction, of the cells. Each section was approximately 1 micron in thickness. The optical sections can be merged together to produce Z stack images and cross sectional views through the thickness of the cells.

Optical Microscopy

Thick sections were cut from the blocks of embedded cells for optical microscopy. The sections were 500 nm thick and mounted on a glass slide and stained with toluidine blue for contrast. An Olympus BX60 Microscope with SPOT Insight Digital Camera was used to image the thick sections. The images provided an overall view of the cells in the thin sections for TEM.

Transmission Electron Microscopy

A JEOL 2010F STEM and JEOL 200CX TEM were used for the TEM analysis. The JEOL 2010F was operated at 200kV. It is equipped with an Oxford INCA Pentafet
EDS system for elemental analysis. It also has a JEOL Brightfield STEM Detector (BF) and High Angle Annular Darkfield (HAADF) STEM Detector. HAADF images are formed by collecting the high angle, elastically scattered electrons which depend on the atomic number of the sample. The higher the atomic number of the sample, the more electrons are scattered at high angles and will be collected by the HAADF detector. The high atomic number regions of the sample appear bright white in the HAADF images. A compositional map of the sample is produced with atomic number contrast in the HAADF images. (Pennycook et al., 1988) The HAADF or Z contrast imaging is used to identify areas of the sample with higher mass thickness or atomic number. EDS analysis was used to confirm the presence of the nanoparticles in the cells through microchemical analysis. EDS analysis is required to determine the composition of the high atomic number areas because the staining process in biological samples may produce nanoscale electron dense precipitates that can be mistaken for the nanoparticles. The JEOL 200cx was used for conventional Brightfield TEM imagining at 120 kV to locate areas of interest in the sample.

**Results**

**LSI Ru(bpy)3 Doped Silica Nanoparticles**

The LSI Ru(bpy)3 doped silica nanoparticles were spherical nanoparticles. They were very uniform in shape and size. A brightfield TEM image of the silica nanoparticles is presented in Figure 2-1. The mean particle size was 16.8 nm with a standard deviation of 3.16 nm.

**Confocal Microscopy Results**

Intracellular localization of the silica nanoparticles was confirmed using the LSCM. Z stack images through entire cells were made from 1 micron thick optical sections. The
thin optical sections were imaged individually to visualize the through thickness contents of a cell. Cross sectional views of the x-z and y-z planes were obtained from the merged z stack images. Nanoparticles were observed within the cell membrane using the cross sectional views.

Confocal images of the control sample of the A549 cells are presented in Figure 2-2. The nucleus was stained blue with Hoechst and the membrane was stained green with Oregon Green 488. An optical section through the cells, with the x-z and y-z planes, or cross sections, on the right and bottom of the main image is presented in Figure 2-2. The x-z and y-z images were formed from the views along the lines that extend horizontally and vertically through the main image. The nuclei are surrounded by the membranes in the plan view and cross section images. Figure 2-3 is a high magnification image of the control cells showing the nuclei inside the cell membranes.

Confocal images of the A549 cells dosed with 20 µg/ml silica nanoparticles for 2 hours are presented in Figure 2-4. The Ru(bpy)3 doped silica nanoparticles appear red in the images. The silica nanoparticles were taken up by the cells in less than 2 hours. The particles were observed in the interior of the cell, but not in the nuclei. The cells dosed with 40 µg/ml silica nanoparticles for 24 hours are presented in Figure 2-4B. A large volume of nanoparticles were taken up into the cells after 24 hours of exposure. The silica nanoparticles were observed inside the cell membrane of all of the dosed cells, but they were taken up into the nuclei. An optical thin section of the cells dosed for 24 hours with 40 µg/ml nanoparticles is presented in Figure 2-5. The x-z and y-z cross sections through the cells are presented to the left and bottom of the main image. The
nanoparticles are present inside the cell membrane and outside the nuclei. The cellular uptake of nanoparticles did not kill the cells.

**Optical Microscopy Results**

Thick sections of the control and dosed A549 cells were examined using transmitted light microscopy to get an overall view of the cells. The control A549 cells are presented in Figure 2-6A. The cells were intact. The nuclei are visible inside the cytoplasm of the cell. The nuclei appeared dark in the images, and the cytoplasm is a uniform lighter grey. There were a few holes present in the interior of the cell membranes. A thick section of the A549 cells dosed with silica nanoparticles for 2 hours is presented in Figure 2-6B. The cells looked similar to the control sample. The cells that were exposed to silica nanoparticles for 24 hours are presented in Figures 2-6C and D. These nuclei are dark grey surrounded by the cytoplasm which is a lighter grey. There are numerous holes of varying sizes in the cytoplasm of these cells, which could be due to nanoparticle pull out during sectioning. The holes in the cytoplasm of the cells are highlighted with arrows in Figures 2-6C and D.

**TEM Results**

Thin sections of the cells were examined using TEM to determine the location of the silica nanoparticles in the ultrastructure of the cells. A conventional brightfield TEM image of one cell is presented in Figure 2-7. The cell contained many open areas or vesicules, which appeared light in contrast in the image. The vesicles in the cell are highlighted with arrows in Figure 2-7. Higher magnification images of these regions are presented in Figure 2-8. Nanoparticles were not observed in the control cells. The control cells contained a few large dark, electron dense regions, which are highlighted
with arrows in Figures 2-8A and B. These regions stained dark from the Os post fixation treatment.

Thin sections of the cells dosed with silica nanoparticles for 2 hours are presented in Figures 2-9A and B. Many silica nanoparticles were observed in and around the edge of the cells. The nanoparticles were taken up in to the cells by phagocytosis. The silica nanoparticles were observed primarily in intracellular compartments or endosomes in the cells. The silica nanoparticles were agglomerated in the endosomes. Movement of the silica nanoparticles in the cells was concentrated in the endosomes. These observations agree with those similar experiments on cellular uptake of Titania nanoparticles by A549 cells. (Stearns et al., 2001)

Silica nanoparticles are engulfed by the plasma membrane, as shown in Figure 2-10. The cell membrane is then pinched off to form a vesicule which contains the nanoparticles and extracellular fluid. The silica nanoparticles are internalized into endosomes or membrane bound vesicules inside the cytoplasm of the cell. The membrane bound vesicules contain agglomerated silica nanoparticles. Cellular uptake of silica nanoparticles occurred by pinocytosis and receptor mediated endocytosis. These processes are used by most eukaryotic cells to continually ingest fluid and solutes.

Higher magnification images of the A549 cells are presented in Figures 2-11A and B. The silica nanoparticles are situated around the cell and inside an endosome. The individual silica nanoparticles are clearly visible in and around the cell in Figures 2-11A and B. The silica nanoparticles appear very uniform in size and shape.
The A549 cells that were exposed the silica nanoparticles for 24 hours are presented in Figures 2-12A and B. There was a higher volume of silica nanoparticles found in endosomes inside the cells and along the outside edge of the cell membranes with the higher dose and longer exposure times. The agglomerated silica nanoparticles inside endosomes are highlighted by arrows in Figures 2-12A and B. Higher magnification images of the nanoparticles around the cell membrane and inside the cell are presented in Figure 2-13A and B. There is a very high density of nanoparticles in and around these cells.

HAADF STEM was used to image thin sections with atomic number thickness contrast. The electron dense regions of the sample, that appear very bright in HAADF STEM images, are due to the Os post fixation treatment. The Ru(bpy)3 doped nanoparticles also appear bright in the HAADF images. EDS analysis was performed to determine the chemical composition of the nanoparticles in the thin sections to confirm that the particles were the dye doped silica particles. EDS analysis of the silica nanoparticles confirmed that the particles were composed of Si and O. The silica nanoparticles also contained Ru(bpy)3 dye, which contains Ru, but Ru did not show up in the EDS spectra. The Ru content must be below the detection limit of the EDS technique. The detection limit for EDS is approximately 0.5 weight percent. An HAADF STEM image of an A549 cell dosed with silica nanoparticles for 24 hours are presented in Figure 2-14A. An EDS spectrum from the silica nanoparticles is presented in Figure 2-14B. The STEM image and EDS spectrum are typical of all the samples that were exposed to the silica nanoparticles.
Discussion

The confocal images of the stained cells clearly show the green stained cell membrane surrounding the blue nuclei. The cell membrane has pseudopod extensions that protrude from the surface as shown in Figure 2-2. The silica nanoparticles were readily taken up by the A549 cells by endocytosis after 2 hours. The nanoparticles can be seen inside the cell membrane in the confocal image in Figure 2-3A. The amount of particle uptake increased with dose and time, as more particles are present around the cell membrane for higher particle doses and longer time exposures. The particles were always observed inside the cell membrane but not inside the nuclei.

The TEM examination confirmed the presence of nanoparticles inside the cells. The particles were located in membrane bound endosomes inside the cells. The particles were agglomerated in the endosomes and were able move in the cytoplasm of the cell, from the outer membrane to the interior of the cell. The nanoparticles can be transferred to lysosomes in the interior of the cell. The take up of the nanoparticles through pseudopods or membrane extensions was observed in the TEM images presented in Figures 2-9 and 2-10. The location of the nanoparticles in the endosomes/lysosomes around the nuclei was confirmed using TEM, as well as EDS analysis.

Summary

Confocal microscopy is a good technique for imaging fluorescent nanoparticles in live cells. Very little sample preparation is required for the analysis. Fluorescent staining of the cell components provides contrast to the structural features of the cells. The staining is necessary to determine if the particles were taken up into the cells.
TEM is required to determine the location of the nanoparticles inside the cells. The same sample that was imaged live using confocal microscopy was prepared for TEM using a pop off technique. This enabled observation of the same sample with both microscopy techniques. The nanoparticles resided in endosomes and lysosomes with the cytoplasm of the cells. The TEM was used to confirm the location and composition of the nanoparticles in the cells through imaging and EDS analysis. The confocal imaging was used to determine that the uncoated silica nanoparticles were readily taken up inside the cells. TEM was used to determine that the nanoparticles were taken into the cells by endocytosis and resided in the endosomes and lysosomes within the cells.
Figure 2-1. Brightfield TEM image of the LSI Ru(bpy)3 doped silica nanoparticles.

Figure 2-2. Live cell confocal images of the control A549 cells stained with Hoechst 33258 and Oregon Green 488. The cells were healthy and alive. A) An optical slice through the cells showing the green membranes surrounding the blue nuclei. The crossed lines label the position of the x-z and y-z images on the right side and bottom of the image.
Figure 2-3. High magnification confocal image of two cells with nuclei stained blue surrounded by the green cell membrane.

Figure 2-4. Live cell confocal images of the A549 cells dosed with LSI Ru(bpy)3 doped silica nanoparticles. The nanoparticles, which are red in the images, were taken up into the cells but were not taken into the nuclei. A) The cells were dosed with 20 µg/ml nanoparticles for 2 hours have a low concentration of nanoparticles. B) The cells were dosed with 40 µg/ml nanoparticles for 24 hours have a high concentration of particles.
Figure 2-5. Confocal image of the A549 cells dosed with 40 μg/ml Ru(bpy)3 doped silica nanoparticles for 24 hours. The x-z and y-z cross sections that correspond to the positions of the lines through the main image are presented at the bottom and right side of the image. The silica nanoparticles are shown inside the cell membrane in both the plan view and cross sectional images.
Figure 2-6. Transmitted light images of the control and dosed A549 cells. A) The cells in the control sample, which contains no nanoparticles, have dark grey nuclei surrounded by lighter grey cytoplasm. B) The cells were dosed with 20 µg/ml silica nanoparticles for 2 hours. They have dark grey nuclei surrounded by lighter grey cytoplasm. C) The cells were dosed with 20 µg/ml silica nanoparticles for 24 hours and have vacuoles in the cytoplasm. D) The cells were dosed with 40 µg/ml silica nanoparticles for 24 hours and have vacuoles in the cytoplasm.
Figure 2-7. Brightfield TEM image of a cell from the control sample. The cell contained endosomes and lysosomes, some of which are highlighted by the arrows. Some of the endosomes and lysosomes contained dark, electron dense features.

Figure 2-8. Brightfield TEM images of the A 549 cell from the control sample. A) There were endosomes in the cell that contained cellular debris. Two of the endosomes are highlighted with arrows. B) The cell contained dark, electron dense features that stained dark from the Os post fixation treatment.
Figure 2-9. Brightfield TEM images of an A549 cell dosed with 20 mg/ml silica nanoparticles for 2 hours. Nanoparticles were observed in endosomes and around the periphery of the cell. A) Silica nanoparticles in an endosome inside the cell. The nanoparticles are highlighted with arrows. B) Nanoparticles around the periphery of a cell, being invaginated by the cell membrane and others inside an endosome. The three areas are highlighted with arrows.

Figure 2-10. Brightfield TEM images of an A549 cell dosed with 20 mg/ml silica nanoparticles for 2 hours. Nanoparticles around the periphery of the cell and at various stages of invagination. A) Nanoparticles around the periphery of the cell. B) Nanoparticles being engulfed by the cell membrane.
Figure 2-11. Brightfield TEM images of the 20 nm diameter silica nanoparticles inside a membrane bound vacuole and outside a cell. A) 20 nm spherical silica nanoparticles inside a membrane bound vacuole. B) 20 nm spherical silica nanoparticles outside the cell membrane.

Figure 2-12. Brightfield TEM images of an A 549 cell dosed with 40 μg/ml silica nanoparticles for 24 hours. A) There were many particles along the outside of the cell membrane. B) There were many large agglomerates of nanoparticles encapsulated in endosomes inside the cell.
Figure 2-13. TEM images of the A549 cell dosed with 40 μg/ml silica nanoparticles for 24 hours. The 16 nm diameter silica nanoparticles are resolved in the images. A) The 16 nm silica nanoparticles were observed outside cell membrane. (B) The silica nanoparticles were agglomerated particles in endosomes inside the cell.

Figure 2-14. HAADF STEM image of the agglomerated silica nanoparticles inside an endosome and the corresponding EDS spectrum. A) The silica nanoparticles appear white in the HAADF image due to the Ru(bpy)3 dye. There were many particles located in endosomes inside the cells. B) EDS spectrum from the silica nanoparticles. The spectrum contains carbon, oxygen, silicon, chlorine, and copper.
CHAPTER 3
INVESTIGATION OF THE EFFECT COATINGS ON THE PROPERTIES OF PLATINUM NANOPARTICLES AND THEIR UPTAKE INTO BEAS CELLS

Platinum (Pt) nanoparticles have been studied for a long time as catalysts. The catalytic properties of Pt are now being explored for medical applications because of their antioxidant, anti-inflammatory and pro-oxidant functions. (Finkel and Holbrook, 2000, Kim et al., 2008, Onizawa et al., 2009, Pelka et al., 2009) These properties are of particular interest as possible therapies for anti-aging and inflammatory lung diseases which are caused by oxidative stress, including chronic obstructive pulmonary disease (COPD). (Kim et al., 2008, Onizawa et al., 2009) Pt nanoparticles can suppress oxidative stress by quenching reactive oxygen species (ROS), without exerting a cytotoxic effect. (Hamasaki et al., 2008) They can act as antioxidants to reduce ROS in living organisms by scavenging $\text{O}_2^-$, $\text{H}_2\text{O}_2$ and OH. (Finkel and Holbrook, 2000, Hamasaki et al., 2008) This behavior mimics that of the antioxidant enzymatic defense systems of most organisms. (Kim et al., 2008)

**Oxidants and ROS**

Oxidants are created as a normal function of the intracellular metabolism. (Sies, 1997) A balance between the levels of oxidants and antioxidants are regulated for normal growth. (Finkel and Holbrook, 2000) Oxidants are also created by exogenous sources, such as UV light, inflammatory cytokines, and environmental toxins, which can create an excess of ROS. Excessive ROS can cause oxidative stress that leads to impaired physiological functions including damage to proteins, lipids and DNA, which can result in cell death, disease and aging. (Finkel and Holbrook, 2000, Kim et al., 2008, Onizawa et al., 2009, Drodege, 2002)
Heme Oxygenase

A cellular antioxidant enzyme system, Heme oxygenase (HO-1), has been associated with lung resistance against oxidative stress. (Fukano et al., 2006, Slebos et al., 2007) The HO-1 enzyme provides protection against oxidative stress conditions, through antioxidant, anti-apoptotic, and anti-inflammatory actions. (Ko et al., 2005) The protection against exogenous environmental stress provided by HO-1 is one of the most important mechanism mammalian cells possess to maintain the balance between the oxidant and antioxidant capacity of cells. (Fukano et al., 2006) An increased level of HO-1 is found in smokers, while the level of HO-1 decreases in patients with severe airway inflammation. (Slebos et al., 2007) The reduced expression of HO-1 may indicate an impaired defense against oxidative stress. (Ko et al., 2005, Maestrelli et al., 2003) The up-regulation of HO-1, in smokers as compared with non-smokers, shows the role of this protein as active defense mechanism against smoke injury. (Maestrelli et al., 2003)

Colloidal Pt Nanoparticles

Colloidal Pt nanoparticles have been studied as oxidation and reduction catalysts and antioxidant potential in various medical applications. (Furlong et al., 1984, Rao and Trivedi, 2005) Pt nanoparticles have been found to prevent pulmonary inflammation in mice due to cigarette smoke exposure. (Onizawa et al., 2009) They have been used to quench ROS, mimicking the enzymatic defense mechanism, and extending the life of worms. (Kim et al., 2008, Hamasaki et al., 2008)

Pt colloids, solid Pt nanoparticles suspended in a liquid phase, were used in this study. They were synthesized by the reduction of a Platinum salt, H$_2$PtCl$_6$, in the presence of a reducing agent, NaBH$_2$, with polyvinylpyrrolidone (PVP) as a protective agent. The amount and composition of the salt, reducing agent, protective agent and
solvent, and the processing temperature control the size and shape of the colloids through the reaction kinetics. (Ahmadi et al., 1996, Teranishi et al., 1999) PVP is an amphiphilic, nonionic polymer that is widely used as a steric stabilizer or capping agent to keep the nanoparticles from agglomerating. (Ahmadi et al., 1996, Teranishi et al., 1999, Xiong et al., 2006) Without a protective agent, such as PVP, larger polycrystalline metallic precipitates form. The molar ratio of PVP to Pt was found to control the dispersion stability and the size of the Pt colloids more than the molecular weight of the PVP. (Teranishi et al., 1999, Sato and Kohnosu, 2001) The higher concentrations of PVP lead to a smaller particle size, due to the increased adsorption of PVP and reduced aggregation of the nuclei. (Cheremisinoff, 1997) Agglomeration of the Pt nanoparticles into clusters and superstructures increases when smaller amounts with of PVP are used. (Shiraishi et al., 2000) Increasing the molecular weight of the PVP increases the viscosity of the solutions. The longer polymer chains increase the stability of the nanoparticles by the addition of a neutrally charged coating that is thick enough to provide steric hindrance. (Sato and Kohnosu, 2001)

**Catalytic Activity of Colloidal Pt**

The catalytic activity of colloidal Pt nanoparticles is related to the particle size, shape, and number of exposed surface atoms located at corners and facets. (Ahmadi et al., 1996, Narayanan and El-Sayed, 2004) The catalytic activity of Pt nanoparticles increases with decreasing particle size and superstructure (or agglomerate) size. (Hamasaki et al., 2008, Shiraishi et al., 2000) The catalytic activity of colloidal Pt nanoparticles decreases as a function of shape, from tetrahedral to near spherical to cubic. (Narayanan and El-Sayed, 2004, Van Hardeveld and Hartog, 1969) This shape effect was related to the number of active sites which were considered to be those at
the corners and edges. The number of corner and edge sites in tetragonal, near spherical and cubic structures is presented in Table 3-1.

Another consideration in assessing the catalytic activity of the PVP and dendrimer encapsulated Pt nanoparticles is the transport of the reactants through the organic surface layers to the surface of the Pt nanoparticles. A sketch of the three types of nanoparticles used in the study is presented in Figure 3-1. The catalytic behavior of dendrimer encapsulated Pt nanoparticles is a strong function of its environment. The catalytic response is much greater in good solvents than in gas phase reactions. (Albiter et al., 2010) The dendrimers are thought to expand in good solvents allowing passage of the molecules through to the Pt surface, while in poor solvents and in the gas phase the dendrimers contract passivating the surface of the Pt nanoparticles. (Lang et al., 2003)

**Expression of HO-1 in BEAS Cells**

In this chapter the relationship between the starting properties of Pt nanoparticles and their cellular uptake characteristics were investigated to determine their effect on the expression of the cytoprotective HO-1 enzyme in BEAS cells. An in depth study of the expression of the HO-1 enzyme and Il-8 cytokine in BEAS cells dosed with colloidal Pt nanoparticles was conducted by Ana Montes-Worboys (Montes-Worboys, 2010). The IL-8, Interleukin 8, is a chemokine that is secreted by cells and associated with inflammation and oxidant stress. Five types of colloidal Pt nanoparticles with different protective or capping agents were tested in this study. The Platinum nanoparticles tested consisted of Pt with no protective agents, Pt with three different molecular weights of PVP, and Pt with a dendrimer agent. The test matrix is presented in Table 3-2. The cell viability was measured using Trypan blue dye exclusion assay and Lactate
dehydrogenase release assay after exposures to the colloidal Pt nanoparticles. The expression of HO-1 and IL-8 genes was determined using Real time polymerase chain reaction and SDS-Polyacrylamide gel electrophoresis and Western Blot analysis. The BEAS cells were incubated with different doses of colloidal Pt nanoparticles (50, 100, 200 and 400 µM), for 4, 8, 24 and 48 hour periods. One type of colloidal Pt nanoparticle, Pt with the 1,300,000 molecular weight PVP coating (Pt-PVP 1.3M), had a significant increase of HO-1 gene expression. The increase in HO-1 gene expression was dependent on both the dose of Pt-PVP 1.3M nanoparticles and exposure time. There was not a significant increase in the levels of IL-8 gene expression in the same samples, signaling low inflammation and cell viability was high after the nanoparticle exposures.

The possible role of Pt-PVP 1.3M nanoparticles to protect the cells against the oxidative stress damage provoked by cigarette smoke was explored through the co-incubation of BEAS cell for 24 and 48 hours with different doses of cigarette smoke extract (CES), ranging from 0.5 to 10%, and the Pt nanoparticles. The levels of HO-1 increased significantly when cells were incubated with Pt-PVP 1.3M but not with the other particles assayed. The HO-1 gene expression was higher after 24 hours that after 48 hours incubation time. When BEAS cells were exposed to smoke extract for a long time period, up to 72 hours, the expression of HO-1 was not increased, indicating the loss of the capacity of protection against the damage provoke by the smoke insult. For the highest dose of smoking extract, the levels of HO-1 expression were the same for all of the particles assayed, probably due to the decrease in the survival rate. The levels of IL-8 expression were increased with the highest dose of smoking extract.
Description of Work

The goal of this work was to investigate the relationship between the physical properties of the colloidal Pt nanoparticles and their effect on the expression of HO-1 enzyme in the BEAS cells. The physical properties of the colloidal Pt nanoparticles were characterized using TEM, EDS, and Dynamic Light Scattering (DLS) measurements. The cellular uptake of the colloidal Pt nanoparticles was investigated through experiments on cultured BEAS cells.

Materials and Methods

Preparation of Platinum Nanoparticles

The Platinum nanoparticles were prepared by Diago Yamamoto, Satoshi Wantanabe, and Minoru T. Miyahara at the Department of Chemical Engineering, Kyoto University, Nishikyo-ku, Kyoto, Japan. Potassium hexachloroplatinate (K₂PtCl₆) or potassium tetrachloroplatinate (K₂PtCl₄) was used as a platinum source, sodium borohydride (NaBH₄) as a reducing agent, and polyvinylpyrrolidone (PVP, average molecular weights of 10000 (10k), 55000 (55k), and 1300000 (1.3M)) or generation-4 hydroxyl-terminated poly(amidoamine) dendrimer (G4-OH) as a protective agent that prevents the aggregation of resultant nanoparticles. K₂PtCl₆ was used for the synthesis of PVP-coated Pt particles, and K₂PtCl₄ for that of dendrimer-encapsulated and uncoated ones. All the chemicals were purchased from Aldrich Chemical Co. The five types of platinum nanoparticles are listed in Table 3-2.

PVP-protected Pt nanoparticles (referred to as Pt-PVP) were synthesized using a K₂PtCl₆ with PVP solution and a NaBH₄ solution was mixed with a micromixer. A central collision micromixer was which realizes perfect mixing by utilizing shearing forces at the collision of fluid segments (Nagasawa, et al, 2005) Reduction proceeded upon mixing,
and then a dispersion of PVP-protected Pt nanoparticles was obtained from the outlet of the mixer. The molar ratio of PVP in the monomer unit to Pt was set as 1:1, while that of NaBH₄ to Pt as 2:7. The dendrimer-encapsulated Pt nanoparticles (referred to as Pt-DEN) were synthesized using the procedure which is previously reported. (Yamamoto, et al, 2009). The molar ratio of dendrimer molecule to Pt was set to be 0.025. Pt nanoparticles without protective polymers were synthesized as a control by just mixing the solutions of K₂PtCl₆ and NaBH₄, which naturally resulted in precipitation due to the aggregation of Pt particles. In the Pt nanoparticle synthesis, the concentration of Pt was set to be 20 mM, and the resultant suspension was diluted to a desired concentration before use.

**Nanoparticle Characterization**

The particle size of the nanoparticles was characterized using a Microtrac Nanotrac Particle Size Analyzer. The Nanotrac works on the principles of dynamic light scattering which can be used to determine the particle size distribution of particles in solutions ranging in size from a few nanometers to a few microns. DLS is based on the detection and correlation of laser light intensity fluctuations from small particles in solution. The laser light is scattered by hitting particles in solution undergoing Brownian motion, called the Doppler shift. The shift in light frequency is related to the size of the particles causing the shift. The particle mobility is dependent on particle size, temperature and viscosity of the liquid. Samples were diluted in DI water and ultrasonicated prior to the DLS measurements.

The zeta potential was measured using the Beckman Coulter Delsa Nano at a pH of 7.4 and the Brookhaven Instruments Corporation Zeta Plus was used to confirm the measurements. The zeta potential is the electrostatic potential at the boundary
between the particle, including its most closely associated ions, and the surrounding media. It is a function of the surface charge of the particles, the adsorbed layers at the interface and the nature and composition of the surrounding suspension medium. It is an indication of the effective charge on the particles and it is related to the electrostatic repulsion between them which is used as a measure of the stability of particle suspensions. The DVLO theory is used to describe the stability of a particle in solution in terms of the total potential energy function which is the sum of the potential energy due to the solvent, the Van der Waals attractive force and double layer repulsive force. Repulsive forces between the nanoparticles are necessary to keep the particles separate. A large negative or positive zeta potential indicates that the particles will remain dispersed, a low Zeta Potential number indicates that there is no force to prevent the particles from aggregating.

The particle size and shape were also characterized using a JEOL JEM 2010F Scanning Transmission Electron Microscope and Oxford INCA EDS system. EDS analysis allows for the detection of x-rays emitted from the sample for determination of chemical composition. The samples were prepared for TEM by placing a drop of nanoparticles on a Copper TEM grid with a carbon support film.

**BEAS Cell Cultures**

Human bronchial airway epithelial cells (BEAS) were purchased from American Type Culture Collection (ATCC). Cells were cultured according to the ATCC prescription in Cambrex Bronchial Epithelial Cell Growth Medium (BEGM), a serum-free medium, in a humidified atmosphere of 5% CO₂/balanced air at 37°C. Cells were cultured in 6 well plates and grown to 90% confluence. The media was changed to Cambrex Bronchial
Epithelial Cell Basal Medium (BEBM), a growth factor-free media, for the treatment experiments with platinum nanoparticles.

**Nanoparticle Dosing Experiments**

Two sets of experiments were run with the cultured BEAS cells and Pt nanoparticles for TEM analysis of the particle uptake. The first experiment consisted of exposing the BEAS cells, passage 44, to the Pt nanoparticles with no agents and Pt–PVP 1.3M nanoparticles for 24 hours. A 100 µM dose (20 µg/ml) of Pt nanoparticles in BEBM media was used for the experiments. The second experiment consisted of exposing the BEAS cells to the Pt nanoparticles with no agents, Pt-PVP 10k, Pt-PVP 55k and Pt-PVP 1.3M, a for 6 hours. A 300 µM (60 µg/ml) dose of Pt nanoparticles in BEBM media was used.

**Sample Preparation for TEM**

Thin sections for TEM were prepared using a microwave procedure. A Pelco Biowave Microwave was used for processing. The BEAS cells in the 6 well plates were washed two times in sodium cacodylate buffer solution to wash away the proteins. The cell medium was removed and the buffer added to the wells. The samples were microwaved for 45 seconds at 180 watts with a 1 minute rest for each wash. The cells were fixed in half strength Karnovsky fixative which consists of 2.5% glutaraldehyde and 2% paraformaldehyde in sodium cacodylate buffer. The samples were fixed in the 6 well plates for 45 seconds in the microwave at 180 watts followed by a 1 minute rest. The cells were washed two times with the sodium cacodylate buffer solution after fixation using the 45 seconds microwave at 180 watts followed by a 1 minute rest after each wash. They were then given a post fixation treatment in 2% osmium tetraoxide (Os) in
sodium cacodylate buffer solution, with a 1 minute bench top rest, 45 second microwave under vacuum at 180 watts followed by a 3 minute bench top rest. The cells were washed three times with buffer solution and two times with DI water after the Os fix using the microwave for 45 seconds under vacuum at 180 watts followed by a 1 minute bench top rest. The cells were then scrapped from the 6 well plates, put in centrifuge tubes with 1 ml buffer and centrifuged to pelletize the cells. The cell pellets were encapsulated in 2% agarose gel and given two water washes with a 45 second microwave at 180 watts and 1 minute rest. The samples were dehydrated using a graded ethanol/ DI water microwave procedure (70%/30%, 50%/50%, 30%/70%, 90%/10%, two times 100% ethanol, and two time in 100% acetone), for 45 seconds in the microwave at 180 watts and 1 minute rest on the bench top. The dehydrated samples were then infiltrated with graded Spurrs’ resin /ethanol mixtures (1:3, 1:1 3:1), followed by 100% Spurrs’ resin in the microwave for 3 minutes under vacuum and 3 minute benchtop rest. The cells in 100% Spurrs’ were then cured overnight. The encapsulated cells were placed in a silicone mold and filled with 100% Spurrs’. The embedded samples were cured for 2 days at 60C and thin sections were cut with a Leica Ultra Cut S Microtome. Semi thin sections, 0.5 micron thick, and mounted on a glass slide. They were stained with toluidine blue and examined using an Olympus optical microscope. The thick sections were used to determine if the sections contained BEAS cells. The thick sections were also imaged using SEM. The thin sections, 80 nm thick, were used for TEM imaging.

**TEM Analysis of BEAS Cells**

The morphology of the BEAS cells and location of nanoparticles inside the cells were characterized using TEM. A JEOL 200cx TEM and JEOL JEM 2010F STEM
equipped with an Oxford INCA EDS system. The JEOL 2010F is equipped with a JEOL Brightfield and High Angle Darkfield Annular Detector (HAADF) for z contrast imaging used to investigate the nanoparticle uptake by the BEAS cells.

Results

Characterization of Platinum Nanoparticles

DLS results

The mean particle size measurements by DLS are listed in Table 3-2. The mean particle size by number was calculated by dividing the sum of the diameters of all of the individual particles by the total number of particles in the distribution. The mean particle size by volume was calculated by dividing the sum of the spherical volumes of all of the particles by the total number of particles. The mean particle size of the Pt-No Agents nanoparticles was 3.3 nm by number and 3.2 microns by volume as measured by DLS. This is a good indication that there are micron sized agglomerated present in the sample analyzed. The mean particle size of the Pt-dendrimer nanoparticles was 2.7 nm by number and 131 nm by volume as measured by DLS. This again is a good indication of particle agglomeration. The particle size of the Pt-PVP nanoparticles decreased with increasing molecular weight of the PVP. The mean particle size of the 10k, 55k and 1.3M Pt-PVP nanoparticles was 3.5 nm, 3.5 nm and 2.2 nm respectively. The particle size by volume the three Pt-PVP nanoparticles was 4.5 nm, 4.1 nm and 2.9 nm. The larger mean particle size by volume indicates that there was some clustering of the PVP coated nanoparticles.

TEM results

The colloidal Pt nanoparticles with no agents formed large agglomerates or superstructures of fine fcc nanoparticles. Brightfield TEM images of the Pt nanoparticles
with no agents are presented in Figures 3-2A and B. A small area of the agglomerated Pt nanoparticles is presented in Figure 3-2A. The agglomerates were many microns in size. A small cluster of nanoparticles is presented in Figure 3-2B. The cluster is approximately 15 nm in size. The primary particle size of the Pt-no agents nanoparticles measured by TEM was 3.3 nm, but it was very difficult to get a size distribution measurement from the images because of the particle agglomeration.

The Pt nanoparticles made with the dendrimer were relatively well dispersed. Brightfield TEM images of the Pt-den nanoparticles are presented in Figures 3-3A, B and C. The nanoparticles are predominantly spherical with some elongated oval shaped nanoparticles. The spherical nanoparticles are fairly uniform in size. A single spherical nanoparticle is presented in the high resolution TEM image in Figure 3-3C. The particle has a [011] orientation with a lattice spacing of 0.226 nm. The primary particle size measured by TEM of the Pt-dendrimer particles was 2.0 nm, the agglomerate size was not measured. The orientation of the particle in Figure 3-3C is [011] as determined from the Fast Fourier Transform (FFT) of the image in Figure 3-3D.

The Pt nanoparticles coated with 10k PVP are presented in Figures 3-4A, B, C and D. These nanoparticles are a bimodal mix of well dispersed spherical nanoparticles and large interconnected superstructures, as shown in Figures 3-4A and B. The well dispersed nanoparticles ranged in size from 1.8 to 8.1 nm. The mean particle size was measured at 3.8 nm. This sample contained large NaCl and KCl crystals which are shown in Figure 3-4B. The composition of the large crystals was confirmed by EDS analysis. Most of the nanoparticles were spherical, although there were some cubes, as
shown in Figure 3-4C. A high resolution lattice image of a nanoparticle is presented in Figure 3-4D. The lattice spacing of the nanoparticle is 0.226 nm.

The Pt nanoparticles coated with 55k PVP are presented in Figures 3-5A and B. These nanoparticles were also a mix of finely dispersed nanoparticles and large interconnected superstructures, similar to the Pt-PVP 10k nanoparticles. The primary particle size measured by TEM was 3.3 nm. A large super structure is presented in Figure 3-5A. The primary nanoparticles were all nearly spherical. This sample also contained large NaCl and KCl crystals. The large NaCl and KCl crystals are presented in Figure 3-5B. The composition of the large crystals was confirmed using EDS analysis. A high resolution lattice of a few nanoparticles is presented in Figure 3-5D. The orientation of the larger particle is [011] and the lattice spacing is 0.226 nm.

The Pt nanoparticles coated 1,300,000 molecular weight PVP were well dispersed and fairly uniform in size and shape. Brightfield TEM images of the Pt-PVP 1.3M nanoparticles are presented in Figures 3-6A through D. The mean primary particle size of the Pt-PVP 1.3M nanoparticles was 2.9 nm. The nanoparticles are predominantly spherical in shape with some elongated oval nanoparticles. A high resolution lattice image of a Pt-PVP 1.3M nanoparticle is presented in Figure 3-6D. The nanoparticle has a lattice spacing of 0.226 nm.

**Zeta potential**

The zeta potential measurements provide an indication of the stability of the particle dispersion in solution. The zeta potential results are presented in Table 3-2. The Pt-den and Pt-PVP nanoparticles all had relatively low zeta potentials which ranged from -26 to -14 mV. These low values indicate low inter particle repulsive forces and similar behavior for all of the nanoparticles.
Particle Up Take by BEAS Cells

The take up of Pt nanoparticles by BEAS cells was investigated after dosing with 100 μM (20 μg/ml) colloidal Pt for 24 hours and 300 μM (60 μg/ml) colloidal Pt for 6 hours. The nanoparticle behavior was similar for both sets of experiments. The Pt nanoparticles were taken up by the BEAS cells without being toxic to the cells. The Pt nanoparticles were taken up into the cells after 6 hour exposures and remained in the cells for 24 hours. The BEAS cells were alive and healthy after the 24 hour exposures the Pt nanoparticles.

BEAS cells are squamous cells. They have a long, flat shape and typically have elliptical nuclei. A diagram of an epithelial cell is presented in Figure 3-7A, showing the nuclei, endoplasmic reticulum, lysosomes, mitochondria and golgi complexes. A transmitted light image of the control BEAS cells is presented in Figure 3-7B. The long flat cells are shown in the figure. Brightfield TEM images of the control BEAS cells are presented in Figures 3-8 and 3-9. The long, flat BEAS cells contained numerous spherical lysosomes, which are shown in Figure 3-8. A few dark, high density features were observed in the cells, they are shown in Figure 3-9. The composition of these features was determined using EDS analysis. They contained osmium from the post fixation treatment. One of the features is presented in Figure 3-9B. The corresponding EDS spectrum is presented in Figure 3-9C. The elements in the spectrum are C, O, Os and Ni. The Ni peaks are a result of scattering from the Ni TEM grid and C and O peaks are from the embedded tissue.

The BEAS cells exposed to 300 μM (60 μg/ml) Pt-no agents nanoparticles for 6 hours are presented in Figures 3-10A and B. These cells were also very healthy. The
cells contain dense agglomerates of Pt nanoparticles. A brightfield STEM image is presented in Figure 3-10A. The corresponding HAADF image is presented in Figure 3-10B. The Pt nanoparticles are bright white in the HAADF images. The composition of agglomerated Pt nanoparticles was confirmed using EDS analysis. The EDS spectrum is presented in Figure 3-10C. The spectrum contains C, O, Os, Pt and Ni. The agglomerated Pt nanoparticles were taken up into the cells without killing the cells.

The BEAS cells exposed to 300 μM Pt-den nanoparticles for 6 hours are presented in Figures 3-11A and B. The cells contained a large number of lysosomes, some of which contained clusters of finely dispersed Pt nanoparticles, as shown in Figure 3-11B. The composition of the nanoparticles was confirmed with EDS analysis. The EDS spectrum is presented in Figure 3-11C. The finely dispersed nanoparticles were taken up into the cells without killing the cells.

The BEAS cells exposed to 300 μM (60 μg/ml) Pt-PVP 10k nanoparticles for 6 hours are presented in Figures 3-12A through C. The Pt nanoparticles were taken into the lysosomes inside the cells. Agglomerated nanoparticles as well as individual and small clusters of nanoparticles were found in the lysosomes. The lysosomes in some of the cells were enlarged. An enlarged lysosome containing agglomerated Pt nanoparticles is presented Figures 3-12B. Small clusters of nanoparticles in a lysosome are presented in Figures 3-12C. The Pt nanoparticles are highlighted by the arrows.

The BEAS cells exposed to 300 μM (60 μg/ml) Pt-PVP 55k nanoparticles for 6 hours are presented in Figures 3-13A through C. The nanoparticles were observed as agglomerates and small clusters inside the cells, as shown in Figure 3-13B. Enlarged lysosomes were not observed in the BEAS cells exposed to the Pt-PVP 55k
nanoparticles. Small clusters of nanoparticles around the outside of a BEAS cells are presented in Figure 3-13C.

The BEAS cells exposed to 300 μM (60 μg/ml) Pt-PVP 1.3M nanoparticles for 6 hours are presented in Figures 3-14A and B. The Pt-PVP 1.3M nanoparticles were dispersed throughout the lysosomes and adjoining areas of the cells. The nanoparticles inside a BEAS cell are highlighted with arrows in Figure 3-14B. The composition of the nanoparticles was confirmed using EDS analysis. The EDS spectrum is presented in Figure 3-14C. Brightfield and HAADF STEM images of the Pt-PVP 1.3M nanoparticles highlighting the dispersed Pt nanoparticles are presented in Figures 3-15A and B. The nanoparticles are located in a membrane bound vesicle and appear black in the BF STEM image and white in the HAADF imaged. Some nanoparticles are located outside the membrane bound vesicle.

**Discussion**

The Pt nanoparticles were very fine in size, the primary particle size by number ranged from 2.0 nm to 3.5 nm. There was some agglomeration of the Pt nanoparticles as seen by the particle size measurements by volume and the TEM images. The Pt nanoparticles were for the most part all the same shape, they were predominantly spherical or nearly spherical. A summary of the particle size data is presented in Table 3-2. The Pt-no agents nanoparticles were very agglomerated due to the preparation without any protecting or capping agents. The agglomeration was detected by the DLS measurements and presented in the TEM image in Figure 3-3A. There were some agglomeration issues with the Pt-dendrimer nanoparticles, as indicated by the larger mean particle size by volume. Minor agglomeration was also seen in the TEM image in
Figure 3-4A. The Pt-PVP 10k and 55k nanoparticles had a larger primary particle size of 3.5 nm by number and smaller particle size, 4.5 and 4.1 nm, by volume, than the Pt-no agents or Pt-den nanoparticles. Agglomerates, smaller than those observed in the Pt-no agents sample were observed in the TEM images of Pt-PVP 10k and 55k nanoparticles in Figures 3-4A and 3-5A. This type of superstructure formation was attributed to too little PVP in solution. (Shiraishi et al., 2000) Large NaCl and KCl crystals were also observed in this sample. The primary particle size of the Pt PVP 1.3M was 2.2 nm by number and 2.9 nm by volume. The Pt-PVP 1.3M nanoparticles were smallest in size of the 5 types of Pt nanoparticles. They were also well dispersed as measured by DLS and shown in Figure 3-6A. The long polymer chains of the 1.3M PVP would provide a thicker coating, providing better dispersion and smaller particle size than the 10k and 55k molecular weight PVPs.

All five types of Pt nanoparticles were taken up into the BEAS cells. The trends of nanoparticle take up into the BEAS cells were similar for the 6 and 24 hour exposures. Neither the dose or exposure time caused the Pt nanoparticles to be cytotoxic to the cells. The nanoparticles were predominantly located in lysosomes, membrane bound vesicules, inside the cells. The Pt nanoparticles were observed as agglomerates in the Pt-no agents and Pt-PVP 10k and 55k samples as shown in Figures 3-10, 3-12 and 3-13. The Pt-den and Pt-PVP 1.3M nanoparticles were well dispersed inside the lysosomes, as shown in Figures 3-11 and 3-14. Individual and small clusters of nanoparticles were observed outside the lysosomes in the BEAS cells.

The increased catalytic activity Pt-PVP 1.3M nanoparticles can be partially attributed to the small particle size and better dispersion as compared to the other Pt
nanoparticles. The Pt-PVP 1.3M nanoparticles would also have a thicker PVP coating than the other PVP coated nanoparticles. The 1.3M PVP has much longer polymer chains than the 10k or 55k PVP which would lead to a thicker PVP coating on the nanoparticles, preventing agglomeration, that may be bound to fewer sites on the nanoparticles, leaving more of the Pt nanoparticle surface sites exposed. The dendrimer did a relatively good job at keeping the Pt nanoparticles dispersed, but it is possible that the dendrimer contracts in aqueous solutions, inhibiting the catalytic properties of the Pt nanoparticles by blocking the surface sites. (Lang et al., 2003)

It is not apparent that the up regulation of HO-1 by BEAS cells exposed to the Pt-PVP 1.3M nanoparticles is due solely to the differences in particle size, agglomeration and cellular uptake characteristics of the Pt nanoparticles by the BEAS cells. There may be other differences in the Pt-PVP nanoparticles besides just the molecular weight of the PVP. The Pt-PVP 1.3M could be a Pt ion PVP complex or there could be some other chemical differences between the nanoparticles. Additional characterization of the PVP coating is required. There is also the possibility that the metal available for catalysis is different for the 3 types of PVP coated nanoparticles, due to tighter coating or oxidized surfaces. Unfortunately there was not enough Pt-PVP 1.3M or Pt-dend nanoparticles to complete additional testing.

**Summary**

The properties and cellular uptake behavior of five types of Pt nanoparticles were investigated to determine why one type of Pt nanoparticle, Pt with a 1.3M molecular weight PVP coating, causes an up regulation of the HO-1 enzyme in BEAS cells. All of the Pt nanoparticles were taken up into the BEAS cells and were not cytotoxic to the cells. The Pt-PVP 1.3 M nanoparticles were very small and well dispersed inside the
cells in comparison to the other types of nanoparticles. The 1.3 M molecular weight PVP coating produces a thicker coating on the nanoparticles but does not seem to affect the catalytic activity of the Pt nanoparticles.

**Future Work**

Additional testing on the PVP coating and surface condition of the Pt nanoparticles is needed to understand the up regulation of HO-1 caused by the Pt PVP 1.3M nanoparticles in BEAS cells. In particular, surface chemical analysis of the particles would be very useful.
Figure 3-1. Diagram of the three types of Pt nanoparticles used in this study, Pt no agents, Pt-den and Pt-PVP.

Table 3-1. The percentage of corner and edge sites in tetragonal, near-spherical and cubic crystals. (Van Hardeveld and Hartog, 1969)

<table>
<thead>
<tr>
<th></th>
<th>Tetragonal</th>
<th>Near-Spherical</th>
<th>Cubic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent of Total Sites</td>
<td>28.0</td>
<td>3.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Percent of Surface Sites</td>
<td>35.0</td>
<td>13.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Table 3-2. Particles size, dispersion and zeta potential measurements for the platinum nanoparticles

<table>
<thead>
<tr>
<th>Nanoparticles Properties</th>
<th>Pt-(no stabilizing agents)</th>
<th>Pt-dendrimer</th>
<th>Pt-PVP 10k MW</th>
<th>Pt-PVP 55k MW</th>
<th>Pt-PVP 1.3M MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Particle Size by Number by DLS</td>
<td>3.0 nm</td>
<td>2.7 nm</td>
<td>3.5 nm</td>
<td>3.5 nm</td>
<td>2.2 nm</td>
</tr>
<tr>
<td>Mean Particle Size by Volume by DLS</td>
<td>3.2 micron</td>
<td>131.3 nm</td>
<td>4.5 nm</td>
<td>4.1 nm</td>
<td>2.9 nm</td>
</tr>
<tr>
<td>Zeta Potential (pH7.4)</td>
<td>-37 mV</td>
<td>-21 mV</td>
<td>-26 mV</td>
<td>-30 mV</td>
<td>+8 mV</td>
</tr>
<tr>
<td>Particle Size Mean Size by TEM Dispersion</td>
<td>3.3 nm</td>
<td>±0.9 nm</td>
<td>2.0 nm</td>
<td>±0.5 nm</td>
<td>3.8 nm</td>
</tr>
<tr>
<td>A: agglomerated</td>
<td>A</td>
<td>FD</td>
<td>A+FD</td>
<td>A+FD</td>
<td>FD</td>
</tr>
<tr>
<td>FD: finely dispersed</td>
<td>A-minor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3-2. Brightfield TEM images of the colloidal Pt nanoparticles made without protective agents. A) A large interconnected superstructure composed of much smaller nanoparticles. B) A small cluster of Pt nanoparticles approximately 15 nm in diameter.
Figure 3-3. Brightfield TEM images of the Pt nanoparticles plus dendrimer. A) The nanoparticles are relatively well dispersed and uniform in size. B) Most of the nanoparticles are near spherical or rod shaped. C) High resolution TEM image of a single Pt nanoparticle lattice spacing of 0.226 nm. D) FFT of nanoparticle image showing a with [011] orientation.
Figure 3-4. Brightfield TEM images of the Pt-PVP 10k nanoparticles. A) The Pt-PVP10k nanoparticles formed large interconnected superstructures. A large NaCl crystal is shown on the right side of the image. B) The nanoparticles have a bimodal distribution of small, well dispersed nanoparticles and large interconnected superstructures. C) The well dispersed nanoparticles are nearly spherical with sizes ranging from 2 to 5 nm. D) High resolution TEM image of a single Pt nanoparticles with a lattice spacing of 0.226 nm.
Figure 3-5. Brightfield TEM images of Pt-PVP 55k nanoparticles. A) The nanoparticles were a mix of large interconnected superstructures and finely dispersed nanoparticles. B) There were large KCl crystals mixed in with the nanoparticles. C) The finely dispersed nanoparticles were nearly spherical. D) High resolution lattice image of a few nanoparticles with a [011] orientation.
Figure 3-6. Brightfield TEM images of Pt-PVP 1.3 M nanoparticles. A) The Pt PVP 1.3M nanoparticles are relatively well dispersed and uniform in size. B) The nanoparticles are predominantly near spherical in shape. C) High resolution TEM image of a few nanoparticles with 0.226 nm lattice spacing.
Figure 3-7. BEAS cells. A) Diagram of an epithelial cell showing the parts of the cell. B) Transmitted light image of many BEAS cells. http://en.wikibooks.org/wiki/A-level_Biology/Biology_Foundation/cell_structure

Figure 3-8. Brightfield TEM images of control BEAS cells. A) The BEAS cells are long and flat and contain many lysosomes. B) The lysosomes range in size up to 0.5 micron in diameter. C) The lysosomes are present in the cells.
Figure 3-9. Brightfield TEM images of the control BEAS cell. A) The control BEAS contained many vesicules. B) The dense feature inside the BEAS cell had a high Os content. C) EDS spectrum from the dark feature in the cell in image B.
Figure 3-10. Brightfield TEM images of BEAS cells exposed to Pt-no agents nanoparticles and the EDS spectrum from Pt nanoparticles. A) Agglomerated Pt nanoparticles are located inside the BEAS cells. B) HAADF STEM image of the agglomerated Pt nanoparticles in the BEAS cells. C) EDS spectrum from an agglomerate of Pt nanoparticles.
Figure 3-11. Brightfield TEM images of a BEAS cell exposed to Pt-den nanoparticles for 6 hours and the corresponding EDS spectrum. A) The BEAS cell has many vesicles. B) Dispersed Pt-den nanoparticles are located inside some of the vesicles. C) EDS spectrum from the Pt-den nanoparticles inside the BEAS cell.
Figure 3-12. Brightfield TEM images of the BEAS cells exposed to Pt-PVP 10k nanoparticles for 6 hours. A) Agglomerated nanoparticles were taken up into vesicules in the cells. B) Agglomerated nanoparticles inside a large vesicule. C) Small clusters of nanoparticles were also found inside vesicules in the BEAS cells. The nanoparticles are highlighted by the arrows.
Figure 3-13. Brightfield TEM images of BEAS cells exposed to Pt-PVP 55k nanoparticles for 6 hours. A) Large agglomerates were observed inside the BEAS cells. B) Higher magnification image showing the small clusters as well as large agglomerates of nanoparticles inside the cell. C) Small clusters of nanoparticles attaché to membrane on the outside of a BEAS cell.
Figure 3-14. Brightfield TEM images of the BEAS cells exposed Pt-PVP 1.3M for 6 hours and the corresponding EDS spectrum. A) BEAS cells have many vesicles and some dense bodies. B) Very small clusters and individual Pt nanoparticles are located in the vesicles of the BEAS cell. C) EDS spectrum from the dark particles in the cell in image B.
Figure 3-15. STEM images of the Pt-PVP 1.3M nanoparticles in a vesicle in a BEAS cell. A) Brightfield STEM image of the dispersed Pt nanoparticles located in and around the vesicle. B) HAADF STEM image of the Pt-PVP 1.3M nanoparticles located in and around the vesicle.
CHAPTER 4
INVESTIGATION OF THE EFFECT OF SURFACE COATING ON THE COAGULATION, CIRCULATION TIME AND ACCUMULATION OF GOLD NANOPARTICLES IN THE LIVER OF MICE

Nanoparticles that enter the body are quickly taken out of circulation by the reticuloendothelial system (RES) or mononuclear phagocytic system. The RES is part of the body’s immune response system that is specifically designed to remove foreign matter from the circulatory system. Polymer coatings are put on nanoparticles to keep them in circulation for longer times through changes in the surface chemistry that prevent absorption of plasma proteins which stimulates take up by the macrophages of the RES. (Otsuka et al., 2003; Owens et al., 2006) The polymer coated particles have been called stealth particles because they go undetected by the macrophages. (Gref et al, 2000) Coatings are used by the pharmaceutical industry for drug applications that require long circulation times. However, even polymer coated nanoparticles are eventually eliminated from the circulation system. The focus of this study was to determine the effect of surface coating on the coagulation, circulation time and accumulation of gold nanoparticles in the liver of mice. The chapter discusses the characterization of coatings on gold nanoparticles and the differences in the distribution of native, or uncoated, gold nanoparticles and polyethylene glycol coated (PEGylated) gold nanoparticles in the liver of mice after intravenous injection.

**Background**

**The Liver Functions**

The liver is the largest organ in the body. It has many functions, including the synthesis of proteins, synthesis and storage of fats, process and storage of carbohydrates, form and secrete bile and eliminate potentially harmful substances.
(Maton et al., 1993) The liver of a mouse is made up of 4 lobes or sections. (Arias et al., 2001) Each lobe contains numerous small sections called lobules. A diagram of a liver is presented in Figure 4-1. Blood enters the liver from the portal vein and hepatic artery. (Maton et al., 1993) The portal vein carries approximately 75% of the blood into the liver from the gastrointestinal tract and the spleen, and 25% of the blood is arterial blood from the hepatic artery. Ingested substances in the blood flow through the portal vein and are processed by the liver. The blood flows from branches of the portal vein and hepatic artery into the small capillaries, called sinusoids, which flow into the central vein of each lobule. (Arias et al., 2001) These veins coalesce into hepatic veins as the blood flows out of the liver and into the circulation system.

**Hepatocytes and Sinusoidal Endothelial Cells**

The main functional cells in the liver are called hepatocytes. (Arias et al., 2001) Approximately 70-80% of the cells in the liver are hepatocytes. The hepatocytes are arranged in plates which are separated by sinusoids. The sinusoids, or small blood vessels, are lined with endothelial cells. (Arii et al., 2000) The arrangement of the hepatocytes and blood vessels in the lobules is presented in the enlarged view of the liver in Figure 4-1. The endothelial cells that line the sinusoids and are in direct contact with the blood. They take up soluble macromolecules, such as proteins, and small particles from the blood by endocytosis and transport white blood cells to the liver. (Arii et al., 2000; Smedsrød et al., 1994) Endocytosis is the uptake of fluid, dissolved solutes and macromolecules into a network of tubules and vesicules called endosomes within the cell.(Karp, 1996) Small particulate and dispersed nanoparticles that are suspended in the fluid are taken up into the endothelial cells by receptor mediated endocytosis and micropinocytosis. (Smedsrød et al., 1994; Widmann et al., 1972) Receptor mediated
endocytosis is the cellular uptake of specific macromolecules after they bind to receptors, or clathrin coated pits, on the outer surface of the plasma membrane. (Karp, 1996) Micropinocytosis is the take up of macromolecules by invagination of the plasma membrane which is pinched off resulting in small vesicles in the cytoplasm. The endothelial cells take up relatively few particles compared to Kupffer cells. (Braet et al., 2002)

**Kupffer Cells**

The Kupffer cells are macrophages of the RES of the liver that ingest foreign materials such as bacteria, cellular debris, and foreign particles by a process called phagocytosis, or “cell eating”. (Aderem et al., 1999; Karp, 1996) Kupffer cells are located on top of and in between the endothelial cells that line the sinusoids. (Arii et al., 2000) The Kupffer cells are also in direct contact with the blood. (Wisse et al., 1974a) (Karp, 1996) The functions of the Kupffer cells are to break down the red blood cells and remove foreign substances by phagocytosis. Approximately 15% of the cells in the liver are Kupffer cells and 25% of the Kupffer cells are located in the periportal region of the liver, where the portal vein enters the liver. (Arii et al., 2000) They are the first cells to come into contact with the blood and ingested material that enters the liver. The Kupffer cells in the periportal region of the liver have the highest phagocytic capacity of all the Kupffer cells in the liver. (Arii et al., 2000; Wisse et al., 1974a) They swell in size as debris is taken in and may eventually affect blood flow.

Initially, the large or agglomerated foreign particles adhere to the Kupffer cell membranes and are then engulfed by extensions of the cell membrane called pseudopods. (Wisse et al., 1974b) The particles are taken up into phagosomes or phagocytic vacuoles, which merge with lysosomes, or spherical organelles, that attempt
to digest their contents. In the case of nanoparticles, they are not destroyed but remain in lysosomes in the Kupffer cells for years. (Wisse et al., 1974a; Widmann et al., 1972; Braet et al., 2002) Kupffer cells have a large number of the lysosomes and can take up a large amount of particles. (Wisse et al., 1974b)

Kupffer cells take up large particles and agglomerated nanoparticles very quickly by phagocytosis. (Wisse et al., 1974a) Experiments on mice and rats have shown that once in the circulatory system, most of the intravenously injected particles accumulate in the Kupffer cells in the liver, regardless of the size of the nanoparticles. (De Jong et al., 2008; Sadauskas et al., 2007; Sonavane et al., 2008; Wisse et al., 1974a) The phagocytic activity of the Kupffer cells in the livers of mice and rats has been reported for many types of particles, including Thortrast (Wisse et al., 1974a), colloidal gold (Sadauskas et al., 2009; Sonavane et al., 2008), colloidal carbon (Widmann et al., 1972) and latex particles (Singer et al., 1969). Single nanoparticles or small groups of nanoparticles are taken up into the Kupffer cells by micropinocytosis. (Sadauskas et al., 2007; Widmann et al., 1972; Wisse et al., 1974a) The Kupffer cells take up many more particles than the endothelial cells. (Smedsrød et al., 1994; Widmann et al., 1972)

The nanoparticles that are taken up in to the liver remain in the liver, but the number of particle containing Kupffer cells decreases over time. (Sadauskas et al., 2009) The Kupffer cells that are damaged by take up of a large number of nanoparticles are phagocytized by other Kupffer cells, resulting in a high concentration of nanoparticles in a fewer number of cells over time. (Sadauskas et al., 2009) It is believed that the nanoparticles remain in the liver for the life span of the mouse.
Particle Properties that Affect Cellular Up Take

The fate of nanoparticles after intravenous injection is partially controlled by their size, surface charge and surface chemistry. (De Jong et al., 2008; Hillyer et al., 2001; Sadauskas et al., 2009; Sadauskas et al., 2007; Sonavane et al., 2008) Nanoparticles that are less than or equal to 10 nm have been found in the blood, liver spleen, kidney, testis, heart lungs and brain of mice and rats after in vivo testing. (De Jong et al., 2008; Hillyer et al., 2001) Nanoparticles that are larger than 10 nm in diameter are predominantly found in the blood and liver, with lesser amounts in the spleen. (De Jong et al., 2008; Sadauskas et al., 2009) Adsorption of plasma proteins, called opsonins, on the surface of nanoparticles, can increase the rate of particle uptake by receptor mediated endocytosis or through reduction of the coulombic repulsion between the particles and negatively charged cell membranes. (Dobrovolskaia et al., 2009b) Surface coatings, such as polyethylene glycol, are used to camouflage the nanoparticles by changing the surface properties, which keeps them in circulation for longer times. (Hillyer et al., 2001; Sheng et al., 2009) Surface coatings on nanoparticles are used to reduce cellular up take by limiting the hydrophobic and electrostatic interactions which enable protein adsorption and particle agglomeration. (Aggarwal et al., 2009; Dobrovolskaia et al., 2009b; Gref et al., 2000; Sheng et al., 2009)

A hydrophilic or neutral polymer is attached to the surfaces of hydrophobic nanoparticles to increase blood circulation times. In addition to hydrophilicity, polymers with flexible chains that constantly change conformations, such as PEG or PVP, are required to reduce plasma protein adsorption. (Gref et al, 2000) Plasma protein adsorption is also dependent on the molecular weight or chain length of the polymer and the density of the coating. A minimum of 2-5% 5000 molecular weight PEG is
necessary to mask the surface recognition of nanoparticles by plasma proteins. (Jeon et al. 1991) The effect of surface coating on coagulation, circulation time and accumulation of nanoparticles in the liver is not known.

**Nanotoxicity Testing**

Risk models for the toxicity of nanoparticles are developed based on in vivo and in vitro testing. In vitro models are based on tests using cultured cells from different organs and cells line to determine toxicity. These tests typically have to be validated with in vivo testing because the in vitro testing does not accurately replicate the in vivo environment. Reliable and reproducible in vitro screening tests are done more often because they are less expensive, take place in a controlled laboratory environment and takes less time then in vivo testing, but they need to be validated by in vivo testing. (Dobrovolskaia, et al., 2009a; Fischer et al., 2007)

**The Advantages of Nanogold for In Vivo Testing**

Gold nanoparticles are used in many studies because they are commercially available, inert, and have good contrast for imaging. (Daniel et al., 2004; Hanfield et al., 2004; Hanfield et al., 2006; Murphy et al., 2008) There is also no background gold content in the body and they are not cytotoxic. (Connor et al., 2005; De Jong et al., 2008) The concentration of gold in the tissue and blood samples is measured by weight and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). (Sadauskas et al., 2009) The distribution of the nanoparticles in the liver has been studied using optical microscopy and Transmission Electron Microscopy (TEM). (De Jong et al., 2008; Dobrovolskaia et al., 2009b; Hanfield et al., 2006; Murphy et al., 2008) A silver enhancement technique was used to enlarge gold nanoparticles for detection in the optical microscope. (Sadauskas et al., 2009; Sadauskas et al., 2007) The silver
enhancement works by the attachment of silver ions to the gold surface by reduction to metallic silver, resulting in enlarged silver coated gold nanoparticles. The distribution of gold nanoparticles in tissue can be evaluated using optical microscopy because the enlargement of the nanoparticles.

TEM is routinely used to image the ultrastructure of the liver. It is also used to resolve single nanoparticles, their size, shape and exact location in the cells. (Widmann et al., 1972; Wisse et al., 1974a) Metallic or high atomic number nanoparticles are electron dense and appear dark in brightfield TEM images. Even though most biological samples are composed of light elements it does not mean that all electron dense features are nanoparticles. Biological samples are usually fixed and or stained with heavy metals which can cross link the tissue and impart contrast to the ultrastructural features of the cells and tissue in the TEM. Therefore not all of the dark features and particles in the cells are nanoparticles. Precipitation of heavy metal salts from the stains can result in dense particulate artifacts in the tissue sections. The chemical composition of the particles can be verified using EDS analysis, it can be used to distinguish between artifacts, stained tissue and nanoparticles in cells and tissues.

**Description of Work**

This study examined the differences in coagulation, circulation time and distribution of native, or uncoated, 40 nm gold particles and 40 nm gold nanoparticles with 5000 MW Polyethylene Glycol (PEG) covalently bound to the surface, also called PEGylated gold, in the liver of mice as a function of time after intravenous injection. The native and PEGylated gold nanoparticles are characterized and the nanoparticle uptake and distribution analyzed as a function of circulation time.
Characterization of the Gold Nanoparticles

TEM

TEM was used for determination of crystallinity, crystal structure, surface roughness and composition of nanoparticles. Polymer coatings on nanoparticles are difficult to characterize using brightfield TEM imaging because the polymers have low molecular weights which result in limited scattering of the electron beam and hence minimum contrast in the images. Heavy metal staining procedures were used to image the polymer coating in the TEM. Negative staining consists of using heavy metal salts that dry from aqueous solution as an amorphous background material on the TEM grid. (Bozzola et al., 1999; Harris, 1991; Sawyer et al., 2008) The nanoparticles are surrounded and embedded in the stain, but the stain does not attach to the polymer. The polymer appears light on a dark background. This technique is used for imaging the size, shape and surface of structure of particulate matter in biological and polymer sciences. (Sawyer et al., 2008) Negative staining has also been used to image the protein coronas on gold particles after incubation in human plasma. (Dobrovolskaia et al., 2009b; López-Viola et al., 2009)

CPS

A model DC24000 CPS Disc Centrifuge was used to determine the particle size distribution of the gold nanoparticles and the coating thickness was determined from the difference in particle size between the PEGylated gold and native gold nanoparticles. A small sample of the gold nanoparticles was dispersed in DI water and ultrasonicated. Standard gold nanoparticles, 95 nm in diameter, were used to calibrate the disc centrifuge.
Characterization of the Gold Nanoparticles in the Mouse Tissue

The microscopy techniques used to characterize the uptake of the gold nanoparticles in the mouse tissue were transmitted light and darkfield reflected light microscopy, Scanning Electron Microscopy (SEM), Ion Abrasion Scanning Electron Microscopy (IASEM) or Focused Ion Beam-SEM (FIB-SEM), Transmission Electron Microscopy (TEM) and Scanning Transmission Electron Microscopy (STEM) with Energy Dispersive X-Ray Analysis (EDS). Light microscopy was used to view large areas of the liver tissue and the distribution of gold nanoparticles in the liver tissue. IASEM or FIB-SEM was used for a three dimensional view of the gold distribution in a small volume of an individual cell. TEM was used to locate the nanoparticles in the cells in liver and STEM-EDS were used for uniquely identifying them by chemical composition.

Optical Microscopy

Bright field transmitted light images are formed by light rays passing through a sample. Differences in refractive index and opacity of a sample alter the speed and path of the light rays passing through it. The interference of these light rays produces contrast in a brightfield image. Most biological specimens have very little inherent contrast. The theoretical resolution of transmitted light microscopy is defined as the smallest distance between two points that can be seen. It is a function of the wavelength of the light source and the numerical aperture of the objective lens. It is calculated by: Resolution = $0.61\lambda/N.A$. $\lambda$ is the wave length of the light used and N.A. is the numerical aperture of the objective lens. The resolution limit of optical microscopy is controlled by the wavelength of the light source and is on the order of 200 to 400 nm.
Agglomerated nanoparticles in the tissue were imaged using brightfield transmitted light microscopy at 1000x magnification.

Darkfield reflected light microscopy proved to be a very useful tool for imaging the gold nanoparticles in liver tissue. The shiney gold nanoparticles have a high albedo coefficient making them easy to see in darkfield with resolutions on the order of 100 nm. They appear orange and yellow in the images due to localized surface plasmon resonance which is an increase in optical scattering at a resonant frequency when the oscillation of electrons is confined by the nanoscale dimensions of the particle. (Wax et al., 2009; Murphy et al., 2008) The nanoparticles were observed in high contrast, darkfield images at magnifications of 100x and 200x. The size, number of agglomerates and area fraction of the gold nanoparticles were determined using ImageJ software to analyze the darkfield images.

**Ion Abraison SEM (IASEM)**

The IASEM was used to characterize the three dimensional distribution of gold nanoparticles in a small volume of a Kupffer cell. A series of secondary electron images were taken of the mouse liver after ion abrasion of approximately 40 nm thick layers of tissue. The series of images were linked together into a movie to present the three dimensional view of the nanoparticle distribution in a Kupffer cell.

**TEM/STEM/EDS**

TEM and STEM-EDS analysis were used to image the ultrastructure of the liver tissue and elemental analysis of individual nanoparticles. The TEM and STEM samples were post fixed with 2% aqueous Osmic Acid. The post fixation treatment cross links the cellular structure and adds contrast to the otherwise low contrast biological specimens. Additional staining of biological TEM samples is routinely done to impart contrast on
otherwise low contrast specimens. The additional staining techniques were not used for the liver samples so the gold nanoparticles would be easier to find in the tissue.

The STEM/EDS techniques were used to uniquely identify the gold nanoparticles in the tissue samples. In a STEM, the electron probe is scanned over the sample, similar to SEM, but the electrons are collected after being transmitted through the sample. Samples must be electron transparent, with thickness on the order of 100 nm. The advantage of STEM/EDS technique is that the resolution is on order of the size of the electron beam, a probe size of 2-3 nanometers was used for the analysis. The High Angle Annular Darkfield STEM detector was used for high resolution atomic number (Z) contrast imaging. The detector collects the electrons that have been scattered at high angles as they go through the sample. High atomic number elements scatter more electrons at high angles, producing an image where the higher Z materials show up brighter in the images. EDS is used to determine the elemental composition of individual nanoparticles.

**Materials and Methods**

**Gold Nanoparticle Synthesis**

The gold particles used in this study were synthesized by Paul Carpinone at PERC. They were synthesized by reducing HAuCl₄ with Tribasic Sodium Citrate at 100°C. The molar concentration of gold was 50 mg/liter and the volume of colloids was 400 ml.

**Gold Nanoparticle Preparation for TEM**

Samples were prepared for TEM by dropping 5 µl of particles in suspension on to a carbon coated TEM grid. The samples were dried overnight. Negative staining was carried out by dropping the 5 µl of particle suspension on the TEM grid and drying for 1
minute. The remaining sample was blotted off the grid with filter paper and placed sample side down on top of a drop of 2% aqueous solution of Uranyl Acetate (UA) for 1 minute. The excess stain was blotted from the grid. The grids were dried in air for approximately 30 minutes followed by examination in the TEM.

**In Vivo Tests**

Native gold and PEG coated gold nanoparticles were administered to ICR mice via tail vein injection at a dose of 1 mg per mouse in 150 μl of DI water. Blood, liver, and spleen samples were removed and prepared for analysis at intervals between 5 minutes to 48 hours after dosing. Particle size analysis was performed in real time with blood lysed, or broken down, prior to the analysis. The test matrix is presented in Table 1. Three experiments were run for each test condition.

**Gold Content by Weight and ICP-MS**

The weight of the liver, the spleen and the blood were measured after each test. The average weight of gold in the liver, spleen and blood was calculated based on the average value from the control mice. The average percent of recovered gold from the liver, spleen and blood at each exposure time is presented in Table 1.

**Sample Fixing after Dissection for Microscopy**

The liver and spleen samples were stored in formalin after dissection for up to 8 hours until the testing was completed at the end of each day. The tissue samples were then placed in 4% paraformaldehyde and 2% glutaraldehyde in sodium cacodylate buffer fixative, with a pH 7.24, and stored at 4 C for up to 4 days. The liver and spleen samples were sectioned into small pieces, a few millimeters in cross section, for TEM. Small pieces of tissue were used because fixative would not penetrate into the larger
pieces of tissue. The tissue samples were fixed in Trump’s solution and washed in sodium cacodylate buffer.

**Histology Sample Preparation**

The liver tissue was prepared for histological examination using the paraffin technique at the Histology Resource laboratory at the McKnight Brain Institute. The histology sections were prepared in 5 steps: dehydration, clearing, infiltrating, embedding and sectioning. The tissue was fixed and stored in Sodium Cacodylate buffer at 4 C. The dehydration of the tissue samples was done in graded water/ethanol solutions (70%/30%, 50%/50%, 30%/70%, 10%/90%, and two times 100% ethanol). The tissue was cleared by multiple soakings in xylene. The samples were vacuum infiltrated with paraffin four times after clearing. The embedded tissue was sectioned with a microtome into 10 micron slices and mounted on glass slides. The sections were stained with Haematoxylin and Eosin (H&E). The Haematoxylin is a basic stain that binds to acidic components of the cell such as the nucleic acids or nucleus of the cell and endoplasmic reticulum. The Eosin is an acidic stain which binds to basic components of the cell and extracellular matrix. Cover slips were glued in place over the sections.

**Sample Preparation for TEM**

The samples were prepared for TEM using a Microwave preparation procedure. A PELCO Biowave Microwave Processor was used for the processing. The dissected tissue was fixed in Trumps Solution for 45 seconds in the microwave at 180 watts. This step was done to ensure fixation through the thickness of samples. The tissue was rinsed two times with cacodylate buffer for 45 seconds in the microwave at 180 watts. The tissue samples were post fixed in 2% osmic acid in 0.1 M phosphate buffer for one.
minute at room temperature and 45 seconds in the microwave under vacuum, followed by a 3 minute stand at room temperature. The tissue samples were rinsed two times with phosphate buffer for 45 seconds in the microwave at 180 watts. Sample dehydration was done in graded water/ethanol solutions (70%/30%, 50%/50%, 30%/70%, 10%/90%, and two times 100% ethanol). Each step was 45 seconds in the microwave at 180 watts followed by 1 min stand at room temperature. The samples were vacuum infiltrated in graded ethanol/Spurrs resin mixtures (70%/30%, 50%/50%, 30%/70%, and 100%) in the microwave at 250 watts for 3 minutes. A second infiltration step with 100% Spurrs resin for 48 hours at room temperature. The samples were put in silicone molds and filled with Spurrs resin. The molds were cured at 68 F for 48 hours.

After curing, the embedded tissue was cut from the blocks with a jewelers saw. The pieces of embedded tissue were glued to cylindrical plugs. The blocks were trimmed with a razor blade and sectioned using the Leica Ultra Cut S Ultramicrotome. Thick sections, 500 nm thick, were cut and mounted on glass slides to check the samples prior to sectioning the thin sections for TEM. The thick sections were stained with toluidine blue and examined using a stereo microscope. Thin sections, 70-100 nm, were cut for TEM.

**Optical Microscopy**

The histology sections were examined with an Olympus BH-2 Microscope with Pixera 120C Digital Camera for brightfield transmitted light microscopy. The images were taken at 200x and 1000x. Transmitted light and darkfield reflected light images were taken with Olympus BX60 with a SPOT Insight Digital Camera. Photoshop was used to superimpose the darkfield images of the gold nanoparticles on to a brightfield transmitted light image of the same area to show the location of the nanoparticles in the
cellular structure of the liver. Darkfield images were taken at 200x and 500x. The superimposed brightfield transmitted light/ darkfield reflected light images were taken at 500x.

**IASEM or FIB-SEM**

IASEM was used to visualize the distribution of gold nanoparticles in a Kupffer cell in three dimensions. A FEI Quant DB 235 Dual Beam FIB was used for the IASEM experiments. The images were taken with the through the lens secondary detector at 5 kV and the sample was ion abraded with a 30 kV gallium ion beam at 50 pA. This technique is called serial sectioning, it combines iterative removal of material from a bulk specimen using focused ion beam milling with SEM imaging of each section. (Heymann et al. 2006, 63-73) Thirty nanometer thick layers were removed from the bulk specimen. A high resolution SEM image was taken of each newly abraded surface. Sixty images were collected from a 2 micron thick region. The total area removed was approximately 2 microns by 2 microns by 2 microns. The image stack was manipulated and made into a movie sequence using AMIRA software.

**TEM**

The thin sections of the liver tissue were examined using a JEOL 200cx TEM at 120 kV and a JEOL JEM 2010F STEM at 200kV. The JEOL 200cx was used for brightfield TEM imaging. The JEOL 2010F was used for STEM imaging and EDS analysis. The STEM is equipped with JEOL brightfield and HAADF STEM detectors. The HAADF images are also called atomic number contrast images because of the sensitivity of this technique to differences in atomic number in the sample. The STEM is also equipped with an Oxford INCA Pentafet EDS detector for elemental analysis.
Results

Gold Nanoparticles

The physical properties of the nanoparticles were characterized by TEM and centrifugal particle sizing (CPS). TEM images of the native gold nanoparticles and PEGylated gold nanoparticles are presented in Figures 4-2A and B respectively. These low magnification TEM images provide an indication of the dispersion of the native and PEGylated gold. The native gold nanoparticles contained many agglomerates, some as large as 500 nm. Some native gold nanoparticles were also dispersed on the TEM grid, as shown in Figure 4-2A. The well dispersed, PEGylated gold nanoparticles are presented in Figure 4-2B. There were no agglomerates observed in the PEG coated gold sample.

Higher magnification TEM images of the native gold and PEGylated gold nanoparticles are presented Figures 4-3A and B. The shape of the particles ranged from spherical to oval with the average length of the longest dimension was 40 nm. Of particular interest in these images is the surface of the gold nanoparticles. A very light halo is present around the native gold nanoparticles in Figure 4-3A. The halo is approximately 5 nm thick and surrounds the three nanoparticles. This halo is often observed in TEM of gold nanoparticles, it is thought to be a drying ring from excess citrate from the colloidal solution. The PEG coated particles are presented in Figure 4-3B. There is a very thin, approximately 2 nm, layer around the PEG coated particles. The PEG coating does not produce very much contrast in the brightfield TEM images and it not uniform around the circumference of the particles. The PEG coated gold nanoparticles were imaged using HAADF STEM detector, but contamination built up.
very quickly on the specimen grids, darkening the background of the TEM grid and concealing the PEG coating.

Brightfield TEM images of negatively stained native and PEGylated gold nanoparticles are presented in Figures 4-4A and B. There were no negatively stained features in the image of the native gold nanoparticles. The PEGylated nanoparticles are surrounded by a white halo, the negatively stained PEG coating, which is surrounded by the dark background. The thickness of the PEG coating is not uniform around the circumference of the particles. It ranges from less than 1 nm to 4 nm. The contrast of the coating also varies around the circumference of the particles indicating that there are local differences in the coating thickness.

High resolution lattice image of a gold nanoparticle is presented in Figure 4-5. The particles appear spherical at low magnifications, but they are actually faceted. This five fold twin structure is typical in gold nanoparticles. Two faces with a [011] orientation and a lattice spacing of 0.235 nm can be seen in Figure 4-5.

The particle size distribution of the native and PEGylated gold nanoparticles in DI water was measured using the CPS Disc Centrifuge. The average diameter based on mass was 40 nm for the native gold and 42 nm for PEGylated gold. The mass basis particle size distribution for the native gold is presented in Figure 4-6. The particle size of the PEGylated gold was approximately 2 nm larger than that of the native gold due to PEG surface coating. The particles are very uniform in size, as indicated by the narrow particle size distribution.

**Gold Content by Weight**

The average percentage of the gold recovered from the liver, spleen and blood is presented in Table 1. This work was done by Paul Carpinone. There was a high
concentration of native gold found in the livers after all of the tests. The native gold particles were taken up into the liver almost immediately after injection, as evidenced by the high percentage of gold found in the liver after the 5 minute exposures. The native gold content in the liver remained high after all of the exposure times with a slight decrease shown in the 6 hour and 48 hour tests. The percentage of recovered native and PEGylated gold nanoparticles in the liver as a function of time is presented in Figure 4-7. The native gold content in the spleen was fairly low after all of the exposures. There was a slight increase in native gold content in the spleen after the 6 hour and 48 hour tests, which corresponds to the decrease in native gold found in livers for those exposure times. The native gold content in the blood was very low for all of the tests.

The concentration of PEGylated gold nanoparticles in the liver increased as a function of exposure time, as shown in Figure 3. The PEGylated gold concentration in the livers was fairly low for the exposure times from 5 minutes to 4 hours. The amount of gold recovered in the liver increased to moderate levels after 8 hours and further increased after the 24 and 48 hour exposures. The PEGylated gold content in the spleen was fairly low after all of the tests. The spleen contained very low amounts of the PEGylated gold for the exposure times from 5 minutes to 4 hours. There was a slight increase in the PEGylated gold content in the spleen for the exposures between 8 hours and 48 hours.

The PEGylated gold content in the blood was very high through the 4 hour exposures. It dropped off dramatically after 24 and 48 hours exposures. This can be
explained by the take up of the gold nanoparticles by the liver after the long exposure times.

**Histology Sections**

Histology sections were prepared from six liver samples, three exposure times for the native gold nanoparticles and PEGylated gold nanoparticles. The samples, exposure times, and percentage of gold recovered from a piece of each liver are presented in Table 2. Histology sections were made from the livers exposed to native gold nanoparticles for 5 minutes, 6 hours and 24 hours. All of the liver sections with native gold nanoparticles contained relatively high concentrations of gold. Histology sections were made from the livers exposed to PEGylated gold nanoparticles for 2 hours, 24 hours and 48 hours. The concentration of gold in the liver exposed to PEGylated particles for 2 hours was low and the livers exposed to PEGylated gold for 24 hours had very high gold concentrations.

**Brightfield Transmitted Light Microscopy**

The histology sections were examined with an Olympus BH-2 Microscope with Pixera 120C Digital Camera. Brightfield images of a liver exposed to native gold nanoparticles for 5 minutes are presented in Figures 4-8A and B respectively. The majority of the cells in the liver are hepatocytes. They are the larger cells with the round, purple nuclei. They are uniform in shape and size. The nuclei of the hepatocytes are bluish purple and the proteins surrounding the nuclei were dyed pink in the images from the H&E stain. The Kupffer cells and endothelial cells are the smaller cells that line the sinusoid and capillaries. The Kupffer cells are smaller than the hepatocytes, they have smaller round or irregularly shaped nuclei. This sample contained many nanoparticles, although they are not seen in the low magnification image in Figure 4-8A. The gold
nanoparticles were observed in the higher magnification image in Figure 4-8B as black dots in the elongated Kupffer cells. Nanoparticles were not observed in the hepatocytes. Many of the gold nanoparticles in the 5 minute sample are clustered around the red blood cells. The particles around the red blood cells are presented in Figure 4-8B. They are also present inside the Kupffer cells.

Brightfield images of a liver exposed the native gold nanoparticles for 24 hours are presented in Figure 4-9A and B. The gold nanoparticles are not visible in the low magnification image in Figure 4-9A. The gold nanoparticles in the 24 hour sample are clustered in the elongated Kupffer cells. The clusters of nanoparticles are presented in Figure 4-9B. The 24 hour sample has larger clusters of gold nanoparticles then the 15 minute sample. The sample exposed for 6 hours was also examined. The distribution of gold nanoparticles in the 6 hour sample was similar to that in the 24 hour sample with many gold nanoparticles in the Kupffer cells.

Brightfield images of a liver exposed to PEGylated gold nanoparticles for 2 hours are presented in Figures 4-10A and B. The sample exposed to PEGylated nanoparticles for 2 hours did not contain any elongated grey features or gold nanoparticles. The higher magnification image of 2 hour sample is presented in Figure 4-10B. There are no gold nanoparticles in the 2 hour sample. The concentration of gold nanoparticles was very high in the 24 hour sample.

Brightfield images of the liver exposed to PEGylated gold nanoparticles for 24 hours are presented in Figures 4-11A and B. There were many elongated Kupffer cells in the 24 hour sample. They are presented in the higher magnification image in Figure 4-11B. The gold nanoparticles were observed as black dots in the elongated cells in the
24 hour sample. These elongated Kupffer cells and dark particles were not observed in the 2 hour sample with the lower gold content, but were observed throughout the 24 and 48 hour samples. Gold nanoparticles were also observed near the blood cells in the 24 hour and 48 hour samples. Gold nanoparticles were not observed in the hepatocytes.

**Darkfield Reflected Light Microscopy**

Darkfield images of the native and PEGylated gold nanoparticles in DI water are presented in Figures 4-12A and B respectively. The gold nanoparticles appeared very bright against the black background in the images. The bright spots in Figure 4-12A which contained the native gold particles were larger than those in Figure 4-12B which contained the PEGylated gold particles. The native gold nanoparticles tend to agglomerate, which would effectively increase the observed particle size. There are fewer agglomerates in the PEGylated sample because PEG helps to keep the nanoparticles dispersed resulting in fewer agglomerates.

Darkfield reflected light microscopy was used to image the liver histology sections of the mice exposed to native gold nanoparticles and PEGylated gold nanoparticles. The gold nanoparticles appeared bright orange and yellow against the dark background in the darkfield images. The resolution of the darkfield technique is approximately 100 nm which would enable imaging groups of 2 or more nanoparticles with the darkfield technique. The relative amount and area distribution of nanoparticles was obtained from the darkfield images are presented in Table 4-3. The distribution of the nanoparticles within cellular structure of the liver was obtained by superimposing the darkfield images of the nanoparticles on the brightfield images of the tissue.

The darkfield reflected light images of the liver exposed to native gold nanoparticles for 5 minutes and 24 hours are presented in Figures 4-13A and B
respectively. The 5 minute sample contained numerous small pockets native gold nanoparticles distributed throughout the section. The 24 hour sample many large and small clusters of native gold nanoparticles. The concentration of native gold nanoparticles in the 24 hour sample appeared higher than that in the 5 minute sample.

The darkfield images of the histology sections of the livers exposed to PEGylated gold nanoparticles for 2 hours and 24 hours are presented in Figures 4-14A and B respectively. The 2 hour sample had a very low concentration of gold nanoparticles. There are only a few small clusters of PEGylated gold nanoparticles in the image of the 2 hour sample presented in Figure 4-14A. An image of the sample exposed the PEGylated gold nanoparticles for 24 hours is presented in Figure 4-14B. There was a high concentration of gold nanoparticles distributed throughout the 24 hour sample. The concentration of gold nanoparticles in the sample exposed for 2 hours was much lower than sample exposed for the 24, as shown by the low number of gold clusters present in Figure 4-14A, as compared to Figure 4-14B.

The darkfield images of the native gold particles were superimposed over the brightfield image to show the nanoparticle distribution in the cellular structure of the liver. The overlaid darkfield/brightfield images of the native gold nanoparticles onto the cellular structure of the livers exposed to native gold nanoparticles for 5 minutes and 24 hours are presented in Figures 4-15A and B respectively. The gold nanoparticles are colored black in the images to highlight the location of the particles in the cells. The gold content of the liver exposed to native gold particles for 24 hours was much higher than that of the liver exposed to native gold nanoparticles for 5 minutes. The greater number
and size of the agglomerated gold nanoparticles for the liver exposed to native gold nanoparticles for 24 hours is presented in Figure 4-15B.

The darkfield images of the gold nanoparticles superimposed on to the brightfield images of the livers exposed to PEGylated gold nanoparticles for 2 hours and 24 hours are presented in Figures 4-16A and B. The 2 hour sample contained very few nanoparticles, as evidenced by the few black areas in the image. The 24 hour PEG coated gold sample contained many nanoparticles, similar to the native gold 24 hour sample.

Information of the distribution of gold nanoparticles in the liver was determined using ImageJ software and the darkfield images. The average number of gold nanoparticle agglomerates, average area of agglomerated gold nanoparticles, the average gold agglomerate size, and area fraction of gold agglomerates per for 10 images for each sample were calculated using ImageJ software. The darkfield images were taken of the histology sections which included the livers exposed to native gold nanoparticles for 5 minutes (5N), 1 hour (6N) and 24 hours (24N) and those exposed to the PEGylated gold nanoparticles for 2 hours (2P), 24 hours (24P) an 48 hours (48P). The results are presented in Table 4-3. The area fraction calculations are based on the area of the 500x images, which was 320 microns x 312 microns, or 99840 square microns. The measurements were made on 10 images of each sample, encompassing a total area of approximately 1 mm². The 5N sample contained the largest number of agglomerates, but they were smaller in size than those found in most of the other samples. The average size of the gold agglomerates for the 6N and 24N samples were the same, 1.2 microns, but there were more gold agglomerates in the 24 hour sample.
The lowest amount and smallest gold agglomerates were found in the 2P sample. The area fraction of gold nanoparticles in the 2P samples was less than 0.1%. The 24P had a similar gold content to the 24N sample and the 48P had a lower area fraction of gold agglomerates.

**IASEM**

The 24 hour sample exposed to PEGylated gold nanoparticles was examined using the FEI DB 235 Strata Dual Beam Focussed Ion Beam (FIB) for serial sectioning. Forty nanometer thick sections were removed from the sample with the ion beam and a high resolution secondary electron image of the exposed face of the sample was recorded. A total of 60 slices were recorded over a 2 micron thickness. The stack of images was put together into a movie using AMIRA software. The movie is presented in Movie 1. The distribution of gold nanoparticles through a 2 micron by 2 micron by 2 micron volume of a cell is presented in the movie. This sample contained many clusters of gold nanoparticles that appeared to encaspsulated in circular features such as endosomes and lysosomes. The gold nanoparticles are located around the inner edge and inside numerous endosomes and lysosomes inside the Kupffer cells.

**TEM/STEM/EDS Analysis**

The livers exposed to native gold and PEGylated gold nanoparticles for 15 minutes and 24 hours were examined using TEM. The sample exposure times and the percentage of recovered gold are listed in Table 4-4. The gold nanoparticles were located in the cells using HAADF imaging and EDS analysis.

The thin sections from the livers exposed to native gold nanoparticles for 15 minutes and 24 minutes had high concentrations of the gold nanoparticles. The particles were present as single particles, small groups of 2 to 4 particles and large, tightly
packed agglomerates. Large agglomerates of the native gold nanoparticles in the liver exposed for 15 minutes are presented in Figures 4-17A and B. There were many large agglomerates of the gold nanoparticles in the 15 minute sample. An agglomerate of gold nanoparticles is shown in Figure 4-17A. The nanoparticles have a characteristic oval shape with the longest dimension approximately 40 nm which can be clearly seen in the figure. The particles in the agglomerate in Figure 4-17B are tightly packed, so the size and shape of the individual particles cannot be seen.

An HAADF STEM image of a single gold nanoparticle is presented in Figure 4-18A. The gold nanoparticles are very dense and scatter a large number of electrons. As a result of the electron scattering, the nanoparticles appear black in the brightfield images and white in the HAADF STEM images. A typical EDS spectrum from the gold particles is presented in Figure 4-18B. EDS analysis confirmed that the bright white particles in the HAADF images were gold nanoparticles. The other elements present in the spectrum are carbon, and copper. Carbon can be from the carbon film on the TEM grid and the tissue and the copper is present due to scattering from the TEM grid. There are some bright white areas in the HAADF STEM images of the samples that are not gold nanoparticles. The bright white artifacts in the 15 minute sample are presented in Figure 4-19A. The corresponding EDS spectra are presented in Figures 4-19B and C, and are a result of the osmium post fixation treatment given to the tissue during TEM preparation. The shape and size of the bright white artifact features are not consistent with oval shape of the 40 nm gold nanoparticles. A typical EDS spectrum from Osmicated tissue is presented in Figure 4-19C. It contains carbon, oxygen, osmium and
copper. The carbon, oxygen and osmium are from the tissue and the carbon and copper are from the grid.

TEM images of agglomerates of gold nanoparticles in the 24 hour sample are presented in Figures 4-20A and B. The gold nanoparticles were present in small clusters of one or more particles and large, tightly packed clusters. A HAADF STEM image of agglomerated gold nanoparticles is presented in Figure 4-21A. The characteristic size and shape of the gold nanoparticles is shown in the HAADF STEM along with the bright white atomic number contrast. The EDS spectrum from the gold nanoparticles in Figure 4-21A is presented in Figure 4-21B. The elemental analysis from the gold nanoparticles contained carbon, oxygen, gold and copper. An EDS spectrum from a dark area of the tissue is presented in Figure 4-21C, it contained carbon, oxygen and copper from the tissue, carbon film and copper grid.

The sample that was exposed to PEGylated nanoparticles for 15 minutes had a low concentration of gold. Large clusters of gold nanoparticles were not observed in this sample in the TEM. The gold nanoparticles were found in small clusters of 1 to 4 particles. Gold nanoparticles around the edge of a sinusoid in the liver are presented in Figures 4-22A and B. There is an erythrocyte or oxygenated white blood cell in the sinusoid. The erythrocyte is the dark feature in the center of Figure 4-22A. Two individual nanoparticles are shown on the edge of the erythrocyte in Figure 4-24B. Four gold nanoparticles in various locations on the edge of the sinusoid and between the sinusoid and endothelial cell, called the space of Disse, are presented in Figure 4-23. The particle labeled 1 is in the intercellular region. The other particles labeled 2, and 4 are located in the Space of Disse. Only a few gold nanoparticles were taken up into the
Kupffer cells in this sample. A small cluster of four gold nanoparticles in an endosome of a Kupffer cell in the sample exposed to PEGylated gold nanoparticles for 15 minutes is presented in Figure 4-24A. The EDS spectrum from the cluster of gold nanoparticles is presented in Figure 4-24B. The spectrum contains gold from the nanoparticles and copper and carbon from the grid and tissue.

Brightfield TEM images of the mouse liver exposed to PEGylated gold particles for 24 hours are presented in Figures 4-25A and B. There is an agglomerate of the gold nanoparticles in a Kupffer cell on the right side of Figure 4-25A. Some of the dark features throughout the rest of the image are osmicated tissue. An agglomerate of gold nanoparticles is presented in Figure 4-25B. The characteristic shape of the gold nanoparticles is evident in Figure 4-25B because the particles are not tightly packed agglomerates. The agglomerated nanoparticles seem to be confined in oval or circular features typical of the endosome/lysosome features.

Brightfield STEM image of the 24 hour sample is presented in Figure 4-26A. This area of the sample contains an agglomerate of gold particles. The gold nanoparticles are dark in the brightfield image. EDS was used to confirm the elemental composition of the particles. A typical EDS spectrum from a gold nanoparticle is presented in Figure 4-26B. The EDS spectrum from the dark features in these images contained gold from the nanoparticles and carbon and copper from the grid. The morphology of the dark features is also consistent with that of the 40 nm gold nanoparticles. There were a number of areas that contained clusters of the gold nanoparticles usually encased in an endosome/lysosome vesicle. EDS of some of the dark, irregularly shaped features
confirmed the fact that they did not contain gold and were most likely artifacts from sample preparation.

**Discussion**

The concentration of gold nanoparticles in the liver of the mice as a function of test time is summarized by the weight data presented in Table 4-1 and the image analysis data presented in Table 4-3. The native gold nanoparticles were taken up into the liver almost immediately after intravenous injection. The native gold concentration remains high throughout all of the exposure times, which ranged from 5 minutes to 48 hours. The cellular take up behavior of the PEGylated gold nanoparticles as a function of time was much different than that of the native gold nanoparticles. Take up of the PEGylated gold nanoparticles by the Kupffer cells was very low for exposure times of 5 minutes to 4 hours. The cellular up take of the PEGylated gold nanoparticles increased with exposure times of 8 to 48 hours. The gold content in the livers exposed to PEGylated gold nanoparticles for the 8 to 48 hours were similar to those the gold content in the livers exposed to the native gold nanoparticles.

Intravenously injected foreign particles are taken up by the Kupffer cells in the liver as part of the body’s immune response system. A key factor for phagocytic recognition by the macrophages is known to be the absorption of plasma proteins, or opsonins, on the particle surface. (Sheng *et al.*, 2009) Particle size also affects cellular up take, the larger or agglomerated particles are taken up faster than the smaller nanoparticles. The large particles are coated quickly by plasma proteins which trigger certain receptors on the surface of the Kupffer cells to initiate phagocytosis. The combined effect of plasma protein adsorption and size of the agglomerated native gold nanoparticles caused increased phagocytic activity by the Kupffer cells which resulted in the take up of a large
number of nanoparticles in a very short amount of time. (Aggarwal et al., 2009; Dobrovolskaia et al., 2009)

The low concentration of the PEGylated gold nanoparticles in the mouse livers after the short test exposures, 5 minutes to 4 hours, signifies that there was low phagocytic activity of the Kupffer cells. This is due to the nonadsorption of the plasma proteins which stimulate phagocytosis and the good dispersion of the PEGylated gold nanoparticles. PEG coatings are known to prevent the adsorption of plasma proteins, or opsonins, and keep the particles in circulation through alteration of the surface charge and improved surface hydrophobicity. (Sheng et al., 2009) The PEG coating prevented detection of the nanoparticles by the Kupffer cells for the test times up to 4 hours. After which, the particles were rapidly taken up by the macrophages. There was a high concentration of gold nanoparticles in samples exposed to PEGylated gold nanoparticles for 16 to 48 hours. The PEGylated gold nanoparticles behaved more like the native gold after long test times. The distribution of the PEGylated gold nanoparticles in the Kupffer cells after the longer test times was similar to that of the native gold. The similarities in particle distribution can be seen in the darkfield optical micrographs presented in Figures 4-12 and 4-13, the superimposed darkfield/ brightfield images in Figures 4-15 and 4-16 and the image analysis data in Table 4-3. It is apparent that the effectiveness of the PEG on the surface of the gold nanoparticles decreased after the 4 hours of circulation time. One theory is that the PEG coating deteriorates with time, the nanoparticles become agglomerated, coated with the plasma proteins, and then get taken up by the Kupffer cells.
Correlative microscopy was used to examine the distribution of the gold nanoparticles in the livers and uniquely identify the gold nanoparticles in the tissue. The overall distribution of native gold and PEGylated gold nanoparticles in the liver was shown in the brightfield and darkfield optical images of the histology sections. The relative amount of gold nanoparticles in the liver sections was evaluated using image analysis on the darkfield images. The overall distribution of gold nanoparticles was best represented in the superimposed darkfield/brightfield images in Figures 4-15 and 4-16 and data presented in Table 4-3. Agglomerates of gold nanoparticles, approximately 100 nm in diameter or the equivalent of 2 nanoparticles, were resolved in the low magnification darkfield images. The enhanced contrast of the gold nanoparticles in the darkfield imaging make it a very useful technique for determining the overall particle distribution in a 10 micron thick section of the tissue. These images confirmed the presence of a large amount of gold nanoparticles in the 5 minute and 24 hour native gold samples and 24 hour PEGylated gold sample. The distribution of gold nanoparticles in all of the native gold samples and the long time test exposures for the PEGylated gold particles looked very similar.

The IASEM technique was used to determine the distribution of nanoparticles in three dimensions in the 24 hour PEGylated sample. The volume distribution of gold nanoparticles is presented in the movie. The structural information cannot be obtained from these images. The particles appeared to be contained in oval shaped features consistent with shape of the lysosomes. There were many clusters of particles located in the 2 micron x 2 micron x 2 micron volume of material most likely from one Kupffer cell. The 3 dimensional view of the nanoparticle distribution presents another way to
assess the amount of nanoparticles taken up by the Kupffer cells. The 3 dimensional view of the gold nanoparticle distribution shows that nanoparticles are present in more than one endosome or lysosomes within the volume of one cell. The distribution of the nanoparticles in the each slice is similar to that in the TEM sections, with addition of distribution in the depth of the sample.

The ultrastructure of the tissue and specific location of the nanoparticles in the cells and tissue were examined using TEM and EDS. The TEM has the resolution for imaging the ultrastructure of the liver and determining the composition of a single nanoparticle. The amount and distribution of gold nanoparticles in the 15 minute and 24 hour native gold and 24 hour PEGylated gold samples were similar. The nanoparticles were agglomerated in lysosomes in the Kupffer cells. The agglomerates varied in size. In many cases, the nanoparticles were very tightly packed in the agglomerates, especially in the native gold samples. The nanoparticles were present in numerous clusters in a single cell.

The tightly packed clusters of nanoparticles in the livers exposed to native gold particles may be due to the agglomeration of the nanoparticles prior to cellular up take. This would also explain the observation of single clusters of native gold nanoparticles in a given cell. The PEGylated gold nanoparticles remain dispersed for longer times and were taken up into the cells in smaller clusters at short time exposures. After long time exposures, the PEGylated nanoparticles behave more like the native gold nanoparticles due to the loss of PEG coating.

**Summary**

A combination of microscopy techniques are required to adequately characterize the distribution of the nanoparticles in the tissue, the location of nanoparticles in cells
and confirm the identity of the nanoparticles through chemical analysis. Low magnification, optical techniques are required for determining the particle distribution over larger areas of the tissue. They are also useful for determining the approximate locations of nanoparticles in the tissue for TEM analysis. TEM and EDS are required to determine the location of the nanoparticles in the ultrastructure of the cells and confirm the elemental composition of the nanoparticles. These TEM and EDS results were used to formulate a hypothesis on the effect of PEG coated nanoparticles on the circulation time and the mechanism of aggregation and uptake of the gold nanoparticles prior to removal by the liver.

Uncoated and polyethylene glycol (PEG) coated gold nanoparticles were intravenously injected into mice and allowed to circulate for 5 minutes to 48 hours. The PEG coating thickness was determined using high resolution TEM and negative staining techniques. The amount and distribution of gold in sections of the liver was determined using darkfield reflected light microscopy. Image analysis was used to determine the gold agglomerate size, number of agglomerates and area fraction of agglomerates in the sections. The three dimensional distribution of gold nanoparticles in a single cell was obtained using ion abrasion scanning electron microscopy. The uncoated gold nanoparticles were taken up almost immediately by the Kupffer cells of the liver. The native gold nanoparticles were observed in large, tightly packed agglomerates in lysosomes inside the cells. The PEG coated nanoparticles were rapidly taken up after 2 hours. Loosely packed clusters of PEG coated gold nanoparticles were observed lining the inner surface and inside of the lysosomes.
Figure 4-1. A) Diagram of a liver with an enlarged section of the showing the cells that surround the interlobular viens. B) Histology section of the liver showing a vien surrounded by endothelial cells, Kupffer cells and Kupffer cells further out.
Table 4-1. Test matrix of mice experiments

<table>
<thead>
<tr>
<th>Test Label</th>
<th>Exposure Time</th>
<th>Gold Surface Coating</th>
<th>Weight of Gold (μg) Average of 3 Tests</th>
<th>Percent of Gold Recovered Average of 3 Tests</th>
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<td>Liver</td>
<td>Spleen</td>
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<td>0</td>
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<td>0.1</td>
</tr>
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<td>38.1</td>
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<tr>
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<td>P</td>
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<tr>
<td>4</td>
<td>4 hours</td>
<td>N</td>
<td>601.3</td>
<td>40.1</td>
</tr>
<tr>
<td>4</td>
<td>4 hours</td>
<td>P</td>
<td>90.6</td>
<td>24.9</td>
</tr>
<tr>
<td>8</td>
<td>8 hours</td>
<td>N</td>
<td>826.9</td>
<td>52.9</td>
</tr>
<tr>
<td>8</td>
<td>8 hours</td>
<td>P</td>
<td>243.4</td>
<td>46.7</td>
</tr>
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<td>24</td>
<td>24 hours</td>
<td>N</td>
<td>913.0</td>
<td>45.8</td>
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<tr>
<td>24</td>
<td>24 hours</td>
<td>P</td>
<td>585.5</td>
<td>78.3</td>
</tr>
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<td>48</td>
<td>48 hours</td>
<td>N</td>
<td>215.0</td>
<td>42.9</td>
</tr>
<tr>
<td>48</td>
<td>48 hours</td>
<td>P</td>
<td>539.4</td>
<td>109.1</td>
</tr>
</tbody>
</table>
Figure 4-2. Brightfield TEM images of the native and PEG coated 40 nm gold particles. 
A) Native gold nanoparticles with agglomerates up to 500 nm in size. B) Well dispersed PEG coated gold nanoparticles.

Figure 4-3 Brightfield TEM images of native and PEG coated gold nanoparticles. A) 40 nm native gold nanoparticles B) 40 nm PEGylated gold nanoparticles with thin polymer coating.
Figure 4-4. Brightfield TEM images of negatively stained native and PEGylated 40 nm gold nanoparticles. A) Native gold nanoparticles B) PEGylated gold nanoparticles

Figure 4-5. High resolution TEM image of a gold nanoparticle. The measured lattice spacing was 0.235 nm which is the (111) spacing. The particles are facitited, with hexagonal symmetry, as evidenced by the six crystal faces, two of which are in focus with the same lattice spacing.
Figure 4-6. A plot of the particle size distribution by mass for the native gold nanoparticles measured by CPS. The average diameter based on the mass of the native Au particles was ~42 microns.

Figure 4-7. Plot of the percentage native and PEGylated gold nanoparticles found in the liver after different exposure times. (data collected by Paul Carpinone)
Table 4-2. Liver histology sections and the percentage of gold recovered from each lobe

<table>
<thead>
<tr>
<th>Sample</th>
<th>Exposure time</th>
<th>Gold Coating</th>
<th>Gold Percentage Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>5N2L</td>
<td>5 min</td>
<td>Native gold</td>
<td>88.5</td>
</tr>
<tr>
<td>6N3L</td>
<td>1 hr</td>
<td>Native gold</td>
<td>94.2</td>
</tr>
<tr>
<td>24N1L</td>
<td>24 hr</td>
<td>Native gold</td>
<td>91.4</td>
</tr>
<tr>
<td>2P1L</td>
<td>2 hr</td>
<td>PEG coated gold</td>
<td>11.5</td>
</tr>
<tr>
<td>24P1L</td>
<td>24 hr</td>
<td>PEG coated gold</td>
<td>96.1</td>
</tr>
<tr>
<td>48P3L</td>
<td>48 hr</td>
<td>PEG coated gold</td>
<td>83.4</td>
</tr>
</tbody>
</table>

Figure 4-8. Transmitted light images of a mouse liver that was exposed to native gold nanoparticles for 5 minutes. A) Low magnification image of the liver tissue. B) Higher magnification image of the liver tissue which gold nanoparticles (black dots) in the Kupffer cells lining the sinusoids.
Figure 4-9. Transmitted light images of a mouse liver that was exposed to native gold nanoparticles for 24 hours. A) Low magnification image of the liver tissue. B) Higher magnification image of the liver tissue which contains agglomerated gold nanoparticles (black dots) in the Kupffer cells lining the sinusoids.

Figure 4-10. Transmitted light images of a mouse liver that was exposed to PEGylated gold nanoparticles for 2 hours. A) Low magnification image of the liver tissue. B) Higher magnification image of the liver tissue which contains two agglomerates of gold nanoparticles (black dots) in the Kupffer cells lining the sinusoids.
Figure 4-11. Transmitted light images of a mouse liver that was exposed to PEGylated gold nanoparticles for 24 hours. A) Low magnification image of the liver tissue. B) Higher magnification image of the liver tissue which contains gold nanoparticles (black dots) in the Kupffer cells.

Figure 4-12. Darkfield Images of the Gold nanoparticles in distilled water. A) Native gold nanoparticles B) PEGylated gold nanoparticles
Figure 4-13. Darkfield images of a liver that was exposed to native gold nanoparticles for 5 minutes and 24 hours. The gold nanoparticles show up orange and yellow, depending upon agglomerate size, in the darkfield images. There are gold nanoparticles throughout sample. A) Section of liver exposed to native gold nanoparticles for 5 minutes contains a high concentration of gold nanoparticles. B) Section of liver exposed to native gold nanoparticles for 24 hours contains a high concentration of gold nanoparticles.

Figure 4-14. Darkfield images of a liver that was exposed to PEGylated gold nanoparticles for 2 hours and 24 hours. A) Section of liver exposed to PEGylated gold nanoparticles for 2 hours contains very few gold nanoparticles. B) Section of liver exposed to native gold nanoparticles for 24 hours contains a high concentration of gold nanoparticles.
Figure 4-15. Darkfield images of the gold nanoparticles superimposed on the transmitted light images of the livers that were exposed to native gold nanoparticles for 5 minutes and 24 hours. The gold nanoparticles were colored black in the images to highlight the particle distribution in the tissue. There are gold nanoparticles throughout both samples. A) The liver exposed to native gold nanoparticles for 5 minutes. B) The liver exposed to native gold nanoparticles for 24 hours.

Figure 4-16. Darkfield images of the gold nanoparticles superimposed on the transmitted light images of the livers that were exposed to PEGylated gold nanoparticles for 2 hours and 24 hours. The gold nanoparticles were colored black in the images. A) The liver exposed to PEGylated gold nanoparticles for 2 hours contains very few nanoparticles. B) The liver exposed to PEGylated gold nanoparticles for 24 hours contains many gold nanoparticles.
Table 4-3. Number, size and area fraction of agglomerated gold nanoparticles

<table>
<thead>
<tr>
<th>Number of Agglomerates</th>
<th>Average Agglomerate Size (microns)</th>
<th>Total Area of Agglomerates (sq microns)</th>
<th>Area Fraction of Agglomerates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Gold</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min (5N)</td>
<td>403</td>
<td>0.4</td>
<td>171</td>
</tr>
<tr>
<td>1 hour (6N)</td>
<td>304</td>
<td>1.2</td>
<td>353</td>
</tr>
<tr>
<td>24 hour (24N)</td>
<td>336</td>
<td>1.2</td>
<td>372</td>
</tr>
<tr>
<td>PEGylated Gold</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hour (2P)</td>
<td>184</td>
<td>0.1</td>
<td>15</td>
</tr>
<tr>
<td>24 hour (24P)</td>
<td>332</td>
<td>0.9</td>
<td>311</td>
</tr>
<tr>
<td>48 hour (48P)</td>
<td>259</td>
<td>0.7</td>
<td>186</td>
</tr>
</tbody>
</table>

Object 4-1. Serial section movie of the liver exposed to PEGylated gold nanoparticles for 24 hours (24P). The three dimensional distribution of nanoparticles in a 2 micron by 2 micron by 2 micron volume is presented in the movie.(.mpg 7MB)

Table 4-4. Samples analyzed by TEM

<table>
<thead>
<tr>
<th>Samples</th>
<th>Tissue</th>
<th>Exposure Times</th>
<th>Weight of Gold in Tissue(µg)</th>
<th>Percent Gold Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>004N</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15N1L</td>
<td>Liver</td>
<td>15 min</td>
<td>107.4</td>
<td>83.4</td>
</tr>
<tr>
<td>15P3L</td>
<td>Liver</td>
<td>15 min</td>
<td>61.4</td>
<td>6.2</td>
</tr>
<tr>
<td>24N1L</td>
<td>Liver</td>
<td>24 hours</td>
<td>671.3</td>
<td>91.4</td>
</tr>
<tr>
<td>24P2L</td>
<td>Liver</td>
<td>24 hours</td>
<td>819.4</td>
<td>86.1</td>
</tr>
<tr>
<td>5N1S</td>
<td>Spleen</td>
<td>5 min</td>
<td>52.8</td>
<td>6.9</td>
</tr>
<tr>
<td>5P1S</td>
<td>Spleen</td>
<td>5 min</td>
<td>6.2</td>
<td>1.3</td>
</tr>
<tr>
<td>24N2S</td>
<td>Spleen</td>
<td>24 hours</td>
<td>44.2</td>
<td>4.4</td>
</tr>
<tr>
<td>24P3S</td>
<td>Spleen</td>
<td>24 hours</td>
<td>105.5</td>
<td>16.6</td>
</tr>
</tbody>
</table>
Figure 4-17. Brightfield TEM images of the livers exposed to native gold nanoparticles for 15 minutes. Large clusters of nanoparticles were found throughout the sample. A) The gold nanoparticles are agglomerated in a cell. B) An agglomerate of gold nanoparticles is present in the cell. The particles are packed very tightly together in the agglomerate.

Figure 4-18. HAADF STEM image and corresponding EDS spectrum from the mouse liver exposed to native gold nanoparticles for 15 minutes. A) HAADF STEM image of a single gold nanoparticle. B) EDS spectrum from area x, which contained gold, carbon, and copper.
Figure 4-19. HAADF STEM images of the mouse liver exposed to native gold nanoparticles for 15 minutes and the corresponding EDS spectrum. A) HAADF STEM image of osmicated tissue labeled x. B) HAADF STEM image of osmicated tissue. C) Typical EDS spectrum from area of osmicated tissue labeled x. The spectrum contains carbon, oxygen, osmium and copper.
Figure 4-20. Brightfield TEM images of the livers exposed to native gold nanoparticles for 24 hours. These sections contained large tightly packed clusters of nanoparticles. A) Large clusters of gold nanoparticles in a Kupffer cell adjacent to a sinusoid. B) Large clusters of gold nanoparticles in the Kupffer cell.
Figure 4-21. HAADF STEM image of a cluster of gold nanoparticles in the mouse liver exposed to native gold nanoparticles for 24 hours and the corresponding EDS spectra. A) HAADF STEM image of a cluster of gold nanoparticles. The particles are 40 nm in diameter with a characteristic egg shape. B) Typical EDS spectrum from areas 1, 2 and 4 which contain the gold nanoparticles. The spectrum contains Carbon, Oxygen, Gold and Copper. C) EDS spectrum from area 3 which contains Carbon, Oxygen, Silicon and Copper.
Figure 4-22. Brightfield TEM images a portion of a liver exposed to PEGylated gold nanoparticles for 15 minutes. A) The dark feature in the center of the image is a red blood cell. B) Two gold nanoparticles on the edge of the red blood cell.

Figure 4-23. Brightfield TEM image of gold nanoparticles in the space of Disse adjacent to a red blood cell.
Figure 4-24. Brightfield TEM image of 40 nm gold nanoparticles in the endosome of a Kupffer cell in the liver exposed to PEGylated gold nanoparticles for 15 minutes and the corresponding EDS spectrum. A) 40 nm gold particles in an endosome. B) EDS spectrum from gold nanoparticle confirming the presence of the gold.

Figure 4-25. Brightfield TEM images of the PEGylated gold nanoparticles in Kupffer cell on the edge of a sinusoid that was exposed to PEGylated gold nanoparticles for 24 hours. A) Large clusters of gold nanoparticles are present in the Kupffer cells. B) Large clusters of PEGylated gold nanoparticles in the endosomes and lysosomes of a Kupffer cell.
Figure 4-26. Brightfield STEM image of the gold nanoparticles in the Kupffer cell of the mouse liver exposed to gold nanoparticles for 24 hours and the corresponding EDS spectrum. A) Brightfield STEM image of the gold nanoparticles. B) EDS spectrum from one of the gold nanoparticles. The spectrum contains carbon, gold and copper.
Nanoparticles are produced in large numbers for applications ranging from electronic materials to diagnostic imaging. Along with the increased manufacture and use of nanoparticles comes increased the risk of environmental exposures. The potential impacts of these materials on the environment are relatively unknown. It has been shown that nanomaterials have the potential to adversely affect aquatic life, including zooplankton such as daphnia. (Petersen et al., 2009; Guan et al., 2004) The Daphnia magna, or water flea, is a small freshwater crustacean that is used as a model organism for investigating environmental exposures. (Barata et al., 2002; Guan et al., 2004; Heugens et al., Lal et al., 1984; 2003; Poynton et al., 2007) They are considered an important part of the fresh water food chain. A picture of a Daphnia magna is presented in Figure 5-1. Daphnia are extremely sensitive to low metal concentrations and environmental pollutants. (Barata et al., 2002; Guan et al., 2004) They are filter feeders that sieve large quantities of water. They also stir up sediment if food is scarce in the water. Suspended nanoparticles can be ingested along with food in the water and sediment. Daphnia are commonly used for ecotoxicology testing because they are sensitive to the environmental pollution, easy to culture, and have short life spans. (Lal et al., 1984; Lovern et al., 2007; Lovern et al., 2008)

In many cases, daphnia are more susceptible to toxicity from nanomaterials than other forms of aquatic life that have been tested. (Lovern et al., 2007; Poynton et al., 2007) It is unclear if this susceptibility results from unique mechanisms of toxicity for nanomaterials that operate in daphnia or if it is due to pharmacodynamic differences that result from filter feeding employed by daphnids that may result in elevated
exposure to small particles. Prior studies have demonstrated that daphnia are capable of ingesting nanomaterials. (Jackson et al., 2009; Lovern et al., 2007; Petersen et al., 2009) The studies have also demonstrated that nanomaterials associated with daphnia can be transferred to higher trophic levels. Because of the diversity of nanomaterials, it is important that we develop an understanding of the fundamental processes that govern ingestion, uptake and accumulation of nanomaterials in living organisms.

The purpose of this study was to quantitatively assess the bioaccumulation potentials of Cadmium Selenide quantum dots (QDs) in aquatic environments, specifically Daphnia magna. The uptake, depuration and biodistribution of charged and neutral quantum dots by Daphnia magna were studied. The effect of surface charge on the uptake of polyethylene glycol (PEG) coated QDs by the cells that line the gut in Daphnia magna was studied using particle size analysis, zeta potential, TEM, STEM and EDS analysis.

Materials and Methods

QDs with ZnS coating purchased from Invitrogen were used in this study. The QDs are considered toxic due to the cadmium. The cytotoxicity of CdSe quantum dots can be altered with ZnS and polymeric surface coatings. (Hardman et al., 2006) The QDs had a final coating of PEG which is used to impart solubility in aqueous environments and prevent aggregation of the quantum dots. The QDs were purchased with amine and carboxyl molecules conjugated to the PEG side chains to obtain a charged surface coating. Three types of quantum dots were purchased from Invitrogen, non-targeted quantum dots (Q tracker 655); Carboxyl functionalized PEG coated QDs (Q tracker 655 ITK) and amine functionalized PEG coated QDs (Q tracker 655 ITK).
Particle Size Characterization

TEM

The size, shape and morphology of the nanoparticles were analyzed using TEM. The TEM samples were prepared by placing a drop of particles in solution on a carbon coated TEM grid.

CPS

The particle size of the quantum dots was measured using Centrifugal Particle Sizing (CPS) Disc Centrifuge. A dilution of 1:1000, sample to filtered water, ratio was used for the analysis.

Zeta potential

The zeta potential is the electrostatic potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particles. (Sze et al., 2003) It provides an indication of the stability of a colloidal dispersion. The zeta potential is obtained by measuring the mobility of fine particles in an electric field. The zeta potential of particles in solution is affected by pH and conductivity. A Coulter Nano Delsa C DLS particle size analyzer with a laser wavelength of 658 nm was used to measure the zeta potential of the QDs. The zeta potential measurements of the QDs were made in both facility water and Nanopure water filtered to 20 nm. The Smoluchowski model, which relates the mobility to the zeta potential at the interface, was used to calculate the zeta potential. (Hunter et al., 1981)

Daphnia Exposures to Nanoparticles

Daphnia magna were exposed to 8nM QDs with various functional groups, nontargeted PEG, carboxyl functionalized PEG and amine functionalized PEG. Twenty animals were included in each treatment beaker, and each treatment was replicated 3
times. Daphnia were exposed in the dark to each treatment group for 4, 12 or 24 hours. Another group of daphnia were exposed for 24 hours, then moved to clean facility water to “depurate” QDs present in their gut for an additional 4 to 24 hours. After each time point, daphnia were washed three times in clean, filtered facility water, then collected with as little wash water carryover as possible and placed into ultrapure Milli-Q water and sonicated using a probe sonicator. Samples were then centrifuged for 2 minutes at 500 rpm to remove any potential leftover debris. The supernatant was read in triplicate with an excitation and emission wavelength of 355 nm and 655 nm. A 6 point standard curve that accounted for auto fluorescence of untreated daphnia was used to calculate amount of QDs present in each treatment group.

**Light Microscopy**

Thick sections were examined using an Olympus BX69 with a SPOT Insight Digital Camera for brightfield imaging.

**Confocal Microscopy**

The Daphnia magna were preserved in 10% formalin after exposures to the QDs and depuration. The Q dot uptake and localization was examined using a Leica SP5 TCS laser scanning confocal microscope. Excitation of QDs was achieved using the violet 405 nm laser, and brightfield illumination was captured using 488nm blue light. The pixel resolution was 1024 x 1024. Digital images were recorded and processed using LAS-AF software version 2.0.

**TEM Sample Preparation**

The Daphnia gut tissue was prepared for TEM by washing two times in Tyrode’s buffer (8.0 g NaCl, 0.2 g KCl, 0.05 g NaH₂PO₄*H₂O, 1.0g NaHCO₃, 1.0 g Glucose, 0.1 g MgCl₂*2H₂O and 0.33 g CaCl₂*2H₂O at pH 7.4). The tissue was then fixed in 1%
glutaraldehyde in Tyrods’s Buffer. The tissue was rinsed with Tyrode’s Buffer for 15
minutes after fixation. The cells were post fixed in 2% osmic acid in 0.1 M phosphate
buffer for 1 hour. The osmic acid was drained and the tissue was rinsed two times with
phosphate buffer. The tissue was dehydrated in graded water/ethanol solutions
(70%/30%, 50%/50%, 30%/70%,10%/90%, and two times 100% ethanol) and then
infiltrated with graded ethanol/Shell EPON® Resin 828 mixtures (1:3, 1:1 3:1, and 100%
EPON). The tissue was then infiltrated with 100% EPON® and cured over night at 68 F.
The gut tissue was sectioned with diamond knife using a Leica Ultra Cut S ultra
microtome.

TEM Analysis

TEM was used to determine cellular and sub cellular localization of the quantum
dots within the GI epithelium and confirm their presence using elemental analysis. The
gut sections of the Daphnia were examined using conventional Brightfield TEM, High
Angle Annular Darkfield STEM (HAADF STEM), and Energy Dispersive X-Ray
(STEM) was used for the TEM analysis. The JEOL 2010F was operated at 200kV. It is
equipped with an Oxford INCA Pentafet EDS System for elemental analysis. It also has
a JEOL Brightfield STEM Detector and HAADF detector. The HAADF images are
formed by collecting the high angle, elastically scattered electrons which depend on the
atomic number of the sample. The resulting image provides a compositional map of the
sample with atomic number contrast. (Pennycook et al.,1988) The HAADF or Z contrast
imaging is used to identify areas of the sample with higher mass thickness or atomic
number because they appear very bright in the images. EDS analysis was used to
uniquely identify the elemental composition of the nanoparticles.
Results

TEM of Particles

A brightfield TEM image of the QDs is presented in Figure 5-2. The QDs are very uniform, rod shaped particles. The particles are approximately 12 nm by 6 nm.

Particle Size Distribution

The particle size distribution of the QDs was measured and the number and mass distribution were calculated. The particles had bimodal size distributions. The results are presented using two size ranges, one to measure the primary particle size distribution and one to measure the agglomerate size distribution. The modes, or the most frequently occurring particle size, of the particle size distributions are presented in Table 5-1. The mode of primary particle size distribution for the non targeted PEG coated particles was 5.6 nm by mass and number. The mode of the primary particle size distribution of the amine terminated, PEG coated particles was 6.6 nm by mass and by number. The mode of the particle size distribution of the carboxyl terminated, PEG coated particles was indeterminate by number and 14.9 nm by mass. These measurements assume that the particles are spherical. The QDs were rod shaped, which may account for the low particle size measurements. The anisotropic particles have a slower settling rate during the analysis then spherical particles. The modes of the agglomerate size distributions were between 109 and 120 nm for the three types of QDs.

Zeta Potential

The zeta potential provides an indication of the stability of the colloidal solution. The zeta potential of Nanopure water and facility water were measured. The zeta potential measurements are presented in Table 5-2. The background of the Nanopure
water ranged from -4.4 mV to -5.33 mV and that of the facility water was -11.30 mV. The zeta potential of the amine functionalized PEG coated QDs and nontargeted PEG coated QDs were -13.8 mV and -14.6 mV respectively in nanopure water. These results are very similar and do not provide a real difference in zeta potential between the two types of particles. The zeta potential of the PEG coated QDs in facility water was -7.9 mV. The zeta potential of the amine terminated and carboxyl terminated PEG coated QDs were -5.0/-3.6 mV and -1.7 mV respectively. All of the solutions had negative zeta potentials. The nontargeted QDs had the most negative zeta potential followed by the amine terminated PEG coated QDs. The carboxyl terminated QDs had the least negative zeta potential. It was very close to 0.

**Confocal Microscopy**

The QDs were ingested by the daphnia. They accumulated in the GI tract of the daphnids. The retention of the QDs following depuration was affected by both the charge of the particles and the exposure time. There was an overall higher affinity of the carboxyl functionalized PEG coated quantum dots for the daphnia after 24 hour exposures compared with amine functionalized PEG coated and the nontargeted PEG coated quantum dots. A table with the weight of material present in the daphnid gut after 24 hours of exposure is presented in Table 5-3. The Daphnia exposed to the nontargeted PEG coated QDs had the lowest concentration of QDs, followed by the amine terminated QDs. The carboxyl QDs had the highest concentration. The light microscopy images obtained at the 24 hour time point showed increased association of QDs to the intestine region of the daphnia exposed to carboxyl conjugated QDs compared to the other two treatments. There was a rapid clearance of all three types of quantum dots after depuration.
TEM was used to determine the location of the retained QDs in the Daphnia gut. The Daphnia gut is lined with columnar to cubical epithelial cells. (Bodar et al., 1990) A brightfield TEM image through the cross section of a Daphnia gut is presented in Figure 5-3. The tubular structure in the center of the gut is called the lumen. The epithelial cells that line the gut have two morphologies, one is more electron dense than other as shown in Figure 5-3. The plasma membrane of the light stained cells has been lysed allowing the cell contents to spill into the lumen. (Schultz et al., 1976) The dark stained cells are not in the secretory phase and have an intact plasma membrane.

Uptake of QDs by the GI epithelium cells appeared to be dependent on the secretory phase of the cell. The cells in the nonsecretory phase, with the intact plasma membrane did not contain any QDs. The QDs were observed in the lysed cells. Within these cells, the QDs were not observed within membrane bound organelles, but appeared to be free in the cytoplasm. There was also evidence that QDs could pass from cell to cell as QDs were also observed in a second layer of cells.

**Daphnia magna exposed to PEG coated quantum dots**

The epithelium cells of the gut were identified by the presence of microvilli around the inside edge of the cells along the gut wall. The microvilli are cellular membrane protrusions that are found on the apical surface of some of the epithelial cells. (Schultz et al., 1976) A brightfield TEM image of the gut tissue is presented in Figure 5-3. The microvilli are present on the surface of the dark grey, nonsecretory epithelial cells. There are also numerous light stained epithelial cells along the gut wall. The nanoparticles were not observed in the gut of the Daphnia exposed to PEG coated
quantum dots. An image of the lumen is presented in Figure 5-4. There are a few dark, electron dense features in the lumen.

BF and HAADF STEM images of the Daphnia gut are presented in Figures 5-5A and B. There are a few black features in the BF STEM image and corresponding white features in the HAADF STEM. EDS analysis was used to determine the elemental composition of the electron dense features in the lumen. A HAADF STEM image and the corresponding EDS spectrum are presented in Figure 5-6. The electron dense features, which show up white in the HAADF images, contained Os from the post fixation treatment. They were artifacts from the sample preparation, not QDs.

**Daphnia magna exposed to amine PEG coated quantum dots**

Quantum dots were observed in the gut tissue of the Daphnia exposed to amine PEG coated quantum dots. A BF TEM image of the gut section of the Daphnia exposed to the Amine PEG coated quantum dots is presented in Figure 5-7. Three areas of interest are labeled in the image. Area A is located in and around the microvilli surrounding the nonsecretory epithelium cells. Area B was located in the light stained, secretory epithelium cells. Area C was located in the dark stained, nonsecretory epithelial cells.

An HAADF STEM image of Area A is presented in Figure 5-8. The nonsecretory cell stained dark with the Os and appears white in the HAADF image. Higher magnification STEM images of the lumen around the microvilli are presented in Figures 5-9 A and B. The QDs appeared bright white in these images. There were many clusters of QDs observed in this area. An HAADF image of the quantum dots and corresponding EDS spectrum is presented in Figure 5-10. The EDS spectrum for the area with the Q dot contained, C, O, Na, Se, S, Os, and Cd.
Area B from Figure 5-7 contained the light stained epithelium cells along the gut wall. HAADF STEM images of the cells in area B are presented in Figures 5-11A and B. The secretory cell borders a nonsecretory cell which is located on the right side of Figure 11A. The QDs were observed throughout the secretory cells. Clusters of QDs are highlighted by the arrows in Figure 5-11A. Within these cells, the QDs were not observed in membrane bound organelles, but appeared to be free in the cytoplasm. A higher magnification image of a typical area containing the QDs is presented in Figure 5-11B. The composition of the QDs was confirmed using EDS.

Area C contained the epithelial cells in the nonsecretory phase. These cells appeared very bright in the HAADF images due to the heavy staining with Os. The ultrastructure of the epithelial cells is presented in Figures 5-12A and B. The epithelial cells in the nonsecretory phase are shown on the right side of Figure 12A and B. A lysed cell is shown on the left side of the Figure 12A and the lower left portion of Figure 12B. There are QDs present in the lysed cell on the lower left portion of Figure 12B that are highlighted with arrows. The nonsecretory cells did not contain any QDs. An HAADF image of a nonsecretory cell is presented Figure 5-13A. A typical EDS spectrum from one of the white features in this area is presented in Figure 5-13B. The elements present in this area were C, O and Os. All of these elements are associated with the fixed tissue. The white features were all identified as Os containing tissue features in this type of cell.

**Daphnia magna exposed to carboxyl PEG coated quantum dots**

BF TEM images of the Daphnia gut exposed to Carboxyl terminated, PEG coated quantum dots are presented in Figures 5-14A and B. A cross section through the gut is presented in Figure 5-14A. The lysed and intact epithelium is also present in this
sample. The three distinct regions of the gut were examined; the lumen (A), the intact cells (B) and the lysed epithelium cells (C). These areas are presented in Figure 5-14B.

HAADF STEM images of the lumen from the gut exposed to carboxyl terminated PEG coated QDs are presented in Figures 5-15A and B. QDs were observed throughout the lumen, they show up bright white in the images. They are highlighted in Figure 15A with arrows. The characteristic shape of the QDs is shown in Figure 15B. HAADF images of the quantum dots in the lysed epithelium cells are presented in Figures 5-16A and B. The QDs were observed throughout the lysed cells. Some of the QDs are highlighted with arrows in Figure 5-16A. A higher magnification image of the QDs in the lysed cell are presented in Figure 5-16B. QDs were not observed in the intact cells.

**Discussion**

QDs were found in the gut and epithelium cells of Daphnia. The amount of QDs found in the gut and epithelial increased with negative charge on the particles. The presence of QDs in the gut was confirmed by confocal microscopy and quantified by weight measurements. TEM was used to determine the cellular and sub cellular localization of QDs within the epithelium and confirm the identity of the QDs using elemental analysis. The three types of QDs were ingested by the daphnia, as evidence by the confocal and weight measurements. However the retention of material following depuration increased with functionalized PEG coatings on the particles and longer exposure times. The functionalized PEG coated QDs appeared to be much more abundant within the epithelial cells than the nontargeted PEG coated QDs as determined by weight and TEM.
The functionalized PEG on the surface of the QDs did not produce large differences in the surface charge of the QDs. The measured zeta potentials for the QDs are presented in Table 5-2. The zeta potential of the all the QDs were slightly negative and very close to zero. The zeta potential of the QDs in facility water range from -1.7 mV for the carboxyl PEG coated QDs, -5.0 mV for the amine PEG coated QDs and -7.9 mV for the nonfunctionalized QDs. The desired effect of having positively charged, neutral and negatively charged QDs was not achieved. The nontargeted PEG coated QDs had the most negative zeta potential and the carboxyl terminated PEG coated QDs had the least negative zeta potential, which was very close to 0. The weight of three types of QDs taken up by the Daphnia are presented in Table 3. The nontargeted PEG coated QDs with the most negative zeta potential had the least amount of particle take up and the carboxyl terminated PEG coated QDs with the zeta potential close to zero were taken up in the largest amounts followed closely by the amine terminated PEG coated QDs. There appears to be a relationship between the zeta potential and particle take up, the more negatively charged particles were taken up into the gut at a slower rate than the less negatively charged particles.

The nontargeted QDs were not observed in the TEM samples, but were observed in the confocal images. A limitation of TEM analysis is that only a small volume of material is examined, and in this case that sample area did not contain any QDs. The functionalized PEG coated QDs were observed in the lumen and lysed epithelium cells of both types of TEM samples. QDs were not observed in wholeintact epithelial or nonsecretory cells. It is not possible to determine if the QDs were taken up through the cell membrane of the lysed cells or were in the lumen and spread throughout the
cytoplasm once the epithelium cells were lysed. It does not appear that the QDs were taken up by endocytosis because the particles were not observed in membrane bound organelles. The QDs were observed in the cytoplasm of the lysed cells and in the lumen.

Summary

QDs were not taken up by nonsecretory epithelium cells in the Daphnia gut and small variations in zeta potential do not appear to make a significant difference in the amount of uptake of the QDs. The QDs were found in the lumen and lysed cells of the gut. There are still questions on whether or nanoparticles are taken up by whole, intact epithelium cells and how cellular uptake is affected by highly charged particles.
Figure 5-1. Daphnia magna or water flea. (Ebert, 2005)

Table 5-1. Particles size distribution measured by centrifugal particle sizing

<table>
<thead>
<tr>
<th></th>
<th>Primary Particle Size, Number Distribution</th>
<th>Agglomerate Size, Number Distribution</th>
<th>Primary Particle Size, Mass Distribution</th>
<th>Agglomerate Size, Mass Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amine PEG QDs</td>
<td>6.6 nm</td>
<td>119.1 nm</td>
<td>6.6 nm</td>
<td>116.3 nm</td>
</tr>
<tr>
<td>PEG QDs</td>
<td>5.6 nm</td>
<td>117.8 nm</td>
<td>5.6 nm</td>
<td>109.7 nm</td>
</tr>
<tr>
<td>Carboxyl PEG QDs</td>
<td>114.0 nm</td>
<td>14.9 nm</td>
<td>115.9 nm</td>
<td></td>
</tr>
</tbody>
</table>

Table 5-2. Zeta potential distribution measurements

<table>
<thead>
<tr>
<th>Water Type for Measurement</th>
<th>Nano Pure Water</th>
<th>Facility Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amine PEG QDs</td>
<td>-13.8 mV</td>
<td>-5.0/-3.6 mV</td>
</tr>
<tr>
<td>PEG QDs</td>
<td>-14.6 mV</td>
<td>-7.9 mV</td>
</tr>
<tr>
<td>Carboxyl PEG QDs</td>
<td></td>
<td>-1.7 mV</td>
</tr>
</tbody>
</table>
Figure 5-2. Brightfield TEM image of the rod shaped Invitrogen QDs.

Table 5-3. Weight of QDs in the daphnia gut

<table>
<thead>
<tr>
<th>Type of Quantum dot</th>
<th>Weight of particles in gut (µg)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non targeted PEG</td>
<td>95.17</td>
<td>9.78</td>
</tr>
<tr>
<td>Carboxy terminatd</td>
<td>259.17</td>
<td>17.70</td>
</tr>
<tr>
<td>Amine terminated</td>
<td>150.01</td>
<td>18.91</td>
</tr>
</tbody>
</table>
Figure 5-3. Brightfield TEM image of the gut tissue from a Daphnia magna that was exposed to PEG coated QDs.

Figure 5-4. Brightfield TEM image of the lumen inside the gut of a Daphnia magna exposed to PEG coated QDs.
Figure 5-5. Brightfield and darkfield STEM images of the gut from Daphnia magna exposed to PEG coated QDs. A) Brightfield STEM of the microvilli and lumen from the gut. The arrow is pointing to an electron dense region of the sample. B) Darkfield STEM image of the gut area with the denser features showing white in the image.

Figure 5-6. HAADF STEM image of an electron dense region in the lumen of a Daphnia gut exposed to PEG coated Q dots and the corresponding EDS spectrum. A) Electron dense regions in the lumen. B) EDS spectrum from the electron dense region contains Carbon, Oxygen, and Osmium from the post fixation treatment.
Figure 5-7. Brightfield TEM image of the gut tissue from a Daphnia magna exposed to amine functionalized, PEG coated QDs. Three distinct areas are identified in the gut tissue along this sector of the gut wall, the lumen (A), the lysed cell (B) and intact cell (C).

Figure 5-8. HAADF STEM image of area A, it includes the nonsecretory endothelial cell, microvilli and lumen of the gut exposed to amine functionalized, PEG coated QDs.
Figure 5-9. HAADF STEM images of QDs in area A the gut of the Daphnia exposed to amine functionalized, PEG coated QDs. A) QDs located in between the microvilli. B) QDs located in the lumen near the microvilli.

Figure 5-10. A HAADF STEM image of amine functionalized, PEG coated QDs in the lumen area of the gut with the corresponding EDS spectrum. A) The HAADF STEM image of the QDs. The QDs are bright white and approximately 20 nm long. B) EDS spectrum from a Q dot. The Q dot area contains Carbon, Oxygen, Sodium, Selenium, Sulfur, Cadmium and Chlorine.
Figure 5-11. HAADF STEM image of area B of the gut tissue exposed to amine functionalized, PEG coated QDs. A) There are many QDs, some of which are highlighted by the arrows, in this region of the gut tissue. B) Higher magnification image of the QDs in the gut tissue.

Figure 5-12. HAADF STEM images of the endothelial cells in the gut exposed to amine functionalize, PEG coated QDs. A) The endothelial cells contained many white features as a result of the Os post fixation treatment. B) There were not any QDs located in the cells. QDs were identified outside the cell membrane and are highlighted by the arrows.
Figure 5-13. HAADF STEM image of the endothelial cell and the corresponding EDS spectrum. A) There were many white dots in the endothelial cell that were not QDs. EDS analysis was performed on the white areas that are highlighted by the arrows in the image. B) Typical EDS spectrum from the white areas in the endothelial cell highlighted by the arrows. They contain carbon and Osmium from the post fixation treatment.

Figure 5-14. Brightfield TEM images of the Daphnia gut exposed to carboxyl functionalized, PEG coated QDs. A) Low magnification image of the gut showing the lumen, microvilli, and epithelium. B) Higher magnification image of the gut showing the lumen and epithelium cells. Three distinct areas are identified in the gut tissue along this sector of the gut wall, the lumen (A), the lysed cell (B) and intact cell (C).
Figure 5-15. HAADF STEM images of the lumen, area A in Figure 5-14B. A) There were many carboxyl functionalized, PEG coated QDs in the lumen. Some of the Qdots are highlighted by the arrows. B) Higher magnification image of the QDs in the lumen.

Figure 5-16. HAADF STEM images of the carboxyl functionalized, PEG coated QDs in the lysed cells lining the epithelium in area C from Figure 5-14B. A) There are many QDs in the cells lining the epithelium, some are highlighted by the arrows. B) Higher magnification image of the QDs in the cells lining the epithelium.
CHAPTER 6
METHODOLOGY FOR THE STUDY OF NANOPARTICLES AND THE CELLULAR UPTAKE OF NANOPARTICLES USING OPTICAL AND ELECTRON MICROSCOPY

Microscopy techniques are essential to the analysis of all aspects of nanotoxicity testing, from particle characterization, to analyzing particle uptake after in vitro and in vivo testing. Correlative microscopy, using multiple microscopic techniques with different resolution limits, is used to solve these complex problems. The mechanistic processes that cause nanotoxicity are difficult to analyze because of the size disparity between nanoparticles, particles less than 100 nm, and biological systems, which have features on the order of many microns. TEM is needed to analyze the properties of nanoparticles such as shape, size, state of agglomeration, crystallinity, coating thickness and chemistry. Information on the interactions of nanoparticles with biological systems after in vitro and in vivo testing is gained through comparison of images taken using multiple techniques. Combinations of optical microscopy techniques and electron microscopy are used to locate the nanoparticles and uniquely identify the composition and location of the nanoparticles in the biological structures.

Finding nanoparticles in biological samples with TEM alone can be as challenging as finding a needle in a haystack. The limited field of view in a TEM sample makes it necessary to use lower magnification screening techniques to determine if the cellular uptake of nanoparticles occurred and locate potential areas of interest. There is no one microscopy technique that works well in every situation, each technique that can be has specific sets of advantages and disadvantages. Fluorescence or confocal microscopy can be used to image fluorescent particles in live or fixed cells and tissue. Brightfield optical techniques can be used to image agglomerates of nanoparticles. Darkfield optical techniques can be used to image metallic nanoparticles in cells and tissue. Once
the nanoparticles are identified in the cells, then TEM/STEM/EDS analysis can be used to uniquely identify the size, shape and composition of the nanoparticles in the cells.

**Description of Techniques**

**Optical Techniques**

Confocal Microscopy is good a technique for imaging whole cells and tissue with fluorescent or fluorescent labeled nanoparticles. It has a wide field of view for imaging multiple cells at one time. Higher power objectives and digital zoom capabilities enable viewing of the structures above 1000x. Live cells, fixed cells, and tissue samples can be imaged with these techniques providing some flexibility in the analysis. In certain instances, the sample preparation is minimal and the same sample can be processed for TEM. Fluorescent stains are used to highlight the cellular features, such as cell membranes and nuclei. Confocal microscopy also has the ability to image optical sections through the cells and tissue so the general location of the nanoparticles inside a cell membrane or nucleus can be determined. This is a good technique to use as a screening process for the presence of fluorescent or fluorescent labeled nanoparticles in cells and tissue samples. Areas of interest can be micro dissected from the larger tissue pieces for more in depth inspection by TEM.

Auto fluorescence of cells and tissue can lead to misinterpretation of confocal Images. The auto fluorescence must be carefully adjusted to below background intensity on control samples before imaging test samples.

Fluorescence microscopy is a quick and easy technique for determining if fluorescent or fluorescent labeled nanoparticles are in or around cells and tissue. The fluorescence technique is similar to confocal microscopy but it does not have the ability to image optical slices through the thickness of the samples. Nanoparticles can be
detected with fluorescence microscopy, but more difficult to pinpoint their location because the images are from the total sample thickness. The ability to image slices in the z direction is lost unless the samples are physically sectioned. In addition, auto fluorescence is also an issue with this technique.

Brightfield Transmitted Light Microscopy is a valuable tool for low magnification imaging of cells and tissue samples, but is not very good for imaging nanoparticles. Changes in the morphology of cells can be monitored using brightfield microscopy. Agglomerates of nanoparticles, with a minimum size of 200 nm are visible at 1000x magnification using brightfield microscopy. Sample preparation for transmitted light microscopy is typically more complicated then imaging live or fixed cells and tissues used for confocal microscopy. It involves dehydration, embedding, sectioning and staining.

Darkfield Reflected Light Microscopy is a great technique for imaging metallic nanoparticles. The metallic nanoparticles are visible in the cells and tissue sections at magnifications around 200x because they reflect and scatter light. These images provide information on the particle distribution throughout large areas of tissue. The background in these images is dark, so no information on the cells and tissue are obtained directly using this technique. The darkfield images of the nanoparticles can be superimposed on the brightfield images to show the nanoparticle distribution in the cells. The particle distribution throughout the cells can be determined using image analysis software. This is a good technique to be used as a screening process for the presence of metallic nanoparticles in cells and tissue.
**Electron Microscopy**

TEM is a great technique for imaging the nanoparticles and ultrastructure of cells and tissue samples. The size and shape of individual nanoparticles can be imaged with TEM. It also provides a level of detail on the structure of cells and tissue that cannot be obtained with any optical technique. There are a few limitations to using TEM to locate nanoparticles in cells and tissue samples. One issue is sample preparation, specifically the time and expertise required to prepare good thin sections. Conventional TEM preparation for biological samples consists of dehydration, fixing, embedding, sectioning and staining. If the nanoparticles were taken up into the cells, then typically they remain in the cells through all of the preparation steps. If the nanoparticles are outside the cells then they can be washed away during some of the preparation steps. Cryo TEM preparation techniques, which are equally difficult, are preferred for these samples.

A second limitation of TEM analysis of nanoparticles in biological samples occurs if the concentration of nanoparticles is very low. The TEM sample and field of view in the TEM are both very small, which can make it difficult to find the nanoparticles in a low concentration sample. A single cell may extend over numerous grid squares and grid bars. It can take some time to examine the entire cell to locate the nanoparticles. Areas of interest can fall on top of grid bars which can block the region from view. HAADF STEM, or Z contrast, imaging facilitated finding high atomic number nanoparticles in unstained biological samples. The high atomic number nanoparticles show up bright white in the HAADF images and can be seen at relatively low magnifications. It is best to use unstained sections of the biological samples because heavy metal stained structural features and artifacts from the staining process can easily be mistaken for nanoparticles. EDS or EELS techniques can be used to confirm the presence of the
nanoparticles by elemental microchemical analysis. It is a great way to distinguish between nanoparticles and artifacts from the staining process.

The TEM/STEM/EDS results should be well documented and checked for reproducibility. If cellular uptake of nanoparticles is confirmed using TEM/STEM/EDS techniques, and the TEM results correlate well with the optical microscopy results, then the optical microscopy technique can be used as a screening process on future samples.

IASEM and TEM tomography are techniques that can be used to determine the 3-D distribution of nanoparticles in biological samples.

**Description of Work**

A methodology for the microscopic analysis of the cellular uptake of nanoparticles is presented in this chapter. It is based on the microscopy results obtained through a number of studies at the Center for Human and Environmental Toxicology at the University of Florida. It is broken down into two sections, Particle Characterization by Electron Microscopy and Characterization of Nanoparticles in Cells and Tissue after In Vitro and In Vivo Testing by Correlative Microscopy.

**Particle Characterization by Electron Microscopy**

The TEM is routinely used to characterize nanoparticles. It is the best way to determine the primary particle size and particle shape of the nanoparticles. TEM can also be used to determine the crystallinity, crystal structure, and phase identification. TEM can also be used to assess the agglomeration state of the particles and agglomerate size. The chemical composition of the nanoparticles can be determined using EDS and EELS. The thickness and morphology of coatings on nanoparticles can also be evaluated using heavy metal staining techniques and energy filtered imaging.
The main limitation of using TEM to characterize the particle size is the small amount of sample that is actually analyzed. The SEM can also be used to determine the particle size and shape, and sample a larger number of particles. Microscopy in conjunction with image analysis or direct particle size measurements can be used to determine particle size distribution.

The particle size distribution can be determined using image analysis and direct particle size measurements from SEM or TEM images. Other techniques such as centrifugal particle size analysis and dynamic light scattering are very useful for determining the particle size distribution from larger sample volumes then can be measured using electron microscopy.

Methodology for Characterizing Nanoparticles Using Electron Microscopy

A flow chart showing the methodology for characterizing nanoparticles using electron microscopy is presented in Figure 6-1.

Sample Preparation

1. Nanoparticles must be washed sufficiently prior to TEM sample preparation.

2. Disperse nanoparticles in a solvent and ultrasonicated for 15 minutes.

3. TEM grids, usually 200-400 mesh, with a carbon or other support film are used to observe powders. Place grid on a piece of filter paper with the support film facing down, or the grid can be held in self clamping tweezers.

4. Dispense nanoparticles onto the grid with a micropipette.
   a. Hold the grids in self clamping tweezers and drop ~20 μl of particle solution on to the grid.
   b. If the grids are placed on filter paper, and the sample concentration are low, then add multiple drops of the particle solution to the sample.

5. Grids must be dry before TEM analysis.
TEM/STEM and SEM analysis

1. Analyze nanoparticles in the TEM or STEM.
   a. Brightfield TEM Imaging: Determine nanoparticle size, shape, state of dispersion, and presence of surface coatings. Images can be used for particle size distribution.
   b. Electron diffraction: Determine the crystal structure and phase of the nanoparticles.
   c. EDS: Determine the microchemical constituents of the particles
   d. EELS: Determine the elemental composition, and information on the electronic and atomic structure of the materials. Imaging techniques that use portions of the energy spectra, such as energy filtered imaging and zero loss imaging, can be used to map the location of certain elements.

2. Analyze nanoparticles in the SEM
   a. SEI – Determine size and shape of the nanoparticles.
   b. EDS – Determine the elemental composition of the nanoparticles.

Characterization of the Cellular Uptake of Nanoparticles in Tissue and Cells

In vitro tests are commonly used as a first assessment in determining nanotoxicity. In vitro tests are usually assays or tests to determine cytotoxicity, proliferation, genotoxicity and altered gene expression for specific cell lines. Cell death, growth, reproduction, and activity in the presence of nanoparticles are determined from assays. Microscopic analysis of the cells from the assay can be analyzed to determine the interactions between the nanoparticles and cells, such as cellular uptake, and confirm the location and composition of the nanoparticles within the cells.

The microscopic evaluation of in vitro cell cultures consists of two parts, an optical technique and TEM/STEM/EDS. The optical technique is used to image the whole cells and determine if nanoparticles are present in and around the cells. The optical technique will depend on the type of nanoparticle being used, fluorescent or a
fluorescent labeled particles, verses a nonfluorescent particle. Confocal or fluorescence microscopy is used to image cells with fluorescent particle or fluorescent labeled particles. An advantage to this technique is that live or fixed cells can be used and so the only preparation required in staining and fixing if desired. Tissue samples can be fixed and sectioned with a cryostat and mounted on a glass slide. Areas of interest can be removed for electron microscopy.

If nonfluorescent particles are used then brightfield, darkfield and differential interference contrast microscopy can be used to locate the particles in the cells and tissue. Thick sections mounted on glass slides are used for imaging with the optical techniques. Samples can be prepared by making histology sections, cryostat sections or plastic embedded sections. If histology sections are used for brightfield and darkfield imaging, then different pieces of tissue have to be used for TEM. Plastic embedded sections can be sliced thick for optical microscopy and followed by thin sectioning for TEM.

TEM/STEM/EDS is used to determine the location, distribution, and composition of nanoparticles within the cells and tissue. It is also used to examine the morphology of the ultrastructure of the tissues and cells following nanoparticle exposure. TEM analysis is best used on samples where nanoparticles have been identified using other techniques, because finding a nanoparticle in a cell can be like trying to find a needle in haystack.

Conventional TEM is used to determine the location of the nanoparticles in the cells, characterize the size, shape and chemistry of the nanoparticles in the cells and assess any changes to the ultrastructure of the tissue or cell sample. The appearance
of nanoparticles can be difficult to distinguish from the electron dense, stained cellular structures in the same size range, such as the mitochondrial matrix granules or ribosomes. When imaged solely with Conventional TEM, it is possible to mistake the stained cellular structure for nanoparticles. Energy dispersive x-ray microanalysis (EDS) and Electron Energy Loss techniques such as Parallel Electron Energy Loss Spectroscopy (PEELS) and Energy filtered TEM (EFTEM) are used to determine the elemental composition of an area in a sample. These techniques can also be used to identify nanoparticles in the cells and tissues.

High Angle Annular Detection (HAADF) in STEM is another technique used to image samples with contrast based on atomic number. The heavier atomic number elements scatter more electrons, which are detected and show up much brighter in the images than the lower atomic number elements.

**Methodology for Characterizing the Cellular Uptake of Nanoparticles in Cells**

Flow charts of the methodology for characterizing the cellular uptake of nanoparticles cells and tissue using optical microscopy and electron microscopy techniques are presented in Figure 6-2 and 6-3 respectively.

**Confocal Microscopy of Cells**

1. Sample preparation for Confocal microscopy of live or fixed cells
   a. Transfer cells from assay dish to a petri dish with 1.5 mm cover slip on bottomor glass slide with 1.5 m cover slip
   b. Wash cells with fresh buffer
   c. Add buffer for imaging
   d. Stain cells to highlight cellular components if desired
2. Image cells with confocal microscopeImage nanoparticles only
a. Image nanoparticles only

1) Set the excitation and emission ranges for the fluorescent nanoparticles

b. Image control sample

1) Set the excitation and emission ranges for the stains used

2) Determine if autofluorescence of the cells is an issue

3) Set gain to show no emission for the fluorescent nanoparticles using the control sample

4) Image the control sample to document the untested condition of cells

a. Image sample exposed to nanoparticles

5) Image the test sample using the conditions set up for the nanoparticles and control sample to limit auto fluorescence and locate nanoparticles

6) Image layers through the thickness of cells, both plan view and cross sections, to determine if particles are located inside the cell

b. Document the nanoparticles if present in the cells

**Brightfield, Darkfield or DIC Microscopy**

1. Prepare cells for Brightfield, Darkfield or DIC microscopy

   a. Remove growth media from cells

   b. Wash with buffer

   c. Add fixative (Karnovsky’s fixative 2.5% glutaraldehyde, 2% paraformaldehyde in sodium cacodylate buffer wash 2 times

   c. Centrifuge, spin down ,to collect cells

   d. Encapsulate cells in 3% agarose gel

   e. Secondary Fixation in 1% OsO4 in buffer

   f. Wash in buffer solution

   g. Wash 2 times in DI water

   h. Dehydrate in graded ethanol/ DI water series (25%/75%, 50%/50%, 25%/75%, 10%/90%, 100%)
i. Dehydrate in 100% acetone 2 times

j. Infiltrated cells with graded Acetone/Spurr’s series (70%/30%, 50%/50%, 30%/70%, 100%)

k. Cure embedded cells over night in 100% Spurr's

l. Section embedded cells with ultramicrotome and collect sections on glass slide

m. Stain sections with toluidine blue or some other stain to show cellular features

2. Image sections with brightfield, darkfield or DIC microscopy

   a. Brightfield transmitted light imaging of cellular structure and agglomerated nanoparticles

   b. Darkfield reflected light imaging of distribution of metallic nanoparticles

3. Image thick section using confocal microscopy if desired

**TEM/STEM/EDS**

1. Prepare Cells for Electron Microscopy

   a. Follow procedure above for brightfield, darkfield and DIC sample preparation through step m

   b. Section embedded cells with ultra microtome, making 70 to 100 nm slices

   c. Capture slices on carbon coated TEM grid or a clean, uncoated grid dipped in ethanol adhesive mixture

2. Image thin sections in TEM

   a. Brightfield imaging to document cellular ultrastructure and locate nanoparticles

   b. HAADF STEM imaging to locate high atomic number nanoparticles

   c. EDS/EELS analysis to confirm presence of nanoparticles by elemental composition
Characterization of the Cellular Uptake of Nanoparticles after In Vivo Testing Using Correlative Microscopy

In vivo tests are conducted to determine the interactions of nanoparticles in the living organism. In some cases, the behavior of nanoparticles in the living organism is not the same as those predicted by an assay. Various organs and blood are typically analyzed for nanoparticle content after in vivo tests. Take up of nanoparticles into various organs can be measured by weight and ICP mass spec. These techniques provide information on the amount and chemistry of the particles taken up into the entire organ. Correlative microscopy techniques are then used to determine the location and distribution of the nanoparticles in the organ.

The microscopic evaluation of organs and tissue is similar to that outlined above for cells. It consists of two parts, an optical technique and TEM/STEM/EDS. The optical technique is used to image large areas of the tissue and determine the areas that contain nanoparticles. The optical technique will depend on the type of nanoparticle being used, fluorescent or a fluorescent labeled particles, verses a nonfluorescent particle. Confocal or fluorescence microscopy is used to image organs or tissue with fluorescent particle or fluorescent labeled particles. An advantage to this technique is that small organs or sections of tissue can be examined with minimum sample preparation. Organs can be sectioned for confocal microscopy. There are a few techniques that can be used to section the organ for confocal microscopy. The thick section preparation techniques are the same for confocal microscopy and brightfield, darkfield and DIC microscopy. Histology sections are a common way to have thick sections prepared from tissue samples. Tissue samples can be fixed and sectioned with a cryostat and mounted on a glass slide. The cryostat sections can be removed from
the glass slides and embedded for TEM. Plastic embedding and microtome sectioning can also be used to prepare samples for optical microscopy followed by TEM.

TEM/STEM/EDS analysis is used for a more detailed analysis of the particle locations and elemental composition. It is important to have identified the general locations of the nanoparticles with an optical microscopy technique to facilitate locating the nanoparticles in the thin sections prepared for TEM. The location of the nanoparticles in the ultrastructure of the tissue can be uniquely identified using TEM. TEM images provide an area distribution of the nanoparticles.

The three dimensional distribution of nanoparticles in tissue samples can be obtained using IASEM. The plastic embedded tissue blocks work well for IASEM.

**Methodology for Characterizing the Cellular Uptake of Nanoparticles after In Vivo Testing Using Correlative Microscopy**

Flow charts of the methodology for characterizing the cellular uptake of nanoparticles after in vitro and in vivo testing using optical microscopy techniques and electron microscopy techniques are presented in Figure 6-2 and 6-3 respectively.

**Confocal Microscopy of Tissue Samples**

1. Sample preparation for Confocal microscopy of live or fixed tissue
   a. Live or fixed tissue
      1) After dissection, immerse the tissue in either buffer or fixative
      2) Wash tissue with fresh buffer
      3) Add buffer for imaging
      4) Stain tissue to highlight cellular components if desired
   b. Crystostat sections
      1) Fix tissue in 10% formalin in PBS or tissue can be frozen directly
2) Remove formalin
3) Wash samples 3 times with Cacodylate buffer
4) Mount sample on chuck with OCT compound
5) Cryo protect tissue in 10% PEG, 30% glycerol mixed 1:1 with buffer
6) Put tissue, glass slides and knife in cryostat to cool to 19°C
7) Section with Leica CM1950 Cryostat at 19°C
8) Transfer slices to glass slide

2. Image tissue samples with confocal microscope
   a. Image nanoparticles
      1) Set the excitation and emission ranges for the fluorescent nanoparticles
   b. Image control sample
      1) Set the excitation and emission ranges for the stains used
      2) Determine if autofluorescence of the cells is an issue
      3) Set gain to show no emission for the fluorescent nanoparticles using the control sample
      4) Image the control sample to document the untested condition of cells
   c. Image sample exposed to nanoparticles
      1) Image the test sample using the conditions set up for the nanoparticles and control sample to limit auto fluorescence and locate nanoparticles
      2) Image layers through the thickness of cells, both plan view and cross sections, to determine if particles are located inside the cell

3. Dissect tissue for TEM sample preparation

Optical Microscopy of Thin Sections
1. Sample Preparation of Histology Sections
   a. Fix tissue
b. Dehydrate tissue in graded water/ethanol solutions (70%/30%, 50%/50%, 30%/70%, 10%/90%, and two times 100% ethanol)

c. Clear the tissue in multiple soakings in xylene.

d. Vacuum infiltrate the samples with paraffin 4 times

e. Section 10 micron slices of embedded tissue with a microtome and mount samples on glass slides

f. Stain the sections with Haematoxylin and Eosin or fluorescent stains if imaging with confocal microscopy

g. Glue cover slips over sections

2. Image tissue sections (histology, cryostat or polymer embedded) with brightfield, darkfield or DIC or confocal microscopy

   a. Brightfield transmitted light imaging of cellular structure and agglomerated nanoparticles

   b. Darkfield reflecdted light imaging of distribution of metallic nanoparticles

TEM/STEM/EDS Analysis of Tissue Sections

1. Preparation of plastic embedded samples from tissue or cryostat sections

   a. Fix tissue in Trump’s solution

   b. Rinse 2 times with cacodylate buffer

   c. Post fixation treatment in 2% OsO4in 0.1 M phosphate buffer

   d. Rinse 2 times in phosphate buffer solution

   e. Dehydrate samples in graded water/ethanol solutions (70%/30%, 50%/50%, 30%/70%, 90%/10%, and two times 100% ethanol)

   f. Vacuum infiltrate samples with graded Ethanol/Spurr’s Resin mixtures (70%/30%, 50%/50%, 30%/70%, and 100%)

   g. Infiltrate with 100% Spurr’s resin for 48 hours

   h. Put infiltrated samples in molds and fill with Spurr’s resin
i. Cure embedded samples for 48 hours

j. Cut embedded tissue from blocks with jeweler’s saw and glue embedded tissue to cylindrical plug

k. Trim block to expose tissue

l. Cut a few thick sections, approximately 500 nm thick and stain with toluidine blue

m. Check thick sections with optical microscope for area of interest

n. Cut thin sections, 70-100 nm, for TEM

2. Preparation of plastic embedded samples from cryostat sections or histology sections

a. Rehydrate tissue sections in decreasing concentrations of ethanol/water (100%, 90%/10%, 70%/30%, 50%/50%, 20%/80%, and two times 100% water) by dropping solutions onto sample

b. Refix tissue in 3% glutaldehyde in 0.1M cacodylate buffer

c. Wash sample 2 times in cacodylate buffer

d. Post fix tissue in 1% OsO4 in 0.1 M cacodylate buffer followed by 2 washes

e. Dehydrate tissue in graded water acetone series

f. Infiltrate sample with EPON® for a minimum of three hours

g. Place open ended beem capsule over area of interest and cure over night at 60 C

h. Add EPON® to fill two thirds of the beem capsule

i. Cure over night at 60 C

j. Remove the beem capsule from the slide by scoring around the base of the block, then heat slide to 100C for 15 s and remove from the slide

k. Trim the block face to the area of interest

l. Section the block with an ultra microtome similar to the procedure outlined above

3. Analyze thin sections in TEM/STEM/EDS
a. Brightfield imaging to document cellular ultrastructure and locate nanoparticles

b. HAADF STEM imaging to locate high atomic number nanoparticles

c. EDS/EELS analysis to confirm presence of nanoparticles by elemental composition

**Conclusions**

The methodologies listed above outline the general procedure for preparation and analysis of cells and tissue specimens that have been dosed with nanoparticles for correlative microscopy techniques. Multiple microscopy techniques are needed for the analysis of nanoparticle take up by cells and tissue because of the size disparity between the nanoparticles and cellular features. Finding nanoparticles in cells and tissue samples can be challenging because it is like finding a needle in a haystack. Nanoparticles are very small compared with cells and tissue samples. The nanoparticles are very difficult to locate in the cells and tissue samples unless they are present in fairly large quantities or they are agglomerated. A methodology like the presented in this chapter should be incorporated into any standardized nanotoxicity testing protocol that is developed.

These methodologies were used to obtain the following new results:

1. Pt nanoparticles with a 1.3M molecular weight PVP coating caused an up regulation of the HO-1 enzyme when exposed to BEAS cells. The Pt-PVP 1.3 M nanoparticles were very small, mean particle size of 2.2 nm, and well dispersed inside the cells in comparison to the other types of nanoparticles investigated.

2. The PEG coating thickness on gold nanoparticles was measured using high resolution TEM and negative staining techniques.

3. The amount and distribution of gold in sections of the liver was determined using darkfield reflected light microscopy. Image analysis was used to determine the gold agglomerate size, number of agglomerates and area fraction of agglomerates in the sections.
4. The three dimensional distribution of gold nanoparticles in a single cell was obtained using ion abrasion scanning electron microscopy.

5. The uncoated gold nanoparticles were taken up almost immediately by the Kupffer cells of the liver. The native gold nanoparticles were observed in large, tightly packed agglomerates in lysosomes inside the cells.

6. The PEG coated nanoparticles were rapidly taken up after 2 hours. Loosely packed clusters of PEG coated gold nanoparticles were observed lining the inner surface and inside of the lysosomes.

7. QDs were not taken up by nonsecretory epithelium cells in the Daphnia gut and small variations in particle surface charge do not appear to make much of a difference in take up behavior.

8. The QDs were found in the lumen and lysed cells of the gut.
Nanoparticle (NP) Characterization

TEM

- Brightfield TEM - NP size, shape, crystallinity, coating, state of dispersion
- Electron Diffraction - crystal structure, phase id
- EDS/EELS – composition
- Zero Loss Imaging – coating morphology
- Negative Staining Techniques – coating thickness and morphology

SEM

- SEI – NP size and shape
- EDS – Bulk chemical composition

Figure 6-1. Flow chart outlining the methodology for nanoparticle characterization using electron microscopy.

Characterization of NPs in Cells and Tissue after In Vitro and In Vivo Testing

Optical Microscopy Techniques

Fluorescent NPs

Confocal (preferred) or Fluorescence

- Image NPs only for calibration
- Stain Cells to visualize cell membrane, nuclei, and other features
- No overlap of the emission wavelength of NPs and stains
- Image control cells to determine gain settings to remove auto fluorescence
- Image NPs in cells at same settings
- Image layers through cell thickness and cross sectional views to determine if NP inside cells (confocal)

Non Fluorescent NPs

BF Transmitted Light

Metallic NP

Histological Sections (H&E stain)
Cryostat Sections
Plastic Embedded Sections

- BF - Image cellular structure over wide field of view
- BF - Difficult to image anything but agglomerated NPs at 1000x
- Determine if sample suitable for TEM
- DF – Image metallic NP distribution at magnifications as low as 200x

Figure 6-2. Flow chart outlining the methodology for characterizing cellular uptake of nanoparticles using optical microscopy.
Characterization of NPs in Cells and Tissue after In Vitro and In Vivo Testing

TEM Sample Preparation

Live or Fixed Cells, Cryostat Sections:
Same cells/tissue imaged for confocal can be prepared for TEM
TEM prep: Fixation, dehydration, embedding and sectioning

Histology Sections:
Prepare new samples for TEM

Plastic Embedded Samples:
Use same sample blocks for TEM

When looking for high Z NPs use Os post fixation treatment but no additional heavy metal stains

TEM Analysis

• BF TEM – Image cellular ultrastructure and size and shape of NPs (high Z NPS appear dark in BF TEM, but not all black dots are NPs)
• HAADF STEM – Image high atomic number NPs
• EDS/EELS – Confirm location of NPS by elemental analysis

SEM Analysis

• BS- Image high Z NPs distribution
• FIB-SEM- slice and view 3 D particle distribution in cells
• Additional stains are needed to image cellular structure

Figure 6-3. Flow chart outlining the methodology for characterizing cellular uptake of nanoparticles using electron microscopy.
LIST OF REFERENCES


183


BIOGRAPHICAL SKETCH

Kerry Siebein graduated from Worcester Polytechnic Institute in Worcester, MA with a bachelor’s degree in mechanical engineering and a master’s degree in materials engineering. After graduation she moved to California and worked for Failure Analysis Associates in Palo Alto, CA and then in Newton, MA. She attended Massachusetts Institute of Technology for one year and then took a job at the Army Materials Testing Laboratory in Watertown, MA. From there she jumped to her next position at the newly formed High Performance Ceramics Division with Norton San Gobain in Northboro, MA. She eventually left Massachusetts with her husband and lived in Connecticut as a stay at home mom for a couple of years. Her next destination was Los Alamos National Laboratory where she worked in the Polymers and Coatings Group for a few years. She followed her husband back to Connecticut again, where she wrote articles for the Copper Development Association website. Then she broke out on her own and moved to Gainesville, Florida, where she started working at Siebein Associates an acoustical consulting firm. After a few years she walked into a position at the Particle Engineering Research Center at the University of Florida. She is currently holding a position of Associate In Engineering at the Major Analytical Instrumentation Center (MAIC) in the College of Engineering at the University of Florida. She is the coordinator for Transmission Electron Microscopy and Field Emission Scanning Electron Microscopy at MAIC.