A CHARACTERIZATION OF GOLD NANOPARTICLE UPTAKE BY MAMMALIAN CELLS

Thomas Garner
Clemson University, tgarner@clemson.edu

Follow this and additional works at: https://tigerprints.clemson.edu/all_dissertations
Part of the Medical Toxicology Commons

Recommended Citation
https://tigerprints.clemson.edu/all_dissertations/1243

This Dissertation is brought to you for free and open access by the Dissertations at TigerPrints. It has been accepted for inclusion in All Dissertations by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.
A CHARACTERIZATION OF GOLD NANOPARTICLE UPTAKE BY MAMMALIAN CELLS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

By
Thomas Ross Garner
December 2013

Accepted By:
Dr. Stephen J. Klaine, Committee Chair
Dr. William S. Baldwin
Dr. Cindy M. Lee
Dr. Andrew S. Mount
Dr. Aaron P. Roberts
ABSTRACT

Over the past decade, there has been an exponential growth in products and applications that utilize nanotechnology, as these materials present exciting opportunities for the development of new products, enhancement of existing products, and the evolution of innovative procedures. However, this recent growth has occurred in the absence of detailed knowledge concerning the interactions of nanoparticles with biological systems. Nanoparticles must usually be bioavailable in order to elicit a biological response, and to reach an active site they must usually cross a membrane. As a result, cell culture studies have proven to be beneficial and are generally one of the first tests conducted upon the synthesis of a new particle type. Furthermore, the development of in vitro assays to predict biological interactions with nanoparticles may facilitate future endeavors towards accurate assessments of the risk that nanoparticles may pose on the environment. The goal of my dissertation was to characterize the uptake of gold nanoparticles by the mammalian cell line A549 through determining the roles of particle characteristics and culture medium components, and by describing potential modes by which particles are endocytosed. To assess the influence of the physical and chemical characteristics of gold nanospheres on cell uptake, a semi-factorial design was followed, examining three different particle sizes (roughly 5nm, 15nm and 50nm) modified by three different surface charges (anionic, cationic, and nonionic). The influence of particle concentration on cellular uptake was analyzed for 5nm and 50nm citrate-
capped spheres over 24 h, and particle uptake increased as exposure concentration increased. The influence of particle size and surface charge on uptake was analyzed for each particle type over 2 h, and with the exception of 50nm nonionic particles, uptake was charge dependent; as cationic particles were taken up to a greater extent than anionic particles, which were taken up more than nonionic particles. To assess the influence of the culture medium components on particle uptake, cells were exposed to 30nm citrate-capped particles in exposure media supplemented with differing concentrations of fetal bovine serum (FBS) and bovine serum albumin (BSA). The uptake of particles exposed in FBS was inversely related to protein exposure concentration, which was not observed with particles in media supplemented with BSA, suggesting that certain components of FBS deter particle uptake. When cells were exposed to particles in media supplemented charcoal/dextran treated FBS, particle uptake increased significantly suggesting that growth factors and steroids may inhibit particle uptake. When cells were exposed to particles in media supplemented with BSA in addition to fetuin, uptake decreased significantly suggesting that fetuin may deter particle uptake. Also, particle uptake decreased when calcium was removed from the exposure media, suggesting that uptake occurs through a calcium dependent process. To assess potential modes of endocytosis utilized in the cellular trafficking of nanoparticles, cells were co-exposed to 30nm citrate-capped nanospheres and known inhibitors of specific endocytic pathways. Filipin and nystatin were used to inhibit caveolae-dependent endocytosis,
chlorpromazine and phenylarsine oxide to inhibit clathrin-dependent endocytosis, and cytochalasin D and 5-(N-ethyl-N-Isopropyl) amiloride (EIPA) were utilized to inhibit macropinocytosis. No statistical decrease in particle uptake, in comparison to controls, was observed following exposure to any of the inhibitors, suggesting that the uptake of nanoparticles was not dependent upon any one specific mode of endocytosis. Overall, the results of my dissertation indicate that uptake is dependent upon both characteristics of the particle and exposure medium, and that the uptake of particles is a complex process which cannot be described solely by pharmacological inhibition.
DEDICATION

This work is dedicated to my parents, Dr. James Samuel Garner, IV and Arden Thomas Garner. I could not have come this far without the guidance, opportunities, love, and support that they have continually bestowed upon me for my entire life. For this I will forever be grateful and will never be able to thank them enough.
ACKNOWLEDGMENTS

I would like to begin by thanking Dr. Steve Klaine for giving me the opportunity to work with him and further develop my skills as an environmental toxicologist and as a person. I could not have asked for a better advisor, mentor, colleague, or friend. I would also like to thank each of my committee members, Dr. Bill Baldwin, Dr. Cindy Lee, Dr. Andy Mount, and Dr. Aaron Roberts, for the support, guidance, time, and patience that they have given me over the years in order to reach this milestone in my life.

I would also like to thank Norm Ellis and John Smink for their continued support throughout my time here at Clemson. Norm was extremely helpful in my analytical analysis, even under the tightest of deadlines when it required working late nights and weekends, and both were always there to discuss science, life, football, and all things between. I would have never made it this far without the advice and support from all members of the Klaine Lab, past and present. I could never ask for better colleagues and friends than those that I had here. Specifically, I am forever indebted to Dr. Aaron Edgington, Dr. Brandon Seda, Dr. Brad Glenn, and Austin Wray for furthering my knowledge on nanoparticles, particle synthesis, particle characterization, and all other nano-related matter.

I must also thank Dr. Catherine Murphy and Dr. Christopher Kitchens, as well as Ashley Hart and Scott Cole from the lab of Dr. Kitchens, for providing me with the array of particles needed in the second chapter of my dissertation. This
work could not have been completed without their collaboration. I would also like to thank all the faculty members of the Environmental Toxicology Graduate Program, Dr. Charlie Rice, Dr. Lisa Bain, and Dr. Kim Paul for always being there to answer any questions and offer suggestions and advice for my work. Also, my cell culture work could never have gone as smoothly without the help of Dr. Gia-Ming Hong, Ben Green, Amy Anderson, Lauren Sweet, and Jui-Tung “Ray” Liu.

I always tell Mrs. Francis Atkinson that everything I ever needed to know, I learned in kindergarten. As this is not completely true, I must also thank each and every teacher that I have had along the way, for I know that I was no simple pupil. I would especially like to thank Susan Smith and Cynthia Collins for teaching me to never accept anything short of excellence, as well as Bunny Beeson, Robert Kirby, and Dr. Alix Darden for making science come to life, which grabbed my heart and soul. I must thank Dr. John Weinstein for believing in me, mentoring me, his wonderful friendship, and for causing me to realize my desired path of study.

Finally, I would not have been able to make it this far without the unconditional love of my family, friends, and Citadel brethren. I would like to graciously thank my grandparents Dr. Jim and Mary Garner and J.B. and Lenora Thomas, my parents Dr. Sandy and Arden Garner, my brother Jay Garner, my god-brother Ryan Brewer, my sister-in-law Whitney Garner, my niece Pressley Anne Garner, and my nephew Samuel Garner for their never-ending love and
support. Especially to my grandfathers who, through their actions, taught me the value of hard work and that if you do what you love, you will do it well and for a lifetime. I must also thank Thomas Hunter, Daniel Isaac, and my brother for always believing in me and pushing me when I no longer believed in myself, and for being my best friends, as well as Thomas and my mother for making me realize that “there’s something in these hills”. Finally, I must thank Dr. Anthony Sowers, Dr. Holly Nance, Dr. Joe Bisesi, Dr. Louis Plough, Aileen Plough, Amy Anderson, Cason Collins, Ira Harper, Jeremy Trammell, and The Sosebees for their friendship, the wonderful memories, and making my time in Clemson an amazing experience.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xvi</td>
</tr>
<tr>
<td>CHAPTER ONE: LITERATURE REVIEW: THE ENVIRONMENTAL FATE AND EFFECTS OF ENGINEERED NANOPARTICLES</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Nanoparticle Fate in the Environment</td>
<td>3</td>
</tr>
<tr>
<td>Nanoparticle Uptake <em>In Vivo</em></td>
<td>5</td>
</tr>
<tr>
<td>Nanoparticle Uptake <em>In Vitro</em></td>
<td>9</td>
</tr>
<tr>
<td>Research Needs and Conclusions</td>
<td>13</td>
</tr>
<tr>
<td>References</td>
<td>15</td>
</tr>
<tr>
<td>CHAPTER TWO: THE INFLUENCE OF PARTICLE SIZE AND SURFACE CHEMISTRY ON THE CELLULAR UPTAKE OF GOLD NANOPARTICLES</td>
<td>22</td>
</tr>
<tr>
<td>Abstract</td>
<td>22</td>
</tr>
<tr>
<td>Introduction</td>
<td>23</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>25</td>
</tr>
<tr>
<td>Particle Synthesis</td>
<td>25</td>
</tr>
<tr>
<td>Particle Characterization</td>
<td>27</td>
</tr>
</tbody>
</table>

ix
TABLE OF CONTENTS (cont.)

Page

Cell Culture ........................................................................................................ 27
Cell Exposures .................................................................................................... 28
Influence of Particle Concentration .............................................................. 29
Influence of Particle Characteristics ............................................................ 29
Analytical Procedure ....................................................................................... 30
Data Analysis .................................................................................................... 30

Results .............................................................................................................. 31
Particle Characterization ............................................................................... 31
Influence of Particle Concentration ............................................................. 32
Influence of Particle Characteristics ............................................................ 35
Influence of Particle Size ............................................................................... 38
Influence of Particle Surface Charge ............................................................ 41
Size and Surface Effects ................................................................................. 48

Discussion ......................................................................................................... 50

Conclusions ....................................................................................................... 52

References ......................................................................................................... 54

CHAPTER THREE: NOVEL APPROACHES TO ELUCIDATE
SERUM COMPONENTS INVOLVED IN THE CELLULAR
UPTAKE OF NANOPARTICLES ........................................................................ 58

Abstract ............................................................................................................. 58

Introduction ....................................................................................................... 59

Materials and Methods .................................................................................. 61
Particle Synthesis ............................................................................................ 61
Particle Characterization ................................................................................. 61
Cell Culture ...................................................................................................... 62
Cell Exposures ................................................................................................. 63
FBS vs. BSA Supplementation ......................................................................... 63
Phase I PIE ....................................................................................................... 64
Phase II PIE ..................................................................................................... 65
Influence of Calcium ......................................................................................... 66
Analytical Procedure ......................................................................................... 66
## TABLE OF CONTENTS (cont.)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data Analysis</td>
<td>67</td>
</tr>
<tr>
<td>Results</td>
<td>67</td>
</tr>
<tr>
<td>Particle Characterization</td>
<td>67</td>
</tr>
<tr>
<td>FBS vs. BSA Supplementation</td>
<td>68</td>
</tr>
<tr>
<td>Phase I PIE</td>
<td>71</td>
</tr>
<tr>
<td>Phase II PIE</td>
<td>74</td>
</tr>
<tr>
<td>Influence of Calcium</td>
<td>80</td>
</tr>
<tr>
<td>Discussion</td>
<td>83</td>
</tr>
<tr>
<td>Conclusions</td>
<td>87</td>
</tr>
<tr>
<td>References</td>
<td>88</td>
</tr>
<tr>
<td>CHAPTER FOUR: EXAMINATION OF POTENTIAL MODES OF NANOPARTICLE UPTAKE THROUGH THE PHARMACOLOGICAL INHIBITION OF ENDOCYTIC PATHWAYS</td>
<td>93</td>
</tr>
<tr>
<td>Abstract</td>
<td>93</td>
</tr>
<tr>
<td>Introduction</td>
<td>94</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>96</td>
</tr>
<tr>
<td>Particle Synthesis</td>
<td>96</td>
</tr>
<tr>
<td>Particle Characterization</td>
<td>96</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>97</td>
</tr>
<tr>
<td>Cell Exposures</td>
<td>98</td>
</tr>
<tr>
<td>Inhibition of Caveolae-Dependent Endocytosis</td>
<td>99</td>
</tr>
<tr>
<td>Inhibition of Clathrin-Dependent Endocytosis</td>
<td>99</td>
</tr>
<tr>
<td>Inhibition of Macropinocytosis</td>
<td>99</td>
</tr>
<tr>
<td>Analytical Procedure</td>
<td>100</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>100</td>
</tr>
<tr>
<td>Results</td>
<td>101</td>
</tr>
<tr>
<td>Particle Characterization</td>
<td>101</td>
</tr>
<tr>
<td>Inhibition of Caveolae-Dependent Endocytosis</td>
<td>101</td>
</tr>
<tr>
<td>Inhibition of Clathrin-Dependent Endocytosis</td>
<td>105</td>
</tr>
<tr>
<td>Inhibition of Macropinocytosis</td>
<td>109</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS (cont.)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discussion</td>
<td>113</td>
</tr>
<tr>
<td>Conclusions</td>
<td>115</td>
</tr>
<tr>
<td>References</td>
<td>117</td>
</tr>
<tr>
<td>CHAPTER FIVE: CONCLUSIONS AND FUTURE RESEARCH</td>
<td>120</td>
</tr>
<tr>
<td>APPENDIX I</td>
<td>124</td>
</tr>
<tr>
<td>APPENDIX II</td>
<td>135</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>Influence of particle concentration on uptake for 5nm citrate-capped spheres</td>
<td>33</td>
</tr>
<tr>
<td>2.2</td>
<td>Influence of particle concentration on uptake for 50nm citrate-capped spheres</td>
<td>34</td>
</tr>
<tr>
<td>2.3</td>
<td>Uptake of citrate-capped spheres</td>
<td>36</td>
</tr>
<tr>
<td>2.4</td>
<td>Uptake of PEG-capped spheres</td>
<td>37</td>
</tr>
<tr>
<td>2.5</td>
<td>Uptake of PEI-capped spheres</td>
<td>39</td>
</tr>
<tr>
<td>2.6</td>
<td>Influence of particle size on the uptake of citrate-capped spheres</td>
<td>40</td>
</tr>
<tr>
<td>2.7</td>
<td>Influence of particle size on the uptake of PEG-capped spheres</td>
<td>42</td>
</tr>
<tr>
<td>2.8</td>
<td>Influence of particle size on the uptake of PEI-capped spheres</td>
<td>43</td>
</tr>
<tr>
<td>2.9</td>
<td>Influence of particle surface charge on the uptake of 5-6nm spheres</td>
<td>44</td>
</tr>
<tr>
<td>2.10</td>
<td>Influence of particle surface charge on the uptake of 13-18nm spheres</td>
<td>46</td>
</tr>
<tr>
<td>2.11</td>
<td>Influence of particle surface charge on the uptake of 50-55nm spheres</td>
<td>47</td>
</tr>
<tr>
<td>2.12</td>
<td>Influence of particle size and surface charge on the uptake of gold nanoparticles</td>
<td>49</td>
</tr>
<tr>
<td>3.1</td>
<td>Influence of FBS on the uptake of gold nanoparticles</td>
<td>69</td>
</tr>
<tr>
<td>3.2</td>
<td>Influence of BSA on the uptake of gold nanoparticles</td>
<td>70</td>
</tr>
<tr>
<td>3.3</td>
<td>Influence of iFBS on the uptake of gold nanoparticles in comparison to FBS uptake</td>
<td>72</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES (cont.)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4</td>
<td>Influence of dFBS on the uptake of gold nanoparticles in comparison to FBS uptake</td>
<td>73</td>
</tr>
<tr>
<td>3.5</td>
<td>Influence of cFBS on the uptake of gold nanoparticles in comparison to FBS uptake</td>
<td>75</td>
</tr>
<tr>
<td>3.6</td>
<td>Influence of aBSA on the uptake of gold nanoparticles in comparison to BSA uptake</td>
<td>76</td>
</tr>
<tr>
<td>3.7</td>
<td>Influence of hBSA on the uptake of gold nanoparticles in comparison to BSA uptake</td>
<td>78</td>
</tr>
<tr>
<td>3.8</td>
<td>Influence of tBSA on the uptake of gold nanoparticles in comparison to BSA uptake</td>
<td>79</td>
</tr>
<tr>
<td>3.9</td>
<td>Influence of fBSA on the uptake of gold nanoparticles in comparison to BSA uptake</td>
<td>81</td>
</tr>
<tr>
<td>3.10</td>
<td>Influence of calcium on the uptake of gold nanoparticles in comparison to BSA uptake</td>
<td>82</td>
</tr>
<tr>
<td>4.1</td>
<td>Inhibition of caveolae-dependent endocytosis when supplemented with FBS</td>
<td>102</td>
</tr>
<tr>
<td>4.2</td>
<td>Inhibition of caveolae-dependent endocytosis when supplemented with BSA</td>
<td>104</td>
</tr>
<tr>
<td>4.3</td>
<td>Inhibition of clathrin-dependent endocytosis when supplemented with FBS</td>
<td>106</td>
</tr>
<tr>
<td>4.4</td>
<td>Inhibition of clathrin-dependent endocytosis when supplemented with BSA</td>
<td>108</td>
</tr>
<tr>
<td>4.5</td>
<td>Inhibition of macropinocytosis when supplemented with FBS</td>
<td>110</td>
</tr>
<tr>
<td>4.6</td>
<td>Inhibition of macropinocytosis when supplemented with BSA</td>
<td>112</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-1</td>
<td>Hyclone Historical Data Sheet: Characterization of Standard FBS</td>
<td>136</td>
</tr>
<tr>
<td>Image</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>-------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>AI-1</td>
<td>TEM characterization of 5nm citrate-capped spheres</td>
<td>125</td>
</tr>
<tr>
<td>AI-2</td>
<td>TEM characterization of 18nm citrate-capped spheres</td>
<td>126</td>
</tr>
<tr>
<td>AI-3</td>
<td>TEM characterization of 50nm citrate-capped spheres</td>
<td>127</td>
</tr>
<tr>
<td>AI-4</td>
<td>TEM characterization of 6nm PEG-capped spheres</td>
<td>128</td>
</tr>
<tr>
<td>AI-5</td>
<td>TEM characterization of 15nm PEG-capped spheres</td>
<td>129</td>
</tr>
<tr>
<td>AI-6</td>
<td>TEM characterization of 55nm PEG-capped spheres</td>
<td>130</td>
</tr>
<tr>
<td>AI-7</td>
<td>TEM characterization of 5nm PEI-capped spheres</td>
<td>131</td>
</tr>
<tr>
<td>AI-8</td>
<td>TEM characterization of 13nm PEI-capped spheres</td>
<td>132</td>
</tr>
<tr>
<td>AI-9</td>
<td>TEM characterization of 30nm citrate-capped spheres</td>
<td>133</td>
</tr>
<tr>
<td>AI-10</td>
<td>TEM characterization of 30nm citrate-capped spheres</td>
<td>134</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

The novel properties that emerge as materials are taken to sizes of a few billionths of a meter have driven the nanotechnology revolution and the exponential growth of products and applications that utilize nanoparticles. These materials present exciting opportunities for the development of new products, enhancement of existing products, and the evolution of innovative procedures from drug delivery to medical imaging. Nanoparticles are being examined for use in various medical areas from imaging to the development of non-viral vectors for gene therapy. At the same time, however, uncertainties regarding potential deleterious effects of the interactions of nanoparticles with biological systems have alarmed scientists and governmental agencies. These uncertainties have the potential to polarize the public against possible benefits of nanotechnology as the lack of adequate knowledge may lead to sensationalized press.

To elicit a direct biological response, nanoparticles must be bioavailable; which usually means they must cross a membrane to reach an active site. Note, however, that some nanoparticles may cause adverse effects through the
release of ions or the generation of oxidizing equivalents without crossing a membrane. In most organisms, the initial biological membrane encountered during exposure is either lungs (gill or gill-like structures for aquatic organisms) or gut tract. Nanoparticles have been shown to cross the blood-brain barrier, move through embryonic zebrafish membranes, and enhance the transport of drugs through membranes [1,2,3]. However, studies that quantitatively characterize the influence of particle characteristics on such transport are currently lacking.

To accurately assess the impacts that nanoparticles may have on biological systems, it is necessary to understand how these materials interact with the environment. While organisms have been in contact with naturally occurring nanoparticles since the beginning of time, there are two key concerns surrounding the recent explosion in their manufacturing [4]. First, physical differences between naturally occurring nanoparticles and engineered nanoparticles must be characterized to determine if these differences might result in adverse responses. Second, the interactions between engineered nanoparticles and environmental and biological ligands must be characterized to assess their influence on bioavailability. The latter concern is particularly important as we move from studying pristine nanoparticles to those that have aged in the environment [4].

Most studies examining the impacts of nanoparticles involve exposures with pristine, freshly prepared products. While these studies prove useful in
understanding possible biological effects under rare conditions, they have done little to further our understanding of what is occurring in the field. A greater understanding of the aqueous solubility, rates of aggregation, reactivity, and degradation of nanoparticles is warranted, and one may suspect that these factors are inherently related to the physical properties of the particle itself. This chapter reviews current research on the influence of particle characteristics on biological uptake and environmental fate, while also identifying research needs in this area.

2. NANOPARTICLE FATE IN THE ENVIRONMENT

To better understand how particles interact with biological systems in the field, we must first comprehend the nature of nanoparticles in environmental settings, as the influence that the environment has on particle characteristics will need to be examined. It is inevitable that when engineered particles enter ecological systems, their characteristics will be altered. What we do not know is how this alteration will affect uptake and biotic responses. It is possible, and even highly probable, that the alterations in particle characteristics, such as aging, agglomeration, and aggregation, will cause different responses than what has been suggested from previous laboratory studies.

Due to the unlimited combinations of nanoparticle characteristics, the scientific community is concerned with the transport ability of these particles. It
has been suggested that due to particle shapes and sizes, nanoparticles will be intercepted, diffuse and settle from solution over time [5]. However, removal from solution may not always occur, as an examination of transport and deposition of fullerenes and oxide particles in porous media revealed that while fullerene and oxide aggregates were efficiently removed from the system, hydroxylated fullerenes remained highly mobile and were only removed at very low percentages [6]. Studies on silica and anatase particles have concluded similar results, as nearly half of the particles remained mobile and in solution [7]. Such results contradict the common notion that all particles will sediment and slowly become covered by organic matter and more sediment. However, it is generally accepted that particles will aggregate and fall out of solution; for this reason there is a need for more research with benthic species.

Apart from entering aquatic systems through run-off and deterioration, nanoparticles used in medical applications or personal care products may find their way into the environment through municipal and industrial wastewater treatment facilities. The ionic strength in freshwaters, and especially in marine environments, would increase the potential for engineered nanoparticles to aggregate and sediment [8]. However, surface modifications can sometimes out-compete forces of Brownian motion size based interactions and remain in solution. As a result, nanoparticle suspensions from wastewater discharges are of great concern, for nanoparticles have been shown to associate with many persistent contaminants of concern, including PAHs, dioxins, PCBs, PBDEs, and
endocrine disruptors. Currently, it is unknown how nanoparticles may affect contaminant bioavailability and transport [5].

3. NANOPARTICLE UPTAKE IN VIVO

In vivo studies present an opportunity for some of the greatest advancements in our understanding of nanoparticle interactions with biological systems. Furthering our knowledge on the influence of particle size, shape, and surface chemistry would benefit applications in human health systems, as well as in settings of environmental health and safety. The current lack of knowledge in these areas has greatly inhibited advancements in both the development of biomedical applications as well the quantitative assessment of the risk of nanoparticles to humans and ecosystems.

How nanoparticles interact with mammalian systems may depend on their physical and chemical characteristics. Human exposures to asbestos particles during mining operations and their widespread use led to a global pandemic of lung diseases [9]. These diseases, including lung cancer and asbestosis, were not only shown to be caused by the interactions of the asbestos fiber with lung tissue, but were also shown to be related to fiber aspect ratio. Specifically, these extreme detrimental effects were only present when initiated by thin, long particles with diameters of 3µm and lengths longer than 20µm [9]. Recent research has demonstrated that carbon nanotubes caused asbestos-like
pathogenicity when introduced into the abdominal cavity of mice, and the response, including inflammation and formation of granulomas, was size dependent, varying as a function of carbon nanotube length [10].

Wittmaack [11] reviewed published dose-response data on lung inflammation in rats and mice exposed to titanium dioxide or carbon nanoparticles and concluded that particle number, joint length, and surface area were useful metrics for data analysis. On the other hand, his results suggested that particle size-based surface area was not a significant metric of analysis. Other investigators have concluded that responses to nanoparticle exposure are size dependent. Inhalation studies involving rats exposed to polytetrafluoroethylene and titanium dioxide nanoparticles have suggested that induced lung inflammation was dependent on particle size [12]. When gold nanoparticles were intratracheal instilled in rats, the response was also size dependent, as smaller particles were removed from the lungs, and larger particles remained sequestered in the lungs [13]. Furthermore, it has been suggested that when exposed intravenously, small particles are easily eliminated through renal excretion pathways and larger particles are localized in marrow, the kidney, stomach, and associated with the phagocytic system and target endothelial systems [14,15,16,17].
While the impacts of particle size on uptake in mammals have been examined with many particle types, the influence of shape is currently lacking in the literature. However, when exposed to symmetrical and irregularly shaped polymer lipid nanoparticles, rats were able to clear standard shapes easily from the liver, while irregularly shaped particles remained localized in the spleen [18]. Such results suggest that particle shape is also an important characteristic that should be considered when trying to develop structure activity relationships.

*Daphnia magna* are freshwater pelagic filter feeders that have been used extensively in aquatic bioassays with nanoparticles. Due to their efficient filtering of the water column, any particles in the water column enter their gut tract. Furthermore, research from our laboratory suggests that effects on these organisms may not be limited to biochemical changes, but may also be induced by physical stressors surrounding the particle obstruction of gut tract function [19].

Aquatic vertebrates, such as the Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), are also subject to nanoparticle exposure. These organisms are standard test species that have been comprehensively examined in traditional solute toxicity tests, and have proven useful in investigating impacts of nanoparticles. Target areas of concern for nanoparticle exposure to these organisms include, but are not limited to, gills, gut, liver, and sometimes brains [20].
The shape of a nanoparticle may also determine how a particle interacts with aquatic organisms. When embryonic zebrafish were exposed to spherical nanoparticles versus dendritic rod shaped particles, there was no difference in accumulation within the lumen with respect to particle shape [21]. However, while the study could not conclusively link uptake to size differences, rod shaped particles were found to be more toxic than their spherical counterparts, which suggests that particle shape does lead to differences in levels of interaction for embryonic fish.

Similarly, research from our laboratory has produced comparable results with *D. magna*. Differences in toxicity were higher when exposed to carbon nanotubes than gold nanospheres [19]. Furthermore, while particles of any shape were not found to cross the lumen, retention times in the gut tract were slower for oblong rods and faster for the symmetrical spheres.

How nanoparticles interact with organisms in aquatic systems may also depend on the charge of the particle and the hydrophilicity of the particle surface. When Japanese medaka were exposed to gold nanospheres of similar size and shape with ligand attachments on their surfaces, uptake and bioaccumulation was greatest in fish intestines and gills, and lower in dorsal fins, brains, hearts, gonads, and livers [22]. More importantly, however, was the charge dependent uptake in these regions, as uptake was highest among cationic hydrophobic particles. Furthermore, cationic hydrophobic particles were widely distributed in
many organs and caused high mortality within 24 hr. All hydrophilic species, on
the other hand, were able to be excreted and are suggested to pose little impact
on environmental health and safety.

4. NANOPARTICLE UPTAKE IN VITRO

Nanoparticles must be bioavailable in order to elicit a biological response,
and to reach an active site they must usually cross a membrane. As a result, cell
culture studies have proven to be beneficial and are generally one of the first
tests conducted upon the synthesis of a new particle type. A major drawback,
however, to current literature involving interactions between cells and
nanoparticles is the lack of information provided, poor techniques, and lack of
transparency in methods of analysis. A standardization of methods and data
analysis involving cells and nanoparticles would facilitate the future
understanding of the underlying mechanisms behind their transport across cell
membranes.

Research has demonstrated that gold nanoparticles approximately 1nm in
diameter can penetrate membranes of HeLa cervical cancer cells, as well as the
nuclear membrane and even attach to DNA [23]. Larger gold nanoparticles have
also been shown to cross membranes. These include 14 nm citrate-coated gold
nanoparticles taken up by human dermal fibroblasts and 18nm particles taken up
by a human leukemia cell line, K562 [24, 25]. Cytotoxicity, as a function of
uptake, is also dependent upon particle size with exposure to smaller particles resulted in more toxicity when tested in four different cell lines [26]. However, there are conflicting results in the literature as to the influence of size on nanoparticle uptake. Thurn et al. [27] suggested that exposure to smaller particles resulted in the greatest uptake while Chithrani et al. [28] reported that larger particles of sizes averaging 50nm were taken up to a greater extent than smaller particles.

The shape of a nanoparticle also determines how it may enter a cell. Simply put, particles of oblong shape may not effectively reach a receptor site on the membrane, require more energy for processes of endocytosis, or would be less favorable to engulfment than more symmetrical shapes. In a study investigating nanorods and nanospheres of varying sizes, Chithrani et al. [28] concluded that levels of interaction were dependent upon shape, as the uptake of rod-shaped nanoparticles was lower than their spherical counterparts. Aside from this study, other studies examining the influence of particle shape on uptake in biological systems are severely lacking [27].

Just as size has an influential role in interactions between nanoparticles and cells, so does the surface chemistry including particle charge. This line of research is extremely important for areas of research involving drug delivery and imaging as there are times when a high level of interaction between cells and particles is desirable, and other times when no interaction is preferred [29].
Chung et al. [30] reported that the uptake of mesoporous silica particles was greatest in human mesenchymal stem cells when the particle surfaces had a positive charge. Positively charged particles are believed to have higher uptake rates in cells than negatively charged particles due to the overall negative net charge of cell membranes [27]. Neutrally charged particles, which resist protein sorption, result in the lowest levels of uptake [29].

Cell culture medium is commonly supplemented with serum proteins, as they are vital components necessary for cell growth and survival. As nanoparticles are introduced into the medium, the supplemented proteins readily sorb to particle surfaces due to hydrophobic and electrostatic interactions [31,32]. These interactions occur rapidly, require little external energy (e.g. mixing) and particles are completely coated with serum proteins within seconds to minutes [33]. Coincidentally, without this interaction nanoparticles would aggregate due to the ionic strength of the culture media [28,34]. Cell uptake of nanoparticles is dependent upon adsorbed proteins on their surface; these proteins interact non-specifically with membrane surfaces resulting in the endocytosis of the particle-protein complex [28]. Fetal bovine serum (FBS), a widely used media supplement, contains an array of different proteins, each of which has the potential to interact differently with a nanoparticle and the interaction may be driven by protein and particle specific characteristics. [33,35]. Since different proteins are known to enter cells through different mechanisms, understanding
the complex protein-particle relationship will help to further understand the processes by which nanoparticles enter cells [28].

Nanoparticles enter cells through three principal routes of endocytosis: clathrin-dependent, caveolae-dependent, and macropinocytosis [36]. Clathrin-dependent endocytosis is the mechanism by which surface proteins are taken up at specialized sites in the cell membrane where clathrin is recruited to form pits that are cleaved by dynamin to localize cargo into endosomes [37]. Caveolae-dependent endocytosis occurs similarly to clathrin-dependent in that surface proteins are collected in invaginations cleaved by dynamin and localized into endosomes; however this mechanism relies on the recruitment of caveolin and presence of cholesterol for internalization [38]. Macropinocytosis is a less understood mechanism of endocytosis in which membrane structures formed by actin filaments, such as lamellipodia, ruffles, and blebs, engulf macromolecules and particles into intracellular macropinosomes.

Previous studies have successfully described the utilization of different endocytotic pathways by nanoparticles through the use of pharmacological inhibitors [39,40,41]. However, all nanoparticles do not follow the same route of uptake. Instead, the mode of particle uptake is dependent upon many factors including, but not limited to, cell type, cell function, available proteins for conjugation, particle type, and particle characteristics. Further research is warranted to decipher how each of these interactions alters the uptake of
nanoparticles to facilitate the advancement of biomedical applications as well as risk assessment endeavors.

5. RESEARCH NEEDS AND CONCLUSIONS

Over the past decade, much effort has been made to investigate the effects of nanoparticles on a parallel level with their increased production. However, with current estimates suggesting less than 10% of all funding in nano-related disciplines is in areas of environmental health and safety, our understanding of their interactions with the environment around us is a mere fraction of the knowledge we now have concerning their possible benefits to society. To decrease the uncertainties currently surrounding the exposure, effects, and risk characterization of nanoparticles, more research must be conducted.

A major drawback to this advancement is not in our inability to detect nanoparticles, but in our inability to distinguish them from bulk materials [42]. With future advancements in analytical techniques and instrumentation, greater steps towards risk characterization will be made. Until then, however, it is necessary to use the tools and methods that are currently available to produce transparent, reproducible, high quality results. As evident in this review, particles characteristics are vital parameters to understanding particle interactions with environmental systems. As a result, it is imperative for future studies to account for these parameters in the analysis of their results.
While there is information concerning the effects of the physical characteristics of nanoparticles, most have done so independently of each other. Research investigating the collective influence that particle size, shape, and surface chemistry have on environmental interactions for a given core particle is both crucial and necessary to advance our understanding of the negative effects of particles, as well as their benefits. A complete characterization of uptake is necessary to accurately predict particle uptake. Furthermore, research investigating the processes by which particles interact with environmental media and processes by which they are taken up is also warranted. As a result, the goal of my dissertation is to characterize the uptake of gold nanoparticles by the mammalian cell line A549 through determining the roles of particle characteristics and culture medium components, and by describing potential modes by which particles are endocytosed. This information will facilitate future advancement in the field of nanotoxicology, while also decreasing the uncertainties currently surrounding the exposure, effects, and risk characterization of nanoparticles [4].
6. REFERENCES


CHAPTER 2

THE INFLUENCE OF PARTICLE SIZE AND SURFACE CHEMISTRY ON THE CELLULAR UPTAKE OF GOLD NANOPARTICLES

ABSTRACT: This study assessed the influence of the physical and chemical characteristics of gold nanospheres on their uptake by the mammalian cell line A549. A semi-factorial design was followed, examining three different particle sizes (roughly 5nm, 15nm and 50nm) modified by three different surface charges (anionic, cationic, and nonionic). The influence of particle concentration on cellular uptake was analyzed for 5nm and 50nm citrate-capped spheres over 24 h. Particle uptake increased as exposure concentration increased. The influence of particle size and surface charge on uptake was analyzed for each particle type over 2 h. With the exception of 50nm nonionic particles, uptake was charge dependent, as cationic particles were taken up to a greater extent than anionic particles, which were taken up more than nonionic particles. The uptake of gold nanoparticles was also dependent on particle size but this relationship may be a function of particle surface charge because particle uptake was greatest for larger negatively charged particles, and for smaller positively charged particles. Results may help to predict particle interactions with biological systems and facilitate future endeavors of quantifying the risk of particle release into the environment, as uptake cell is dependent on an array of particle characteristics.
1. INTRODUCTION

The recent growth of products and applications that contain, or utilize, nanoparticles has occurred in the absence of detailed knowledge of the interactions of these particles with environmental processes. According to the Woodrow Wilson Center’s Project on Emerging Nanotechnologies, there are over 1,000 consumer products on the market that contain or utilize nanoparticles. This number is expected to grow exponentially, as the potential use of these materials is unlimited. Currently, there is a need for quantitative research that provides a foundation upon which to predict biological interactions with nanoparticles. This information would facilitate both product development and future endeavors towards accurate assessments to the risk that nanoparticles may pose on the environment.

Due to their ability to cross cell membranes, gold nanoparticles have been proposed for detection of viruses, bacteria, and toxins; medical imaging; drug delivery; and cancer therapy applications [1]. Gold nanoparticles also serve as ideal model particles in research endeavors on particle uptake due to their stability and relatively low toxicity [2]. Gold particles are also easily synthesized in a variety of shapes, including, but not limited to, spheres, rods, cones, diamonds, and sheets. Also, the relative sizes of the shapes are able to be controlled, resulting in monodisperse solutions [3]. Furthermore, it is relatively straightforward to modify the surfaces of these particles with an array of coatings that result in differences in charge and polarity that could influence their
interactions with biological membranes. However, studies that quantitatively characterize the influence of particle size and surface chemistry are currently lacking.

Nanoparticles must be bioavailable to result in a biological response, and to reach an active site they must usually cross a membrane. Many biomedical applications of nanoparticles rely on their ability to cross membranes; therefore, the ability to predict particle movement based on physical characteristics would enhance both product development and product application. Similarly, environmental regulators are also interested in the potential for particles to cross membranes since these particles may pose the greatest risk.

The goal of this study was to investigate the influence of particle characteristics on the uptake of gold nanoparticles in mammalian cells. This study tests the hypothesis that particle uptake is a function of a particle’s chemical and physical characteristics. Potentially, each change elicits a new material that has the potential to interact differently with biological systems. This study is the first to collectively investigate the role of varying particle characteristics for a given core chemistry under the same conditions within the same cell line, which is critical to accurately model particle uptake. With the recent explosion of commercial products utilizing nanotechnology, it is imperative that predictive relationships be developed between particle characteristics and particle fate and effects to facilitate quantitative evaluation of risk.
2. MATERIALS AND METHODS

2.1 Particle Synthesis

Citrate-capped gold nanospheres, 5nm, 18nm, and 50nm, were obtained from the laboratory of Professor Catherine Murphy, University of Illinois at Urbana–Champaign. Particles were synthesized by methods previously described [4]. Briefly, 5nm citrate-capped particles were prepared by adding 0.5ml 0.01M HAuCl$_4$ and 0.5ml 0.01M citrate solution to 19ml ultrapure water. The mixture was stirred as 0.1M NaBH$_4$ was added, and the solution was allowed to mix for 2-3 hours. The 18nm particles were synthesized by adding 2.5ml 0.01M HAuCl$_4$ to 100ml ultrapure water while stirring, brought to a boil, and a 1% citrate solution was added to the mixture. The solution was stirred and boiled for 15-20 minutes until the solution was a deep red. The 50nm particles were synthesized by combining 2.25ml 18nm particles, 2.44ml 0.01M HAuCl$_4$, and 250ml ultrapure water, brought to a boil, and 4ml of 0.01M ascorbic acid solution was added. The solution was stirred and boiled for 1 hour until it turned purple with a brown tint.

Polyethylene glycol-capped nanoparticles (PEG) of 6, 15 and 55nm were obtained from the laboratory of Professor Christopher Kitchens, Clemson University. HAuCl$_4$ of 99.995% purity was purchased from Alfa Aesar; sodium borohydride was purchased from EMD Chemicals; both were used as received. Polyethylene glycol-thiol, PEG-SH, 1000 MW, was purchased from Laysan Bio,
Inc. Particles were synthesized using a modified procedure described previously [4,5]. The 6nm particles were synthesized using a modification of the citrate synthesis in which a 50 mL solution of 0.25mM HAuCl$_4$ was reduced with 0.5 mL of iced 0.1M NaBH$_4$ in the presence of PEG-SH (0.25mM) instead of citrate. The 15 nm particles were synthesized using a citrate seeding procedure in which 1mL of 0.1M aqueous citrate was added to 49mL of DI H$_2$O and brought to a boil while stirring. Once a rolling boil was reached, 0.5 mL of 0.05M aqueous HAuCl$_4$ was added and the solution was boiled for 10 minutes until the solution turned a bright red color. The solution was then allowed to cool and the citrate was ligand exchanged with PEG-SH. The 55 nm particles were synthesized by bringing a 50 mL solution of 0.25 mM HAuCl$_4$ to a rolling boil, then adding 0.25 mL of 20 nm gold citrate particles and 0.05 mL of 0.05mM citrate. The solution boiled for 15 minutes and then stirred for an additional 10 minutes without heat. The citrate was then ligand exchanged with PEG-SH.

Polyethyleneimine-capped nanoparticles (PEI) of 5 and 13nm were obtained from the laboratory of Professor Christopher Kitchens, Clemson University. Particles were synthesized using a modified procedure described previously [6]. Briefly, 5nm particles were synthesized by adding 2wt% PEI and 1mM HCl to 20ml ultrapure water while stirring, followed by the addition of 0.05M NaBH$_4$. The solution was stirred for 15 minutes. The 13nm particles were synthesized by adding 2wt% PEI and 1mM HCl to 20ml ultrapure water.
were gradually synthesized over the several days through the slow process of PEI reduction.

2.2 Particle Characterization

Particles were characterized in ultra-pure water for size and morphology using transmission electron microscopy (TEM) techniques (Clemson University Electron Microscopy Laboratory). Relative particle sizes were obtained by dynamic light scattering (DLS), and a measure of the electrokinetic potential (zeta potential) for each particle type was obtained. DLS and zeta measurements for citrate and PEG particles were analyzed using a Zetasizer (Malvern), while PEI particles were analyzed with a 90Plus Particle Size Analyzer (Brookhaven). Particles were analyzed after digestion in aqua regia (nitric acid:hydrochloric acid, 1:3 respectively), and dilution to a 5% acid solution. Gold mass was analyzed via inductively coupled plasma-mass spectrometry (ICP-MS) (Thermo Scientific XSeries 2).

2.3 Cell Culture

A549 adenocarcinomic human alveolar basal epithelial cells (ATCC, Rockville MD) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose, 584mg/l L-glutamine, and 110mg/l sodium pyruvate (Mediatech, Inc., Manassas, VA). DMEM was supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin/streptomycin (HyClone), and
2% non-essential amino acids (Sigma-Aldrich, St. Louis, MO) to form complete growth medium. Cells were cultured in 75cm² sterile, polystyrene, tissue culture-treated, vented flasks under clean conditions (Corning, Corning, NY), and incubated under 5% CO₂ at 37°C as described previously [7, 8].

For subculturing, cells in flasks were rinsed with 1X PBS (Fisher Scientific, Waltham, MA), and incubated in a 0.25% trypsin (Sigma-Aldrich) solution for 5-15 minutes, or until cells detached from the flask surface. Complete growth media was then added to inhibit trypsin activity, and cells were aspirated and transferred to sterile centrifuge tubes (Corning). Cells were centrifuged at 3,000 rpm (1,400 g) for three minutes, after which the supernatant was removed and discarded. Cells were then resuspended in complete growth medium, diluted, and transferred to sterile flasks. When not in use, cells were cryopreserved at -80°C in complete growth media supplemented with 5% dimethyl sulfoxide (DMSO) (Fisher Scientific).

2.4 Cell Exposures

Cells were removed from flasks using a 0.25% trypsin solution, and plated in sterile, polystyrene, tissue culture-treated 12-well plates (Costar, Corning, NY) at 100,000 cells per well. Cells were plated in complete growth medium and allowed 24 h for attachment to the plate surface [9]. After 24 h, the complete growth media was aspirated, and exposure medium containing gold nanoparticles was carefully added to each well.
2.4.1 Influence of Particle Concentration

Cells were exposed to increasing gold concentration of 5nm and 50nm negatively charged citrate-capped gold nanospheres for 24 h (n=3). Specifically, cells were exposed to 5nm particles at concentrations of 0.04 mg/l, 0.08mg/l, 0.16mg/l, and 0.32 mg/l, and 50nm particles at concentrations of 0.8mg/l, 1.6mg/l, 3.2mg/l, and 6.4mg/l.

2.4.2 Influence of Particle Characteristics

Cells were exposed to eight forms of gold nanospheres of differing size and surface charge (n=3). These particle types included 5nm negatively charged citrate-capped spheres, 18nm negatively charged citrate-capped spheres, 50nm negatively charged citrate-capped spheres, 6nm neutrally charged PEG-capped spheres, 15nm neutrally charged PEG-capped spheres, 55nm neutrally charged EPG-capped spheres, 5nm positively charged PEI-capped spheres, and 13nm positively charged PEI-capped spheres. Cells were exposed to each particle type at a concentration of 1mg/l for 0.5, 1.0, 1.5, and 2.0 h. The majority of previous examining the uptake of nanoparticles by cells have focused on 24 h uptake; however, many cells, including A549, double within 24 h. In an effort to limit the variability and impact of cell growth on my results, exposures were shortened to 2 h.
2.5 Analytical Procedure

At the end of each time-point, exposure medium was removed and cells were carefully rinsed with 1X PBS to remove residual particles. Gold nanospheres were then digested within each well following the addition of 1ml 30% aqua regia, and plates were incubated at 37°C under 5% CO₂ for 30 minutes. Samples were removed from each well and diluted to a 5% acid solution. Gold uptake was analyzed via inductively coupled plasma-mass spectrometry (ICP-MS) (Thermo Scientific XSeries 2).

2.6 Data Analysis

Data presented are in a mass-basis format (Au ng/10⁵ Cells) and represent the average and standard deviation of triplicate samples. All statistical analysis was performed using SAS and JMP Pro 10 (SAS Institute, Cary, NC, USA). Statistically significant differences were determined by a one way analysis of variance (ANOVA) in conjunction with Tukey-Kramer’s test for post hoc analysis or a t-test. A p value less than 0.05 was considered statistically significant.
3. RESULTS

3.1 Particle Characterization

Using TEM, citrate-capped gold nanoparticles were found to be spherical in shape with diameters of 5nm (4.97±1.48), 18nm (18.06±3.77), and 50nm (49.87±12.26) (Appendix I: Image AI-1, Image AI-2, and Image AI-3, respectively). DLS estimated that 5nm citrate particles had a diameter of 11nm (10.69±0.27), 18nm citrate particles had a diameter of 20nm (20.13±0.49), and 50nm citrate particles had a diameter of 60nm (59.89±0.11).

TEM analysis revealed that PEG-capped gold nanoparticles were spherical in shape with diameters of 6nm (6.16±1.94), 15nm (15.10±2.01), and 55nm (54.84±22.31) (Appendix I: Image AI-4, Image AI-5, and Image AI-6, respectively). DLS estimated that 6nm PEG particles had a diameter of 41nm (41.39±0.55), 15nm PEG particles had a diameter of 22nm (22.15±0.39), and 55nm PEG particles had a diameter of 60nm (60.86±0.41).

TEM analysis revealed that PEI-capped gold particles were spherical in shape with diameters of 5nm (4.77±2.14), and 13nm (13.38±4.49) (Appendix I: Image AI-7 and Image AI-8, respectively). DLS estimated that 5nm PEI particles were 18nm (17.52±0.80) in diameter and that 13nm PEI particles were 25nm (24.79±0.65) in diameter. While a degree of polydispersity is inevitable in particle synthesis, particles were monodisperse with respect to average particle sizes described.
Zeta potential measurements for citrate-capped spheres were -30mV for 5nm particles, -45mV for 18nm particles, and -40mV for 50nm gold nanospheres. Zeta potential measurements for PEG-capped spheres were -12mV for 6nm particles, -18mV for 15nm particles, and -8mV for 55nm particles. Zeta potential measurements for PEI-capped particles were 34mV for 5nm particles and 32 mV for 13nm particles. The measurements suggest that particles used in this study were appropriately charged and stable in suspension.

### 3.2 Influence of Particle Concentration

Cells were exposed to increasing concentration of 5nm and 50nm negatively charged citrate-capped gold nanospheres for 24 h. Uptake of 5nm citrate-capped spheres was concentration dependent and was positively related to exposure concentration (Figure 2.1). After 24 h, gold uptake in $10^5$ cells was 0.17±0.03ng when exposed to 0.04mg/l, 0.30±0.05ng when exposed to 0.08mg/l, 0.63±0.07ng when exposed to 0.16mg/l, and 0.91±0.08ng when exposed to 0.32mg/l 5nm citrate-capped gold nanospheres.

Uptake of 50nm citrate-capped spheres was also concentration dependent and was positively related to exposure concentration (Figure 2.2). After 24 h, gold uptake in $10^5$ cells was 2.56±0.33ng when exposed to 0.8mg/l, 52.44±0.79ng when exposed to 1.6mg/l, 179.82±6.80 when exposed to 3.2mg/l, and 457.28±24.56ng when exposed to 6.4mg/l 5nm citrate-capped gold nanospheres.
Figure 2.1: The uptake of 5nm citrate-capped spheres at increasing concentrations over 24 h (mean ± standard deviation, n=3). Uptake was dose-dependent, and increased as the concentration of particles increased.
Figure 2.2: The uptake of 50nm citrate-capped spheres at increasing concentrations over 24 h (mean ± standard deviation, n=3). Uptake was dose-dependent, and increased as the concentration of particles increased.
3.3 Influence of Particle Characteristics

Cells were exposed to eight forms of gold nanospheres of differing size and surface charge. Cells were exposed to each particle type for a period of 0.5, 1.0, 1.5, and 2.0 h, and uptake was positively related to exposure time, with the exception of 5nm PEG and 50nm PEG particles for which uptake was extremely low and variable.

Cells were first exposed to 5nm, 18nm, and 50nm citrate-capped gold nanospheres (Figure 2.3). Gold uptake of 5nm particles in 10^5 cells was 2.42±1.20ng after 0.5 h, 8.31±1.27ng after 1.0 h, 12.12±2.63ng after 1.5 h, and 12.96±2.90ng after 2.0 h of exposure. Gold uptake of 18nm particles in 10^5 cells was 5.31±0.71ng after 0.5 h, 11.79±1.77ng after 1.0 h, 17.98±4.32ng after 1.5 h, and 23.70±0.47ng after 2.0 h of exposure. Gold uptake of 50nm particles in 10^5 cells was 1.15±0.18ng after 0.5 h, 10.06±3.73ng after 1.0 h, 14.98±0.97ng after 1.5 h, and 25.66±1.90ng after 2.0 h of exposure.

Cells were exposed to 6nm, 15nm, and 55nm PEG-capped gold nanospheres (Figure 2.4). Gold uptake of 6nm particles in 10^5 cells was below the limit of detection (LOD, 0.1ng/10^5 cells) after 0.5 h, 0.71±0.62ng after 1.0 h, 0.36±0.35ng after 1.5 h, and 0.78±0.10ng after 2.0 h of exposure. Uptake of 15nm particles in 10^5 cells was below the LOD after 0.5 h, 1.40±0.73ng after 1.0 h, 0.66±0.10ng after 1.5 h, and 0.27±0.33ng after 2.0 h of exposure. Gold uptake
Figure 2.3: The uptake of citrate-capped spheres over 2 h (mean ± standard deviation, n=3), when exposed to 1mg/l gold concentration.
Figure 2.4: The uptake of PEG-capped spheres over 2 h (mean ± standard deviation, n=3), when exposed to 1mg/l gold concentration.
of 55nm particles in $10^5$ cells was 7.18±1.10ng after 0.5 h, 54.11±2.00ng after 1.0 h, 110.88±3.50ng after 1.5 h, and 173.02±2.29ng after 2.0 h of exposure.

Cells were exposed to 5nm and 13nm PEI-capped gold nanospheres (Figure 2.5). Gold uptake of 5nm particles in $10^5$ cells was 18.81±1.68ng after 0.5 h, 33.13±0.38ng after 1.0 h, 38.50±1.68ng after 1.5 h, and 45.84±1.26ng after 2.0 h of exposure. Gold uptake of 13nm particles in $10^5$ cells was 16.32±0.89ng after 0.5 h, 28.40±0.50ng after 1.0 h, 36.62±1.09ng after 1.5 h, and 40.75±1.77ng after 2.0 h of exposure.

### 3.4 Influence of Particle Size

To investigate the influence of particle size on the uptake of gold nanoparticles, comparisons among levels of uptake after 2 h were conducted among particles of the same surface chemistry. Average particle uptake of citrate-capped gold nanospheres was 12.96±2.90ng for 5nm particles, 23.70±0.47ng for 18nm particles, and 25.66±1.90ng for 50nm particles when $10^5$ cells were exposed (Figure 2.6). An analysis of variance (ANOVA) in conjunction with a Tukey-Kramer HSD post hoc test was conducted to compare means of each particle type. The uptake of 5nm particles was significantly lower than the uptake of 18nm particles ($p=0.0015$), as well as significantly lower than the uptake of 50nm particles ($p=0.0006$). There was no significant difference between the uptake of 18nm and 50nm citrate-capped gold nanospheres ($p=0.5014$).
Figure 2.5: The uptake of PEI-capped spheres over 2 h (mean ± standard deviation, n=3), when exposed to 1mg/l gold concentration.
Figure 2.6: Comparison of particle size dependence on citrate-capped particle uptake after 2 h exposure to 1mg/l gold (mean ± standard deviation, n=3). Values with the same letter are not statistically different from each other.
The average particle uptake of PEG-capped gold nanospheres after 2 h exposure was 0.78±0.10ng for 6nm particles, 0.27±0.33ng for 15nm particles, and 173.02±2.29ng for 55nm particles when $10^5$ cells were exposed (Figure 2.7). An analysis of variance (ANOVA) in conjunction with a Tukey-Kramer HSD post hoc test was conducted to compare means of each particle type. There was no significant difference between the uptake of 6nm and 15nm particles ($p=0.8907$). The uptake of 55nm particles was significantly greater than the uptake of 6nm particles ($p<0.0001$), as well as significantly greater than the uptake of 15nm particles ($p<0.0001$).

The average particle uptake of PEI-capped gold nanospheres after 2 h exposure was 45.84±1.26ng for 5nm particles and 40.75±1.77ng for 13nm particles when $10^5$ cells were exposed (Figure 2.8). A t-test was conducted to compare means of the two particle types. The uptake of 5nm particles was significantly greater than the uptake of 13nm particles ($p=0.0190$).

### 3.5 Influence of Particle Surface Charge

To investigate the influence of surface chemistry differences on the uptake of gold nanoparticles, comparisons between levels of uptake after 2 h were conducted among particles of similar size and different surface charges. Average particle uptake for 5nm citrate-capped spheres was 12.96±2.90ng, 6nm PEG-capped spheres was 0.78±0.10ng, and 5nm PEI-capped spheres was 45.84±1.26ng when $10^5$ cells were exposed (Figure 2.9). An analysis of variance
Figure 2.7: Comparison of particle size dependence on PEG-capped particle uptake after 2 h exposure to 1mg/l gold (mean ± standard deviation, n=3). Values with the same letter are not statistically different from each other.
Figure 2.8: Comparison of particle size dependence on PEI-capped particle uptake after 2 h exposure to 1mg/l gold (mean ± standard deviation, n=3). Values with the same letter are not statistically different from each other.
Figure 2.9: Comparison of particle charge dependence on 5-6nm particle uptake after 2 h exposure to 1mg/l gold (mean ± standard deviation, n=3). Values with the same letter are not statistically different from each other.
(ANOVA) in conjunction with a Tukey-Kramer HSD post hoc test was conducted to compare means of each particle type. For the smallest particles, the uptake of cationic PEI-capped particles was significantly greater than the uptake of anionic citrate-capped particles (p<0.0001), as well as significantly greater than the uptake of nonionic PEG-capped particles (p<0.0001). Furthermore, the uptake of nonionic PEG-capped particles was significantly lower than the uptake of anionic citrate-capped particles (p=0.0004).

Average particle uptake for 18nm citrate-capped spheres was 23.70±0.47ng, 15nm PEG-capped spheres was 0.27±0.33ng, and 13nm PEI-capped spheres was 40.75±1.77ng when 10^5 cells were exposed for 2 h (Figure 2.10). An analysis of variance (ANOVA) in conjunction with a Tukey-Kramer HSD post hoc test was conducted to compare means of each particle type. For the 14-18nm particles, the uptake of cationic PEI-capped particles was significantly greater than the uptake of anionic citrate-capped particles (p<0.0001), as well as significantly greater than the uptake of nonionic PEG-capped particles (p<0.0001). Once again, the uptake of nonionic PEG-capped particles was significantly lower than the uptake of anionic citrate-capped particles (p<0.0001).

The average particle uptake for 50nm citrate-capped spheres was 25.66±1.90ng and 55nm PEG-capped spheres was 173.02±2.29ng when 10^5 cells were exposed for 2 h (Figure 2.11). A t-test was conducted to compare means of the two particle types, and the uptake of nonionic PEG-capped
Figure 2.10: Comparison of particle charge dependence on 14-18nm particle uptake after 2 h exposure to 1mg/l gold (mean ± standard deviation, n=3). Values with the same letter are not statistically different from each other.
Figure 2.11: Comparison of particle charge dependence on 50-55nm particle uptake after 2 h exposure to 1mg/l gold (mean ± standard deviation, n=3). Values with the same letter are not statistically different from each other.
particles was significantly greater than the uptake of 50nm anionic citrate-capped particles (p<0.0001).

3.6 Size and Surface Effects

To examine the combined roles that particle size and surface charge, an analysis of variance (ANOVA) in conjunction with a Tukey-Kramer HSD post hoc test was conducted to compare means of each particle type that were tested (Figure 2.12). Overall, uptake was greatest among 55nm PEG-capped particles (173.02±2.29ng, p<0.0001). With the exception of 55nm PEG-capped particles, surface charge was the determining factor in uptake as cationic particles were taken up to a greater extent than anionic particles, which were taken up to a greater extent than nonionic particles.

Within the set of cationic particles, uptake of 5nm PEI-capped particles (45.84±1.26ng) was significantly greater than that of 13nm PEI-capped particles (p=0.0303). There was no significant difference among anionic 50nm citrate-capped particles (25.66±1.90ng) and 18nm citrate capped particles (23.70±0.47ng) (p=0.8280); however, both were taken up significantly more than the uptake of 5nm citrate-capped particles (12.96±2.90ng) (p<0.0001). Finally, uptake was lowest among nonionic 6nm PEG-capped particles (0.78±0.10ng) and 15nm PEG-capped particles (0.27±0.33ng), but there was no significant difference between the two particle types (p=0.9999).
Figure 2.12: Collective comparison of particle charge and size dependence on uptake after 2 h exposure of 1mg/l gold (mean ± standard deviation, n=3). Values with the same letter are not statistically different from each other.
4. DISCUSSION

In my study, the uptake of both 5nm and 50nm citrate-capped gold nanospheres exhibited a dose dependent relationship, as uptake increased with an increase in exposure concentration. Chithrani et al. [10] have also examined the dependence of particle concentration on the uptake of citrate-capped nanospheres by HeLa cells. Their analysis revealed that the uptake of 14nm, 50nm and 74nm particles was also dose-dependent. However, in their study, uptake plateaud. Furthermore, they estimated that HeLa cells were only able to take up 3000 14nm particles, 6160 50nm particles, and 2988 74nm particles.

Chithrani et al. [10] also conducted studies in which they examined the dependence of particle size and shape on the uptake of citrate-capped nanospheres by HeLa cells. In their study, they compared the uptake of 14nm, 30nm, 50nm, 74nm, and 100nm particles. Uptake increased as particle size increased from 14nm-50nm. However, uptake declined as particle size increased over 50nm, such that the uptake of 74nm particles was similar to that of 30nm particles, and the uptake of 100nm particles was lower than the uptake of 14nm particles. While we I did not see a significant difference in the uptake of 50nm citrate particles in comparison to 18nm citrate particles, my results are similar in that the uptake of 50nm particles was greater than that of smaller, 5nm particles.

PEG coated particles are designed to resist protein adsorption, which could greatly alter their levels of uptake as many studies suggest protein sorption
is a major facilitator of particle uptake [10,11]. Nativo et al. [12] found that gold nanoparticles coated in PEG are not taken up by HeLa cells. My results are similar to their study in that minimal uptake by 6nm and 15nm particles was observed. However, in my study uptake was greatest, for all particle types, with 50nm PEG-coated gold nanospheres. This response could be due to differences in the spatial configurational freedom of the PEG chains on the surface of the particle types, as alterations in the architectural structure of the chains can lead to protein interaction, thus enhancing uptake [11, 13, 14].

With exception of 50nm PEG-coated gold nanospheres, I observed the greatest uptake in positively charged particles, followed by negatively charged particles, and finally, smaller particles with a neutral charge. A thorough review by Thurn et al. [15] examined the uptake of particles of different core chemistries, including titanium dioxide, quantum dots and gold, and varying surface charges. They describe that cells preferentially took up positively charged particles in comparison to negatively charged particles. A review by Verma and Stellacci [11] arrived at the same conclusion that the uptake was greatest in cells exposed to positively charged particles. Thurn et al. [15] attributed this phenomenon to the negative charge of the cell membrane.

Results from this study suggest that the charge of the particles surface is the most important factor contributing to enhanced gold nanoparticle uptake, as positively charged particles were taken up greater than negatively charged
particles. Investigations into the effect of surface charge on the uptake of mesoporous silica nanoparticles by mesenchymal stem cells suggest this as well; as uptake was correlated to positively charged particles [16]. These results have also been observed in vivo with gold nanoparticles, as the bioaccumulation of particles in medaka fish intestines and gills was highest among those with positively charged surfaces [17]. This further validates the need for accurate modeling of particle interactions with membrane surfaces, as in vitro testing may prove capable of predicting in vivo responses to nanoparticle exposures.

However, it is quite possible that these results are not simply due to the negative charge of the cell membrane [15]. Instead, cells and tissues likely recognize the proteins that readily and rapidly sorb to particle surfaces due to hydrophobic and electrostatic interactions [18,19,20]. Hence, it is critical to further investigate the particle-protein interactions involved and the formation of protein coronas, as the surface charge of the particle determines the proteins involved in corona formation; which may ultimately regulate biological responses [21].

5. CONCLUSIONS

Nanoparticle uptake was dependent upon both concentration of exposure, as well as length of exposure. The uptake of gold nanoparticles was also dependent on particle size but this relationship is a function of particle surface
charge; as particle uptake was greatest in larger negatively charged particles and in smaller positively charged particles. With exception to 55nm PEG particles, uptake was ultimately dependent upon particle surface charge; as cells preferentially took up positively charged particles over negatively charged particles, followed by neutrally charged particles. However, further investigation is needed to accurately predict the roles of particle characteristics to facilitate future assessments of risk that these particles may have on biological systems.
6. REFERENCES


CHAPTER 3

NOVEL APPROACHES TO ELUCIDATE SERUM COMPONENTS INVOLVED IN THE CELLULAR UPTAKE OF NANOPARTICLES

ABSTRACT: This study assessed the influence of the serum components of cell growth media on the uptake of 30nm gold nanospheres by the mammalian cell line A549. Cells were exposed to particles with exposure media supplemented with differing concentrations of fetal bovine serum (FBS) and bovine serum albumin (BSA). The uptake of particles exposed in FBS was inversely related to protein exposure concentration, which was not observed with particles in media supplemented with BSA, suggesting that certain components of FBS deter particle uptake. Cells were also exposed in media supplemented with dialyzed FBS, charcoal/dextran treated FBS, and FBS that contained lower concentrations of immunoglobulin G (IgG) than found in standard FBS. Particle uptake increased significantly following charcoal/dextran treatment suggesting that growth factors and steroids may inhibit particle uptake. Cells were exposed in media supplemented with BSA in addition to apolipoproteins, fetuin, hemoglobin, and transferrin. Uptake decreased significantly when cells were exposed with BSA and fetuin, suggesting that fetuin may deter particle uptake. Finally, particle uptake decreased when calcium was removed from the exposure media, suggesting that uptake occurs through a calcium dependent process.
1. INTRODUCTION

The recent growth of products and applications that contain, or utilize, nanomaterials has occurred in the absence of detailed knowledge of the interactions of nanoparticles with environmental processes. At the core of this lack of understanding are the processes by which nanoparticles interact with biological membranes. While studies have attempted to elucidate the modes by which particles are endocytosed, no direct mechanistic processes have been described.

A review of the literature on NP uptake revealed that the vast majority of the work has been performed using in vitro cell culture assays. Results of the research efforts to date can be contradictory, as no definitive conclusions have been agreed on about what particle types result in the greatest degree of uptake [1,2]. One potential reason for the inconsistencies is the complex cell culture medium used in the bioassays. Few, if any, studies have quantified the interactions of the NP of interest with the components of the medium; in spite of the fact that several investigators have reported on the role of protein corona formation on the surfaces of nanoparticles [3,4,5]. Hence, it is critical to better understand particle-protein interactions and the formation of protein coronas, for the cell likely recognizes the proteins that readily and rapidly sorb to particle surfaces due to hydrophobic and electrostatic interactions [6,7,8].
The challenge in undertaking a study to elucidate which protein components of a complex culture medium enhance or inhibit nanoparticle uptake is that there are many combinations to investigate. To reduce the number of assays I have taken a simplified approach called a protein identification evaluation (PIE). This process is similar to that used to identify the toxic component(s) of complex effluents, through Toxicity Identification Evaluations (TIEs) [9,10]. The experimental design for my research began with the entire cell culture medium, and then used physical and chemical separation techniques to remove certain components. After each separation, another assay is performed and the effect of the separation on NP uptake is quantified. Additional separation techniques are then applied to the fractions and bioassays repeated. After several iterations the protein(s) remaining in each fraction will be identified. Using this procedure, it is possible to isolate and identify proteins that enhance or inhibit NP uptake.

The goal of this research was to identify the components of the cell culture medium that enhance the cell uptake of gold nanoparticles. Fetal bovine serum (FBS), a widely used media supplement, contains an array of different proteins, each of which has the potential to interact with particle surfaces [1]. The affinity of a given protein to interact with a nanoparticle may be dependent upon the physical characteristics of that particle [11,12]. As different proteins are known to enter cells through different mechanisms, an understanding of particle characteristics, as well as surface-bound proteins, will help to further understand
the processes by which nanoparticles enter cells [1]. I hypothesize that the uptake of gold nanospheres by A549 cells is dependent upon the interactions of the particles with serum supplements in the exposure medium, and these interactions mediate the transport of particles across membranes.

2. MATERIALS AND METHODS

2.1 Particle Synthesis

Citrate-capped gold nanospheres, 30nm, were produced in-house by methods previously described [13]. Briefly, particles were synthesized by adding 2.5ml 0.01M HAuCl$_4$ to 100ml ultrapure water while stirring. The solution was brought to a boil, and a 1% citrate solution was added to the mixture. The solution was then allowed to stir and continue boiling for 15-20 minutes until the solution was a deep red.

2.2 Particle Characterization

Particles were characterized in ultra-pure water for size and morphology using transmission electron microscopy (TEM) techniques (Clemson University Electron Microscopy Laboratory). Relative particle sizes were obtained by dynamic light scattering (DLS), and a measure of the electrokinetic potential (zeta potential) for each particle type was obtained. DLS and zeta measurements
were analyzed using a 90Plus Particle Size Analyzer (Brookhaven). Particles were analyzed after digestion in aqua regia (nitric acid:hydrochloric acid, 1:3 respectively), and dilution to a 5% acid solution. Gold mass was analyzed via inductively coupled plasma-mass spectrometry (ICP-MS) (Thermo Scientific XSeries 2).

2.3 Cell Culture

A549 adenocarcinomic human alveolar basal epithelial cells (ATCC, Rockville MD) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose, 584mg/l L-glutamine, and 110mg/l sodium pyruvate (Mediatech, Inc., Manassas, VA). DMEM was supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin/streptomycin (HyClone), and 2% non-essential amino acids (Sigma-Aldrich, St. Louis, MO) to form complete growth medium. Cells were cultured in 75cm² sterile, polystyrene, tissue culture-treated, vented flasks under clean conditions (Corning, Corning, NY), and incubated under 5% CO₂ at 37°C as described previously [14, 15].

For subculturing, cells in flasks were rinsed with 1X PBS (Fisher Scientific, Waltham, MA), and incubated in a 0.25% trypsin (Sigma-Aldrich) solution for 5-15 minutes, or until cells detached from the flask surface. Complete growth media was then added to inhibit trypsin activity, and cells were aspirated and transferred to sterile centrifuge tubes (Corning). Cells were centrifuged at 3,000 rpm (1,400 g) for three minutes, after which the supernatant was removed and
discarded. Cells were then resuspended in complete growth medium, diluted, and transferred to sterile flasks. When not in use, cells were cryopreserved at -80 °C in complete growth media supplemented with 5% dimethyl sulfoxide (DMSO) (Fisher Scientific).

2.4 Cell Exposures

Cells were removed from flasks using a 0.25% trypsin solution, and plated in sterile, polystyrene, tissue culture-treated 12-well plates (Costar, Corning, NY) at 100,000 cells per well. Cells were plated in complete growth medium and allowed 24 h for attachment to the plate surface [16]. After 24 h, complete growth media was aspirated, and exposure medium containing gold nanoparticles was carefully added to each well. Cell viability was examined and confirmed for each exposure medium using the Promega™ CellTiter 96™ AQueous One Solution Cell Proliferation Assay (MTS) (Fisher Scientific). Viability was not significantly different from that of controls under any of the conditions tested.

2.4.1 FBS vs. BSA Supplementation

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with FBS (30.3g/l) and DMEM supplemented with bovine serum albumin (BSA) (Fisher Scientific). A BSA solution was formulated by diluting BSA in 1X PBS to a concentration of 22.6g/l, to mimic the average albumin
concentration of Standard FBS (HyClone Historical Data Sheet, Appendix II). Complete exposure medium was formulated to contain 1% FBS, 5% FBS, 10% FBS, 20% FBS, 1% BSA, 5% BSA, 10% BSA, and 20% BSA. Cells were exposed to 1mg/l gold nanospheres in each exposure medium for 2 h. At the end of each time-point, exposure medium was removed and cells were carefully rinsed with 1X PBS to remove residual particles.

### 2.4.2 Phase I PIE

A Phase I Particle Interaction Evaluation (PIE) was conducted through the use of different forms of FBS that remove specific components from the serum. Cells were exposed to increasing concentrations of Standard FBS (FBS) (30.3g/l), Charcoal/Dextran Treated FBS (cFBS) (25.7g/l), Dialyzed FBS (dFBS) (29.5 g/l), and Super-Low immunoglobulin G (IgG) FBS (iFBS) (29.9g/l).

Complete exposure medium was formulated to contain 1%, 5%, 10%, and 20% of each type of FBS. Cells were exposed to 1mg/l 30nm citrate-capped gold nanospheres in each exposure medium for 2 h. At the end of each time-point, exposure medium was removed and cells were carefully rinsed with 1X PBS to remove residual particles.
2.4.3 Phase II PIE

A Phase II Particle Interaction Evaluation (PIE) was conducted through the addition of FBS components to BSA. Concentrations of each component were determined from HyClone’s Historical Data Sheet for Standard FBS (Appendix II). Specifically, a 320mg/l cholesterol-rich apolipoprotein solution was prepared in BSA (aBSA), an 81mg/l hemoglobin solution was prepared in BSA (hBSA), and a 1.79 g/l transferrin solution was prepared in BSA (tBSA) by diluting the apo-transferrin concentration to 0.38g/l and the holo-transferrin concentration to 1.41 g/l. HyClone’s Historical Data Sheet does not quantify levels of fetuin, however, based on the average total protein concentration, and protein concentrations listed, as well as values in the literature, fetuin concentrations were estimated to be 10g/l [17]. As such, a 10g/l fetuin solution was prepared in BSA (fBSA). All proteins were purchased from Sigma-Aldrich.

Complete exposure medium was formulated to contain 1%, 5%, 10%, and 20% of BSA, and each protein-BSA complex. Cells were exposed to 1mg/l 30nm citrate-capped gold nanospheres in each exposure medium for 2 h. At the end of each time-point, exposure medium was removed and cells were carefully rinsed with 1X PBS to remove residual particles. Cell viability was examined and confirmed for each exposure medium using the Promega™ CellTiter 96™ AQueous One Solution Cell Proliferation Assay (MTS) (Fisher Scientific).
2.4.4 Influence of Calcium

The influence of calcium on particle uptake was examined by exposing cells to 30nm gold nanospheres in standard DMEM which contains calcium (200mg/l) (Mediatech), and calcium free DMEM (Invitrogen). Calcium free DMEM was supplemented with L-glutamine and sodium pyruvate, at concentrations of 584mg/l and 110mg/l, respectively, to mimic complete growth medium (Invitrogen). Complete exposure medium was formulated to contain 10% FBS in standard DMEM, 10% FBS in calcium free DMEM, 10% BSA in standard DMEM, and 10% BSA in calcium free DMEM. Cells were exposed to 1mg/l gold nanospheres in each exposure medium for 2 h. At the end of each time-point, exposure medium was removed and cells were carefully rinsed with 1X PBS to remove residual particles.

2.5 Analytical Procedure

At the end of each time-point, exposure medium was removed and cells were carefully rinsed with 1X PBS to remove residual particles. Gold nanospheres were digested within each well following the addition of 1ml 30% aqua regia, and plates were incubated at 37°C under 5% CO₂ for 30 minutes. Samples were removed from each well and diluted to a 5% acid solution. Gold uptake was analyzed via inductively coupled ICP-MS (Thermo Scientific XSeries 2).
2.6 Data Analysis

Data presented are in a mass-basis format (Au ng/10^5 Cells) and represent the average and standard deviation of triplicate samples. All statistical analysis was performed using SAS and JMP Pro 10 (SAS Institute, Cary, NC, USA). Statistically significant differences were determined by a t-test and relationships were determined by simple linear regression. Statistical differences between relationships were determined by an analysis of covariance (ANCOVA). A p value less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Particle Characterization

Particles were characterized in ultrapure water by TEM for size and morphology. Measurements were quantified at random with TEM software, as well as with ImageJ (National Institutes of Health). Citrate-capped gold nanoparticles were found to be spherical in shape with a diameter of 30nm (29.57±8.78) (Appendix I: Image AI-9). DLS estimated that 30nm citrate particles had a diameter of 36nm (35.74±0.65). Zeta potential measurements were also quantified and used to predict particle charge and stability in solution. The average zeta potential for 30nm citrate-capped spheres was -45mV (-45.19±5.87). These results reveal that the particles used in these experiments were monodispersed and stable in suspension.
3.2 FBS vs. BSA Supplementation

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with increasing concentrations of FBS. After 2h, gold uptake in $10^5$ cells was 148.64±39.43ng when exposed in exposure medium containing 1% FBS, 64.45±9.41ng when exposed in exposure medium containing 5% FBS, 30.01±5.20ng when exposed in exposure medium containing 10% FBS, and 19.04±2.49ng when exposed in exposure medium containing 20% FBS (Figure 3.1). Uptake was negatively correlated to FBS concentration, as uptake decreased with an increase in protein concentration ($R^2=0.85$, $p<0.0001$).

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with increasing concentrations of BSA. After 2h, gold uptake in $10^5$ cells was 119.90±16.59ng when exposed in exposure medium containing 1% BSA, 129.98±4.48ng when exposed in exposure medium containing 5% BSA, 134.68±9.57ng when exposed in exposure medium containing 10% BSA, and 132.94±5.55ng when exposed in exposure medium containing 20% BSA (Figure 3.2). Regression analysis confirmed that there was no relationship between nanoparticle uptake and BSA concentration ($R^2=0.27$, $p=0.0858$).
Figure 3.1: The influence of FBS on the uptake of gold nanoparticles by A549 Cells.
Figure 3.2: The influence of BSA on the uptake of gold nanoparticles by A549 Cells.
3.3 Phase I PIE

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with increasing concentrations of Super-Low IgG FBS (iFBS). After 2h, gold uptake in $10^5$ cells was $128.46\pm13.15$ng when exposed in exposure medium containing 1% iFBS, $72.60\pm4.27$ng when exposed in exposure medium containing 5% iFBS, $33.94\pm6.10$ng when exposed in exposure medium containing 10% iFBS, and $21.80\pm1.68$ng when exposed in exposure medium containing 20% iFBS (Figure 3.3). Uptake was negatively correlated to iFBS concentration, as uptake decreased with an increase in protein concentration ($R^2=0.89$, $p<0.0001$). An analysis of covariance (ANCOVA) was utilized to examine differences between the uptake of particles exposed in iFBS in comparison to FBS. There was no statistical difference in the regression slopes ($p=0.4478$).

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with increasing concentrations of Dialyzed FBS (dFBS). After 2h, gold uptake in $10^5$ cells was $175.66\pm2.36$ng when exposed in exposure medium containing 1% dFBS, $68.38\pm13.65$ng when exposed in exposure medium containing 5% dFBS, $48.20\pm4.50$ng when exposed in exposure medium containing 10% dFBS, and $36.56\pm7.09$ng when exposed in exposure medium containing 20% dFBS (Figure 3.4). Uptake was negatively correlated to dFBS concentration, as uptake decreased with an increase in protein concentration ($R^2=0.74$, $p=0.0003$). An ANCOVA confirmed that there was no statistical
Figure 3.3: The influence of FBS on the uptake of gold nanoparticles by A549 Cells, compared to that of iFBS. Statistical analysis revealed that there was no difference when media was supplemented with iFBS.
Figure 3.4: The influence of FBS on the uptake of gold nanoparticles by A549 Cells, compared to that of dFBS. Statistical analysis revealed that there was no difference when media was supplemented with dFBS.
difference in the regression slopes ($p=0.1522$) between the uptake of particles in dFBS and FBS.

Cells were also exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with increasing concentrations of Charcoal/Dextran Treated FBS (cFBS). After 2h, gold uptake in $10^5$ cells was $162.14\pm32.38$ng in exposure medium containing 1% cFBS, $71.70\pm12.78$ng in exposure medium containing 5% cFBS, $49.29\pm10.41$ng in exposure medium containing 10% cFBS, and $45.59\pm5.95$ng in exposure medium containing 20% cFBS (Figure 3.5). Uptake was negatively correlated to cFBS concentration, as uptake decreased with an increase in protein concentration ($R^2=0.63$, $p=0.0020$). An ANCOVA indicated that there was a statistical difference in the regression slopes ($p=0.0366$) between the uptake of particles in cFBS compared to FBS.

3.4 Phase II PIE

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with increasing concentrations of BSA and cholesterol-rich apolipoproteins (aBSA). After 2h, gold uptake in $10^5$ cells was $56.86\pm3.50$ng in exposure medium containing 1% aBSA, $66.56\pm9.55$ng in exposure medium containing 5% aBSA, $67.74\pm2.83$ng in exposure medium containing 10% aBSA, and $71.88\pm10.29$ng in exposure medium containing 20% aBSA (Figure 3.6). Uptake was positively correlated to aBSA concentration, as uptake increased
Figure 3.5: The influence of FBS on the uptake of gold nanoparticles by A549 Cells, compared to that of cFBS. Statistical analysis revealed that there was a significant increase in uptake when media was supplemented with cFBS.
Figure 3.6: The influence of BSA on the uptake of gold nanoparticles by A549 Cells, compared to that of aBSA. Statistical analysis revealed that there was no difference when media was supplemented with aBSA.
with an increase in protein concentration \((R^2=0.36, p=0.0404)\). An analysis of covariance (ANCOVA) showed there was no statistical difference in the regression slopes \((p=0.6241)\) between the uptake of particles in aBSA compared to BSA.

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with increasing concentrations of BSA and hemoglobin (hBSA). After 2h, gold uptake in \(10^5\) cells was 97.08±3.06ng in exposure medium containing 1% hBSA, 89.84±4.50ng in exposure medium containing 5% hBSA, 84.98±3.90ng in exposure medium containing 10% hBSA, and 96.14±14.74ng in exposure medium containing 20% hBSA (Figure 3.7). There was no correlation between uptake and hBSA concentration, as uptake remained the same as protein concentrations increased \((R^2=0.00, p=1.0000)\). An ANCOVA found there was no statistical difference in the regression slopes \((p=0.1906)\) between the uptake of particles in hBSA compared to BSA.

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with increasing concentrations of BSA and transferrin (tBSA). After 2h, gold uptake in \(10^5\) cells was 83.16±14.55ng in exposure medium containing 1% tBSA, 100.14±8.50ng in exposure medium containing 5% tBSA, 92.36±5.47ng in exposure medium containing 10% tBSA, and 119.24±11.27ng in exposure medium containing 20% tBSA (Figure 3.8). Uptake was positively correlated to tBSA concentration, as uptake increased with an increase in protein
Figure 3.7: The influence of BSA on the uptake of gold nanoparticles by A549 Cells, compared to that of hBSA. Statistical analysis revealed that there was no difference when media was supplemented with hBSA.
Figure 3.8: The influence of BSA on the uptake of gold nanoparticles by A549 Cells, compared to that of tBSA. Statistical analysis revealed that there was no difference when media was supplemented with tBSA.
concentration ($R^2=0.53$, $p=0.0073$). An ANCOVA showed that there was no statistical difference in the regression slopes ($p=0.1538$) between the uptake of particles in tBSA and BSA.

Finally, cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with increasing concentrations of BSA and fetuin (fBSA). After 2h, gold uptake in $10^5$ cells was 53.04±2.59ng in exposure medium containing 1% fBSA, 42.19±0.08ng in exposure medium containing 5% fBSA, 33.85±1.30ng in exposure medium containing 10% fBSA, and 26.29±1.85ng in exposure medium containing 20% fBSA (Figure 3.9). Uptake was negatively correlated to fBSA concentration, as uptake decreased with an increase in protein concentration ($R^2=0.53$, $p=0.0073$). An analysis of covariance (ANCOVA) indicated that there was a statistical difference in the regression slopes ($p<0.0001$) between uptake of particles in fBSA compared to BSA.

### 3.5 Influence of Calcium

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM containing calcium supplemented 10% FBS, DMEM free of calcium supplemented with 10% FBS, DMEM containing calcium supplemented with 10% BSA, and DMEM free of calcium supplemented with 10% BSA (Figure 3.10). After 2h, gold uptake in $10^5$ cells was 17.52±0.87ng in exposure medium calcium with 10% FBS and 13.12±0.82ng in exposure medium free of calcium with 10% FBS. A t-test was conducted to examine differences in uptake in media
Figure 3.9: The Influence of BSA on the uptake of gold nanoparticles by A549 Cells, compared to that of fBSA. Statistical analysis revealed that there was a significant decrease in uptake when media was supplemented with fBSA.
Figure 3.10: Comparison of calcium dependence on particle uptake at 2 h. Values with the same letter are not statistically different from each other. Statistical comparisons between FBS treatments (left) and BSA treatments (right), while represented together, were conducted independently of each other.
supplemented with FBS in the presence and absence of calcium, and found that uptake significantly decreased when calcium was removed from the system (p=0.0031).

Gold uptake in $10^5$ cells was 161.22±13.68ng in exposure medium calcium with 10% BSA and 130.34±9.26ng in exposure medium free of calcium with 10% BSA. Once again, a t-test showed that uptake significantly decreased when calcium was removed from the system (p=0.0382). The data further support the notion that albumin was highly involved in the uptake of 20nm citrate-capped spheres by A549 cells, while also suggesting that calcium was involved in this process when exposure medium was supplemented with both FBS and BSA.

4. DISCUSSION

The uptake of gold nanoparticles was inversely related to the concentration of supplemented FBS. It was first believed that this response was due to competition for receptor binding sites, and that higher concentrations of FBS were simply flooding the system with available proteins. However, examination of exposures supplemented with BSA suggested that competition was not a likely mechanism, as a similar inverse relationship was not observed. Albumin accounts for roughly 65% of FBS, by mass, and is by far the most concentrated component of FBS. As a result of the drastic differences in these relationships observed, I hypothesized that albumin does not deter the uptake of
nanoparticles by mammalian cells. Furthermore, the absence of calcium from the media had a significant effect on particle uptake, suggesting that calcium is necessary for the transfer of particles across membranes.

A Phase I PIE investigation involved the removal of constituents from the serum supplement, and examining cellular response. Charcoal/Dextran FBS removed lipophilic non-polar compounds such as growth factors, hormones, cytokines and steroids; Dialyzed FBS removed hormones and cytokines; Super-low IgG FBS decreased IgG concentrations from 0.2g/l to less than 0.005g/l. No significant difference in the uptake of gold nanoparticles was observed following the reduction of IgG in the supplemented media. Removing hormones and cytokines from supplemented media through the use of Dialyzed FBS also had no effect on particle uptake. However, the removal of lipophilic non-polar components such as steroids, growth factors, hormones and cytokines did have a significant effect on particle uptake. The removal of these components caused a significant increase in the uptake of 30nm citrate-capped spheres. As a result, I suggest that the inhibition is due mainly to growth factors and steroids, as the removal of hormones and cytokines had no effect in the Dialyzed FBS exposed treatments.

A Phase II PIE involved the addition of FBS components to bovine serum albumin, and examining cellular response. In knowing the components that cause an alteration in cellular response, an understanding of the interactions
involved in particle uptake, as well as deterrence from uptake, may be gained. The addition of cholesterol-rich apolipoproteins, hemoglobin, and transferrin, revealed no significant difference in the regression slope in comparison to standard BSA. However, the addition of fetuin to BSA, at concentrations intended to mimic those commonly found in FBS, had a significant negative impact on the uptake of gold nanoparticles. As a result, I suggest that the deterrence from particle uptake, observed when cells were exposed in media supplemented with FBS, is also due to particle interactions with fetuin.

Previous research identified the presence of apolipoproteins and hemoglobin in protein coronas of nanoparticles, increasing their potential to affect the intracellular trafficking, fate, and transport of nanoparticles in cells [18]. Furthermore, current literature suggests that particles conjugated with apolipoproteins and transferrin facilitates the uptake of nanoparticles to which they are conjugated [19, 20]. However, other studies indicated that fetuin was involved in the hepatic uptake of negatively charged particles by rat livers. Fetuin is highly expressed in hepatocytes and is involved with the regulation of bone growth, which could explain why a similar response was not observed in lung cells in my study [21]. This information, coupled with that of my study, suggests that predictive modeling of particle uptake, based on protein-particle interactions, cannot be accomplished through the analysis of a single cell line [22].
While albumin has been shown to interact with various particle types, results from this study suggest that particles may be interacting with proteins other than albumin, and that these interactions have a significant impact on particle uptake [23]. Another possibility is that proteins present in FBS, such as apolipoproteins, hemoglobin, and transferrin, may be interacting with albumin. The interactions may decrease the potential for particle-albumin binding, resulting in a slight decrease in particle uptake. However, the lack of significant difference in the relationship of uptake collectively among different concentrations leads me to believe these protein-protein interactions do not have a major impact on particle uptake.

The possibility exists that these results are confounded by overall cell health through the exposure period. Cells are cultured in FBS in the presence of nutrients essential to their health, replication, integrity, and survival. However, the experiments described above were designed to extrapolate the involvement of specific protein-particle interactions on uptake and involved exposing cells in the absence of these essential nutrients. While the MTS assay is commonly used to test for cell viability in in vitro assays, viability is assumed through its measure of metabolic activity. While exposed cells were not less active in comparison to controls, there exists the potential that the MTS assay lacked the necessary accuracy to detect differences in membrane activity during these 2 h exposures [24]. As a result, alterations in particle uptake may be a function of cells actively
scavenging for unavailable nutrients that were absent from the exposure medium.

5. CONCLUSIONS

To understand the mechanisms behind the transfer of particles across membranes it is necessary to understand the vectors that mediate this process. I conclude from this study that particle uptake is dependent upon the components that exist within a given exposure medium. Specifically, protein-particle interactions, that occur readily and rapidly when in contact with each other, influence the cellular yield of particle uptake. Fetuin, growth factors, and steroids, commonly present in exposure mediums of studies involving nanoparticle uptake, had a negative impact on particle uptake. Because fetuin exists in relatively high concentrations in FBS, I hypothesize that fetuin may be responsible for the decrease in uptake observed in exposures in FBS [17]. Furthermore, because fetuin levels decrease substantially following development, and are lower in humans than bovines, the current body of in vitro bioassay research that has been conducted using FBS may be inadequate to predict particle uptake in post-fetal, non-bovine biota [17, 25]. However, further research is warranted to better understand these interactions and their impact on the cellular trafficking of engineered nanoparticles.
6. REFERENCES


CHAPTER 4

EXAMINATION OF POTENTIAL MODES OF NANOPARTICLE UPTAKE THROUGH THE PHARMACOLOGICAL INHIBITION OF ENDOCYTIC PATHWAYS

ABSTRACT: This study attempted to assess the modes of endocytosis involved in the uptake of 30nm citrate-capped gold nanospheres by the mammalian cell line A549. Cells were pre-incubated for 0.5 h in the presence of pharmacological inhibitors, after which the exposure medium was spiked with gold nanoparticles at a final concentration of 1mg/l, and cells were exposed for 1 h. Filipin and nystatin were used to inhibit caveolae-dependent endocytosis, chlorpromazine and phenylarsine oxide to inhibit clathrin-dependent endocytosis, and cytochalasin D and 5-(N-ethyl-N-Isopropyl) amiloride (EIPA) were utilized to inhibit macropinocytosis. No statistical decrease in particle uptake, in comparison to controls, was observed following exposure to any of the inhibitors. Particle uptake actually increased significantly when exposed to inhibitors of caveolae-dependent endocytosis and macropinocytosis. My data suggest that the uptake of nanoparticles was not dependent upon any one specific mode of endocytosis. Furthermore, it is likely that all three modes were utilized in the cellular trafficking of engineered nanoparticles.
1. INTRODUCTION

The explosion of products that contain or utilize nanoparticles has occurred in the absence of detailed knowledge of how these materials will interact with biological systems. As of 2006, less than 3% of research funding for the advancement of nanotechnologies was awarded to investigate the health and safety of these materials, and President Obama requested that 7% be awarded to this investigation in 2014 [1,2]. Fundamental knowledge concerning how biota will respond to nanoparticle exposures involves the interactions of these particles with cell membranes. Currently, research describing the specific mechanisms involved in the uptake of nanoparticles is lacking.

Previously, research from our lab investigated the role that particle size and surface charge have on the uptake of gold nanospheres. Gold nanoparticles were chosen for these studies as they are an ideal model particle due to their stability and relatively low toxicity [3]. I focused my endeavors on the interactions between particles and components of supplemented serum in cell growth medium in an attempt to better understand factors involved in the movement of particles across membranes. For these studies, 30nm citrate-capped gold nanospheres were utilized due to their ease of synthesis and the abundance of research in the scientific community on citrate-capped gold spheres. Our interests are now focused on how particles cross cell membranes.
Currently, it has been suggested that nanoparticles may enter cells through three modes of endocytosis: caveola-dependent endocytosis, clathrin-dependent endocytosis, and macropinocytosis. Caveola-dependent endocytosis involves the formation of caveolae by the recruitment of caveolin to lipid rafts in the plasma membrane which are rich in cholesterol and sphingolipids [4]. Clathrin-dependent endocytosis involves the formation of clathrin-coated vesicles formed by the recruitment of adaptor protein-2 (AP-2) and clathrin to synaptotagmin rich regions of the plasma membrane [5]. Macropinocytosis involves the formation of macropinosomes by budding ruffles, or lamellipodia, constructed by actin polymerization [6].

The goal of my project was to determine the mode(s) by which 30nm citrate-capped gold nanospheres are endocytosed by mammalian cells. The analysis was conducted by exposing cells to pharmacological inhibitors of caveola-dependent endocytosis, clathrin-dependent endocytosis, and macropinocytosis, and comparing the degree of particle particle uptake to cells not exposed to these inhibitors. Understanding how these materials cross membranes will enhance the development of products relying on particle uptake for biomedical purposes, while also facilitating future endeavors of accurately characterizing the risk that these materials may pose to biota and the environment.
2. MATERIALS AND METHODS

2.1 Particle Synthesis

Citrate-capped gold nanospheres, 30nm, were produced in-house by methods previously described [7]. Briefly, particles were synthesized by adding 2.5ml 0.01M HAuCl₄ to 100ml ultrapure water while stirring. The solution was brought to a boil, and a 1% citrate solution was added to the mixture. The solution was then allowed to stir and continue boiling for 15-20 minutes until the solution was a deep red.

2.2 Particle Characterization

Particles were characterized in ultra-pure water for size and morphology using transmission electron microscopy (TEM) techniques (Clemson University Electron Microscopy Laboratory). Relative particle sizes were obtained by dynamic light scattering (DLS), and a measure of the electrokinetic potential (zeta potential) for each particle type was obtained. DLS and zeta measurements were analyzed with a 90Plus Particle Size Analyzer (Brookhaven). Particles were analyzed after digestion in aqua regia (nitric acid:hydrochloric acid, 1:3 respectively), and dilution to a 5% acid solution. Gold mass was analyzed via ICP-MS (Thermo Scientific XSeries 2).
2.3 Cell Culture

A549 adenocarcinomic human alveolar basal epithelial cells (ATCC, Rockville MD) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose, 584mg/l L-glutamine, and 110mg/l sodium pyruvate (Mediatech, Inc., Manassas, VA). DMEM was supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin/streptomycin (HyClone), and 2% non-essential amino acids (Sigma-Aldrich, St. Louis, MO) to form complete growth medium. Cells were cultured in 75cm² sterile, polystyrene, tissue culture-treated, vented flasks under clean conditions (Corning, Corning, NY), and incubated under 5% CO₂ at 37°C as described previously [8, 9].

For subculturing, cells in flasks were rinsed with 1X PBS (Fisher Scientific, Waltham, MA), and incubated in a 0.25% trypsin (Sigma-Aldrich) solution for 5-15 minutes, or until cells detached from the flask surface. Complete growth media was then added to inhibit trypsin activity, and cells were aspirated and transferred to sterile centrifuge tubes (Corning). Cells were centrifuged at 3,000 rpm (1,400 g) for three minutes, after which the supernatant was removed and discarded. Cells were then resuspended in complete growth medium, diluted, and transferred to sterile flasks. When not in use, cells were cryopreserved at -80°C in complete growth media supplemented with 5% dimethyl sulfoxide (DMSO) (Fisher Scientific).
2.4 Cell Exposures

Cells were removed from flasks using a 0.25% trypsin solution, and plated in sterile, polystyrene, tissue culture-treated 12-well plates (Costar, Corning, NY) at 100,000 cells per well. Cells were plated in complete growth medium and allowed 24 h for attachment to the plate surface [10]. After 24 h, complete growth media was aspirated, and exposure medium containing gold nanoparticles was carefully added to each well.

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with FBS (30.3g/l) and DMEM supplemented with bovine serum albumin (BSA) (Fisher Scientific) in the presence and absence of known inhibitors of specific modes of endocytosis. All inhibitors were purchased from Sigma-Aldrich. A BSA solution was formulated by diluting BSA in 1X PBS to a concentration of 22.6g/l, to mimic the average albumin concentration of Standard FBS (HyClone Historical Data Sheet, Appendix II). Cell viability was examined and confirmed for each exposure medium using the Promega™ CellTiter 96™ AQueous One Solution Cell Proliferation Assay (MTS) (Fisher Scientific). Viability was not significantly different from that of controls under any of the conditions tested.
2.4.1 Inhibition of Caveolae-Dependent Endocytosis

Cells were pre-incubated in exposure medium supplemented with 10% FBS, 10% BSA, 10% FBS and filipin (1, 5, and 10 mg/l in 1X PBS containing 0.05% DMSO), 10% FBS and nystatin (5, 25, and 50 mg/l in 1X PBS containing 0.05% DMSO), 10% BSA and filipin (1, 5, and 10 mg/l in 1X PBS containing 0.05% DMSO), and 10% BSA and nystatin (5, 25, and 50 mg/l in 1X PBS containing 0.05% DMSO) for 30 minutes. Each well was spiked with 30nm citrate-capped gold nanospheres at an exposure concentration of 1mg/l, and exposed for 1 h.

2.4.2 Inhibition of Clathrin-Dependent Endocytosis

Cells were pre-incubated in exposure medium supplemented with 10% FBS, 10% BSA, 10% FBS and chlorpromazine (0.5, 2.5, and 5 mg/l in 1X PBS), 10% FBS and phenylarsine oxide (5, 25, and 50 µg/l in 1X PBS containing 0.05% DMSO), 10% BSA and chlorpromazine (0.5, 2.5, and 5 mg/l in 1X PBS), and 10% BSA and phenylarsine oxide (5, 25, and 50 µg/l in 1X PBS containing 0.05% DMSO) for 30 minutes. Each well was spiked with 30nm citrate-capped gold nanospheres at an exposure concentration of 1mg/l, and exposed for 1 h.

2.4.3 Inhibition of Macropinocytosis

Cells were pre-incubated in exposure medium supplemented with 10% FBS, 10% BSA, 10% FBS and cytochalasin D (0.5, 2.5, and 5 mg/l in 1X PBS
containing 0.05% DMSO), 10% FBS and 5-(N-ethyl-N-Isopropyl) amiloride (EIPA) (1, 5, and 10 mg/l in 1X PBS containing 0.05% DMSO), 10% BSA and cytochalasin D (0.5, 2.5, and 5 mg/l in 1X PBS containing 0.05% DMSO), and 10% BSA and EIPA (1, 5, and 10 mg/l in 1X PBS containing 0.05% DMSO) for 30 minutes. Each well was spiked with 30nm citrate-capped gold nanospheres at an exposure concentration of 1mg/l, and exposed for 1 h.

2.5 Analytical Procedure

At the end of each time-point, exposure medium was removed and cells were carefully rinsed with 1X PBS to remove residual particles. Gold nanospheres were digested within each well following the addition of 1ml 30% aqua regia, and plates were incubated at 37°C under 5% CO₂ for 30 minutes. Samples were removed from each well and diluted to a 5% acid solution. Gold uptake was analyzed via ICP-MS (Thermo Scientific XSeries 2).

2.6 Data Analysis

Data presented are in a mass-basis format (Au ng/10^5 Cells) and represent the average and standard deviation of triplicate samples. All statistical analysis was performed using SAS and JMP Pro 10 (SAS Institute, Cary, NC, USA). Statistically significant differences were determined by a one way analysis of variance (ANOVA) with Dunnett’s means comparisons test. A p value less than 0.05 was considered statistically significant.
3. RESULTS

3.1 Particle Characterization

Particles were characterized by TEM for size and morphology. Measurements were quantified at random with TEM software, as well as with ImageJ (National Institutes of Health). Citrate-capped gold nanoparticles were found to be spherical in shape with a diameter of 30nm (28.73±6.64) (Appendix I: Image AI-10). DLS estimated that 30nm citrate particles had a diameter of 26nm (26.06±0.34). Zeta potential measurements were also quantified and used to predict particle charge and stability in solution. The average zeta potential for 30nm citrate-capped spheres was -50mV (-50.11±8.03). These results reveal that the particles used in these experiments were monodispersed and stable in suspension.

3.2 Inhibition of Caveolae-Dependent Endocytosis

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with 10% FBS, and 10% FBS with increasing concentrations of filipin and nystatin (Figure 4.1). After 1h, gold uptake in $10^5$ cells was 17.63±6.96ng when exposed in exposure medium containing 10% FBS. Gold uptake was 32.00±2.93ng in exposure medium containing 10% FBS and 1mg/l filipin, 25.63±2.74ng in exposure medium containing 10% FBS and 5mg/l filipin, and 30.43±1.18ng in exposure medium containing 10% FBS and 10mg/l filipin. Gold uptake was 21.30±0.73ng in exposure medium containing 10% FBS and
Figure 4.1: Uptake of 30nm citrate-capped spheres when exposed in 10% FBS in the presence of filipin and nystatin (mean ± standard deviation, n=3). Concentrations with an asterisk are significantly different than that of the controls.
5mg/l nystatin, 22.33±3.84ng in exposure medium containing 10% FBS and 25mg/l nystatin, and 25.98±2.13ng in exposure medium containing 10% FBS and 50mg/l nystatin.

An analysis of variance (ANOVA) with Dunnett’s means comparisons test was utilized to analyze differences between cells exposed to each chemical inhibitor concentration in relation to cells that were not exposed. Uptake increased significantly when cells were exposed to 30nm citrate-capped spheres in the presence of 1mg/l and 10mg/l filipin (p=0.0054 and p=0.0015, respectively), but not when exposed to 5mg/l filipin (p=0.1175). Cells showed no significant difference in uptake when exposed to 30nm citrate-capped spheres in the presence of 5mg/l, 25mg/l, and 50mg/l nystatin (p=0.6522, p=0.4749, and p=0.1031, respectively).

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with 10% BSA as controls, and 10% BSA with increasing concentrations of filipin and nystatin (Figure 4.2). After 1h, gold uptake in 10^5 cells was 81.00±17.56ng when exposed to medium containing 10% BSA. Gold uptake was 92.30±1.08ng when exposed to medium containing 10% BSA and 1mg/l filipin, 76.42±5.08ng when exposed to medium containing 10% BSA and 5mg/l filipin, and 70.70±1.98ng when exposed to medium containing 10% BSA and 10mg/l filipin. Gold uptake was 107.32±5.42ng when exposed to medium containing 10% BSA and 5mg/l nystatin, 90.82±5.15ng when exposed to medium
Figure 4.2: Uptake of 30nm citrate-capped spheres when exposed in 10% BSA in the presence of filipin and nystatin (mean ± standard deviation, n=3). Concentrations with an asterisk are significantly different than that of the controls.
containing 10% BSA and 25mg/l nystatin, and 101.68±7.02ng when exposed to medium containing 10% BSA and 50mg/l nystatin.

An ANOVA with Dunnett’s means comparisons test revealed no significant difference in the uptake of 30nm citrate-capped spheres when exposed to 1mg/l, 5mg/l and 10mg/l filipin compared to control values (p=0.4679, p=0.9211, and p=0.5378, respectively). Uptake increased significantly when cells were exposed to 30nm citrate-capped spheres in the presence of 5mg/l nystatin (p=0.0357), but not when exposed to 25mg/l or 50mg/l nystatin (p=0.6042 and p=0.1056, respectively).

3.3 Inhibition of Clathrin-Dependent Endocytosis

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with 10% FBS as controls, and 10% FBS with increasing concentrations of chlorpromazine and phenylarsine oxide (Figure 4.3). After 1h, gold uptake in \(10^5\) cells was 24.17±6.72ng in medium containing 10% FBS. Gold uptake was 25.70±3.28ng in medium containing 10% FBS and 0.5mg/l chlorpromazine, 20.28±3.04ng in medium containing 10% FBS and 2.5mg/l chlorpromazine, and 22.97±2.41ng in medium containing 10% FBS and 5mg/l chlorpromazine. Gold uptake was 23.11±2.75ng in medium containing 10% FBS and 5µg/l phenylarsine oxide, 22.15±2.68ng in medium containing 10% FBS and 25µg/l phenylarsine oxide, and 25.88±2.78ng in medium containing 10% FBS and 50µg/l phenylarsine oxide.
Figure 4.3: Uptake of 30nm citrate-capped spheres when exposed in 10% FBS in the presence of chlorpromazine and phenylarsine oxide (mean ± standard deviation, n=3). Concentrations with an asterisk are significantly different than that of the controls.
An ANOVA with Dunnett’s means comparisons test revealed no significant difference in the uptake of 30nm citrate-capped spheres when exposed to 0.5mg/l, 2.5mg/l and 5mg/l chlorpromazine compared to control values (p=0.9555, p=0.6078, and p=0.9781, respectively). Also, there was no significant difference in the uptake of 30nm citrate-capped spheres when exposed to 5µg/l, 25µg/l, and 50µg/l phenylarsnine oxide compared to control values (p=0.9842, p=0.9058, and p=0.9380, respectively).

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with 10% BSA as controls, and 10% BSA with increasing concentrations of chlorpromazine and phenylarsnine oxide (Figure 4.4). After 1h, gold uptake in $10^5$ cells was 110.96±37.55ng in medium containing 10% BSA. Gold uptake was 90.28±2.94ng in medium containing 10% BSA and 0.5mg/l chlorpromazine, 98.98±9.94ng in containing 10% BSA and 2.5mg/l chlorpromazine, and 99.84±8.38ng in medium containing 10% BSA and 5mg/l chlorpromazine. Gold uptake was 81.66±4.01ng in medium containing 10% BSA and 5µg/l phenylarsnine oxide, 75.96±4.78ng in medium containing 10% BSA and 25µg/l phenylarsnine oxide, and 103.38±3.41ng in medium containing 10% BSA and 50µg/l phenylarsnine oxide.

An ANOVA with Dunnett’s means comparisons test revealed no significant difference in the uptake of 30nm citrate-capped spheres when exposed to 0.5mg/l, 2.5mg/l and 5mg/l chlorpromazine compared to control values (p=0.5873, p=0.8702, and p=0.8921, respectively). Also, there was no significant
Figure 4.4: Uptake of 30nm citrate-capped spheres when exposed in 10% BSA in the presence of chlorpromazine and phenylarsine oxide (mean ± standard deviation, n=3). Concentrations with an asterisk are significantly different than that of the controls.
difference in the uptake of 30nm citrate-capped spheres when exposed to 5µg/l, 25µg/l, and 50µg/l phenylarsine oxide compared to control values (p=0.3127, p=0.1945, and p=0.9590, respectively).

### 3.4 Inhibition of Macropinocytosis

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with 10% FBS as controls, and 10% FBS with increasing concentrations of cytochalasin D and EIPA (Figure 4.5). After 1h, gold uptake in 10^5 cells was 13.46±2.25ng in exposure medium containing 10% FBS. Gold uptake was 20.41±4.80ng in exposure medium containing 10% FBS and 0.5mg/l cytochalasin D, 20.94±1.70ng in exposure medium containing 10% FBS and 2.5mg/l cytochalasin D, and 26.99±2.59ng in exposure medium containing 10% FBS and 5mg/l cytochalasin D. Gold uptake was 23.24±2.34ng in exposure medium containing 10% FBS and 1mg/l EIPA, 20.24±1.19ng in exposure medium containing 10% FBS and 5mg/l EIPA, and 20.27±1.31ng in exposure medium containing 10% FBS and 10mg/l EIPA.

An ANOVA with Dunnett’s means comparisons test revealed a significant increase in the uptake of 30nm citrate-capped spheres when exposed to 0.5mg/l, 2.5mg/l and 5mg/l cytochalasin D compared to control values (p=0.0157, p=0.0099, and p=0.0001, respectively). Also, there was a significant increase in the uptake of 30nm citrate-capped spheres when exposed to 1mg/l, 5mg/l, and
Figure 4.5: Uptake of 30nm citrate-capped spheres when exposed in 10% FBS in the presence of cytochalasin D and EIPA (mean ± standard deviation, n=3). Concentrations with an asterisk are significantly different than that of the controls.
10mg/l EIPA compared to control values (p<0.0001, p=0.0014, and p=0.0013, respectively).

Cells were exposed to 30nm citrate-capped gold nanospheres in DMEM supplemented with 10% BSA as controls, and 10% BSA with increasing concentrations of cytochalasin D and EIPA (Figure 4.6). After 1h, gold uptake in $10^5$ cells was 70.32±2.92ng in exposure medium containing 10% BSA. Gold uptake was 110.94±5.00ng in exposure medium containing 10% BSA and 0.5mg/l cytochalasin D, 107.42±10.23ng in exposure medium containing 10% BSA and 2.5mg/l cytochalasin D, and 133.82±13.41ng in exposure medium containing 10% BSA and 5mg/l cytochalasin D. Gold uptake was 73.54±42.32ng in exposure medium containing 10% BSA and 1mg/l EIPA, 96.32±9.69ng in exposure medium containing 10% BSA and 5mg/l EIPA, and 114.50±13.11ng in exposure medium containing 10% BSA and 10mg/l EIPA.

An ANOVA with Dunnett’s means comparisons test revealed a significant increase in the uptake of 30nm citrate-capped spheres when exposed to 0.5mg/l, 2.5mg/l and 5mg/l cytochalasin D compared to control values (p<0.0001, p<0.0001, and p<0.0001, respectively). Also, there was a significant increase in the uptake of 30nm citrate-capped spheres when exposed to 10mg/l EIPA (p=0.0225) compared to control values, but not when exposed to 1mg/l and 5mg/l EIPA (p=0.9923 and p=0.2100, respectively).
Figure 4.6: Uptake of 30nm citrate-capped spheres when exposed in 10% BSA in the presence of cytochalasin D and EIPA (mean ± standard deviation, n=3). Concentrations with an asterisk are significantly different than that of the controls.
4. DISCUSSION

Filipin and nystatin are sterol-binding agents that inhibit caveolae-dependent endocytosis through the depletion of cholesterol from the plasma membrane, which is required to maintain the structural integrity of the caveolae [11]. Following co-exposure with filipin or nystatin and gold nanoparticles, the uptake of gold did not significantly decrease in comparison to those not treated with inhibitors, which suggests that uptake does not occur through a caveolae-dependent process. Uptake actually increased significantly when cells were exposed to particles in the presence of filipin when the media was supplemented with FBS, and increased significantly when cells were exposed to particles in the presence of nystatin when media was supplemented with BSA. An increase in uptake in the presence of these inhibitors suggests that exocytosis of these particles may be cholesterol-dependent, as Dombu et al. [12] observed an increase in particle uptake in airway epithelium cells when cholesterol was depleted by filipin and nystatin.

Chlorpromazine and phenylarsine oxide inhibit clathrin-dependent endocytosis by reacting with vicinal sulfhydryls to form stable ring structures and associating with AP-2, thus removing coated pits from the plasma membrane [13]. The treatment of cells with chlorpromazine and phenylarsine oxide had no effect on the uptake of gold nanoparticles when the exposure medium was supplemented with FBS or BSA, which suggests that the uptake of 30nm citrate-
capped spheres does not occur through a clathrin-dependent process in A549 cells.

Cytochalasin D and EIPA inhibit macropinocytosis though inhibiting sodium-proton exchange and the depolymerization of actin, both required for membrane ruffling and macropinosome formation [14,15]. Exposing cells to these inhibitors in the presence of gold nanoparticles resulted in significant increases in uptake when exposed in medium containing FBS as well as BSA. If macropinocytosis was a mode of uptake of 30nm citrate-capped spheres, a decrease in uptake in comparison to controls would have been expected. As a result, I do not believe that macropinocytosis is a major route by which these particles enter cells. The observed increase in uptake, in the presence of these inhibitors, suggests that exocytosis of these particles may also be actin-dependent [16].

Huang et al. [17] examined the uptake of FITC-chitosan nanoparticles by A549 cells and uptake was inhibited by chlorpromazine, suggesting clathrin-dependent endocytosis as a possibly mode of uptake. Mo and Lim [18] examined the uptake of wheat germ agglutinin-conjugated PLGA nanoparticles, once again by A549 cells, and observed a decrease in particle uptake when exposed to filipin, suggesting caveolae-dependent endocytosis as a possible mode of uptake. dos Santos et al. [19] reported that the uptake of polystyrene nanoparticles was inhibited by cytochalasin A and nocodazole, but not by
chlorpromazine or genistein in A549 cells, suggesting macropinocytosis as a possible mode of uptake. Finally, Brandenberger et al. [20] investigated the uptake of 15nm citrate-capped gold nanospheres, and while inhibition of caveolae-dependent and clathrin-dependent resulted in a decrease in particle uptake, TEM data revealed that particles were in large intracellular vesicles, suggesting uptake by macropinocytosis.

These four studies examined potential modes of uptake in the same cell line as that of my analysis. While I was not able to pinpoint one direct mode of uptake, it is apparent that all three modes have been implicated for involvement in the uptake of nanoparticles. It is likely that cells actually utilize all three modes of endocytosis in particle uptake, and particles enter through non-specific mechanisms [21]. Furthermore, the increased degree of particle uptake when exposed to inhibitors of caveolae-dependent and macropinocytosis may have been a compensatory response to the blockage or preferred routes of uptake; however validation of this hypothesized response requires additional evaluation.

5. CONCLUSIONS

I conclude that the uptake of nanoparticles is not dependent upon any one specific mode of endocytosis. Current literature suggests modes of uptake may change for differing particle types and cell types; or, as Brandenberger et al. [20] suggested, include all three of the modes discussed here. Furthermore, my data
suggest that research into the uptake of 30nm citrate-capped spheres requires
the use of tools other than just pharmaceutical inhibitors.
6. REFERENCES


CHAPTER 5

CONCLUSIONS AND FUTURE RESEARCH

This study investigated the influence on particle characteristics and media composition on the uptake of gold nanoparticles attempted to elucidate the modes by which particles are endocytosed. From the results of this study, we conclude the following:

1. The uptake of gold nanoparticles by A549 cells is dependent upon both particle size and particle surface chemistry.
   - With the exception of 55nm PEG-coated particles, uptake was greatest with cationic particles, followed by anionic particles, and nonionic particles.
   - Uptake was greatest with larger anionic and nonionic particles but with smaller cationic particles.

2. The uptake of gold nanoparticles by A549 cells is dependent upon the composition of the medium in which they are exposed.
   - The removal of steroids and growth factors in supplemented serum had a positive effect on particle uptake, suggesting these components deter uptake.
The addition of fetuin to media supplemented with albumin had a negative effect on particle uptake, suggesting fetuin deters particle uptake.

The removal of calcium from the media had a negative impact on particle uptake, suggesting particle uptake is calcium dependent.

3. The modes of gold nanoparticle uptake by A549 cells cannot be determined through the use of pharmacological inhibitors alone.

- Exposures to known inhibitors of caveolae-dependent endocytosis, clathrin-dependent endocytosis, and macropinocytosis did not have impact on particle uptake, suggesting no single pathway is utilized.

In order to accurately predict particle uptake based on particle characteristics, future research will need to include additional factors than those included in this work. These efforts will need to include more particle sizes, more particle shapes, and more particle surface chemistry modifications, as more parameters will strengthen potential uptake models. Particle sizes greater than 50nm and smaller than 5nm must be included, and multiple ligand attachments for any given charge must also be examined, as different ligands of the same charge may result in uptake differences. While gold nanoparticles serve as great model particles for uptake studies, due to their low toxicity, particles of other core chemistries must also be examined to ensure elemental composition does not
affect uptake. The impacts of these characteristics must also be examined on a wide range of cell lines, as no one line can predict particle uptake for all mammalian cell types. Finally, whole organisms, such as rats and mice, would need to be utilized to test the accuracy of model predictions based upon in vitro results.

To better understand the protein-particle interactions involved in the uptake of nanoparticles, extensions to this work may also be performed. In examining a Phase I PIE, we relied on a variety of FBS types that were purchasable from scientific suppliers. As standard FBS is fairly well characterized, future researchers could formulate specific FBS compositions that exclude any one component or class of components, as well as to employ other methods of component removal from standard FBS. In our examination of a Phase II PIE, we relied on the addition of the most concentrated proteins, other than albumin, to solutions of BSA that mimicked albumin concentrations in standard FBS. Future research should examine the additions of less concentrated proteins to BSA, as well as examine specific serum proteins alone in the absence of BSA. This information would directly investigate the roles of single proteins, and reduce variability of interactions between these proteins, as well as particles, with albumin. Finally, this area of research would benefit from future endeavors to examine the affinities of protein-particle interactions to determine the proteins found in FBS that particles most likely associate with.
To accurately determine the processes by which nanoparticles enter cells, future research will need to utilize a variety of tools and not simply rely on mere pharmacological inhibition. Transmission electron microscopy, environmental scanning electron microscopy, and light microscopy may prove useful in depicting actions taking place on the plasma membrane, as well as vesicles utilized in the storage of nanoparticles once they enter the cell. Furthermore, the use of small interfering RNA (siRNA) may be a more accurate tool in suppressing modes of endocytosis in comparison to chemical inhibition. Chemical inhibitors have the potential to impact various pathways involved in the cell cycle, other than just endocytosis, while siRNAs are designed to decrease the expression of specific genes, resulting in the inhibition of specific pathways. As a result, the use of these techniques would help to validate how particles enter cells.
APPENDIX I
Image AI-1: TEM analysis of 5nm citrate-capped spheres
Image Al-2: TEM analysis of 18nm citrate-capped spheres
Image AI-3: TEM analysis of 50nm citrate-capped spheres
Image Al-4: TEM analysis of 6nm PEG-capped spheres
Image AI-5: TEM analysis of 15nm PEG-capped spheres
Image Al-6: TEM analysis of 55nm PEG-capped spheres
Image Al-7: TEM analysis of 5nm PEI-capped spheres
Image Al-8: TEM analysis of 13nm PEI-capped spheres
Image AI-9: TEM analysis of 30nm citrate-capped spheres
Image AI-10: TEM analysis of 30nm citrate-capped spheres
APPENDIX II
Table AII-1: Hyclone Historical Data Sheet: Characterization of Standard FBS

<table>
<thead>
<tr>
<th>Date</th>
<th>Protein (mg/dL)</th>
<th>Lipid (mg/dL)</th>
<th>Cholesterol (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>Glucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1/2020</td>
<td>10</td>
<td>5</td>
<td>20</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>2/1/2020</td>
<td>15</td>
<td>10</td>
<td>25</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>3/1/2020</td>
<td>20</td>
<td>15</td>
<td>30</td>
<td>200</td>
<td>70</td>
</tr>
<tr>
<td>4/1/2020</td>
<td>25</td>
<td>20</td>
<td>35</td>
<td>250</td>
<td>80</td>
</tr>
<tr>
<td>5/1/2020</td>
<td>30</td>
<td>25</td>
<td>40</td>
<td>300</td>
<td>90</td>
</tr>
<tr>
<td>6/1/2020</td>
<td>35</td>
<td>30</td>
<td>45</td>
<td>350</td>
<td>100</td>
</tr>
<tr>
<td>7/1/2020</td>
<td>40</td>
<td>35</td>
<td>50</td>
<td>400</td>
<td>110</td>
</tr>
<tr>
<td>8/1/2020</td>
<td>45</td>
<td>40</td>
<td>55</td>
<td>450</td>
<td>120</td>
</tr>
<tr>
<td>9/1/2020</td>
<td>50</td>
<td>45</td>
<td>60</td>
<td>500</td>
<td>130</td>
</tr>
<tr>
<td>10/1/2020</td>
<td>55</td>
<td>50</td>
<td>65</td>
<td>550</td>
<td>140</td>
</tr>
<tr>
<td>11/1/2020</td>
<td>60</td>
<td>55</td>
<td>70</td>
<td>600</td>
<td>150</td>
</tr>
</tbody>
</table>