BIOACCUMULATION AND NEUROINFLAMMATION OF GOLD NANOPARTICLES IN THE CENTRAL NERVOUS SYSTEM

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

FAHIMEH FALLAHI
M.S., Azad University, 2007

2013
Wright State University
December 17, 2012

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Fahimeh Fallahi ENTITLED Bioaccumulation and Neuroinflammation of Gold Nanoparticles in the Central Nervous System BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

Mariana Morris, Ph.D.
Thesis Director

Norma C. Adragna, Ph.D.
Chair, Department of Pharmacology and Toxicology

Committee on Final Examination

Mariana Morris, Ph.D.

Saber M. Hussain, Ph.D.

Ioana E. Pavel Sizemore, Ph.D.

R. William Ayres, Ph.D.
Interim Dean, Graduate School
Abstract


Gold nanoparticles (GNPs) possess unique physicochemical properties that may facilitate entry into the central nervous system (CNS) where they may act therapeutically. There is little information on biodistribution or inflammatory effects of GNPs in specific brain regions. Brain Localization and neuroinflammatory response to citrate-capped spherical GNP (10 nm) was determined 24 hours after intravenous (IV) injection in male C57Bl mice. A known inflammogen, lipopolysaccharide (LPS, 2 mg kg\(^{-1}\), SC), was tested as a positive control supplement. Aggregation of GNPs was measured using various Phosphate-Buffered Saline (PBS) concentrations (10, 1, 0.1, 0.01 X) to determine the optimal buffer concentration to maintain GNP solubility. 0.01 X PBS was used in all studies, since it produced the least amount of GNP aggregation. The next experiment verified entry of GNPs into the CNS. Mice were injected IV (200 µg mL\(^{-1}\) 10 nm GNP in 0.01 X PBS) via the tail vein. After 24 hours mice were euthanized (with 130 mg ml\(^{-1}\) Euthasol), and perfused transcardially (with 2% glutaraldehyde and 2% paraformaldehyde), then brains were removed. GNP concentrations were measured using inductively coupled plasma mass spectrometry in whole brain homogenates. To specifically localize accumulation of GNPs in brain, septum, caudate, hippocampus, hypothalamus, cortex, frontal cortex, and spinal cord regions were micro dissected.
Hypothalamus, hippocampus, and septum had the highest GNP levels (6.7, 6.2, and 4.6 µg Au g\(^{-1}\) tissue respectively). To evaluate brain inflammation, we used q-PCR analysis of frozen brain regions for study of pro-inflammatory mediators, Leukemia inhibitory factor (LIF), CC chemokine ligand 2 (CCL2) and Interleukine-1 β (IL-1β). GNPs did not affect cytokine/chemokine expression in cortex, frontal cortex or hippocampus. LPS (positive control), as expected, caused a marked (100-fold) increase in the same cytokines. Results show that GNPs enter brain and concentrate in specific regions without eliciting an inflammatory response. Data raise the possibility of usefulness of GNPs in drug delivery and therapeutic treatment of CNS diseases.
TABLE OF CONTENTS

I. INTRODUCTION .................................................................................................................. 1
   I.1. Nanomaterials ........................................................................................................... 1
   I.2. Gold Nanoparticles ................................................................................................. 3
   I.3. Toxicity of Gold Nanoparticles ............................................................................... 4
   I.4. Neurodegenerative Diseases .................................................................................. 6
   I.5. Neuroinflammatory Response ............................................................................... 7
       I.5.1. Cytokines ....................................................................................................... 7
       I.5.2. Chemokine (C-C motif) Ligand 2(CCL2) ..................................................... 9
   I.6. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) ............................... 9
   I.7. Blood Brain Barrier ............................................................................................ 10

II. HYPOTHESIS and SPECIFIC AIMS ............................................................................ 14
   II.1. Specific Aim 1 ...................................................................................................... 15
       II.1.1. Method for Specific Aim 1 ....................................................................... 15
   II.2. Specific Aim 2 ...................................................................................................... 15
       II.2.1. Method for Specific Aim 2 ....................................................................... 15
   II.3. Specific Aim 3 ...................................................................................................... 15
       II.3.1. Method for Specific Aim 3 ....................................................................... 15
   II.4. Specific Aim 4 ...................................................................................................... 16
II.4.1. Method for Specific Aim 4 ............................................................................. 16

III. MATERIALS and METHODS ............................................................................. 17
III.1. Synthesis and Characterization of GNPs ............................................................. 17

III.2. In Vitro Studies .................................................................................................... 19

III.2.1. UV-Vis Absorption Spectrophotometry (UV-Vis)........................................ 19

III.2.2. Sample Preparation........................................................................................ 20

III.3. In Vivo Studies ..................................................................................................... 21

III.3.1. Animals.......................................................................................................... 21

III.3.2. Intravenous Tail Vein Injection ..................................................................... 22

III.3.3. Perfusion ........................................................................................................ 23

III.3.4. Collection of the Brain Tissue ....................................................................... 24

III.3.5. Brain Dissection ............................................................................................ 25

III.3.6. Digestion Protocol for Whole Brain .............................................................. 25

III.3.6.1 Aqua Regia Protocol ............................................................................... 25

III.3.6.2 Modification and Optimization of Digestion Protocol ........................... 25

III.3.7. Inductively Coupled Plasma Mass Spectroscopy ........................................ 31

III.3.8. Proinflammatory Cytokine Expression ......................................................... 32

III.4. Data and Statistical Analysis ................................................................................ 33
IV. RESULTS .......................................................................................................................... 34
  IV.1. In Vitro Studies ........................................................................................................ 34
    IV.1.1. Optimal PBS Concentration for IV Injection of 10 nm GNPs ...................... 34
  IV.2. In Vivo Studies ....................................................................................................... 41
    IV.2.1. Biodistribution of GNPs in Whole Brain ...................................................... 41
    IV.2.2. Localization of GNPs in Specific CNS Regions ........................................... 43
    IV.2.3. CNS Inflammatory Response to GNPs ......................................................... 45

V. DISCUSSIONS ............................................................................................................. 58

VI. CONCLUSION ............................................................................................................ 63

VII. REFERENCES .......................................................................................................... 65
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Figure I.1: Size Comparison</td>
<td>2</td>
</tr>
<tr>
<td>2. Figure I.2 Barriers in CNS</td>
<td>11</td>
</tr>
<tr>
<td>3. Figure I.3: Transport Pathways</td>
<td>12</td>
</tr>
<tr>
<td>4. Figure III. 1: 10 nm Spherical GNP</td>
<td>17</td>
</tr>
<tr>
<td>5. Figure III. 2: TEM of ~10 nm GNPs</td>
<td>17</td>
</tr>
<tr>
<td>6. Figure III. 3: Dynamic Light Scattering Data</td>
<td>18</td>
</tr>
<tr>
<td>7. Figure III.4: UV-Vis Spectroscopy</td>
<td>18</td>
</tr>
<tr>
<td>8. Figure III.5: Cary 50 UV-Vis Absorption Spectrophotometer</td>
<td>20</td>
</tr>
<tr>
<td>9. Figure III.6: C57Bl/6 Male Mice</td>
<td>21</td>
</tr>
<tr>
<td>10. Figure III.7: IV Injection of 10 nm GNPs</td>
<td>23</td>
</tr>
<tr>
<td>11. Figure III.8: Mouse after Perfusion</td>
<td>24</td>
</tr>
<tr>
<td>12. Figure III.9: Brain Before and After Perfusion</td>
<td>24</td>
</tr>
<tr>
<td>13. Figure III.10 &amp; III.11: Collecting Brain after Perfusion</td>
<td>25</td>
</tr>
<tr>
<td>14. Figure III.12: Microwave Digestion</td>
<td>28</td>
</tr>
<tr>
<td>15. Figure III.13 &amp; III.14: Chunk of Tissue Prior to Modification</td>
<td>28</td>
</tr>
<tr>
<td>16. Figure III.15: Clear Solution after Modification</td>
<td>30</td>
</tr>
<tr>
<td>17. Figure III.16: Perkin Elmer Nexion 300D ICP-MS</td>
<td>31</td>
</tr>
<tr>
<td>18. Figure IV.1: UV-Vis Spectrum of GNP</td>
<td>35</td>
</tr>
</tbody>
</table>
### LIST of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>19. Figure IV.2: UV-Vis Spectrum of Control GNP</td>
<td>35</td>
</tr>
<tr>
<td>20. Figure IV.3: UV-Vis Spectra of the Control 0.01 X &amp; 10.0 X PBS</td>
<td>36</td>
</tr>
<tr>
<td>21. Figure IV.4: UV-Vis Spectrum of the Normal Treatment</td>
<td>36</td>
</tr>
<tr>
<td>22. Figure IV.5: UV-Vis Spectrum of Varied Treatment GNPs and 0.1 X PBS</td>
<td>37</td>
</tr>
<tr>
<td>23. Figure IV.6: UV-Vis Spectrum of Varied Treatment GNPs and 1.0 X PBS</td>
<td>37</td>
</tr>
<tr>
<td>24. Figure IV.7: UV-Vis Spectrum of Varied Treatment GNPs and 10 X PBS</td>
<td>38</td>
</tr>
<tr>
<td>25. Figure IV.8: UV-Vis Spectra of the GNP with Different PBS Conc</td>
<td>39</td>
</tr>
<tr>
<td>26. Figure IV.9: Calibration Curve in ICP-MS</td>
<td>42</td>
</tr>
<tr>
<td>27. Figure IV.10: Gold Accumulation in Whole Brain</td>
<td>43</td>
</tr>
<tr>
<td>28. Figure IV.11: Calibration Curve in ICP-MS</td>
<td>44</td>
</tr>
<tr>
<td>29. Figure IV.12: Profile of GNPs in Different Regions of Brain</td>
<td>45</td>
</tr>
<tr>
<td>30. Figure I.13: LPS (2 mg kg(^{-1}) SC) Caused an Increase of the Same Cytokines</td>
<td>47</td>
</tr>
<tr>
<td>31. Figure IV.14: LPS (2 mg kg(^{-1}) SC) Caused an Increase of the Same Cytokines</td>
<td>48</td>
</tr>
<tr>
<td>32. Figure IV.15: Hypothalamus IL-1(\beta)</td>
<td>49</td>
</tr>
<tr>
<td>33. Figure IV.16: Hypothalamus Lif</td>
<td>49</td>
</tr>
<tr>
<td>34. Figure IV.17: Hypothalamus CCL2</td>
<td>50</td>
</tr>
</tbody>
</table>
# LIST of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>35. Figure IV.18: Hippocampus IL-1β</td>
<td>50</td>
</tr>
<tr>
<td>36. Figure IV.19: Hippocampus Lif</td>
<td>51</td>
</tr>
<tr>
<td>37. Figure IV.20: Hippocampus CCL2</td>
<td>51</td>
</tr>
<tr>
<td>38. Figure IV.21: Caudate IL-1β</td>
<td>52</td>
</tr>
<tr>
<td>39. Figure IV.22: Caudate Lif</td>
<td>52</td>
</tr>
<tr>
<td>40. Figure IV.23: Caudate CCL2</td>
<td>53</td>
</tr>
<tr>
<td>41. Figure IV.24: Frontal Cortex IL-1β</td>
<td>54</td>
</tr>
<tr>
<td>42. Figure IV.25: Frontal Cortex Lif</td>
<td>54</td>
</tr>
<tr>
<td>43. Figure IV.26: Frontal Cortex CCL2</td>
<td>55</td>
</tr>
<tr>
<td>44. Figure IV.27: Cortex IL-1β</td>
<td>56</td>
</tr>
<tr>
<td>45. Figure IV.28: Cortex Lif</td>
<td>56</td>
</tr>
<tr>
<td>46. Figure IV.29: Cortex CCL2</td>
<td>57</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>1. Table 1. Gold Nanoparticle Specifications</td>
<td>19</td>
</tr>
<tr>
<td>2. Table 2. First Stage Microwave Digestion Program</td>
<td>27</td>
</tr>
<tr>
<td>3. Table 3. Second Stage Microwave Digestion Program</td>
<td>27</td>
</tr>
<tr>
<td>4. Table 4. 3rd Stage Microwave Digestion Program</td>
<td>27</td>
</tr>
<tr>
<td>5. Table 5. Gold Accumulation in Whole Brain</td>
<td>42</td>
</tr>
<tr>
<td>6. Table 6. Gold Accumulation in Specific Regions of Brain</td>
<td>45</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to all those who have provided me with the opportunity to complete this thesis. I am indebted to everyone who has helped me through this journey of graduate studies. Most importantly, I am grateful for the constant support, guidance, and encouragement of Dr. Marriana Morris, my research advisor and the chairman of Pharmacology and Toxicology department, who constantly challenged me to improve. I would like to express my gratitude to Dr. Saber Hussain, for his valuable guidance and insight at various stage of this project. I am very honored and grateful for his support through ORISE/AFRL. I am deeply obliged for the scholarship provided by graduate department at Wright State University. It is my earnest desire to continue my passion in research and to be able to give back to society in the near future. I would like to specially thank Dr. Ioana Pavel Sizemore from the committee for all her help and time. Above all many thanks go to Mrs. Terry Oroszi, director of master program, for her continuous encouragement and support. She believed in me even when I did not believe in myself. Furthermore, I would like to acknowledge with much appreciation the crucial role of the faculty and staff of the many departments involved in my research: Dr. Morris’ lab members, Dr. Hussain’s lab members, Dr. Chen’s lab members, Dr. Lucot’s Lab members, Dr. Povel’s lab members, Dr. Ketcha’s lab members, Dr. Adragna, Dr. Leefmans, Mrs. Winslow, Mrs. Schoettinger, Mrs. Hendry, and Ms. Dagher who have supported me in accomplishing my thesis. In particular, I
would like to thank both my collaborators, Dr. James P. O'Callaghan at the Molecular Neurotoxicology Laboratory (Centers for Disease Control and Prevention), and Ryan T. Saadawi in Dr. Joseph A. Caruso’s Research Group at University of Cincinnati. The unconditional love and support of my husband was instrumental in helping me stay focused on the project. Finally, I would like to thank my family and friends for their steadfast support.
“Dedicated to my loving husband and parents”
I. INTRODUCTION

I.1. Nanomaterials:

Nanomaterials are defined as materials having at least one dimension within the range of 1-100 nm. They are produced from manipulation of material at the nanoscale. Engineering of size at this scale creates new materials with novel functionality and physicochemical properties. The various forms of nanomaterials include nanotubes, nanowires, fullerene derivatives (buckyballs), and quantum dots. The nanoparticles have obtained considerable recognition and have contributed to the improvement of new analytical apparatus used in either physical or life sciences (Cui & Gao, 2005; Wu & Bruchez, 2004). The United States Air Force is interested in nanomaterials due to their possible application in multiple advanced systems used as electronics, sensors, and energy production (Hussain, et al., 2006). Organelle or DNA damage, oxidative stress, apoptosis (programmed cell death), mutagenesis, and protein up/down regulation are destructive effects of the interaction of nanoparticles and cellular components such as the membrane, mitochondria, or nuclei (Unfried, et al., 2007; Aillon, et al., 2009; Jia, et al., 2009; Pan, et al., 2009). Since 2000, a growing number of researchers have targeted the effects of nanomaterials on biological interfaces and environmental health. One of the main objectives of research was to comprehend the relationship of size, shape, and surface chemistry to interaction of variety of nanomaterials with biological systems.
(Lewinski, et al., 2008). Due to diverse variable component such as physical and chemical properties of the nanoparticles, cell variety, dosing, and the type of biochemical test used, the outcome is not standardized and therefore inconclusive. An important characteristic of nanoparticles is protein adsorption which facilitates passage of nanomaterial via receptor-mediated endocytosis (Conner and Schmid, 2003). Research on the cellular effects of nanomaterials in vitro has been the predominant method; it is imperative that to study in vivo effects of nanomaterials (Fischer and Chan, 2007) as well.

![Figure I.1: A Size Comparison of Biological Systems and Nanoparticles (Modified from Kattumuri, et al., 2006).](image-url)
I.2. Gold nanoparticles:

Gold nanoparticles (GNPs) possess a multitude of unique characteristics such as stability and distinct optical properties that make them useful for biological imaging by electron microscopy and chemical sensing (Lasagna-Reeves, et al., 2010). Depending on size, charge, shape, and dose, the GNPs have different properties. Due to their straightforward preparation and the selective adsorption to recognition molecules GNPs have been successfully used as a basis for drug delivery (Guerrero, et al., 2010). In addition, their ability to intensely react to time-varying magnetic field, allows for their use in thermal therapy to targeted bodies and ultimately for treatment of cancer, degenerative disorders, and other chronic diseases (Lasagna-Reeves, et al., 2010). Bulk gold is well known to be “safe” and chemically inert, and gold-based compounds have been used in the clinic as anti-inflammatory agents to treat rheumatoid arthritis (Finkelstein, et al., 1976). Gold microparticles have been successfully used in local radioisotope cancer therapy (Metz, et al., 1982). In addition, gold nanoparticles are good candidates for photothermal therapy and imaging agents for in vivo studies (Turner, et al., 2008).
I.3. Toxicity of Gold Nanoparticles:

Distinctive characteristics of GNPs have created technological and scientific interests. It is vital to understand GNPs potential toxicity and health impact prior to their application in clinical settings (Alkilany, et al., 2010). Functionalization of nanoparticles with polyethylene glycol (PEG) will increase their circulatory time by preventing their blood protein adsorption which is useful in treatment (Eghtedari, et al., 2009 and von Maltzahn, et al., 2009). It is important to consider local and systemic toxicity of GNPs in drug delivery (Lasagna-Reeves, et al., 2010). Many studies verify the nontoxic nature of GNPs. An MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colorimetric metabolic assay and is a “gold standard for cytotoxicity. It measures the enzymatic activity of cellular mitochondria and index of cellular activity (Marquis, et al., 2009). An MTT colorimetric assay using a human leukemia cell line indicated that 4, 12, and 18 nm gold nanospheres and capping agents (citrate, cysteine, glucose, biotin, and cetyltrimethylammonium bromide) were nontoxic (Connor, et al., 2005). Likewise, citrate-capped gold nanospheres of 10 nm were not toxic and did not induce activation or change phenotype of dendritic cells of human immune system (Villiers, et al., 2010). Comparable results were obtained using nanospheres of 3.5 nm on immune system cell line (Shukla, et al., 2005). 3.5 nm GNPs endocytosed did not reduce reactive oxygen species level or cause toxicity (Alkilany, et al., 2010). Other groups found contradictory results (Alkilany, et al., 2010) i.e. 2 nm cationic gold nanospheres appeared toxic at
certain doses, but at the same concentration with the same cell line with the negatively charged surface were nontoxic (Goodman, et al., 2004). This was due to interaction of cationic GNPs and disruption of negatively charged cellular membrane (Goodman, et al., 2004). 1.4 nm gold nanospheres caused necrosis, mitochondrial damage, oxidative stress on all cell lines, but no cellular damage resulted from 15 nm gold nanospheres having same surface properties. This was indicative of size-dependent toxicity of GNPs (Pan, et al., 2009). The chemical reactivity exhibited by GNPs smaller than 2 nm was not observed with larger sizes (Turner, et al., 2008). These contradictions are possibly related to variation of toxicity assays, cell lines, and physicochemical properties of nanoparticles. In addition, due to difference in dosing parameters and exposure time, it is difficult to make comparisons (Alkilany, et al., 2010). There are many commercial kits available for quantifying cellular health. Some examples include ROS (Reactive Oxygen Specie) assays for monitoring oxidative stress through ROS level, real-time polymerase chain reaction amplification and DNA micro- array analysis which determine level of gene expression (Marquis, et al., 2009). Intracellular oxidative stress is quantified through GSH (Glutathione) depletion, MDA (3,4-Methylenedioxyamphetamine) production, SOD (Superoxide dismutases) inhibition, and ROS generation which are indicatives of cytotoxicity. GSH is a critical antioxidant which stabilizes cellular redox status by preventing lipid peroxidation of radicals. MDA deletion is a method of measuring lipid peroxidation (Wu, et al., 2011). ROS and oxidative stress have shown to be a good indicator for assessment of cellular cytotoxicity by nanoparticles (Xia, et al., 2006).
I.4. Neurodegenerative Diseases:

The mechanisms of neurodegenerative diseases have not been fully understood. In neurodegeneration, neurons, microglia, and astroglia may be the targets or the source of cytokines and chemokines. Microglia and astroglia activation, immune response, and inflammatory response (i.e. up-regulation of tumor necrosis factor α, interleukin-1β, β-chemokine monocyte chemoattractant protein-1) are linked with neurodegenerative diseases such as Alzheimer's and Parkinson's diseases as well as Multiple Sclerosis. It is not clear whether these responses initiate the disease or they are just body’s response to it (O'Callaghan, et al., 2008; Little, et al., 2002). Parkinson’s disease is a neurodegenerative disease of the central nervous system. Specifically neurodegeneration of the nigrostriatal tract results in death of dopamine-generating neurons in the substantia-nigra, loss of striatal tyrosine hydroxylase containing nerve endings, and reduced striatal dopamine production causing irregular motor symptoms (Pardridge, 2005). In neurodegenerative diseases such as Parkinson's disease, Hallervorden-Spatz syndrome, and Alzheimer's disease lesion, which leads to loss of function or structure, plays a crucial role in causation of diseases (Tansey, et al., 2008).
I.5. Neuroinflammatory Response:

1.5.1. Cytokines:

Cytokines are small short acting proteinaceous messenger molecules that are used in cell communications. They play an important role in mediating inflammatory and immune response. Specific cytokines participate in brain signaling and are involved in production of neurochemical, neuroendocrine, neuroimmune, and behavioral changes of the normal brain and may become highly elevated in certain pathological disorders. These diverse groups of proteins act in a cascade, regulating the immune and inflammatory response as well as certain pathological and physiological conditions. Cytokines are pleotropic and have multiple overlapping biological activities. For example IL-1 may produce immunological effect (production of IL-1, IL-6, and TNFα), neurochemical effect, neuroendocrine effect (increase in hypothalamic-pituitary-adrenal (HPA) activity), and behavioral effect (increase sleep, decrease appetite and sexual drive). It is also known that different cytokines may produce identical biological effects. Upregulation and downregulation of cytokines is highly dependent on stimulation by preceding cytokines (Kronfol & Remick, 2000). Conventionally inflammation outside of CNS involves production of proinflammatory cytokines and chemokines by neutrophils and macrophages, activation of complement proteins and production of reactive oxygen species (ROS) at the site of injury. In brain inflammation microglia and astrocytes are not only source of proinflammatory cytokines but also targets of it (O'Callaghan et al., 2008).
Additionally under certain conditions, neurons have the ability to produce cytokines (Freidin, et al., 1992). IL-1 has been localized in hippocampus and specific hypothalamic areas. Additionally, hypothalamus has been identified as a specific region for IL-1 immunoreactive nerve in human brain (Breder, et al., 1988). There are also extensive immunoreactive regions of TNF and other cytokines in the murine brain (Breder, et al., 1993). Low levels of constitutive expression of certain cytokines have been detected in brain’s blood vessel in addition to the expression of cytokines in response to stimuli (Licinio, et al., 1998). Five major hypothesized mechanisms of cytokine signaling in brain are as follows:

1. Passive transport through circumventricular sites or damaged blood–brain barrier (BBB)
2. Activation of brain’s endothelial cells and inducing production of secondary messengers like nitric oxide or prostaglandins that can diffuse through BBB
3. Active carrier mediated transport
4. Activation of afferent nerves of autonomic nervous system at periphery
5. Activation of afferent vagus nerves (Watkins, et al., 1995).

Leukemia inhibitory factor (LIF) is a multifunctional glycoprotein cytokine of the interleukin 6 (IL-6) family. It binds to gp190 (its specific receptor LIFR-a) and gp130 (common to all members of IL-6 family of cytokines) receptors and signals through JAK-STAT (janus-kinase signal transducer and activator of transcription) and MAPK (mitogen activated protein kinase) pathways. The activation is terminated by SOCS (supressor-of
cytokine signaling) and PIAS (protein inhibitors of activated STAT) proteins. Some of its more common functions are pro and anti-inflammatory, hematopoietic (regulation of differentiation and proliferation) and neuronal actions, inhibition of cell growth and differentiation, and endocrine activity in hypothalamic-pituitary-adrenal axis (neuroimmune mediating effects), bone cell remodeling and metabolism, energy regulation and homeostasis, hormone responsive tumors, uteroplacental unit, and embryogenesis (Auernhammer, et al., 2000)

1.5.2. **Chemokine (C-C motif) ligand 2 (CCL2):**

CC chemokine ligand 2 (CCL2) or monocyte chemoattractant protein 1 (MCP-1) are part of the CC chemokine family. They have a potent ability to stimulate migration of monocytes, dendritic cells, natural killer (NK) cells, and memory T cells from blood to the inflammation site (Deshmane, et al., 2009). Astrocytes, microglial cells, and neurons are a source of CCL2 in brain. Expression of CCL2 in brain demonstrates highly localized dispersion in several specific regions (Banisadr, et al., 2005).

1.6. **Inductively coupled plasma mass spectrometry (ICP-MS):**

Inductively coupled plasma mass spectrometry (ICP-MS) is an accurate quantification method of cellular uptake of GNPs, because of its high specificity and low limits of detection (18 parts per trillion for gold) (Alkilany, et al., 2009). ICP-MS detected 10 nm GNPs in blood, spleen, liver, testis, lung, and brain of rats, 24 hours after injection with a
dose range of 10, 50, 100, and 250 nm GNPs (De Jong, et al., 2008). To determine biodistribution of GNPs the target organs must be isolated, gold ions extracted and oxidized via acid digestion, and then measured with ICP-MS (Cho, et al., 2009). Interference of GNPs through the absorption of visible light should be accounted for while conducting the above mentioned colorimetric assays or other assays that rely of fluorescence changes (AshaRani, et al., 2009). Molecules from immediate region (i.e. indicator dyes) are adsorbed by GNPs (Alkilany, et al., 2008) and result mask their fluorescence (Willets and Van Duyne, 2007).

I.7. Blood Brain Barrier:

The blood–brain barrier (BBB) limits the passage of substances from the systemic circulation into the CNS. Only passage of small high lipid soluble substances with the molecular weight of 400-500 Da is allowed (Pardridge, 2003). It is crucial to verify that GNPs pass the BBB and reach the neuronal tissue of CNS, if we are to look for uses of GNP in the brain (Lasagna-Reeves, et al., 2010). Entrapment of nanoparticles (NPs) by reticuloendothelial system (RES) impedes their delivery to the target organ. It should be noted that the main interest for biological interfaces is in 1-100 nm diameter, and BBB only allows passage of objects smaller than 12 nm (Oberdorster, et al., 2004; Sarin, et al., 2008; Sonavane, et al., 2008). The amphipathic peptide conjugate improved the brain delivery (without affecting the integrity of the BBB and cell viability); increasing the
concentration of gold by fourfold (while reducing its entrapment by spleen) (Guerrero, et al., 2010).

**Figure I.2:** Three main sites of barriers in CNS: 1) Brain endothelium capillaries (forming BBB) 2) The arachnoid epithelium (middle layer of the meninges) 3) The choroid plexus epithelium (secreting CSF). Tight junctions create a barrier and decrease permeability of paracellular pathways. BBB is absent at circumventricular organs (CVO), specialized organs of chemosensitivity and neurosecretion. CVOs are separated from the rest of the brain and CSF by external glial barrier at the ependymal (Modified from Abbott, et al., 2006).
**Figure I.3:** Transport pathways across the cerebrovascular endothelial cells: a) Paracellular transport only allows diffusion of water soluble compounds due to strict restriction of tight junction e.g. polar drugs b) Passive diffusion of lipid soluble amphiphilic agents e.g. alcohol c). Transport protein (carriers) e.g. glucose, amino acids, choline, purine bases, and other substances d). Receptor mediated transcytosis of certain proteins such as insulin and transferrin e). Adsorptive endocytosis and transcytosis; cationization of native plasma proteins like albumin facilitate their uptake (Modified from Abbott, et al. 2006).
Circumventricular organs (CVO) and choroid plexus lack tight junctions and their blood vessels are easily permeable; however, they are isolated by a similar epithelial formation. Tight junctions of the ependymal cells, surrounding choroid plexus and CVOs limits further diffusion of exogenous substances into brain. Choroid plexus produces CSF and controls passages of majority of substances through its polarized epithelial. CVO provides equilibrium between plasma and CSF. CVOs are divided into sensory and secretory organs (Begley, 2004).
II. HYPOTHESIS AND SPECIFIC AIMS

Our central hypothesis is that citrate-capped spherical GNPs (10 nm) cross the BBB and enters the CNS. To test this hypothesis the following experiments are performed.

- The primary set of experiments in this research project determines the optimal concentration of phosphate buffered saline (PBS) that produces the least amount of aggregation.
- The second set of experiments verifies the existence of 10 nm GNPs in the whole brain 24 hours after IV injection in C57BL/6 male mice.
- The third set of experiments is based on developing a novel method focused on localization and bioaccumulation of GNPs in specific areas of CNS.
- The final set of experiments analyzes the pro-inflammatory cytokines expression in different regions of brain.

The main purpose of the research is to localize and quantify GNPs in order to measure the neuroinflammatory response in brain of the treated mice. The proposed work is innovative, because there is little information on the transport of GNPs into CNS and the brain’s inflammatory response.
II.1. **Specific Aim 1:**

To determine the optimal buffer concentration of PBS for IV injection of GNPs

**II.1.1. Method for Specific Aim 1:**

Aggregation of 200 µg mL\(^{-1}\) 10 nm GNPs in various concentration of PBS (10 X, 1 X, 0.1 X, and 0.01 X) are measured via ultraviolet-visible spectroscopy.

II.2. **Specific Aim 2:**

To determine whether peripherally injected 10 nm citrate capped spherical GNP enters the brain (quantification in whole brain)

**II.2.1. Method for Specific Aim 2:**

Inductively coupled plasma-mass spectrometry (ICP-MS) is utilized to detect distribution and accumulation of GNPs 24 hours after IV injection in C57Bl/6 male mice.

II.3. **Specific Aim 3:**

After peripherally injection of GNPs, regions of the CNS that have the high concentration of GNPs are identified (quantification and regional localization of GNPs in brain).

**II.3.1. Method for Specific Aim 3:**

Inductively coupled plasma-mass spectrometry (ICP-MS) is used to localize the accumulation of GNPs 24 hours after IV injection in C57Bl/6 male mice. This is done by examining different sections of brain tissue and spinal cord.
II.4. Specific Aim 4:

To verify the brain’s inflammatory response of C57Bl/6 male mice 24 hours after IV injection of 10 nm GNPs

II.4.1. Method for Specific Aim 4:

Real-time PCR analyses of frozen brain regions are performed to verify mRNA expressions of pro-inflammatory mediators (brain inflammation). A known inflammogen, lipopolysaccharide (LPS 2 mg kg\(^{-1}\), SC) is used at this stage of experiment.
III. MATERIALS AND METHODS

III.1. Synthesis and characterization of GNPs:

The 10 nm spherical GNP was synthesized using the citrate reduction method. GNP particle sizes were analysed via UV-Vis absorption spectrophotometry and dynamic light scattering analysis. Size and shape of GNPs were examined using transmission electron microscopy. Dr. Saber Hussain’s laboratory at Wright Patterson Air force (711 HPW/RHDJ, AFRL) synthesized the 200 µg mL⁻¹ citrate-capped spherical 10 nm GNPs throughout the project, and provided the following characterization analysis, data, and graphs.

Figure III. 1: 200 µg mL⁻¹ citrate-capped 10 nm Spherical GNP

Figure III. 2: Transmission Electron Microscopy image of ~ 10 nm GNPs synthesized using citrate reduction method.
Figure III.3: Dynamic Light Scattering Data for citrate-capped 10 nm spherical GNPs

Figure III.4: UV-Vis absorption spectrum of the citrate-capped 10 nm spherical GNPs
Table 1. Gold nanoparticle specifications

<table>
<thead>
<tr>
<th>Method Used</th>
<th>Size</th>
<th>Morphology</th>
<th>Charge</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate Reduction</td>
<td>~10 nm</td>
<td>Spheres</td>
<td>-33.9 mV</td>
<td>200 µg mL⁻¹</td>
</tr>
</tbody>
</table>

III.2. In Vitro Studies:

III.2.1. UV-Vis Absorption Spectrophotometry (UV-Vis):

In order to verify the optimum buffer concentration of PBS for IV injection of GNP, the UV-Visible absorption measurements were carried out using Varian Cary 50 Bio spectrophotometer. The instrument was set to the following parameters: 190-1100 nm range, dual beam laser mode, 4800 nm min⁻¹ scan rate, and 1 nm intervals. Double deionized water was put into the cuvette as a baseline. Handling the cuvette by the clear side it was checked for scratches. Then the clear side was gently wiped using a Kimwipe, and the cuvette was put in the cuvette holder of the spectrometer with the clear side of it facing the direction of the incident light. After sample preparation the 3 mL disposable cuvettes were filled with the prepared samples to the mark. Finally the samples were put into the cuvette and placed inside the spectrometer. All the measurements were done using double deionized water as the baseline blank. When the run button was pressed the wavelength scan on the computer monitor showed the recording of the absorbance changes during the wavelength scan. This procedure was repeated for each sample.
III.2.2. Sample preparation:

400 µg mL\(^{-1}\) of 10 nm spherical GNPs was diluted to 200 µg mL\(^{-1}\) using double deionized water. The synthesized GNPs were individually characterized by adding 0.30 mL of the 200 µg mL\(^{-1}\) GNP to 2.7 mL of double-deionized water. To obtain the ideal buffer solution before IV injection, 0.270 mL of the 200 µg mL\(^{-1}\) 10 nm GNP was added to 0.03 mL of different concentrations of PBS 10 X, 1 X, 0.1 X, and 0.01 X (9:1 volume ratio), and then diluted in the cuvette with 2.70 mL of double-deionized water. Subsequently the sample was put into cuvette, and was run in UV-visible equipment. Three control measurements were performed for 0.1X and 10.0X PBS (3.00 mL of PBS), and for 180.00 µg mL\(^{-1}\) nanogold (0.270 mL of 200 µg mL\(^{-1}\) and 2.73 mL of double-deionized water) individually.

Figure III.5: Cary 50 UV-Vis Absorption Spectrophotometer by Varian (Dr. Ioana Pavel Sizemore’s Laboratory- Wright State University)
III.3. In Vivo Studies:

III.3.1. Animals:

C57Bl/6 male mice were randomly divided into two groups: control and experimental. The mice had free access to food and water and were maintained on a standard pellet diet [Harlan/ Teklad 8640 Rodent Diet (54% carbohydrate, 29% protein, 17% fat), (Teklad Animal Diets, USA)] and tap water ad libitum. Mice were housed under a 12 hour light /12 hour dark cycle (6:30-18:30, lights on) at a controlled temperature 22 ± 2 °C. The animals’ health was examined routinely. All protocols for research activities were approved by Wright State University Laboratory Animal Care and Use Committee (LACUC).

Figure III.6: C57Bl/6 Male Mice
**III.3.2. Intravenous Tail Vein Injection:**

Mice were injected IV with GNPs via tail vein. To facilitate tail vein visualization and ensure optimal injections, the tail vessels were dilated prior to injection. In order to achieve vessel dilation, housing cages were put on heating pad and under the heating lamp (60W) for about 10 minutes. Then, the mouse was restrained and transferred into a restraining device. A 26 G needle was used for injection. After locating the vein, the needle was inserted into the vein at an angle of approximately 20 degrees with its bevel facing upward. The needle was inserted slowly near the distal end (tip) of the tail. The needle was visualized as it enters the vein. Once the vein wall had been penetrated, the needle’s angle was decreased and the needle was directed approximately 2 mm cranially. Blood was aspirated into the needles’ hub just before injection. During material administration the vein should blanch and no material and swelling should be detectable at the injection site. Material was administered slowly to avoid vascular overload or rupture of the vein from excess pressure. Pressure was applied over the injection site by gently holding a piece of gauze over the injection site for approximately 30 seconds to prevent hematoma formation. The needle was inserted midway into the vein down the tail, permitting additional attempts for venipuncture proximally when the initial attempt was unsuccessful.
III.3.2.1. **Experimental Groups:**

- Treated group: 100 μl of 180 μg mL\(^{-1}\) 10 nm spherical GNPs were intravenously injected through the tail vein (the optimal volume for IV injection). In order to obtain a physiological solution for IV injection, GNP suspension was diluted by adding one part of 0.01 X PBS (10 μL) to nine parts of 200 μg mL\(^{-1}\) 10 nm GNPs (90 μL).

- Control group: for intravenous injection of this group one part of 0.01 X (10 μL) was added to nine parts of double deionized water (90 μL) to obtain a physiological solution.

III.3.3. **Perfusion:**

**Perfusion after Euthanasia with Euthasol:** 24 hours after IV injection of GNPs mice were perfused. A 130 mg mL\(^{-1}\) Euthasol solution was used to euthanize the mice. Mice were injected intraperitoneally (IP) with 1.25 μl g\(^{-1}\) of Euthasol (which corresponds to a
dose of 150 mg kg\(^{-1}\)). Once unresponsive to tail pinch, the abdomen was opened and thorax accessed through the diaphragm. A hypodermic needle was inserted into the left ventricle and the heart’s right atrium was nicked. Slowly 30-40 mL PBS was pushed until fluids draining from heart were clear. Then it was switched to a container of fixative, e.g. a combination of formaldehyde (2%) and glutaraldehyde (2%) in phosphate buffer (pH 7.4) and was perfused~50 mL until the desired amount of fixative was reached.

**III.3.4. Collection of the Brain Tissue:**

Immediately after perfusion whole brains (except brainstems) were collected and kept at -80°C.
III.3.5. Brain Dissection:

Different regions of the CNS such as septum, caudate, hippocampus, hypothalamus, cortex, frontal cortex, and spinal cord were dissected to specifically localize areas of accumulated GNPs. Dissection was performed using 1mm brain block (Ted Pella) and areas were selected by using stereotaxic atlas.

III.3.6. Digestion Protocol for Whole Brain:

The digestion process was performed using Explorer Hybrid pressurized microwave digestion system. Microwave digestion with aqua regia is the preferred method for digestion of mouse brain. In this experiment certain modifications of the digestion protocol were needed to customize the protocol for brain tissue. This modification helped improve the brain digestion process. For each control and treated brain tissue at least 4 vials were needed: 3 vials contained wet mass tissue sample (mg of brain) and 1 vial with all reagents (= blank: no tissue). There were three reasons why spiked GNPs were
omitted from ICP-MS quantification: first they cause saturation of the ICP-MS second existence of a GNP standard reference which represented GNP in samples, and finally the fact that the instrument measures the concentration of GNP three times (for each sample).
All vials were washed with 10% HNO₃ and rinsed with double deionized water three times. Prior to digestion, vials and samples were weighed. First the brain tissues were homogenized manually with “Potter -Elvehjem homogenizer”, and the homogenate tissues were transferred to the vials via double deionized water. Second 2 mL of 30% aqua regia and 200 µl of 500 ppb of Rh (internal standard) were added to each vial. Then vials were placed on the heat block at 60 °C for 1 hour and 90 °C for 3 hours (predigestion). The vials were vented during heating with heating block or microwave (to remove generated vapors). Subsequently Samples were placed in the heating microwave and heated up to 120 °C (table 2 and 3). 300-400 mL of H₂O₂ was added to each vial, and heated till 150 °C (table 4). Finally all samples were diluted to 10 mL. Brain tissue mass and total volumes are very important for this experiment. Double deionized water was added to the vials up to 10 g for whole brain (or 5 g for a specific area of the brain) before performing the ICP-MS. Since all organic molecules were destroyed in digestion process, there was no need to keep the vials cold before ICP-MS. Heating microwave digestion was conducted by Ryan T. Saadawi in collaboration with University of Cincinnati department of chemistry (Joseph A. Caruso Research Group).
The following table provides the digestion time and temperature setting:
Table 2. First stage microwave digestion program

<table>
<thead>
<tr>
<th>Temp °C</th>
<th>Ramp time Min</th>
<th>Hold time Min</th>
<th>Max pressure Bar</th>
<th>Power Watt</th>
<th>Power Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>3</td>
<td>1:0</td>
<td>190</td>
<td>50</td>
<td>off</td>
</tr>
<tr>
<td>90</td>
<td>2</td>
<td>5</td>
<td>190</td>
<td>75</td>
<td>off</td>
</tr>
</tbody>
</table>

*The vials are vent in this stage.*

Table 3. Second stage microwave digestion program

<table>
<thead>
<tr>
<th>Temp °C</th>
<th>Ramp time Min</th>
<th>Hold time Min</th>
<th>Max pressure Bar</th>
<th>Power Watt</th>
<th>Power Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>3</td>
<td>1</td>
<td>190</td>
<td>50</td>
<td>off</td>
</tr>
<tr>
<td>110</td>
<td>2</td>
<td>1</td>
<td>190</td>
<td>50</td>
<td>off</td>
</tr>
<tr>
<td>120</td>
<td>10</td>
<td>5</td>
<td>190</td>
<td>75</td>
<td>off</td>
</tr>
</tbody>
</table>

*There should not be any visible chunks of tissue in the vial before adding H₂O₂ and proceeding to the next step (table 4). Otherwise stage 1 and 2 of digestion program should be repeated one more time (H₂O₂ should only be added when the sample looks semi-clear).*

Table 4. 3rd stage microwave digestion program

<table>
<thead>
<tr>
<th>Temp °C</th>
<th>Ramp time Min</th>
<th>Hold time Min</th>
<th>Max pressure Bar</th>
<th>Power Watt</th>
<th>Power Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>3</td>
<td>1</td>
<td>190</td>
<td>80</td>
<td>off</td>
</tr>
<tr>
<td>130</td>
<td>2</td>
<td>1</td>
<td>190</td>
<td>75</td>
<td>off</td>
</tr>
<tr>
<td>150</td>
<td>2</td>
<td>3</td>
<td>190</td>
<td>75</td>
<td>off</td>
</tr>
</tbody>
</table>
Figure III.12: Explorer Hybrid Pressurized Microwave Digestion System

Figure III.13& III.14: Observation of Chunk of Tissue after Digestion Prior to Modification
**III.3.6.1. Aqua Regia Protocol:**

Aqua regia or nitro-hydrochloric acid is a mixture of 3 part of concentrated hydrochloric acid (HCl) to 1 part of concentrated nitric acid (HNO₃). This mixture results in an extremely corrosive solution which can dissolve even gold. In this experiment chemical splash goggles, face shield, lab coat and appropriate gloves were worn. The solution was mixed in a hood with the sash between me and the solution. First 150mL concentrated HCl was poured in a polypropylene container. Then 50mL of concentrated HNO₃ was added to the HCl carefully and slowly. Afterwards for dilution few milliliters of this mixture were added to water. It is very likely to become hot, more than 100 °C. As a result it should be handled with care. Aqua regia solutions should never be stored.

**III.3.6.2. Modification and Optimization of Digestion Protocol:**

To achieve the best result, several experiments were conducted and the results were compared with each other. In predigestion step (before applying heating block), instead of using aqua regia, different concentration of HNO₃ was utilized. For this experiment the optimal concentration was 35% (rather than using concentrated HNO₃). After adding 35% HNO₃ and Rh to the samples first and second stage of microwave digestion program was performed (table 2 and 3). Then H₂O₂ was added at the third stage of microwave digestion program (table 4) was run and finally concentrated HCl was added and again first stage of microwave digestion program (table 2) was run. Ultimately a clear solution without existence of any tissue in vials was developed.
0.5 mL of 35% Nitric Acid was added to each sample plus 100μL of 500ppb Rh to each sample. Vials were placed on the heat block at 60 ºC for 30 minutes, at 90 ºC for 30 minutes, and then at 110 C for 1 hour. Samples were placed in the heating microwave up to 120 C. 100μL H2O2 was added and heated till 150 ºC. Then 0.75mL of 34% HCL was added to all samples and heated to 90-100 ºC. Finally all samples are diluted to10.0 g (for whole brain) or 5.0 g (for a specific area of the brain). Another modification performed to make the result more reliable was omission of the third stage of microwave digestion program (table 4) for the blank vials.

**Figure III.15:** Using Modified Protocol the Solutions Were Completely Clear after Digestion
III.3.7. Inductively Coupled Plasma Mass Spectroscopy:

Inductively coupled plasma-mass spectrometry (ICP-MS) was utilized to quantify and localize GNPs in the CNS. The instrument used is the Elmer Nexion 300D ICP-MS (Waltham, MA).

8 µL of internal standard mix (Perkin Elmer) was added to each sample. Samples were vortexed and placed in the auto-sampler. After conducting optimization of the instrument to ensure accurate readings, 1 blank and 4 Gold standard solutions were read to obtain a calibration curve. Samples were then run to obtain all concentrations of the digested brain samples. The instrument was set to monitor metal 197Au.

Figure III.16: PerkinElmer® NexION® 300 D ICP-MS in WPAFB, 711 HPW/RHDJ, AFRL (Reproduced with Permission from http://www.perkinelmer.com)
**III.3.8. Proinflammatory Cytokine Expression:**

Mice were sacrificed, 24 hours after IV injection of 10 nm GNPs. Whole brains (except brainstems) were collected and kept at -80°C.

**Real-time PCR:** Total RNA from several brain areas (Cortex, frontal cortex, hippocampus, caudate, and hypothalamus) were isolated using Trizol® reagent (Invitrogen) and Phase-lock heavy gel (Eppendorf AG, Hamburg, Germany). The RNA was further cleaned using RNeasy mini spin column (Qiagen, Valencia, CA). Total RNA (4 µg) were then be reverse-transcribed to cDNA using SuperScript™ III RNase H- and oligo (dT)12-18 primers (Invitrogen) in a 20 µL reaction. Real-time PCR analysis of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Chemokine (C-C motif) ligand 2 (CCL-2), Interleukin-1 beta (IL-1β), Leukemia inhibitory factor (LIF), Interleukin-6(IL-6), Tumor necrosis factor-alpha (TNF-α), and Oncostatin M (OSM) were performed in an ABI 7500 sequence detection system (Applied Biosystems) in combination with TaqMan® chemistry. Specific primers and dual labeled internal fluorogenic (FAM reporter dye, Taqman MGB Probe, nonfluorescent quencher) probe sets (TaqMan® Gene Expression Assays) for these genes were procured from Applied Biosystems and used according to the manufacturer’s recommendations. All PCR amplifications (40-60 cycles) were performed in a total volume of 50 µL, containing 1 µL cDNA, 2.5 µL of the specific Assay of Demand primer/probe mix and 25 µL of Taqman® Universal master mix. Sequence detection software (version 1.7; Applied Biosystems) results were exported as tab-delimited text files and imported into Microsoft Excel for further
analysis. Relative quantification of gene expression was performed using the comparative threshold (CT) method as described by the manufacturer (Applied Biosystems; User Bulletin 2). Changes in mRNA expression levels were calculated after normalization to GAPDH. The ratios obtained after normalization were expressed as fold change over corresponding saline-treated controls. Additionally, a known inflammogen, lipopolysaccharide (LPS, 2 mg kg$^{-1}$, SC), was tested as a supplement. Real time PCR was conducted in collaboration with the Centers for Disease Control and Prevention by Dr. James P. O'Callaghan at the Molecular Neurotoxicology Laboratory.

III.4. Data and Statistical Analysis:

All data are expressed as mean ± SEM. Data were analyzed using STATISTICA® 7.1 (StatSoft Inc., Tulsa, OK). T ANOVA or un-paired t-test was used. Significance was achieved when p<0.05. All data was expressed as mean ± SEM. Data were expressed as mean and standard deviation.
IV. RESULTS

IV.1. In Vitro Studies:

IV.1.1. Optimal PBS Concentration for IV Injection of 10 nm GNPs:

In order to acquire an ideal physiological solution for IV injection of 10 nm GNPs, different concentration of PBS (10 X, 1 X, 0.1 X, 0.01 X) were mixed with 200 µg mL⁻¹ 10 nm GNPs (9 to 1 part). The aggregation of GNPs with these solutions was measured using UV-Visible Spectroscopy. 10 nm GNP in 0.01 X PBS had the least level of aggregation. The result of this study showed that mixture of 10 nm GNP and 0.01 X PBS was the most favorable physiological solution for IV injection. 0.01 X PBS and 10 nm GNPs were used separately as controls to compare the aggregation of 10 nm GNPs with different concentration of PBS by UV-visible Spectroscopy.
Figure IV.1: Ultraviolet-visible (UV-Vis) absorption spectrum of the spherical GNPs containing 200 µg mL\(^{-1}\) of the spherical GNP that was diluted by 10 fold.

Figure IV.2: UV-Vis absorption spectrum of the control 200 µg mL\(^{-1}\) spherical GNP that was diluted by 11.11 fold for measurement
**Figure IV.3**: UV-Vis absorption spectra of the control 0.01 X and 10.0 X PBS

**Figure IV.4**: UV-Vis absorption spectrum of the normal treatment using 200 μg mL⁻¹ of the spherical GNPs and 0.01 X PBS
Figure IV.5: UV-Vis absorption spectrum of varied treatment using 200 µg mL\(^{-1}\) of the spherical GNPs and 0.1 X PBS

Figure IV.6: UV-Vis absorption spectrum of the varied treatment using 200 µg mL\(^{-1}\) of the spherical GNPs and 1.0 X PBS
Figure IV.7: UV-Vis absorption spectrum of the varied treatment using 200 µg mL$^{-1}$ of the spherical GNPs and 10.0 X PBS
Figure IV.8: UV-Vis absorption spectra of the GNP and PBS controls, the normal treatment, and the varied treatment solutions
Figure IV.1 shows the characteristic UV-Vis spectrum of the GNP suspension of 200 µg mL\(^{-1}\) that was diluted 10 fold to a concentration of 20 µg L\(^{-1}\). This ensured that the GNP concentrations in the samples did not exceed the detection range of the instrument. The peak height of 0.44 a. u. corresponds to absorption at 525 nm. Figure IV.2 shows the control spectrum of the GNP suspension that was prepared as it is in a typical injection solution and then diluted by 11.11 folds to a concentration of 18.00 µg L\(^{-1}\). Figure IV.3 shows the control spectra of 0.01 X, and 10.0 X PBS. No absorption features were observed in the spectra of 0.1 X and 10.0 X of the PBS control. Figures IV.4, IV.5, IV.6, and IV.7 show the normal and varied treatments. All normal and varied treatment solutions contained 0.03 mL of PBS, an overall 18.00 ppm concentration of GNPs and a total volume of 3.0 mL in the cuvette. The normal treatment originally consisted of 0.3 mL solution with the same concentration and volume ration as in the actual 0.1 mL injection that was administered to the mice specimen. The normal injection contained 0.01 X PBS that was diluted 100 fold in the cuvette for measurement. The varied treatment contained 0.1 X PBS, 1.0 X PBS, 10.0 X PBS that were also diluted 100 fold in the cuvette for spectrum collection.
IV.2. In Vivo Studies:

IV.2.1. Biodistribution of GNP\textsubscript{s} in Whole Brain:

All procedures including injection, digestion, perfusion, and ICP-MS were combined to investigate the entry of GNP\textsubscript{s} through the BBB. 90 µL of 200 µg mL\textsuperscript{-1} 10 nm GNP\textsubscript{s} plus 10 µL of 0.01 X PBS was injected IV to the tail vein. 24 hours after IV injection mice were perfused; then the brains were collected, weighed, and digested. The ICP-MS results indicated that GNP\textsubscript{s} crossed the BBB, and were distributed throughout the brain. Treated mice had significantly higher concentration of GNP\textsubscript{s} compared to control mice. The ICP-MS measurements showed the control mice had the lowest concentration of GNP in their brain. Although the control mice were injected with PBS and double deionized water, this low concentration may be the incidental artifact or result of widespread existence of GNP in the environment.
Figure IV.9: Calibration Curve in ICP-MS for Whole Brain Samples. 1 blank and 4 Gold standard solutions were read to obtain a calibration curve.

Table 5. Gold accumulation in whole brain after 24 hours IV injection of gold nanoparticle

<table>
<thead>
<tr>
<th></th>
<th>ICP-MS Raw (µg l⁻¹)</th>
<th>µg mL⁻¹</th>
<th>ICP-MS volume</th>
<th>Total Au (µg)</th>
<th>ppm Conc. (µg Au g⁻¹ sample)</th>
<th>STD (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.52</td>
<td>0.000520</td>
<td>5</td>
<td>0.00</td>
<td>-----</td>
<td>0.021</td>
</tr>
<tr>
<td>Control 1</td>
<td>0.52</td>
<td>0.000528</td>
<td>5</td>
<td>0.00</td>
<td>0.0096</td>
<td>0.008</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.34</td>
<td>0.000344</td>
<td>5</td>
<td>0.00</td>
<td>0.0060</td>
<td>0.004</td>
</tr>
<tr>
<td>Control 3</td>
<td>0.52</td>
<td>0.000520</td>
<td>5</td>
<td>0.00</td>
<td>0.0060</td>
<td>0.083</td>
</tr>
<tr>
<td>Treated 1</td>
<td>4.66</td>
<td>0.004658</td>
<td>5</td>
<td>0.02</td>
<td>0.0097</td>
<td>0.000</td>
</tr>
<tr>
<td>Treated 2</td>
<td>4.72</td>
<td>0.004720</td>
<td>5</td>
<td>0.02</td>
<td>0.0098</td>
<td>0.029</td>
</tr>
<tr>
<td>Treated 3</td>
<td>6.70</td>
<td>0.006700</td>
<td>5</td>
<td>0.03</td>
<td>0.0134</td>
<td>0.041</td>
</tr>
</tbody>
</table>
Figure IV.10: Gold accumulation in whole brain 24 hours after IV injection of GNPs (10 nm). Concentration of GNPs in treated mice was significantly high in comparison to the controls. Data were analyzed using unpaired student t-test. Data are expressed as mean± SEM with 3 mice per group. * P<0.05 vs. control.

IV.2.2. Localization of GNPs in Specific CNS Regions:

To specifically localize and quantify the bioaccumulation of GNPs in CNS, specific areas of brain including septum, caudate, hippocampus, hypothalamus, cortex, frontal cortex, and spinal cord were collected (24 hours after IV injection of GNP) and tested. Subsequently areas were digested and the concentration of GNPs was quantified using ICP-MS. The localization and bioaccumulation assay successfully established that hypothalamus, hippocampus, and septum had the highest levels of GNPs (6.7, 6.2, and 4.6 µg Au g⁻¹, respectively).
Figure IV.11: ICP-MS calibration curve for brain regions. 1 blank and 4 Gold standard solutions were read to obtain a calibration curve.

Table 6. Gold accumulation in specific regions of brain 24 hours after IV injection of GNP

<table>
<thead>
<tr>
<th>Sample</th>
<th>ICP-MS Raw (µg L⁻¹ total)</th>
<th>Total Au (µg mL⁻¹)</th>
<th>Sample Volume (mL)</th>
<th>Total Au (µg)</th>
<th>ppm Conc. (µg Au g⁻¹ sample)</th>
<th>STD (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td>29.77</td>
<td>0.029770</td>
<td>5.0000</td>
<td>0.14885</td>
<td>6.15</td>
<td>0.0034</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>16.26</td>
<td>0.016260</td>
<td>5.0000</td>
<td>0.0813</td>
<td>6.66</td>
<td>0.0008</td>
</tr>
<tr>
<td>Septum</td>
<td>11.58</td>
<td>0.011580</td>
<td>5.0000</td>
<td>0.0579</td>
<td>4.56</td>
<td>0.0005</td>
</tr>
<tr>
<td>Caudate</td>
<td>9.28</td>
<td>0.009280</td>
<td>5.0000</td>
<td>0.0464</td>
<td>1.95</td>
<td>0.0005</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>8.01</td>
<td>0.008010</td>
<td>5.0000</td>
<td>0.04005</td>
<td>1.51</td>
<td>0.0002</td>
</tr>
<tr>
<td>Spinal</td>
<td>6.79</td>
<td>0.006790</td>
<td>5.0000</td>
<td>0.03395</td>
<td>1.32</td>
<td>0.0004</td>
</tr>
<tr>
<td>Cortex</td>
<td>6.14</td>
<td>0.006140</td>
<td>5.0000</td>
<td>0.0307</td>
<td>0.64</td>
<td>0.0005</td>
</tr>
<tr>
<td>Blank 1</td>
<td>4.23</td>
<td>0.004230</td>
<td>5.0000</td>
<td>0.02115</td>
<td>-----</td>
<td>0.0001</td>
</tr>
<tr>
<td>Blank2</td>
<td>3.57</td>
<td>0.003570</td>
<td>5.0000</td>
<td>0.01785</td>
<td>-----</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Figure IV.1IV2: Profile of gold concentrations in different regions of brain 24 hours after IV injection of GNP (10 nm).

IV.2.3. CNS Inflammatory Response to GNPs:

Considering the extensive applications of GNPs, it is essential to investigate the harmful neuroinflammatory effects of GNPs in specific regions of brain. Real-time PCR analysis of frozen brain regions was utilized to evaluate brain inflammation by quantification of pro-inflammatory mediators, LIF, CCL2 and IL1β. GNPs did not induce a significant increase of cytokine/chemokine expression in hypothalamus, hippocampus, caudate, cortex, or frontal cortex. LPS, as expected, caused a marked (100-fold) increase of the same cytokines. Surprisingly, there was not any noticeable upregulation of cytokine...
expression (mRNA) in these areas, but there was a significant downregulation of IL-1β expression in frontal cortex. Results revealed that GNPs enter brain and concentrate in specific regions without eliciting an inflammatory response.
Figure IV.13: LPS (2 mg kg\(^{-1}\) SC), as expected, caused a marked increase of the same cytokines.
Figure IV.14: LPS (2 mg kg\(^{-1}\) SC), as expected, caused a marked increase of the same cytokines.
Figure IV.15: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Interleukine-1 β (IL-1β) in hypothalamus did not indicate significant change of IL-1β level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean± SEM.

Figure IV.16: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Leukemia inhibitory factor (Lif) in hypothalamus did not indicate significant change of Lif level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean± SEM.
Figure IV.17: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Chemokine (C-C motif) ligand 2 (CCL2) in hypothalamus did not indicate significant change of CCL2 level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean±SEM.

Figure IV.18: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Interleukine-1 β (IL-1β) in hippocampus did not indicate significant change of IL-1β level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean±SEM.
**Figure IV.19:** Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Leukemia inhibitory factor (Lif) in hippocampus did not indicate significant change of Lif level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean±SEM.

**Figure IV.20:** Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Chemokine (C-C motif) ligand 2(CCL2) in hippocampus did not indicate significant change of CCL2 level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean±SEM.
Figure IV.21: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Interleukine-1 β (IL-1 β) in caudate did not indicate significant change of IL-1β level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean± SEM.

Figure IV.22: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Leukemia inhibitory factor (Lif) in caudate did not indicate significant change of Lif level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean± SEM.
Figure IV.23: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Chemokine (C-C motif) ligand 2 (CCL2) in caudate did not indicate significant change of CCL2 level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean±SEM.
Figure IV.24: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Interleukine-1 β (IL-1 β) in frontal cortex indicated significant change of IL-1β level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean± SEM, ** P<0.01 vs. control.

Figure IV.25: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Leukemia inhibitory factor (Lif) in frontal cortex did not indicate significant change of Lif level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean± SEM.
Figure IV.26: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Chemokine (C-C motif) ligand 2 (CCL2) in frontal cortex did not indicate significant change of CCL2 level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean±SEM.
Figure IV.27: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Interleukine-1 β (IL-1 β) in cortex did not indicate significant change of IL-1β level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean± SEM.

Figure IV.28: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Leukemia inhibitory factor (Lif) in cortex did not indicate significant change of Lif level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean± SEM.
Figure IV.29: Mice were sacrificed, 24 hours after IV injection (GNP (10 nm) + PBS in treated mice, and double deionized water + PBS in controls). Real-time PCR analysis of Chemokine (C-C motif) ligand 2 (CCL2) in cortex did not indicate significant change of CCL2 level in the treated group. Data were analyzed using unpaired student t-test. Data are expressed as mean± SEM.
V. DISCUSSIONS:

Initially, the optimal PBS concentration for IV injection of 10 nm spherical GNP was verified using in vitro tests (10 nm GNP in 0.01 X PBS showed the least amount of aggregation). Due to non-absorptive property of PBS, it did not cause any overlap or interference with the gold spectrum. De Jong, et al. (2008) demonstrated that addition of one part 10 X PBS to 9 part 10, 50, 100, and 250 nm GNP (1.0 X concentration of PBS) caused agglomeration of GNP. Similarly, our results indicated PBS concentrations of 1.0 X and above (10.0 X) caused gradual aggregation of the GNPs. Increasing concentration of PBS caused further aggregation of the GNPs (observed as a change in the GNP peak profile). Notably in the case of the 10 X PBS modified treatment, the GNP absorption peak became broader, the peak height decreased, and the wavelength slightly shifted toward the longer wavelength through a shoulder around 650 nm. This indicated a deformation of the spherical shape (perhaps change of the nanogold into other metallic or ionic forms in the solution).

In order to utilize 10 nm GNPs in drug delivery, diagnosis, and treatment of CNS diseases, it is essential to analyze its distribution, bioaccumulation, and neurotoxicity in specific brain regions. De Jong et al. (2008) demonstrated that distribution of injected GNPs is size dependent. His investigation proved 10 nm GNP had the most tissue distribution (24 hour after IV injection). 10 nm GNP showed the highest concentration in liver followed by blood, spleen, kidney, lungs, brain, reproductive organs, thymus, and
heart. However, 50, 100, and 250 nm GNP were just distributed in liver, spleen, and blood. Investigation of passage of GNP through the blood brain barrier and its entry to the brain, proved the 10 nm spherical GNP (24 hours after IV injection) is capable of passing the blood–brain barrier. The significant increase of GNP concentration in brain of the treated group compared to control group is proportionate to the dose administered and its biodistribution in different organs. Subsequently, to assess the bioaccumulation of GNP in specific regions of brain, different areas of brain were examined. Hypothalamus, hippocampus, and septum had the highest levels of GNPs. This indicates the possibility of passage of GNPs from choroid plexus and CVOs to the brain. Final investigation measured the inflammatory response of GNPs in CNS. No significant increase of proinflammatory cytokine/chemokine expressions were detected in the above mentioned regions of brain. Interestingly the results revealed significant downregulation of IL-1β expression in frontal cortex. The effect of GNP on IL-1β- dependent inflammatory response is unclear. Sumbayev, et al. (2012) discovered the mechanism of suppression of IL-1β-induced inflammatory reaction via citrate stabilized GNPs (in vivo and in vitro) for the first time. Downregulation of IL-1β-induced inflammatory response indicated that IL-1β molecules were accumulated near the surface of GNPs, which reduce the interaction of IL-1β with interleukin receptors. Consequently, citrate stabilized GNPs revealed the anti-inflammation response of IL-1β cytokines in a size dependent manner (5 nm GNP having the strongest effect).
Understanding the effects of NPs on the immune system is an essential step in accurate evaluation of its risk assessment. Generalization of toxic effects of GNPs is difficult due to influence of many factors such as chemical nature, size, roughness, form, aggregation state, coating, etc. Substantial accumulation of citrate GNPs (10 nm) in dendritic cells did not activate cell death. Results were measured in 4, 24, and 48 hour incubations. Further assessment of cytokine secretion showed significant downregulation of IL-12 p70 (is involved in T lymphocyte activation and regulation of antigen specific immune response) and unaffected IL-6 secretion in presence of 10 nm citrate GNPs. These results revealed that it is not possible to evaluate the effect of NPs in different organs by just measuring their cytotoxicity. In brief, the implemented cytotoxic analysis is not sufficient for determination of long term harmful effects of accumulation of GNPs in dendritic cells. Consequently, further analyses are needed to determine the extent of alteration of the cell functionality (Villiers, et al., 2010).

Although bulk gold is considered safe, biocompatibility and environmental impact of GNPs must be investigated. Despite cell uptake and accumulation of spherical GNPs, the MTT assay (mitochondrial activity) of the K562 cell line indicated no cytotoxicity up to ~ 100 μM. Due to toxicity of GNP precursors such as HAuCl4, its proper purification is important. It should be noted that because of the interaction of cationic particles with negatively charged cell membrane, they are toxic at much lower concentrations than their anionic counterparts. Failure to find toxicity does not eliminate ability of GNPs to cause
severe cellular damage i.e. 13 nm citrate capped GNP was not cytotoxic to skin cells, but promoted formation of abnormal actin filament and diminished cell proliferation, adhesion, and motility (Murphy, et al., 2008).

Proof of entry and accumulation of GNP in specific regions of brain raise the possibility of usefulness of GNP in drug delivery and therapeutic treatment of CNS diseases. Moreover, the discovery of anti-inflammatory effects of GNP will open a new window for further therapeutic application of GNP in CNS and autoimmune disorders that are reliant on specific cytokines specially IL-1β. However, it is essential to investigate the long term effect of GNPs in different organs, and then the pathway of their clearance from body.

Alteration of GNP surface area and charge are the main determinant of biokinetic fate of GNPs in the organism. Biodistribution of GNP is highly dependent on its charge. Accumulation of GNP in liver is size dependent (larger size having the highest accumulation). Liver is the main organ for accumulation of positively charged GNPs and kidney is the main organ of accumulation for negatively charged GNPs. Highly positive charged NPs have the fastest elimination rate in liver (Hirn, et al., 2011). Most studied nanoparticles are eliminated from circulatory system through reticuloendothelial system (RES) and are eliminated in liver and spleen (Semmler-Behnke, et al. 2008). Exogenous molecules and particles are taken up by resident macrophages of reticuloendothelial system (called: kupffer cells in liver, microglia in brain, alveolar cells in lungs, and reticular cells in lymph node, bone marrow, and spleen) (Abdelhalim, et al., 2011). In
order for GNP to be eliminated through renal excretion, they must have hydrodynamic
diameter of 5.5 nm or smaller. Excretion of injected 18 nm GNP through hepatobiliary
system is only 0.5%, while renal excretion is extremely low (Semmler-Behnke, et al.
2008; Choi, et al., 2007).

Knowing nanoparticles are less prone to elimination routes, their elimination must be
studied very carefully (Ballou, et al., 2007). Because GNPs are mainly larger than 5.5
nm; their elimination from blood is mainly via reticuloendothelial system (RES).

Consequently, they are accumulated in liver and spleen (De Jong, et al., 2008 and von
Maltzahn, et al., 2009). Additional experiment and further studies are required to measure
the toxicity dose and potential toxicokinetic effect of various GNPs (different shape, size,
and surface charge) in different organs, especially in brain. Finally, it is imperative to
thoroughly investigate GNP biodistribution, long-term effects, clearance, and cytotoxicity
in brain and other vital organs before any clinical application.
VI. CONCLUSION:

Initially we performed in vitro investigations, to determine the optimal buffer concentration of GNP for IV injection (10 nm GNP in 0.01 X PBS had the least level of aggregation). Furthermore, GNP in specific regions of brain were quantified and localized. At the completion of this project, neuroinflammatory response of cytokines was detected. Remarkably there was no upregulation of cytokines. On the contrary, IL-1β in frontal cortex was significantly decreased, which is a sign of suppression of inflammation. This effect is very important for the implication of GNP in clinical diagnosis and therapeutic treatment of neurodegenerative disorders and especially IL-1β-dependent neuroimmune disorders.

This novel approach provides a basis for further utilization of GNP in drug delivery, detection, and therapeutic treatment of a number of CNS and neuroimmune diseases. However to ensure its safety, there is need for extensive investigation on effective dosage, duration of treatment, and on GNP clearance from the brain. Additionally, further investigations are needed to assess the effects of different size, shape, charge, and functionalized GNP to improve their biomedical applications in chemical sensing, biological imaging, drug delivery, treatment of neurodegenerative diseases, cancer treatment, etc. Since this research is still in its early developmental stage, it is vital to check the clearance, neuroinflammatory, and toxicity of various sizes and concentration
of GNP s in a longer duration of treatment. Hopefully, this may provide the basis for targeted treatment of diseases in future.
VII. REFERENCES


doi:10.1210/er.21.3.313


doi:10.1016/j.pharmthera.2004.08.001


doi:10.1002/cne.903370403


66


repeated administration in mice. *Biochemical and Biophysical Research Communications*, 393(4), 649-55. doi:10.1016/j.bbrc.2010.02.046


