Short- and Long-Term Effects of Commercially Available Gold Nanoparticles in Rodents

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biomedical and Veterinary Sciences

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December 11th, 2013
Blacksburg, VA

Keywords: gold nanoparticles, nanotoxicity, mice, rats, chronic, commercially available

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ABSTRACT

Gold nanoparticles (GNPs) are currently being intensely investigated for their potential use in biomedical applications. Nanotoxicity studies are urgently needed to validate their safety in clinical practice. The objective of this research was to assess the acute, subacute, and chronic effects of a single intravenous exposure to commercially available GNPs in two *in vivo* models, mice and rats. Gold nanoparticles were purchased and independently characterized. Animals were exposed to either 1000 mg GNPs/kg body weight (GNP group) or an equivalent volume of phosphate buffered saline (PBS group) intravenously via the tail vein. Subsets of animals were euthanized 1, 7, 14, 21, 28 days (female BALB/c mice and female F344 rats) or 20 weeks (female and male C57BL/6 mice) post-exposure and samples were collected for biochemistry, histopathology, electron microscopy, and atomic absorption spectrometry analysis. Independent characterization demonstrated that the physicochemical properties of the purchased GNPs were in good agreement with the information provided by the supplier. Important differences in GNP-induced immune responses were identified when comparing mice and rats 1 to 28 days post-exposure. Gold nanoparticles stimulated the formation of liver microgranulomas in mice, along with transiently increased serum levels of the proinflammatory cytokine interleukin-18. No such alterations were found in rats. Species differences in GNP biodistribution and excretion were also detected, with higher relative accumulation of GNPs in spleen and longer fecal excretion in rats. In the

long-term (20 weeks after dosing), exposure to GNPs incited chronic inflammation in mice, characterized by the persistence of microgranulomas in liver, spleen, and lymph nodes, as well as further increased serum levels of interleukin-18. Impairment of body weight gain was also observed in the GNP-exposed group. No sex differences were detected. In conclusion, GNPs are not innocuous and have the ability to incite a robust macrophage response in mice. However, considering the mildness of the toxic effects identified despite the high dose selected for the study, GNPs continue to have great potential for biomedical uses. Further studies are needed in order to determine specific mechanisms of toxicity and the role of chronic inflammation in the development of adverse effects after co- or post-exposures.

DEDICATION

I dedicate this work to my beloved husband, Marcelo. His infinite love and unconditional support have been essential throughout this life experience. Te amo!!

ACKNOWLEDGMENTS

I am deeply thankful to everybody that collaborated in some way to the completion and success of this challenging path towards a PhD degree:

All animals utilized in this research

Advisory Committee Members: Renee Prater, Marion Ehrich, Bill Huckle, and Claudio Gutierrez

Labmate: Bonnie Brenseke

Collaborators at the Department of Civil and Environmental Engineering: Matthew Chan, Ronald Kent, and Peter Vikesland

Statistical assistance: Ana María Ortega

Funding: Edward Via College of Osteopathic Medicine (VCOM) and Institute of Critical Technology and Applied Science (ICTAS) at Virginia Tech

Teaching and Research Animal Care Support Service (TRACSS): Lynn Chipkin, Michelle Dobbins, Karen Hall, Peter Jobst, Connie Kingrea, Betsy Midkiff, Dana Reynolds, Dee Snider, and Marlice Vonck

Laboratory specialists: Jen Averill, Kristel Fuhrman, and Delbert Jones

Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) Histology Laboratory: Jenn Rudd and Barbara Wheeler

VMRCVM Morphology Laboratory: Kathy Lowe

VMRCVM Toxicology Laboratory: Geraldine Magnin-Bissel and Barbara Wise

VMRCVM Necropsy Laboratory: Daniel Armstrong

VMRCVM Laboratory for Neurotoxicity Studies: Sandy Hancock, Jonathan Hinckley, and Bernard Jortner

VMRCVM Clinical Pathology Laboratory

ICTAS Nanoscale Characterization and Fabrication Laboratory: Stephen McCartney

Virginia Tech Center for Sustainable Nanotechnology and ICTAS: Marina Quadros

VMRCVM administrators and staff: Gerhardt Schurig, Roger Avery, Ansar Ahmed, Becky Jones, Monica Taylor, Cyndi Booth, Patricia Goudy, and Janet Bramlett

Virginia Tech Graduate School: Karen DePauw and Justin Shanks

Virginia Tech Cranwell International Center

ATTRIBUTION

Several colleagues contributed significantly in the preparation and completion of the research presented throughout this dissertation. Their current affiliation along with a brief description of their contributions is provided here.

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LIST OF ABBREVIATIONS

11-MUA	11-Mercaptoundecanoic Acid
AAS	Atomic Absorption Spectrometry
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ANACOVA	Analysis of Covariance
ANOVA	Analysis of Variance
AST	Aspartate Transaminase
BBB	Blood-Brain Barrier
BCA	Biobarcode Amplification Assay
BTB	Blood-Testis Barrier
CALND	Pentapeptide Cys-Ala-Leu-Asn-Asp
CALNN	Pentapeptide Cys-Ala-Leu-Asn-Asn
CALNS	Pentapeptide Cys-Ala-Leu-Asn-Ser
CNS	Central Nervous System
ELISAs	Enzyme-Linked Immunosorbent Assays
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
GGT	Gamma-Glutamyl Transpeptidase
GNPs	Gold Nanoparticles
GSH	Glutathione
H&E	Hematoxylin-Eosin
HIV	Human Immunodeficiency Virus

ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICTAS	Institute of Critical Technology and Applied Science
IFN-γ	Interferon-gamma
IL	Interleukin
IM	Intramuscular(ly)
IP	Intraperitoneal(ly)
IT	Intratracheal Instillation
IV	Intravenous(ly)
MDA	Malondialdehyde
MEEE	Mercaptoethoxyethoxyethanol
MES	Mercaptoethanesulfonate
MINChar	Minimum Information on Nanoparticle Characterization
NIST	National Institute of Standards and Technology
NO	Nitric Oxide
OECD	Organization for Economic Cooperation and Development
PBS	Phosphate Buffered Saline
PCL	Polycaprolactone
PCR	Polymerase Chain Reaction
PDI	Polydispersity Index
PEG	Polyethylene Glycol
PEGylated	Polyethylene Glycol-Coated
PO	*Per Os* (oral administration)
PVP	Polyvinylpimolidone

RoA	Route of Administration
ROS	Reactive Oxygen Species
SC	Subcutaneous(ly)
SEM	Scanning Electron Microscopy
SEM-EDX	Scanning Electron Microscopy coupled with Energy Dispersive X-ray Spectroscopy
SPR	Surface Plasmon Resonance
TEM	Transmission Electron Microscopy
TMAT	Trimethylammoniumethanethiol
TNF	Tumor-Necrosis Factor
TPPMS	Triphenylphosphine Monosulfonate
TPPTS	Triphenylphosphine Trisulfonate
VMRCVM	Virginia-Maryland Regional College of Veterinary Medicine
Z_{ave}	Average Particle Hydrodynamic Diameter

CHAPTER I: Literature Review

A. Introduction

Nanotechnology is defined by the U.S. National Nanotechnology Initiative as "the understanding and control of matter at the nanoscale, at dimensions between 1 and 100 nanometers approximately".[1] Nanomaterials have unique physicochemical properties (nano-characteristics) such as ultrahigh reactivity, huge specific surface area, special electronic and optical properties, and easy surface modification.[2-5] These characteristics are highly useful and carry the potential of solving many of the current problems in key areas of science and technology, including food science, materials science, environmental science, electrical engineering, pharmacology, and medicine.[2,4-12] Unfortunately, the rapid development of nanotechnology worldwide has not been accompanied by an equally fast development of research about the health impacts of nanomaterials, and, with the subsequent increase in generation and usage of engineered nanoparticles, exposure and toxicity remain a matter of concern.[2,3,6,13-16] Gold nanoparticles (GNPs) in particular are currently used in products such as cosmetics, hair tonics, ink, and lubricant oil.[13] More importantly, they have been suggested as one of the top candidates for biomedical uses, mainly due to their low biological reactivity, easy surface functionalization, fluorescence, photothermal properties, and other unique optical properties arising from their strong and size-tunable surface plasmon resonance (see below, section C).[7,17,18] Gold nanoparticles were believed to be chemically inert,[6] but recent studies have reported toxicity *in vitro*[19,20] and *in vivo.*[7,17,21,22] Genuine comparison among studies is, however, seldom possible, considering that 1) the reactivity and toxicity of nanomaterials depends

largely on their physicochemical characteristics such as size and surface coating,[23,24] 2) the nanoparticles studied are often not well-characterized[25] and different research groups frequently have assessed GNPs with dissimilar physicochemical features, and 3) that different studies used diverse animal models, doses, routes of administration, and endpoints. Furthermore, most *in vivo* studies are short term, albeit it is now well-known that GNPs accumulate in tissues and that clearance is very inefficient,[6,13,26] which could lead to toxic effects emerging long after a single exposure.[7] Our research mimics the intended use of GNPs in biomedical applications, and aims to further elucidate the acute as well as chronic effects of these nanoparticles in two animal models. The results of this study will contribute greatly to the progress of nanotechnology in the biomedical field, by uncovering essential information about GNP safety.

B. Nanotoxicology: History and Current Challenges

In 2004, Donaldson et al.[27] coined the term "nanotoxicology", a new discipline within toxicology, destined to "address gaps in knowledge and to specifically address the special problems likely to be caused by nanoparticles" as a response to evidence suggesting that nanoparticles could have adverse effects not only at their portal of entry, but they could also translocate and affect distant target organs. They recognized that nanomaterials could have very unusual biological effects when compared to conventional particles. These includes their ability to escape the normal detection and defense mechanisms, their possible role as haptens (modifying proteins and causing dysfunction or autoimmune effects), and their great potential for interaction with fluids, cells, and tissues, especially at the site of final retention where they could trigger inflammatory or

immunological responses.[27] The idea was supported by Oberdörster and colleagues in 2005,[24] and since then nanotoxicology has become a recognized discipline.[3]

In their review, Oberdörster et al.[24] highlight the importance of surface chemistry in determining any material's properties. Taking a substance to nanoparticle form increases exponentially the ratio of surface to total atoms or molecules, conferring new and desirable properties to the substance, hence the extraordinary interest in nanotechnology development in recent years. It follows that these same new and unique properties may have positive and desirable effects when in contact with biological systems, but they can, at the same time, be responsible for negative and undesirable effects that remain to be determined.[23,24,28,29] Therefore, the potential risks of nanomaterials are not the same as those of the bulk material of identical chemical composition, and nanotoxicology studies are needed.[2,14,24,30]

Some of the major concerns regarding nanomaterials' potential adverse health effects result from their ability to enter cells by non-endocytic pathways, their ability to translocate and appear in unexpected locations in the body, and their ability to elicit unexpected biological consequences.[10,14,16,25,31,32] Nanoparticles larger than 50 nm are usually found within cells (mostly macrophages) enclosed in phagolysosomes. As particle size decreases, the probability of nanoparticles entering cells free and unbound or via small vesicles or endosomes increases.[14] Nanoparticles that enter cells by non-endocytic mechanisms appear in the cytoplasm of phagocytic as well as non-phagocytic cells, and they are not enclosed by membranes. This means that they stand in direct contact with intracellular proteins, DNA, and other intracellular molecules and structures, increasing their potential for interaction and toxicity.[33,34] On the other hand, it has been

demonstrated that nanostructures have restricted access to some body and/or cell compartments, such as the brain and the nucleus respectively, a restriction that depends largely on their chemical makeup, surface characteristics, and functionalization (conjugation with chemicals and/or biomolecules).[16,35] Furthermore, nanoparticles can now be designed to reach specific compartments, but their final metabolic fate and secondary unintentional effects are still generally unknown.[35] The matching of scales between biological machinery and nanomaterials allows novel macromolecule-particle interactions, and the immunological consequences of these interactions also remain to be determined.[30,34] In this context, it is important to bear in mind that not only direct toxicity on immune cells, but even small alterations on the normal defense mechanisms and responses could lead to a higher risk of developing diseases.[36]

Although there is still a long way to go, there have been advances in the identification of general mechanisms of toxicity of nanomaterials. *In vitro* and *in vivo* studies have shown that nanosized particles of diverse characteristics have higher inflammatory potential per given mass when compared to bigger particles with the same chemical composition, and have identified oxidative stress as one of the underlying mechanisms of toxicity.[10,24,37-46] The exact mechanism by which diverse nanoparticles cause the formation of reactive oxygen species (ROS) is not yet fully understood, but suggested mechanisms include 1) photoexcitation of nanomaterials that causes the release of free electrons, 2) metabolism of nanoparticles that creates redox active intermediates, and 3) inflammatory responses that cause free radicals discharge by macrophages.[24] Inflammation may lead to numerous pathophysiological states that end in acute inflammatory disease or act as a subchronic/chronic initiator/promoter of more harmful

degenerative or neoplastic diseases.[34] Accordingly, it has been recommended that inflammation and oxidative stress indicators should be included as endpoints in all nanotoxicology studies.[42]

Toxicity, including nanotoxicity, depends on the properties of the toxic compound (nanomaterial), the characteristics of the recipient (cell or organism), and the conditions of exposure (Figure 1). Therefore, changes in any of those factors can influence the toxicity outcome. Despite a growing body of evidence about potential toxic effects of nanoparticles, no well defined and accepted guidelines nor standardized methods for nanotoxicology assessment exist, and the selections are often based on the investigator's resources and training. Often different techniques give different results when measuring the same parameter, providing conflicting information and making extrapolation of conclusions and comparison among studies very challenging.[15,36,47-49] There is even an ongoing debate regarding the best dose metrics for nanotoxicity studies. Surface area and number of nanoparticles in exposure medium have been suggested as better alternatives to the classic and still generally used dose metric based on mass of the sample, under the belief that the alternatives offer better dose-response correlations.[15,41-43,50] However, most of these studies have assessed inhaled insoluble low-toxicity nanoparticles, and it is not clear if this better correlation is a general rule that can be applied to all nanomaterials and exposure routes.[28,34] Despite an international research effort, results are frequently contradictory and there are still insufficient data available to make knowledgeable decisions about nanomaterials safety.[31,32,36,42] Current major issues with nanoparticles research include 1) lack of adequate material characterization, 2) irrelevant cell types used for *in vitro* studies, 3) no pertinent routes of exposure and difficulty in the

determination of the appropriate dose(s) used for *in vivo* studies, and 4) lack of chronic exposure studies (personal communication, Roundtable Session: A Decade of Nanotoxicology: Where Do We Stand Now?, 52nd meeting of the Society of Toxicology, March 2013).

1) Selection and characterization of nanomaterials for toxicology testing

Due to the great advance of nanotechnology in recent years there is a vast and constantly expanding number of nanoparticles to be tested for potential toxic effects, which represents a major challenge in hazard assessment of nanotechnology.[51,52] It has been estimated that more than 1,300 consumer products currently on the market incorporate nanoscale materials,[53] and only a few have been tested for nanotoxicity.[42] It is, however, completely unfeasible to execute toxicology studies for all existing and yet to be developed nanomaterials, hence, in order to aid researchers and funding agencies in the selection of nanomaterials for toxicological assessment, the Organization for Economic Cooperation and Development (OECD) published a list of representative nanomaterials that should be tested with highest priority.[54] The main criterion they used to select these nanomaterials was predicted high level of production and use, but they also considered other factors such as existing evidence of toxicity. Nevertheless, it has been recommended that toxicological testing should not be conducted exclusively on these reference nanomaterials, as they do not reflect the great diversity of available and developing nanomaterials.[42]

It is recognized that the biologic activity and biokinetics of nanomaterials are dependent on many parameters, including size, shape, chemistry, surface properties

(area, reactivity, coating, charge, surface modifications, weathering of coating), agglomeration state, biopersistence, and dose,[10,23,24,28,32,50,55] but the relative importance of each one of these features with respect to toxicity is not clear.[42,48,56] In fact, the only feature all nanomaterials have in common is their small size,[42] but even if we standardize every other parameter and only consider size as a variable, there is not a sudden change in properties or fate when particles fall below the arbitrary 100 nm limit.[9,14,29,34] On the contrary, significant differences often exist between the behavior of 10 nm and 90 nm particles, or even between ~1 nm and ~20 nm, although both materials officially fit the current criteria for nanoparticles.[14,34] Actually, the fact that a material is in the nanoscale range does not necessarily mean that it will exhibit nanoscale-specific toxicity.[34,56] When considering all possible variations in physicochemical parameters that could influence the potential for toxicity of nanomaterials, it becomes clear that it is imperative that nanoparticles should be properly characterized in nanotoxicology studies in order to interpret, compare, and reproduce data.[15,23,34,47-49,51,56,57] But how much is enough in terms of physicochemical characterization? There have been several recommendations[15,28,48,49,56,57] and some efforts to develop guidelines for the minimum characterization required for nanotoxicity studies, such as the "Recommended Minimum Physical and Chemical Parameters for Characterizing Nanomaterials on Toxicology Studies" created by the Minimum Information on Nanoparticle Characterization (MINChar) Initiative on 2008,[58] but to date none of these recommendations or guidelines have been consensually accepted and followed by the research community.[42]

One of the advantages of assessing toxicity of engineered nanomaterials is that, because they are purposefully made, they can be uniform (monodisperse) and have precise chemical characteristics, in contrast with unintentional anthropogenic nanoparticles, that are generally non-uniform (polydisperse) and chemically complex.[24,45,49] Yet, not all engineered nanomaterials are standardized, due to variability in the production methods or post-production modifications.[28,34,36,47] Moreover, nanomaterials can even vary with time (e.g., during storage) and from batch to batch from the same provider, so samples used in research can differ greatly from one another.[34,36,47,48] This fact, that may be seen as a problem for the development of toxicological assessments, may actually reflect real-life scenarios, as it is expected that most nanomaterials will be manufactured within a range of physicochemical properties that ensures functionality without over-costly production processes.[34] At this point it is important to note that significant differences have been observed between data from independent characterization of purchased nanomaterials and the information reported by the manufacturer, so independent characterization is still a must in all toxicology studies, including those that utilize commercially manufactured nanomaterials.[47,59]

In spite of the recognized importance of the relationship between physicochemical features and toxicity, currently one of the biggest issues with nanoparticle research is the lack of adequate material characterization.[23,25] This problem prevails in part due to lack of consensus on which parameters need to be included, financial and time limitations, the extensive instrumentation and skilled manpower necessary for throughout nanomaterial characterization, and, in many cases, to the inability of

current technologies to make appropriate measurements. Thus, complete characterization recommended in theory is almost never practically feasible.[15,31,34] Many of the currently available characterization techniques have limitations, and different methods often produce different results when measuring the same parameter, encouraging researchers to use more than one technique (when accessible) for each parameter of interest.[42,48,49] Until a consensus is reached about the properties of nanomaterials that compel assessment, and the appropriate analytical techniques to do so (considering that many of these techniques are still to be devised), a flexible approach is recommended. This implies the specific goals of each study and the feasibility of conducting the analysis dictating the level of characterization required, instead of demanding rigorous physicochemical characterization of nanomaterials for every toxicological investigation.[42,48]

Another challenge for nanotoxicology comes from the stability, or lack thereof, of nanomaterials within biological systems.[48,49] It has been demonstrated that surface coatings and covalent modifications can be altered under environmental conditions, specifically exposure to oxygen and UV light, resulting in augmented production of ROS and cytotoxicity.[60,61] It is also known that nanoparticle properties in liquid suspensions are dynamic,[15] so it is reasonable to think that coatings may as well not be bio-persistent, or they could be modified or metabolized to expose the core nanoparticle material.[24,35] Also, nanomaterials have a strong tendency to agglomerate (particles held together mainly due to weak Van der Waal's forces)[28,48,49] and/or aggregate (particles held together by stronger chemical bonds or sintered)[28] when suspended in biological media such as phosphate buffered saline.[10,15,42,45,51] This

phenomenon changes their size, surface area, shape, and number concentration,[15,34] as well as their ability to enter cells by endocytic pathways versus non-endocytic pathways.[33,62] As already mentioned, nanomaterials' behavior and biologic reactivity are dependent on their physicochemical characteristics, so the ability to distinguish the effect of nanoparticles versus bigger particles of similar composition depends on the use of well dispersed nanoparticles in nanotoxicology studies.[30,51] Yet, the degree of dispersion desired for any nanomaterial hazard investigation should reflect the specific goals of the study (e.g., to identify specific structure-toxicity relationships versus simulate real-life exposure scenarios where the nanoparticles may not necessarily be monodispersed).[42] Strategies to disperse particles usually modify their surface, modifying at the same time their biologic activity and kinetics.[14,42,63] One of the most popular methods to prevent agglomeration is to coat the nanoparticles with a stabilizing agent, for instance polymers or oligomers, that impedes close proximity of particles, therefore hindering attraction forces from becoming effective.[64] Other methods include electrostatic stabilization and sonication, but even after succeeding in separating nanoparticles, the dispersed material may be unstable and re-agglomerate.[14,48] Furthermore, as soon as nanoparticles come in contact with biological fluids or cell culture medium, they become spontaneously coated with biological molecules, forming what is called a "protein corona".[34,36,59,65-67] This additional structure has a dynamic ("soft") and a more stable ("hard") component, with higher and lower exchange rates of proteins between the nanoparticle's surface and media, respectively.[59] For instance, the composition of the protein corona, in particular the soft component, changes depending on the media (i.e., the biological

compartment) into which the nanoparticles are immersed. The specific factors that govern adsorption affinity or selectivity are not clear,[68] although it has been reported that particle size and surface properties influence the composition of proteins that bind to nanoparticles to form the hard component of the protein corona.[59] It has been hypothesized that, since the protein corona influences what surface is actually perceived by cells, nanoparticle-protein interactions would govern the nanoparticle's behavior and cellular response.[34,36,65,66] The impact of these interactions on protein availability, conformational changes (including the constitution or exposure of otherwise hidden immune epitopes)[30], and function are also expected to significantly contribute to toxicity and still need to be addressed.[25,65,66] On the whole, any of these *in vivo* changes would alter the biocompatibility and potential for toxicity of the nanomaterial in question. Subsequently, for *in vivo* studies it is highly desirable to characterize the nanoparticles before, during, and after the exposure(s), or as much as possible considering the current limitations on appropriate and readily available characterization methods, especially in complex biological media.[34,42,48,49]

2) Substantiated alternatives to *in vivo* studies

In vitro studies can provide invaluable information about biologic interactions of nanomaterials with specific types of cells and mechanisms of toxicity, but they are generally unable to replicate complex organic or systemic reactions like inflammation, as well as chronic consequences of exposure, and their ability to predict the pathogenicity of nanomaterials is often doubted.[17,28,34,42,56] They require an initial *in vivo* screening to shed light on the mechanisms of induction of biological

responses and to determine relevant cell types and doses.[28,35,51] Validated means of extrapolating acute *in vitro* results for predicting chronic *in vivo* effects have not been developed.[28] Also, there are extra challenges for *in vitro* analyses of cytotoxicity, such as the ability of nanoparticles to adsorb biomolecules and chemicals, which can result in artifactual cell death or survival, produced by the nanoparticles binding essential nutrients and/or detection reagents.[36,51] Another example is the problem of dose, as exposed cells may receive a very different dose than intended due to the non-uniform spatial distribution of nanoparticles within the suspension medium.[34] These problems must be solved in order to develop reliable *in vitro* assays for the assessment of nanomaterials safety.[36]

Due to the high cost and labor-intensive quality of experimental means for toxicological evaluation of nanomaterials, there have been efforts to develop *in silico* alternatives (e.g., Quantitative Nanostructure-Activity Relationship models) to predict the toxicity of manufactured nanoparticles in biological systems based on their physical, chemical and/or geometrical properties.[68-70] However, the development of structure-toxicity models requires large amounts of data, including careful and systematic physicochemical characterization of the nanomaterials.[34,40,70] Thus this line of research (along with the field of nanoinformatics in general) is at its earliest stage and these models are not yet widely available for application in nanotoxicology assessment.[23,70,71] Owing to the ever increasing number of nanoparticles to be toxicologically tested and to the impossibility of doing so for every single one of them, the development and validation of simple non *in vivo* assays, including *in vitro* and *in silico* tests, is of utmost importance.[11,28,34,56,71] It is

expected that *in vitro* nanotoxicology studies will play an essential role in elucidating character-specific mechanisms of toxicity and means of interaction of nanomaterials with cells and molecules, providing the information necessary to develop those models.[42] But in order to be useful, *in vitro* studies must have *in vivo* substantiation.[35,56] In fields such as nanomedicine, which involves intentional administration of nanomaterials to the body, *in vivo* studies are still considered indispensable[17,28,30] and a key component of the hazard identification process.[17,35,56,72] On the other hand, animal experiments also have significant limitations, particularly the potential differences in reactions of animal models (usually rodents) versus humans to the same material or substance. Human cell and tissue studies could be conducted in addition to animal studies in order to detect any potential species-related differences.[73] In conclusion, both *in vitro* and *in vivo* studies are essential for the advancement of nanotoxicology and nanotechnology, provided that they are conscientiously used to provide feedback to each other, and that the limitations of each of them are carefully considered when drawing and extrapolating conclusions.

3) Pertinent levels and routes of exposure for nanotoxicity studies

Humans have always been exposed to nanoparticles, mostly via inhalation of particles of dust and debris. Such exposures have increased dramatically since the industrial revolution. Now, with the rapid progression of nanotechnology, there is not only increased risk of exposure, but also exposure by different routes including intravenous (IV), subcutaneous (SC), or intramuscular (IM) administration for

diagnostic and therapeutic purposes.[6,14,24,29,36,40,41] Important aspects to consider when designing nanotoxicology studies, as with any toxicology study, are dose and route of exposure. They should be carefully selected to reflect the expected human exposure route and levels.[15,24,28,51,56] Most nanotoxicology studies have focused on accidental exposure via inhalation or dermal contact,[35] but given the increased interest in biomedical applications of nanotechnology, *in vivo* studies taking into account intentional exposures must be performed.[25,28] In this last case, defining the most relevant route of exposure may not be as difficult, as intentional exposures to nanoparticles in biomedicine are generally in the form of IV injections.[5,36,40,41] However, determining the appropriate dose or range of doses may prove to be very challenging, as there is an almost complete lack of data for human exposure levels and biokinetics of nanoparticles.[24,28,36,42,51,56] Differences in tissue exposure should also be considered when selecting models and appropriate endpoints. Vascular endothelial cells may be directly exposed to a higher dose of nanoparticles when administered as IV injections, therefore having increased risk of suffering detrimental effects.[40,41] Effects on the liver and spleen, especially on Kupffer cells and macrophages, also need to be intently scrutinized due to bioaccumulation in blood filtering organs, in particular for the numerous non-degradable nanoparticles currently in development.[25] In conclusion, it is recommended that researchers select the dose(s) to be used based on the goals of their particular study, including the rationale and justification of the selection when publishing their results,[42,66] and that they provide the concentrations on a mass and surface area basis, when possible.[42]

4) Chronic exposure to nanomaterials

Even though nanoparticles are already being applied in nanomedicine and, consequently, people are being intentionally exposed, there is no standardized test system for "medical" nanomaterials, and they are tested borrowing protocols from biomaterials testing and drug testing.[40,41] Nanoparticles for application in the medical field are often approved for development on the belief that they are biodegradable and/or eliminable, with no great potential for accumulation,[40,41] and most nanotoxicology studies performed are high dose, acute studies.[42,56,73] Still, it is known that many nanomaterials undergo little to no metabolism or excretion, accumulate in several organs and tissues and have the potential to trigger chronic inflammatory or immunological responses.[27] Persistence in biological systems and long-term effects are important aspects of general nanoparticle toxicity,[34] raising concerns about chronic effects of non-biodegradable, non-eliminable nanoparticles applied in biomedicine.[40,41] Henceforth, it is imperative to investigate not just the acute, but also the chronic effects of exposure to nanomaterials,[42] especially for biopersistent nanoparticles expected to be purposefully administered to humans.[28]

Nanotoxicology is a young discipline and brings with it high doses of uncertainty, conflicting information, and debate to the research community, industry, and regulatory agencies. Multidisciplinary cooperation, careful design of studies, and thoughtful allocation of resources are essential for the advance of this field.[25,28,42] Researchers should report and discuss in the literature the challenges faced when carrying out nanotoxicology research, for this will be of great help to other investigators working in

the same field, and will aid in filling the knowledge gaps and reaching a consensus on experimental approaches, guidelines, and standard protocols.[15] Maybe more important is that when conducting nanotoxicity research and especially when drawing conclusions from these studies, we need to keep in mind that all drugs, treatments, and most diagnostic procedures carry some risk. Nanotechnology has great potential benefits. When evaluating toxicity, we need to determine if the expected benefits outweigh the potential risks.[14,28,73] Then, and only then, can we make the appropriate recommendations.

C. Gold Nanoparticles: Strong Candidates for Biomedical Uses

It is expected that nanotechnology will have a profound impact on the medical field, with potential applications in disease prevention, diagnosis, and treatment.[9,12,74] Nanoscale properties allow diagnostic and therapeutic interventions in real-time, with minimum invasiveness, increased specificity of delivery and action, decreased side effects, and the capability to respond to internal or external stimuli and report to external receivers.[9,12]

Gold nanoparticles are one of the most popular noble metal nanoparticles due to their stability and high biocompatibility, as well as their easy functionalization and characterization.[75-77] One of the features that place them as a favorite among candidates for biomedical applications is their unique chemical property of being in the unoxidized state at the nano level, unlike most less noble metal nanoparticles. This results in a highly biocompatible and reactive nanoparticle, ideal characteristics for such a purpose.[76-79] Due to their exorbitantly rich surface chemistry, they can be easily conjugated with many

chemicals and biological molecules, generally through thiol (-SH) groups, resulting in the creation of numerous biocompatible, targeted, and controlled diagnostic, treatment, and delivery systems.[32,76] The easy functionalization also allows control over the solubility of the nanoparticles in aqueous and/or organic phases.[80] Gold nanoparticles on the same size range as macromolecules are capable of direct interactions, enabling the production of novel biomolecule-nanoparticle hybrid materials.[81] They also have intrinsic tunable optical properties (see below) that can be exploited directly or indirectly for diagnostics and treatments.[82] Spherical GNPs have absorption peaks near 520-540 nm, but this peak can be adjusted towards the optical window of tissue (700 to 800 nm) by modifying their size and shape.[83,84] Surface plasmon resonance (SPR) is a phenomenon that occurs when a beam of light (e.g., electromagnetic radiation) of an appropriate wavelength falls on the surface of a metal at a particular angle and distance, which causes the free/conduction electrons of the metal to oscillate collectively. This results in part of the light being absorbed (energy is transferred to the metal, generally producing heat) and the rest being scattered (re-irradiation at the same wavelength in all directions), with a gradual reduction in intensity of the reflected light. At the same time, a strong electromagnetic field is generated adjacent to the surface of the particle. All these aspects can be harnessed for multiple biomedical applications related to diagnostics and therapy.[80,82,85,86] The SPR phenomenon is absent in nanoparticles smaller than 2-3 nm[80,86] as well as in bulk materials,[86] and the SPR peaks and proportions of absorption/scattering can be tuned in GNPs by manipulating their size and shape, which is extremely useful for the biomedical exploitation of this phenomenon.[80,82,85]

Even though GNPs can be easily synthesized in a variety of shapes, most research has been focused on nanospheres and nanorods.[77] Cellular uptake of the former appears to be noticeably easier than the latter,[77,87] which may have important toxicological implications and needs to be considered when developing GNP-based biomedical devices and processes. Biosensors and diagnostics, drug delivery and therapeutics, imaging, and tissue engineering and biomaterials have been identified as the four most promising areas of discovery and application of bionanotechnology.[9] Relevant aspects of GNP employment in each of these areas are briefly described below, with special emphasis in spherical and rod-shaped particles:

1) Biosensors and diagnostics

In general, sensors are composed of a recognition element (for binding to the target) and a transduction element (for signaling the binding event).[86] Gold nanoparticles can be functionalized with a wide variety of receptors for the specific detection of targets of interest, and changes in the physicochemical, optical, and/or electronic properties of GNPs triggered by the binding event can be detected and measured.[86] In a sense, GNPs may act as both recognizer and transducer, and can be utilized for highly sensitive detection of numerous targets, including metal ions, organic compounds, oligonucleotides, and proteins, among others.[86,88] The successful use of GNPs in the design of biosensors is currently being investigated, with promising SPR biosensor systems now in development. This technology is based on the conversion of a specific biological interaction into an optical signal, and has applications in immunoanalysis and detection of nucleotide sequences.[89]

It has been stated that GNPs are the perfect materials to develop superior diagnostic methods for the early detection of important diseases such as cancer and human immunodeficiency virus (HIV) infection,[82] as well as Alzheimer, hepatitis B, and tuberculosis.[80] They can serve as signal enhancers for enzyme-linked immunosorbent assays (ELISAs), probes for cancer detection based on imaging, highly sensitive biosensors for Alzheimer disease markers, and probes for direct detection of hepatitis-B virus and *Mycobacterium tuberculosis*.[80]

Gold nanoparticles have a natural tendency to accumulate in tumors, partly due to the characteristic "leaky vasculature" (enhanced permeability and retention effect) of cancerous tissue.[75,76,78,90] Wang et al.[91] demonstrated that polyethylene glycol (PEG)-coated (PEGylated), non-functionalized GNPs accumulate in tumors up to 25 times more than in the surrounding muscle tissue. PEGylation of GNPs is widely used for *in vivo* applications, as it is known to improve the stability of GNPs in solutions, preventing aggregation and non-specific protein adsorption. It also helps avoid detection and phagocytosis by the mononuclear phagocyte system by forming hydrophilic brushes that prevent opsonins and phagocytic cells from recognizing the particles.[15,60,79,85,89-93] This increases the circulation time and the consequent passive accumulation in tumors.[89,90,92,93] Gold nanoparticles can also be conjugated with biomarkers specifically related to cancer cells for more reliable and efficient accumulation in cancerous tissue and, therefore, reduced accumulation in normal tissue.[82,89,90] Furthermore, antibodies and other biomarkers can be easily attached to the terminal carboxyl groups of PEGylated particles,[84] or both components can adhere independently and share the available binding sites on the GNP surface,[79]

combining active targeting with the stabilizing and stealth effect provided by the PEG coating. The optical properties of GNPs and their natural or targeted accumulation within tumors render them highly useful for cancer diagnostic imaging, as well as cancer therapy.[82,91,94] A protocol has already been published[84] for the synthesis of antibody-functionalized PEGylated GNPs for labeling of human pancreatic adenocarcinoma, and the prompt extension of this method into clinical practice is expected. GNPs can also be used for direct detection of targeted circulating tumor cells from blood samples, as carriers and enhancers in optical-based enzyme-linked immunosorbent assays (ELISAs), and for the detection of cancerous cells via dark-field microscopy.[82] Specific imaging and therapeutic techniques and mechanisms are discussed in the following sections.

Important advances in diagnostic assays for HIV have also been accomplished. In 2010, Tang and Hewlett[95] developed a GNP-based biobarcode amplification assay (BCA) that can detect HIV-1 p24 antigen at concentrations as low as 0.1 pg/mL. This assay is 100-150 times more sensitive than the traditional ELISA method (lower detection limit 10-15 pg/mL). Other promising techniques are the direct detection of HIV-1 via visual DNA microarray, which is based on GNP-labeled silver staining and coupled with polymerase chain reaction (PCR), and the possibility of tailoring HIV inhibitor-loaded GNPs towards HIV molecules, in a combination of diagnosis and therapy.[82]

A very useful characteristic of colloidal GNPs for diagnostic applications is that the solution shifts color from red to purple/blue in response to a change in the refractive index produced in the vicinity of the GNP surface when the particles are in

close proximity with each other.[82,86] Thus, when a specific event (e.g., interaction of target analyte with antibodies attached to the GNP surface, or hybridization of complementary oligonucleotides) produces close proximity/aggregation of the particles, there is a near-field surface plasmon coupling between the particles,[18] and the color changes in proportion to the analyte concentration.[82] This shift in color can be easily perceived by the naked eye even at nanomolar concentrations, resulting in a very useful, sensitive, and straightforward colorimetric sensing assay.[80,82,86,89] GNP-based colorimetric diagnostic assays have already been developed for direct detection of biomolecules, including a home pregnancy test currently in the market[53] and two recently developed field tests: one for the detection of *Melissococcus plutonius*, the causative agent of a severe bacterial disease of honey bees (*Apis mellifera*),[96] and another one for the detection of spring viremia of carp virus, which affects cyprinid fish and causes severe economic losses.[97] These last two take advantage of the color shift of colloidal solutions of spherical GNPs from red to blue upon nanoparticle aggregation, which is prevented by single-stranded DNA in the absence of the target nucleic acid.[96,97] This kind of diagnostic assays are specific, sensitive, fast, and cost-effective,[96] carrying the potential of having a great impact not only in human health, but also animal health, animal science, food science, and environmental science, among others.

2) Drug delivery and therapeutics

Current issues in the area of drug delivery and therapeutics include low bioavailability, quick clearance, and high toxicity of some therapeutics such as

nucleic acids, proteins and peptides, vaccines, cancer chemotherapeutics, and other drugs.[98] These problems can be alleviated by nanocarriers, which can be designed to control drug release and/or increase selective cell targeting, cellular uptake, drug solubility, and circulation time.[98] Gold nanoparticles have several advantages that make them attractive for delivery of small drugs and biomolecules. These include easy preparation of nanoparticles of the specifically desired size (between 1 and 150 nm), low toxicity, easy functionalization with therapeutic and targeting moieties (with relative optimal loading capacity),[75,88] natural cellular uptake (that can also be tailored and increased by conjugation with specific proteins),[99] and possibility of remotely controlled release of their load by exploiting their photo-physical properties.[98] Also, thiolated GNPs possess a zwitterionic layer that can be used to encapsulate hydrophobic drugs and deliver them efficiently inside the cell, without functionalization of the drug.[90] For these reasons, GNPs are currently being extensively investigated as carriers of diverse drugs (especially antibiotics and anti-cancer drugs) and biomolecules (including peptides, proteins, DNA, and RNA).[88,89,98]

The main idea of targeted drug delivery systems is to obtain a high concentration of the therapeutic compound in the tissues/cells of interest (increased efficacy), with minimum allocation of drug in other tissues (decreased toxicity).[75,92] In order to avoid accumulation and side effects, the ideal nanocarrier should have a core material that is non-toxic and non-immunogenic, as well as easily eliminated. At the same time, it should be able to evade rapid renal clearance and uptake by the mononuclear phagocytic system, in order to increase the circulation time and the

likelihood of reaching target cells.[98] Most of these characteristics can be achieved through manipulation of size, shape, and surface modifications of nanoparticles; changes that are particularly easy to control with GNPs.

In the specific case of cancer treatment, one of the biggest problems with current cancer therapies is toxicity due to indiscriminate effects on both healthy and malignant tissues.[92] Targeted drug delivery with nanoparticles is, therefore, one of the most promising research areas. Tumor endothelial cells express several tumor-specific receptors that differentiate them from normal cells, and the nucleus of cancerous cells also expresses various tumor-specific receptors on the cell surface. All these abnormal receptors (e.g., epidermal growth factor, folate, transferrin, and tumor-necrosis factor (TNF) receptors)[78,89,90] can be used as targets for tumor-directed drug delivery.[75,76] At least one of these nanotherapeutics has already been developed and completed a phase 1 clinical trial:[100] TNF-bound PEGylated GNPs (termed CYT-6091) combine stabilization, stealth effect, and passive targeting (provided by PEG) with active targeting and therapy (provided by TNF).[79,92] As already discussed, GNPs have a natural tendency to accumulate in tumors (passive vectorization),[88,90,92] can gain access to intracellular compartments (with uptake and intracellular location depending partly on size and shape of the particles, as well as functionalization)[75,78], and are easily adorned with target moieties and therapeutics (active vectorization).[90,92] Additionally, the existence of antiangiogenic properties of GNPs is being investigated.[78,89] All these features make them great candidates for tailored treatment of cancer.

Targeted GNPs can also be employed for other types of therapy, including photothermal therapy, photodynamic therapy, x-ray therapy, radiotherapy, gene therapy, and anti-bacterial therapy.[76,78,80,83,88-90,101] Photothermal therapy with GNPs consists of irradiating a GNP-containing tumor (after passive or active accumulation of GNPs) with the light wavelength at which GNPs reach their maximum absorption (visible to near-infrared region), in order to heat the GNPs and kill the malignant cells. The use of this therapy without GNPs is considerably limited by the damage to healthy tissues, but the introduction of GNPs as localized dose enhancers seems very promising.[76,89] Photodynamic therapy is a similar method (with similar issues and potential role of GNPs), but additionally induces the formation of highly active radicals that damage the tumor microvessels, resulting in tumor malnutrition and extermination.[89] Gold nanoparticles have also been proposed as novel radiosensitizing agents for cancer therapy due to their strong photoelectric absorption coefficient,[102] and intensive preclinical research has provided exciting results and high hopes on the prompt advance of this procedure to clinical research and successful transition to widespread medical practice.[99,101] Experimental evidence shows radiotherapy enhancements much higher than predicted, suggesting that besides the ability to absorb substantially more energy than soft tissue, other mechanisms are involved in the radiosensitization by GNPs.[102] Oxidative stress, cell cycle alterations, and nanoscale dose effects have been proposed to play important roles,[102] and it has been determined that GNPs need to be present within target cells to achieve radiosensitization, with larger particles (or aggregates) providing greater effects.[99]

Besides cancer, GNPs have also shown potential therapeutic applications for other currently critical diseases, for instance HIV infection. They have been demonstrated to convert a biologically and therapeutically inactive compound to a highly active anti-HIV agent upon conjugation with the particles.[103] The therapeutic potential of GNPs is subject of extensive investigation and it is constantly expanding, with new possibilities likely to emerge in the near future.

3) Imaging

Gold nanoparticles have attributes that make them suitable labels and contrast agents for various forms of advanced imaging. Gold nanoparticles are intrinsically fluorescent, have strong magnetic properties, and well-known optical absorption properties in the visible region. Current forms of advanced imaging that make use of GNPs are fluorescence spectroscopy, fluorescence correlation spectroscopy, fluorescence microscopy, magnetic resonance imaging, x-ray imaging, two-photon luminescence, dark-field microscopy, confocal microscopy, electron microscopy, dark-field light scattering, computed tomography, Raman spectroscopy, photoacoustic tomography, and optical coherence tomography.[76,80,82-84,89,90,94]

Metallic nanoparticles offer valuable advantages when compared to other existing probes. For instance, they do not exhibit photo-bleaching or intermittency, allowing visualization during extended periods of time.[80,84] Furthermore, several existent imaging technologies have not made it into mainstream clinical practice due to technical difficulties, some of which can be overcome with the introduction of GNPs. Raman spectroscopy, for example, has limited depth penetration of the optical beam

that carries the Raman signal, and the intrinsic signal produced by pathological tissues is weak. Gold nanoparticles coated with a Raman organic molecule can amplify significantly the intensity of the Raman signal, solving the latter of these problems.[80,83] Photoacoustic tomography (pulsed laser radiation is absorbed and transformed into heat, resulting in thermo-elastic expansion that generates acoustic waves, which can be directly measured and converted into an image)[76,85] presents a similar issue: many diseases do not display a natural photoacoustic contrast. This drawback can be solved by the administration of a photoacoustic contrast agent, and GNPs show great promise for this purpose.[76,83] Also, some significant limitations of imaging techniques that are widely used in clinical practice and research can be greatly overcome by the introduction of GNPs as novel contrast agents, like in the case of x-ray imaging[76,94] and *in vivo* vascular casting.[104]

4) Tissue engineering and biomaterials

Tissue engineering and biomaterials are probably the areas relatively less investigated, so far, with respect to the use of GNPs in particular, but some efforts have been made with promising results. Türk et al.[105] created and evaluated a GNP crosslinked chitosan composite film for its use as a scaffold for fibroblasts. Their results indicate that the combination of chitosan with GNPs, when compared to chitosan alone, promotes cell attachment, growth, and proliferation with improved stability and minimal cytotoxicity.[105] The benefits of GNPs as a material for tissue engineering are just starting to be explored, thus more studies involving the use of GNPs in this area of bionanotechnology are likely to be conducted.

There are many more potential applications of GNPs in biomedicine, some of which do not fall into the previous categories. For example, regarding prevention and immune-modulation, it has been shown that certain haptens conjugated with or adsorbed on GNPs can induce the formation of antibodies with a high titer; therefore their potential dual role as antigen carriers and adjuvants in vaccines is currently under investigation.[89] Maybe the most attractive aspect of GNPs for biomedical uses is that, considering all their features together, they provide a great opportunity for the development of "theranostics" (therapeutics + diagnostics).[75,78,83] For instance, they can be tailored to detect tumor cells through imaging (diagnostic phase), and, if a tumor is identified, they can be remotely activated to deliver a drug load and/or exploit their photothermal properties to destroy the cancer cells (therapeutic phase).

Gold has been used in the past to treat several diseases, especially rheumatoid arthritis, but newer drugs have largely replaced it.[83] Now, advances in the understanding of GNP properties and their potential exploitation in biomedical applications have created excitement and expectation for the return of gold use in humans.[83] Although gold is recognizably an expensive material, the minute amounts needed and the substantial benefits should enable its widespread utilization.[94] In fact, GNPs are already being used effectively in laboratory based clinical diagnostic methodologies, but the greater expectation comes from their potential to expand into clinical practice in the very near future.[83] There have been at least 3 clinical trials in the U.S. that involve the use of GNPs in medicine with direct administration of GNPs compounds to patients: one related to drug delivery (TNF) for the treatment of cancer (phase 1 completed) and two related to

plasmonic photothermal therapy of atherosclerosis (phase 1 and 2 completed).[100] Considering that the study of GNPs as a potential material for medical applications is relatively new,[98] the number of clinical trials involving GNPs compounds is likely to increase in the near future, making imperative the prompt elucidation of the potential adverse effects of this nanomaterial.

D. Toxicity of Gold Nanoparticles

As previously discussed, nanotechnologies represent unique opportunities for biomedical applications, but understanding the fate and potential adverse effects of the nanomaterials after they have fulfilled their therapeutic or diagnostic purpose is critical.[25,106] It has been stated that the lack of knowledge about the potential risks, toxicity mechanisms, and effects of nanomaterials in human beings is currently the most critical problem faced by nanomedical technology.[71,73] Yet, most of the federal research funds for nanotechnology advancement are directed towards nanomaterials applications research, as opposed to investigations regarding nanomaterials implications.[56] The result is that despite all the efforts dedicated towards the development of nanomaterials for biomedical applications, the safety of these materials remains unknown or incompletely understood.[75] In order to take full advantage of the promising utilizations of nanomaterials in biomedicine, we need to understand both applications and implications of nanomaterials.

Due to their great potential for biomedical uses, along with the likeliness of future human exposures, GNPs have recently been added to the Organization for Economic Cooperation and Development (OECD) list of reference materials to be more urgently

tested for toxicity.[54,107] In general, GNPs are considered biocompatible,[67,92,108] but drawing generalized conclusions about their safety is currently an extraordinary challenge, as the available data on their biodistribution and toxicity remains scarce and inconsistent, especially for *in vivo* studies.[66,67,81,89,106,109,110] There is high variability in experimental design when trying to compare experiments from different research groups, resulting in serious discrepancies in their outcomes and conclusions.[66,67,89,106,110-112] To help solve this problem, at least one protocol has been recently published for *in vivo* toxicological evaluation of GNPs,[113] but its widespread acceptance and implementation without modifications may be restricted by some important limitations, namely: it does not include any nanomaterial characterization steps, it considers only SC or intraperitoneal (IP) routes of exposure, it includes only urine for clearance assessment, its toxicity endpoints are limited to body weight loss, clinical signs of distress, and histopathology, and it does not consider long-term evaluation. In spite of these and other pitfalls, protocols are greatly needed to harmonize nanotoxicological studies, and it is expected that research groups would adopt these as initial general guidelines, modifying, complementing, and continuously improving the available protocols. Protocols have also been published for *in vitro* assessment of GNP toxicity,[30] but they are not exempt from debate. One of the greatest disagreements is about the methods chosen to assess cytotoxicity, because many of the recommended cell viability tests are colorimetric dye-based assays, and it is known that GNPs may directly interfere with the results of this kind of assays, producing inconsistencies due to overestimation or underestimation of the toxicity of the particles.[114]

Despite these difficulties and disagreements, Dykman and Khlebtsov[89,106] proposed some tentative general conclusions, namely that 1) the organs of the mononuclear phagocyte system (primarily liver and secondarily spleen) are the main accumulation targets for 10-100 nm GNPs, with prolonged retention and consequent concerns about chronic toxic effects; 2) only very small GNPs (less than 20 nm) are able to cross the blood-brain barrier; 3) 1-2 nm GNPs could have higher toxicity due to potential irreversible binding to biomolecules such as DNA; and 4) no evident acute toxicity appears when the GNPs dose is lower than 0.5 mg/kg/day (or 10^{12} particles/mL for *in vitro* studies). Nevertheless, the same and other authors stress the fact that there is still not sufficient information to make definite wide inferences on the behavior and interactions of various GNPs with biological systems, implying that individual *in vivo* investigation of systemic performance for each GNP-based system is, up to the present time, essential for the advancement of these technologies into clinical practice.[78,80,106,110,115] An up-to-date literature review regarding *in vitro* and *in vivo* studies on spherical GNP toxicity is presented below.

1) *In vitro* studies

It has been stated that thorough GNP cytotoxicity studies should include assessment of cell morphology, viability/membrane integrity, cell cycle arrest/proliferation inhibition, cell death (apoptosis and necrosis), and oxidative stress,[30] but in practice most research efforts only investigate a few of these parameters, also differing widely in the methods chosen and results obtained.

Several groups have found no evidence of GNP-induced cellular toxicity, proclaiming GNPs as inert and extremely biocompatible. For instance, no evidence of cytotoxicity or biologically significant oxidative DNA damage was found when HepG2 cells (human liver carcinoma) were exposed to low doses (≤0.2 μg/mL) of the citrate-capped 10, 30, or 60 nm NIST (National Institute of Standards and Technology) GNP reference materials for up to 24 hours.[116] The authors confirmed cell uptake and intracellular location of GNPs, which were visualized not aggregated and enclosed in vesicles dispersed throughout the cytoplasm but not in organelles. Another group[52] obtained somewhat similar results when they exposed precision-cut slices of liver (male Wistar rats) to significantly higher doses (~1-100 μg/mL) of 5 nm polyvinylpymolidone (PVP)-coated GNPs for up to 24 hours. Gold nanoparticles did not exert toxic effects (as evaluated by viability, oxidative stress, and metabolic performance parameters) despite being readily internalized by Kupffer cells, endothelial cells, monocytes, and hepatocytes. It was determined that the cellular uptake of GNPs was an active process, and their intracellular location was observed as aggregates inside uncoated vesicles, early endosome-like vesicles, late endosomes, and lysosomes, but never inside organelles like the nucleus or mitochondria. These results are in agreement with a study by Chithrani et al.,[87] in which they exposed HeLa (human cervix epithelial carcinoma) cells to citrate-capped GNPs of various sizes (14, 30, 50, 74, or 100 nm) for up to 6 hours. They did not observe changes in cell viability, but documented size-dependent uptake of the particles (with 50 nm GNPs having the maximum cell internalization) and proposed receptor-mediated endocytosis as the uptake mechanism. A different study[117] did not find evidence of

cytotoxicity after exposing COS-1 (African green monkey kidney) cells for 2 hours to 0.0006-0.04 μg/mL of GNPs with core sizes 2.4, 5.5, or 8.2 nm in diameter and coated with a PEG-cell penetrating peptide composite. Yet they detected differential intracellular location, with the smaller particles concentrated in the nucleus and the bigger GNPs confined to the cytoplasm. They also reported that exposure to larger particles (16, 38, and 89 nm) did not result in detectable cell internalization of GNPs in this model. In order to assess the influence of GNP size and different capping agents in cellular uptake and toxicity, Freese et al.[5] performed a systematic study with primary human dermal microvascular endothelial cells exposed to a library of 15 polymer-coated GNPs (18, 35, or 65 nm; coated with PEG, glucosamine-, hydroxypropylamine-, ethanediamine-, or taurine-functional polymethacrylates) at concentrations ranging from 10 to 250 μg/mL for up to 48 hours. No evidence of cytotoxicity was found for any of the GNPs and concentrations tested, although significant differences in cellular uptake were observed: smaller particles were generally internalized in higher numbers than larger ones (but the internalized fraction of the total dose was always the lowest), and positively-charged (ethanediamine) GNPs were taken up by cells in a very high degree while PEG-coated GNPs (neutral-charged) were very poorly internalized.[5]

Conversely, other researchers have found clear evidence of cellular alterations following GNP exposure, indicating that these particles are not innocuous. Nanoparticle size appears to be one of the most important factors influencing their toxic effects *in vitro.* In a cytotoxicity study,[108] three different cell lines (PC-3, human prostate cancer; MCF-7, human breast cancer; and CHO22, Chinese hamster

ovary) were exposed to increasing concentrations (10, 40, 70, 100, and 130 μg/mL) of 3, 5, 6, 8, 10, 17, 30, or 45 nm citrate-capped GNPs for 24, 48, or 72 hours. Cell viability was assessed via three independent assays, and their results consistently demonstrated dose-dependent (directly proportional) mild toxicity for 3, 8, and 30 nm GNPs, but not for the other sizes tested. Somehow similar results were found in a study by Pan et al.[118] in which they exposed four cell types (SK-Mel-28, human melanoma; HeLa, human cervix epithelial carcinoma; L929, mouse fibroblasts; and J774A1, mouse macrophages) to increasing concentrations of 0.8, 1.2, 1.4, 1.8, or 15 nm triphenylphosphine monosulfonate (TPPMS)- or triphenylphosphine trisulfonate (TPPTS)-coated GNPs for up to 48 hours. Their results demonstrate strong size-dependent cytotoxicity, with 1-2 nm GNPs being highly toxic and specifically 1.4 nm particles being markedly more cytotoxic than all other particles, irrespective of coating or cell type tested. In a later study,[46] the same group exposed HeLa cells to 1.4 (20 μg/mL) or 15 nm (200 μg/mL) TPPMS-coated GNPs for up to 48 hours. The cells exposed to the smaller particles showed marked cytotoxicity, generation of ROS, oxidative stress responses, mitochondrial damage, and necrosis. The cells exposed to the larger particles did not show signs of toxicity, even at the overtly higher concentration used. Coradeghini and colleagues[107] also demonstrated size-dependent cytotoxicity of citrate-capped GNPs, with 5 nm particles causing dose- and time-dependent toxicity in BALB/3T3 cells (immortalized mouse fibroblasts) while 15 nm particles did not cause any observable cytotoxicity at the same concentrations and exposure times. They verified cellular internalization (possibly via more than one mechanism, namely clathrin mediated uptake as well as

macropinocytosis) and storage in membrane-bound vesicles for both sizes of GNPs, and found evidence of autophagosome formation and damage to the cytoskeleton (F-actin fibers) more evident in 5 nm particles compared to 15 nm particles. Analogous results were observed in another study,[119] in which a pulsed laser irradiation-induced reduction in size of citrate-coated GNPs was observed (from 15 nm to 3-4 nm), along with dose- and time-dependent cytotoxic effects (impaired cell viability) in primary rat kidney cell culture exposed to 0.0002, 0.0004 or 0.0008 μg/mL of irradiated GNPs for 24, 48, or 72 hours, compared to no toxicity observed when non-irradiated 15 nm GNPs were used at equal concentrations and exposure times.

Likewise, the capping agents and surface charge also appear to exert great influence on nanoparticle-cell interactions and toxicity.[83] One study[46] demonstrated great differences in toxicity between 1.4 nm GNPs coated with TPPMS and comparably sized (1.1 nm) GNPs coated with glutathione (GSH). HeLa cells exposed to 20 μg/mL of the TPPMS-coated GNPs for up to 48 hours showed markedly decreased viability, which was not observed when the cells were exposed to GSH-coated GNPs, even at tenfold higher concentration (200 μg/mL). The mechanism of toxicity was found to be intracellular formation of ROS, leading to a strong oxidative stress response, mitochondrial damage, and cell death by necrosis. Another investigation[19] exposed ECV-304 cells (human umbilical vein endothelial cells) to increasing concentrations (50, 250, 500, or 1000 μg/mL) of 100 nm bare or polycaprolactone (PCL)-coated GNPs for up to 4 days. The bare particles were not stable in solution, and they caused cell morphological alterations and concentration-dependent cytotoxicity. The PCL coating increased stability of the particles in

solution and significantly reduced their cytotoxicity. Schaeublin and colleagues[112] exposed HaCaT cells (human keratinocytes) to 10, 25, 50, or 100 μg/mL of 1.5 nm GNPs capped with trimethylammoniumethanethiol (TMAT, positively charged), mercaptoethanesulfonate (MES, negatively charged), or mercaptoethoxyethoxye-thanol (MEEE, neutral) for 24 hours. Their results show cellular uptake, ROS production, and dose-dependent toxicity regardless of charge, but apparently the mechanism of cell death was different for charged particles (apoptosis) than for neutral ones (necrosis). Another study[63] demonstrated notable dissimilarities in GNP-protein interactions, GNP-cell interactions, and cell uptake between 10 nm citric acid- and tannic acid-capped GNPs (similar negative surface charge). When A549 (human lung epithelial adenocarcinoma) cells where exposed to 10 μg/mL of the different GNPs for 24 hours, high uptake of large agglomerates of citric acid-capped GNPs were observed, while tannic acid-capped GNPs were poorly internalized as discrete particles, suggesting distinct GNP uptake mechanisms depending on their capping agents. In another study,[120] phagocytic RAW 264.7 (mouse blood macrophages) and non-phagocytic HepG2 cells were exposed to 10 μg/mL of negatively charged (sodium 11-mercaptoundecanoic acid-coated) or positively charged ([10-mercaptodecyl]-trimethyl-ammonium bromide-coated) 16, 26, 40, or 58 nm GNPs for 12-24 hours. The objective of the study was to compare the effect of nanoparticle size and charge, as well as cell type, on GNP uptake, intracellular fate, and cytotoxicity. Their results show significant differences in uptake and intracellular fate between the two cell lines, with phagocytic cells internalizing both positively and negatively charged GNPs in comparable rates (likely via phagocytosis

as demonstrated by their observation within large phagosomes) and non-phagocytic cells internalizing significantly more positively charged GNPs than negatively charged ones (possibly via endocytosis as demonstrated by their visualization within secondary lysosomes). Differences in cytotoxicity between the cell types for the oppositely charged GNPs were also detected, mainly correlated to the disparities in uptake, but also dependent on the method used to assess cellular damage. Overall, this study indicates that cell interactions with GNPs are not only influenced by size and surface charge of the particles, but also intensely depend on cell types.[120] Another study[121] found cytotoxicity with decrease in cell viability, evidence of ROS generation, and DNA damage in HepG2 and human peripheral blood mononuclear cells after exposure to ~18 nm citrate-capped (negatively charged) or ~11 nm polyamidoamine dendrimer-capped (positively charged) GNPs at concentrations ranging from 0.002 to 10 μg/mL.

It is important to keep in mind that cell viability and proliferation are not the only toxic effects that may arise as consequences of GNP exposure, as they may be causing serious cellular damage and affecting the proper specific functions of the cells without necessarily killing them.[115,122] A study by Kohl et al.[123] highlights the importance of assessing the potential effects of GNPs in cell differentiation. They exposed human mesenchymal stem cells to increasing concentrations (5.8 to 22.8 μg/mL) of 9 or 95 nm citrate-coated GNPs during adipogenic differentiation (up to 21 days), and reported dose-dependent cytotoxicity with impaired differentiation (decreased fat storage and mitochondrial activity) induced by both sizes of GNPs. Another group[122] studied cell morphology, microfilament disruption, and

extracellular matrix proteins expression along with cell proliferation, apoptosis, and GNP uptake in CF-31 cells (primary human dermal fibroblasts). Their results show that 13 nm citrate-coated GNPs are internalized via phagocytosis, while 45 nm citrate-coated GNPs undergo clathrin-mediated endocytosis as a primary internalization mechanism and phagocytosis as a secondary mechanism. Both types of particles were localized in cytoplasmic vacuoles and caused cellular toxicity (decreased proliferation, increased apoptosis, altered cell morphology, actin filament disruption, and decrease in collagen and fibronectin production) in a dose-, time-, and GNP size-dependent manner, with 45 nm particles inducing greater toxicity. Interestingly, an almost complete recovery was noted when cells were allowed to grow in a GNP-free media after a 3-day GNP exposure.[122]

Finally, interaction with proteins and aggregation may play relevant roles in determining GNP toxicity. Citrate-coated 15 and 50 nm GNPs have been demonstrated to interact with γ-globulin but not with albumin *in vitro*;[124] polyacrylic acid-conjugated 5 and 20 nm GNPs (negatively charged) are able to interact with fibrinogen and induce unfolding of this protein, with consequent activation of the Mac-1 pathway and release of inflammatory cytokines in THP-1 (human monocytic leukemia) cells;[125] and aggregation of transferrin-coated GNPs reduces uptake in HeLa and A549 cells, but not in MDA-MB-435 cells (human melanoma), indicating different uptake mechanisms by different cell types.[62] It has also been hypothesized that nanoparticle aggregation following systemic administration may lead to organ damage from capillary occlusion,[83] but no articles investigating this possibility were found.

Not much is known about the specific mechanisms by which GNPs induce toxicity, but one of the potential mechanisms is oxidative stress through production of ROS. Li and colleagues[20] demonstrated GNP-induced oxidative damage and autophagy in MRC-5 (human fetal lung fibroblast) cells after exposure to a very low concentration (0.0002 μg/mL) of 20 nm fetal bovine serum-coated GNPs for 72 hours. GNPs have also been demonstrated to directly generate ROS when exposed to x-rays[126] and UV irradiation[126,127] *in vitro*. Other mechanisms of oxidative stress generation may play important roles *in vivo*, such as macrophage activation with subsequent release of endogenous free radicals, or generation of redox active intermediates product of GNP metabolism.[24] It has been demonstrated that citrate-capped 13 nm GNPs induce release of nitric oxide (NO) from mouse blood macrophages (RAW 264.7 cell line) *in vitro* in a dose-dependent manner (concentrations of GNPs between 3 and 18 μg/mL were tested), which is alleviated when the coating agent is changed to PEG,[128] but due to conflicting results in the literature there is an ongoing debate on whether GNPs activate or actually inhibit macrophagic NO production.[129]

2) *In vivo* studies (Table 1)

In order to take full advantage of GNPs in biomedicine and minimize their potential toxic effects, it is critical to understand their biodistribution and accumulation in living creatures.[78] *In vivo* studies on biodistribution report that the main sites of GNP accumulation are the liver and spleen, with very poor elimination from the organism.[4,6-8,13,22,26,29,66,106,110,130-132] Changes in size and capping agents

may induce differences in the biodistribution profile to a certain extent (for instance, smaller particles have wider organ distribution than larger ones[4,29,66,110,132] and PEGylation increases circulation times[130]), but the general biodistribution pattern remains the same (i.e., the highest accumulation of GNPs occurs in liver, followed by spleen).[4,8,13,29,130] Within liver, GNPs are actively internalized primarily by Kupffer cells, but also by endothelial cells and hepatocytes (likely via endocytosis)[52], with extremely slow elimination through the bile excretory system.[4,26,52] Nanoparticles with extremely slow clearance rates might be retained in the organism for years, carrying the potential to generate adverse effects long after the initial exposure. Thus, it is of utmost importance to determine the safety of all components of potentially accumulated/retained nanomaterials.[6,12] In the case of GNPs, their preferential accumulation within blood filtration organs, especially liver, and the implications for normal organ function and potential inflammatory responses are particularly relevant.[66] It is also important to discern between the toxic effects of the nanoparticle core and those of the capping agent(s) and ligands,[78,80] especially if the binding between the gold surface and the coating is not too strong and thus they are likely to be separated in the biological environment.[81] While liver and spleen may be the main targets for toxicity due to slow clearance and tissue accumulation of GNPs, the high blood flow through other organs such as the kidney and lungs also place them at high risk of damage.[83] Also, it has been recommended that special attention be paid to potential interactions between nanoparticles and the immune system, since this is likely to define some nonspecific immune responses.[106]

As with *in vitro* studies, there are many *in vivo* investigations that have found no evidence of toxicity after GNP administration. One of them[131] exposed male C57BL/6 mice to 0.04, 0.2, or 0.4 mg/kg/day IP of 12.5 nm citrate-coated GNPs for 8 days, reporting no evidence of toxicity 1 day after the last injection, despite rising accumulation of GNPs with increasing dose in all organs examined. Hainfeld and colleagues[94] exposed tumor-burdened BALB/c mice to 2700 mg/kg IV of commercially available 1.9 nm GNPs as an x-ray contrast agent, and reported that the mice survived with no signs of disease for over 1 year after the exposure. They also administered different doses (7 mg/kg, 70 mg/kg, or 700 mg/kg) of the same GNPs to male and female CD1 mice, and evaluated toxicity (hematology, blood chemistry, and histopathology) 1, 11, and 30 days post-exposure, finding no evidence of toxicity in any of the animals. In a different article,[101] the same group reports no overt clinical signs in BALB/c mice exposed to 2700 mg/kg IV of the same GNPs one year after the exposure, as well as normal hematology and blood chemistry 2 weeks after IV injection of 800 mg GNPs/kg. Glutathione-coated 1.2 nm GNPs (0.02, 0.04, 0.06, 0.08, or 0.12 mg/kg) have also been evaluated *in vivo*,[133] with no evidence of toxicity up to 4 weeks post-SC administration. This investigation focused more on biodistribution, demonstrating rapid renal excretion of the particles along with some chronic accumulation in liver and spleen, especially at the higher doses used. Some important pitfalls of this specific study include: doses are reported in micromolar, hindering straightforward comparison with other *in vivo* studies; only kidneys were sampled for histopathology and considered for toxicity assessment along with survival; and only blood, urine, and some organs (spleen, liver, heart,

kidneys, and lungs) were considered for the biodistribution analysis. Therefore, some caution should be used in the interpretation of their results. Another group[77] found citrate-coated 21 nm GNPs (7.85 mg/kg IP) nontoxic in male C57BL/6 mice, up to 72 hours post-administration. This study, however, demonstrated significant loss of abdominal adipose tissue, accompanied by an apparent inhibition of proinflammatory cytokine expression (TNF-α and interleukin [IL]-6) within the same tissue, suggesting a potential role of GNPs for therapy of obesity-related disorders. Cho and colleagues[26] did not find evidence of toxicity upon histopathological examination of tissues of male BALB/c mice exposed to 4, 13, or 100 nm PEGylated GNPs at a dose of 0.85 mg/kg IV, even up to 6 months after exposure, although they found a transient increase in indicators of oxidative stress and liver damage related to the 4 and 13 nm particles. These results are in agreement, to an extent, with a study by Balasubramanian et al.[13] in which male Wistar rats were exposed to a very low dose (0.01 mg/kg IV) of 20 nm citrate-coated GNPs. In this experiment, microarray analyses revealed significant changes in expression of genes related to detoxification, lipid metabolism, and cell cycle in the liver (2 months post-exposure), as well as genes related to defense response in the spleen (1 month post-exposure) and circadian rhythm in both liver and spleen. Yang et al.[134] reported no evidence of GNP-induced cardiac adverse effects in male BALB/c mice with or without a cardiac pathological condition, one day after the animals were exposed to 13 nm PEG-coated GNPs at a dose of 0.81 mg/kg IV daily for 7 days, despite increased accumulation of GNPs in the pathological hearts. Mutagenic activity of GNPs has also been investigated,[135] with no evidence of mutagenic effect on polychromatic erythrocytes

of the bone marrow of male outbred rats (no strain specified) 1 day after the animals had received the last of 7 doses of 16 or 55 nm PEGylated GNPs (0.25 mg/kg/day, orally [PO]). Finally, Wang et al.[91] demonstrated passive accumulation of 6 nm PEG-coated GNPs in mice tumors after IV injection of 260 mg/kg, with no pathological changes observed after 12 hours. They reported increasing accumulation of GNPs with time in spleen, liver, and cancerous tissue, with concentrations two to three times higher in liver and spleen than in tumors.

In view of the expected importance of nanoparticle size and surface modifications, efforts have been made to define the toxicological role of these parameters *in vivo*. One study[22] exposed male mice (strain not specified) to 4 mg/kg IP of 5, 10, 30, or 60 nm PEGylated GNPs, and reported toxicity associated only to 10 and 60 nm GNPs. Another study[136] found size-dependent toxicity after exposing male BALB/c mice to 3, 5, 8, 12, 17, 37, 50, or 100 nm citrate-coated GNPs at a dose of 8 mg/kg/week IP, with nanoparticles between 8 and 37 nm causing alterations in fur and skin, severe behavioral changes ("camel-like back and crooked spine"), death (half of the animals receiving these nanoparticles died by approximately 21 days of treatment), and histopathological alterations in liver, lung, and spleen. Cho and colleagues[137] did not find distinct biological effects of 4 and 100 nm PEGylated GNPs. They administered 4.26 mg/kg IV of the different size particles to male BALB/c mice and euthanized the animals 30 minutes after the injection. No alterations were observed upon histological examination, but exposure to any of the particles induced significant changes in the expression of genes related to apoptosis, cell cycle, inflammation/immune response, and metabolic processes. It

is pertinent to mention that the same group found a size-dependent transient increase in indicators of oxidative stress and liver damage when mice were exposed to 4, 13, and 100 nm GNPs, and evaluation was made up to 6 months post-exposure[26] (see above). Likewise, they found dose-dependent acute liver toxicity after exposure to 13 nm GNPs, with the highest dose being 4.26 mg/kg[7] (see below). Interestingly, a transient increase in hepatic and renal expression of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) was observed in a different study,[138] after male Wistar-Kyoto rats were exposed to 0.022 mg/kg/day IP of 10 or 50 nm citrate-coated GNPs for 1 or 5 days. The effects were greater in liver and the 50 nm particles caused a more severe impact than the 10 nm particles, but all the expression levels returned to normal after repeated exposure. In a different study,[8] signs of hepatic necrosis (specifically in Kupffer cells) were observed in male Wistar rats only 24 hours after the animals were exposed to 0.6-1 mg/kg IV of 18 nm GNPs capped with five different agents. Abdelhalim and Moussa[139] recently reported increased serum levels of the enzyme aspartate transaminase (AST) in male Wistar-Kyoto rats after IP exposure to 10 or 50 nm GNPs for 3 days, and concluded that the particles may cause liver damage. However, the authors did not find increases in other enzymes that indicate liver injury (alanine transaminase [ALT], γ-glutamyl transpeptidase [GGT], and alkaline phosphatase [ALP] levels were in fact decreased after GNP exposure). Also, comparison of their experiment with other studies is obstructed by the fact that their report does not provide a clear view on their methodology, lacking information on GNPs characteristics (other than size), dose, sampling time points, and statistical procedure. The same group reported liver,[140-142] heart,[143,144] and

blood[145] alterations after IP administration of 10, 20, or 50 nm GNPs to male Wistar-Kyoto rats for 3 or 7 days, with smaller particles exerting a greater effect, but the same issue of lack of crucial methodological information (in particular the dose administered) is encountered when intending to compare their results with other research efforts. Size is also an important factor regarding nanoparticles' ability to cross the blood-brain barrier (BBB), with smaller particles entering the brain to a greater extent than larger ones, although in general a very small percentage of the exposure dose gains access to the nervous system.[64] There are several studies reporting GNPs in brain and suggesting that these particles, when small in size, are able to cross the BBB.[7,26,131,132] But the methods used in these experiments to detect the GNPs include tissue homogenization, so it is possible that the gold was localized in blood and/or inside the cells that constitute the BBB, instead of in the brain parenchyma.

The fact that toxicity may be determined in part by the size of the GNPs suggests that with proper size selection the adverse effects could be avoided.[75] Commonly, particles larger than 10 nm are considered low- or non-toxic, mostly independent of the particular ligand molecules,[81] although some studies (*in vitro* and *in vivo*) reveal a non-linear relationship between nanoparticle size and toxicity[22,93,108] or all the opposite, i.e., that bigger particles may generate greater adverse effects than smaller ones.[122,138] This suggests that although size is a key parameter, there are other variables playing important roles in determining the toxicity of GNPs. Surface modification has also been proposed as a method to diminish the toxicity profile of

GNPs, and PEG is endorsed as one of the most promising coatings to ensure high biocompatibility.[78,83]

Gold nanoparticle size and surface functionalization are key elements governing biodistribution and, consequently, potential adverse effects *in vivo*.[17,106] However, the toxicity of GNPs in complex organisms is not only determined by the particles' properties, but is also greatly influenced by dose, route and frequency of exposure, metabolic pathways, excretion, and immune response.[17,74] Unfortunately, the few available *in vivo* studies differ widely on their experimental design, and detailed information is not always clearly stated by the authors.[66] Cho et al.[7] demonstrated that IV exposure of male BALB/c mice to 13 nm PEG-coated GNPs induced dose-dependent (0.17, 0.85, and 4.26 mg/kg were tested) acute liver inflammation and increased apoptosis, with Kupffer cells playing a significant role in the induction of inflammation. A recent study[111] reported dose-, time-, and frequency of exposure-dependent toxicity of 50 nm GNPs in Swiss albino mice (acute exposure: 1, 2, or 10 mg/kg IV; chronic exposure: 1 or 2 mg/kg IP daily for 90 days). Particularly, they observed hematuria at the higher dose of acute exposure, skin flaccidity and darkening along with significant changes in tissue architecture after chronic exposure, and death of all animals receiving the higher dose of chronic exposure within 30 days of the treatment schedule. They also demonstrated immune stimulation as shown mainly by an increase in white blood cell count, specifically lymphocytes, in all GNP-exposed groups. In another investigation,[17] male ICR mice were exposed to 1.1 mg/kg/day for 28 days (or exposed once and examined 28 days later; the information is not clear in the paper) of 13.5 nm citrate-coated GNPs by

three different routes, namely PO, IP, or IV. The animals receiving the nanoparticles PO showed the highest toxicity, followed by the ones receiving IP injections, while the lowest toxicity was seen with IV injections. Translocation rates of GNPs into the central nervous system (CNS) are generally considered very low, but this does not necessarily mean that GNPs will not exert an effect in the CNS. Indeed, significant oxidative stress, impairment of the antioxidant enzyme glutathione peroxidase, evidence of inflammation, DNA damage, and apoptosis, as well as decreased levels of neurotransmitters serotonin and dopamine were found in brain of male Wistar rats 24 hours after the last repeated exposure to a low dose (0.02 mg/kg IP per day for 3 days) of citrate-coated 20 nm GNPs.[74] The authors did not investigate the occurrence of GNPs in the studied brains, so it is unclear if the observed effects were induced directly by GNPs that crossed the blood-brain barrier, or indirectly through the generation of damage/noxious substances outside of the CNS.

The health state may be an important factor influencing the toxicity potential of GNPs, as recently demonstrated by Hwang and colleagues.[21] They found highly increased liver toxicity in a mouse model of nonalcoholic steatohepatitis (male C57BL/6J mice fed a methionine- and choline-deficient diet for four weeks) 1 and 7 days after IV administration of 5 mg/kg of 15 nm PEG-coated GNPs (versus PEG as the control). No noticeable toxicity was found in healthy mice exposed to the same dose of GNPs. The observed GNPs-induced toxicity in the stressed liver model appears to be the result of stimulation of the inflammatory response along with hastening of stress-induced apoptosis.[21]

At least one study considered sex differences in GNP induced toxicity:[93] male and female C57BL/6 mice were exposed to 4 mg/kg IP of PEG-coated 4.4, 22.5, 29.3, or 36.1 nm GNPs for 28 days. Their results show size- and sex-dependent toxicity and immune stimulation, with 22.5 nm particles inducing the highest toxicity, only males presenting significant liver damage, and activation of immune response for females (higher spleen index) and males (increases in white blood cell and red blood cell counts).

Lastly, a recently published paper[146] demonstrated GNP-induced mutagenesis (with significant phenotypic alterations) and the origination of nano-mutants in *Drosophila melanogaster*. The scientists exposed *Drosophila* to 3 mg/kg per day of 15 nm citrate-capped GNPs in food, for an entire life cycle (F0), and analyzed alterations in this and subsequent generations (F1 and F2). The treatment resulted in considerably impaired reproductive performance (F0), substantial genotoxic effects (as demonstrated by greatly increased apoptosis and necrosis of hemocytes, as well as overexpression of p53 gene in F1 flies), and occurrence of abnormal phenotypes in F1 and F2 generations. Notably, the researchers were able to generate and maintain a mutated strain ("nanomaterial-mutated" or NM-mut), which represents the very first nanomaterial-mutated organism. It is also important to highlight that the same group found no evidence of genotoxicity when the GNPs were coated with PEG.[146]

Although important advances have been made to elucidate the toxic potential of GNPs, there is still much to do in order to close the vast currently existent knowledge

gaps. For instance, not much is known regarding the ability of GNPs to cross reproductive-relevant physiological barriers or their effect on gametes, and the few existing studies have focused only on males.[109] Furthermore, there is no agreement on whether GNPs are able to cross the placenta, or whether they have an effect on the developing embryo or fetus.[109] Likewise, almost nothing is known about the long-term consequences of exposure to GNPs or possible species differences in susceptibility.

E. Hypotheses

The purpose of this research project was to further elucidate and characterize the acute, subacute, and chronic effects of a single intentional exposure to a high dose of commercially available nanomaterials, specifically GNPs, utilizing two *in vivo* models. The **key additions** of this novel research to the knowledge on GNP toxicity are: 1) an experimental design that includes **chronic *in vivo*** assessment after a single exposure to GNPs, which mimics potential diagnostic or therapeutic applications in which the GNPs would be administered only once and the route of administration would be IV injection. This format addresses the issue of GNPs systemic and localized effects long after accumulation and retention in specific tissues or cells. 2) The use of **commercially available GNPs**, which represents the real-life scenario of potential uses of GNPs in biomedicine, in which particle production will be industrialized, as opposed to each user synthetizing their own GNPs, and 3) the use of **two distinct animal species** in parallel experiments permits the detection of potential species-specific reactions to GNPs exposure. Mice and rats were chosen as models in order to fulfill this objective while at the same time allow comparison of the results of this project with other research efforts.

Our overall hypotheses were:

1) Single exposure to a high dose of commercially available GNPs will result in GNP sequestration within macrophages of blood filtration organs, such as liver and spleen, and will negatively impact short-, medium-, and/or long-term health of mice and/or rats.

2) Mice and rats will present differences in type and/or severity of adverse effects observed after the exposure to GNPs.

3) Upon independent characterization, commercially available GNPs will have physicochemical characteristics in good agreement with the information provided by the supplier.

Better knowledge about the potential toxic effects of GNPs will greatly contribute to the development of safe nanotechnology-based biomedical devices and techniques, with high hopes of important progress in early diagnosis and treatment of diverse pathologies.

F. Tables

Table 1. Comparison of GNP toxicity studies *in vivo*

GNPs diameter/ coating (media)	Animal model	RoA	Dose in mg/kg	Time Points	End Points	Main toxicological conclusion	Reference
1.4, 5, 18, 80, or 200 nm/ mono-sulfonated triphenylphosphine (water); 2.8 nm/ cysteamine or thioglycolic acid (water)	Wistar-Kyoto rats (females)	IV	0.006-0.17	1 day	Biodistribution	Size and surface charge significantly influence biodistribution. Higher accumulation in liver for all GNPs tested	* Hirn et al., 2011[4]
1.9 ± 0.1 nm/? (PBS)	CD1 mice (males and females)	IV	7, 70, and 700	1, 11, and 30 days	Hematology, blood chemistry, and histopathology (24 tissues)	No evidence of toxicity, renal clearance	Hainfeld et al., 2006[94]
1.9 ± 0.1 nm/? (PBS)	Tumor-burdened BALB/c mice	IV	2700	>1 year	Clinical signs of illness	No evidence of toxicity, renal clearance	Hainfeld et al., 2006[94]
1.9 ± 0.1 nm/? (PBS)	Tumor-burdened BALB/c mice	IV	800 and 2700	14 days and >1 year	Hematology and blood chemistry (800 mg/kg, 2 weeks), clinical signs of illness (2700 mg/kg, 1 year)	No evidence of toxicity, renal clearance	Hainfeld et al., 2004[101]
4, 13, and 100 nm/ PEG (PBS)	BALB/c mice (males)	IV	0.85	30 minutes, 4 hours, 1 and 7 days, 1, 3, and 6 months	Biodistribution (blood, liver, spleen, mesenteric lymph nodes, lung, kidney, heart, brain, testis, bile, and urine), intracellular location, histopathology, and liver function	No evidence of toxicity, but size-dependent transient increase in indicators of oxidative stress and liver damage. Higher accumulation in liver, spleen, and mesenteric lymph node (within macrophage's cytoplasmic vesicles and lysosomes) for up to 6 months, with very slow elimination in urine and bile	* Cho et al., 2010[26]
4 and 100 nm/ PEG (PBS)	BALB/c mice (males)	IV	4.26	30 minutes	Histopathology (liver, spleen, kidney, heart, lung, testis, and brain) and changes in gene expression (liver)	No histopathological alterations. Significant induction of genes related to apoptosis and inflammatory/immune response, and downregulation of genes related to cell cycle and metabolic process in liver, independent of nanoparticle size	* Cho et al., 2009[137]
10, 50, 100, and 250 nm/ ? (PBS)	WU Wistar-derived rats (males)	IV	0.308-0.432[#]	1 day	Biodistribution (blood, brain, heart, kidney, liver, lung, spleen, testis, and thymus)	Size-dependent distribution of GNPs, with smaller ones showing wider distribution. However, all GNP sizes accumulated mostly in liver and spleen	* De Jong et al., 2008[29]

Table 1. Comparison of GNP toxicity studies *in vivo* (continued)

GNPs diameter/ coating (media)	Animal model	RoA	Dose in mg/kg	Time Points	End Points	Main toxicological conclusion	Reference
13 nm/ PEG (PBS)	BALB/c mice (males) with and without cardiac remodeling (isoproterenol 5 mg/kg/day SC)	IV	0.81 daily for 7 days	1 day (after last injection)	Cardiac function, structure and remodeling, inflammation, biodistribution, and myocardial apoptosis	No evidence of GNPs-induced adverse effects on physiological or pathological heart, despite six-fold more GNPs accumulation in the latter	* Yang et al., 2013[134]
13 ± 2 nm/ PEG (PBS)	BALB/c mice (males)	IV	0.17, 0.85, or 4.26	5 and 30 minutes, 4 hours, 1 and 7 days	Histopathology, apoptosis, inflammation, biodistribution (blood, liver, spleen, kidneys, lung, brain, and testis), and pharmacokinetics	Dose-dependent acute inflammation and apoptosis in liver. Higher accumulation in liver and spleen	* Cho et al., 2009[7]
15 ± 2.3, 50 ± 5.65, 100 ± 5.56, and 200 ± 7.56 nm/ citrate (0.5% sodium alginate solution)	ddY mice (males)	IV	1000	1 day	Biodistribution (blood, heart, liver, lung, spleen, kidney, stomach, pancreas, and brain)	Size-dependent distribution of GNPs, with smaller ones showing greater distribution. However, all GNP sizes accumulated mostly in liver.	* Sonavane et al., 2008[132]
15.5 ± 1.3 nm/ PEG (PBS)	C57BL/6J mice (males) with and without nonalcoholic steatohepatitis (methionine- and choline-deficient diet)	IV	5 (vs. PEG as control)	1 and 7 days	Liver: GNPs localization, fat accumulation, plasma ALT and ASP, inflammation, apoptosis, and oxidative stress	No GNPs-induced toxicity in normal livers, but highly increased toxicity in the stressed liver environment (via stimulation of inflammatory response and acceleration of stress-induced apoptosis)	* Hwang et al., 2012[21]
18.4 ± 4.9 nm/ citrate, 11-MUA, or pentapeptides CALNN, CALNS, or CALND (water)	Wistar rats (males)	IV	0.6-1	0.5, 2, 6, and 24 hours	Biodistribution (GFAAS: blood, heart, liver, lung, spleen, kidney, testis, duodenum, shaft of femur, skeletal muscle, brain, tail, urine, and feces; TEM: liver)	Biodistribution profile remains constant for the first 24 hours, with preferential accumulation in liver and spleen. Pentapeptide coating increases hepatic accumulation. Signs of necrosis in Kupffer cells	* Morais et al., 2012[8]
20 nm/ citrate (water)	Wistar rats (males)	IV	0.01	1 and 7 days, 1 and 2 months	Biodistribution (more than 25 tissues), excretion (feces and urine), and changes in gene expression (liver and spleen)	Persistent accumulation of GNPs in liver and spleen up to 2 months. Inefficient clearance through urine and feces. Significant effects on genes related to detoxification, lipid metabolism, cell cycle, defense response, and circadian rhythm	* Balasubramanian et al., 2010[13]
32.6 ± 3 nm/ TNF and PEG (?)	Tumor-burdened C57BL/6 mice	IV	0.375 and 0.75	16 days	Survival	100% survival	Paciotti et al., 2004[79]

Table 1. Comparison of GNP toxicity studies *in vivo* (continued)

GNPs diameter/ coating (media)	Animal model	RoA	Dose in mg/kg	Time Points	End Points	Main toxicological conclusion	Reference
40 nm/ citrate (?)	C57BL mice (females)	IV	? (0.5 mL of solution containing $9x10^{10}$ particles/mL)	1 day, 1, 3, and 6 months	Liver: GNPs localization and amount	GNP accumulation in Kupffer cells, inside lysosome/endosome-like vesicles. Extremely slow elimination	* Sadauskas et al., 2009[6]
50 nm/ citrate (?)	Swiss mice (males)	IV	1, 2, and 10	6, 12, 24, 48, and 72 hours	Urinalysis, hematology, and blood chemistry	Dose- and time-dependent toxicity and immune stimulation	* Sengupta et al., 2013[111]
3, 5, 8, 12, 17, 37, 50, and 100 nm/ citrate (PBS)	BALB/c mice (males)	IP	8 weekly for ? (up to 50 days?)	?	Survival, behavior, and histopathology (liver, lung, brain, heart, and spleen)	Size-dependent toxicity, with GNPs between 8 and 37 nm causing behavioral alterations ("crooked spine"), death (LD50 was 21 days) and histological alterations in liver, lung, and spleen	* Chen et al., 2009[136]
4.4, 22.5, 29.3, and 36.1 nm/ PEG (?)	C57BL/6 mice (males and females)	IP	4	28 days	Hematology, blood chemistry, and histopathology (uterus, ovaries, and testis)	Size- and sex-dependent toxicity (especially in liver) and immune stimulation	* Chen et al., 2013[93]
5 nm/ PVP (?)	Wistar rats (males)	IP	10	12 hours	Liver: GNPs localization, amount, and excretion in bile	All cell types in liver are able to internalize GNPs (endocytosis), very slow excretion in bile	Dragoni et al., 2012[52]
5 ± 1.1, 10 ± 1.6, 30 ± 4.3, or 60 ± 12.1 nm/ PEG (?)	Mice (males, strain not specified)	IP	4	28 days	Survival, body and organ weight (liver, kidneys, spleen, heart, lungs, and thymus), biodistribution (heart, liver, spleen, and kidney), hematology, blood chemistry	GNPs accumulate mostly in liver and spleen. Toxicity associated with 10 and 60 nm GNPs, but not with 5 and 30 nm particles	* Zhang et al., 2011[22]
9.45 ± 1.33, 20.18 ± 1.8, or 50.73 ± 3.58 nm/ ? (?)	Wistar-Kyoto rats (males)	IP	? (50 or 100 μL) daily for 3 or 7 days	3 and 7 days	Liver histopathology	Size- and dose-dependent histological alterations (smaller GNPs caused more damage)	* Abdelhalim & Jarrar, 2012[140]
10 and 50 nm/ citrate (water)	Wistar-Kyoto rats (males)	IP	0.022 daily for 1 or 5 days	1 day (after last injection)	Expression (mRNA) of proinflammatory cytokines IL-1β, IL-6, and TNF-α in liver and kidney	Transient increase in expression of proinflammatory cytokines in liver and kidney, with 50 nm GNPs causing greater induction than 10 nm GNPs	* Khan et al., 2013[138]
10 and 50 nm/ ? (?)	Wistar-Kyoto rats (males)	IP	? (50 μL) daily for 3 days	?	Blood chemistry (AST, GGT, ALT, ALP, urea, and creatinine)	Slight liver toxicity (increased AST)	* Abdelhalim & Moussa, 2013[139]

Table 1. Comparison of GNP toxicity studies *in vivo* (continued)

GNPs diameter/ coating (media)	Animal model	RoA	Dose in mg/kg	Time Points	End Points	Main toxicological conclusion	Reference
10, 20, and 50 nm/ citrate (water)	Wistar-Kyoto rats (males)	IP	~0.043[†] daily for 3 or 7 days	?	Heart histopathology	Size- and dose-dependent histological alterations (smaller GNPs caused more damage)	* Abdelhalim, 2011[144]
10, 20, and 50 nm/ ? (?)	Wistar-Kyoto rats (males)	IP	? (50 μL) daily for 3 or 7 days	?	Heart histopathology	Size- and dose-dependent histological alterations (smaller GNPs caused more damage)	* Abdelhalim, 2011[143]
10, 20, and 50 nm/ ? (?)	Wistar-Kyoto rats (males)	IP	? (50 or 100 μL) daily for 3 or 7 days	?	Liver histopathology	Size- and dose-dependent histological alterations (smaller GNPs caused more damage)	* Abdelhalim & Jarrar, 2011[142]
10, 20, and 50 nm/ ? (?)	Wistar-Kyoto rats (males)	IP	? (50 or 100 μL) daily for 3 or 7 days	3 and 7 days	Liver histopathology	Size- and dose-dependent histological alterations (smaller GNPs caused more damage)	* Abdelhalim & Jarrar, 2011[141]
10 and 50 nm/ citrate (water)	Wistar-Kyoto rats (males)	IP	~0.04[‡] daily for 3 or 7 days	?	Blood rheological parameters	No evidence of alterations in rheological parameters	* Abdelhalim, 2011[145]
12.5 ± 1.7 nm/ citrate (sodium citrate solution)	C57BL/6 mice (males)	IP	0.04, 0.2, or 0.4 daily for 8 days	1 day (after last injection)	Biodistribution (blood, liver, spleen, kidney, lungs, and brain), survival, behavior, body and organ weight, blood chemistry, hematology, and histopathology	No evidence of toxicity, dose-dependent accumulation in all organs examined but not in blood	* Lasagna-Reeves et al., 2010[131]
20 nm/ citrate (water)	Wistar rats (males)	IP	0.02 daily for 3 days	1 day (after last injection)	Oxidative stress, DNA damage and apoptosis, neurotransmitters, and inflammation in brain	Generation of oxidative stress, impairment of antioxidant enzyme glutathione peroxidase, and potential for inflammation and DNA damage/cell death in brain	* Siddiqi et al., 2012[74]
21.3 ± 0.7 nm/ citrate (Milli-Q water)	C57BL/6 mice (males)	IP	7.85	1, 24, and 72 hours	Blood chemistry (glucose and ALT), urinalysis, biodistribution (SEM: brain, heart, spleen, kidneys, liver, and fat), and histopathology (kidneys)	No evidence of toxicity, but significant fat loss and inhibition of inflammatory cytokines mRNA expression in fat	* Chen et al., 2013[77]

Table 1. Comparison of GNP toxicity studies *in vivo* (continued)

GNPs diameter/ coating (media)	Animal model	RoA	Dose in mg/kg	Time Points	End Points	Main toxicological conclusion	Reference
50 nm/ citrate (?)	Swiss mice (males)	IP	1 and 2, daily for 90 days	15, 30, 60, and 90 days	Urinalysis, hematology, blood chemistry, and histopathology (lungs, kidneys, liver, spleen, and testis)	Dose- and time-dependent toxicity and immune stimulation (0% survival at higher dose level)	* Sengupta et al., 2013[111]
13.5 nm/ citrate (water)	ICR mice (males)	PO	0.135, 0.275, 0.55, 1.1 or 2.2 daily for 14 days?	14 days	Survival, body and organ weight (liver, kidneys, spleen, heart, lungs, brain, colon, muscle, thyroid, lymph nodes, and bone marrow), hematology	Dose-dependent toxicity (although no significant differences were found).	* Zhang et al., 2010[17]
15 nm/ citrate (food)	*Drosophila melanogaster*	PO	3 per day	F0, F1, and F2 generations	Reproductive performance, apoptosis/necrosis on hemocytes, expression of p53 gene, and phenotypic observations	Significant reproductive impairment and genotoxic effects, with mutagenesis (important phenotypic alterations) that can be transmitted to progeny (creation of aberrant strain NM-mut)	* Vecchio et al., 2012[146]
16 and 55 nm/ PEG (0.9% NaCl)	Outbred rats (males, strain not specified)	PO	0.25 daily for 7 days	1 day (after last dose)	Mutagenesis (micronucleus assay: bone marrow polychromatic erythrocytes)	No evidence of mutagenic activity	* Jumagazieva et al. 2011[135]
1.2 ± 0.9 nm/ glutathione (PBS)	BALB/c mice (females)	SC	0.02, 0.04, 0.06, 0.08, and 0.12^	1, 14, and 28 days	Biodistribution (ICP-MS: blood, urine, spleen, liver, heart, kidneys, and lungs), complete blood count, and histopathology (kidneys, 28 days only)	No evidence of toxicity, renal clearance	* Simpson et al., 2013[133]
1.4 and 18 nm/ mono-sulfonated triphenylphosphine (?)	Wistar-Kyoto rats (females)	IV or IT	0.106 (1.4 nm GNPs) and 0.0108 (18 nm GNPs)[§]	1 day	Biodistribution (urine, feces, liver, lung, spleen, kidneys, brain, heart, uterus, blood, skin, gastrointestinal tract, and carcass)	RoA- and size-dependent distribution of GNPs, with smaller ones showing wider distribution and higher translocation (for IT). However, main accumulation in liver for both GNP sizes administered IV	* Semmler-Behnke et al., 2008[110]

Table 1. Comparison of GNP toxicity studies *in vivo* (continued)

GNPs diameter/ coating (media)	Animal model	RoA	Dose in mg/kg	Time Points	End Points	Main toxicological conclusion	Reference
5 nm/ bis(p-sulfonatophenyl) phenylphosphine, PEG 750 g/mol, or PEG 10,000 g/mol (water)	Wistar-Kyoto rats (females)	IV or IT	0.07-0.3	1 and 24 hours	Biodistribution	PEGylation significantly influences biodistribution and prolongs circulation times, with longer PEG chains having greater effects. Higher accumulation in liver and spleen (for IV).	* Lipka et al., 2010[130]
13.5 nm/ citrate (water)	ICR mice (males)	PO, IP, or IV	1.1 daily for 28 days?	28 days	Survival, body and organ weight (liver, kidneys, spleen, heart, lungs, brain, colon, muscle, thyroid, lymph nodes, and bone marrow), hematology	RoA-dependent toxicity (PO>IP>IV)	* Zhang et al., 2010[17]

GNPs, gold nanoparticles; RoA, route of administration; IV, intravenous; IP, intraperitoneal; PO, oral; SC, subcutaneous; IT, intratracheal instillation; PEG, polyethylene glycol; PVP, polyvinylpymolidone; 11-MUA, 11-mercaptoundecanoic acid; CALNN, pentapeptide Cys-Ala-Leu-Asn-Asn; CALNS, pentapeptide Cys-Ala-Leu-Asn-Ser; CALND, pentapeptide Cys-Ala-Leu-Asn-Asp; TNF, tumor-necrosis factor; PBS, phosphate buffered saline; GFAAS, graphite furnace atomic absorption spectrometry; TEM, transmission electron microscopy; SEM, scanning electron microscopy; ICP-MS, inductively coupled plasma mass spectrometry; ALT, alanine transaminase; AST, aspartate transaminase; GGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase. * indicates studies with toxicity assessment as main objective. ? denotes information not provided or nor clear. [#] Doses were calculated for a 250 g rat (the paper reports 6-8 week old rats and 1 mL/rat of 77-108 μg/mL solutions). [†] Doses were calculated for a 230 g rat (the paper reports rats 220-240 g and 100 μL/rat of 0.01% solution). [‡] Doses were calculated for a 250 g rat (the paper reports 250 g rats and 100 μL/rat of 0.01% solution). ^ Doses were calculated for a 20 g mouse (the paper reports doses in μM). [§] Doses were calculated for a 250 g rat (the paper reports 250 g rats and 26.5 [1.4 nm GNPs] or 2.7 [18 nm GNPs] μg/50μL concentrations).

F. Figures

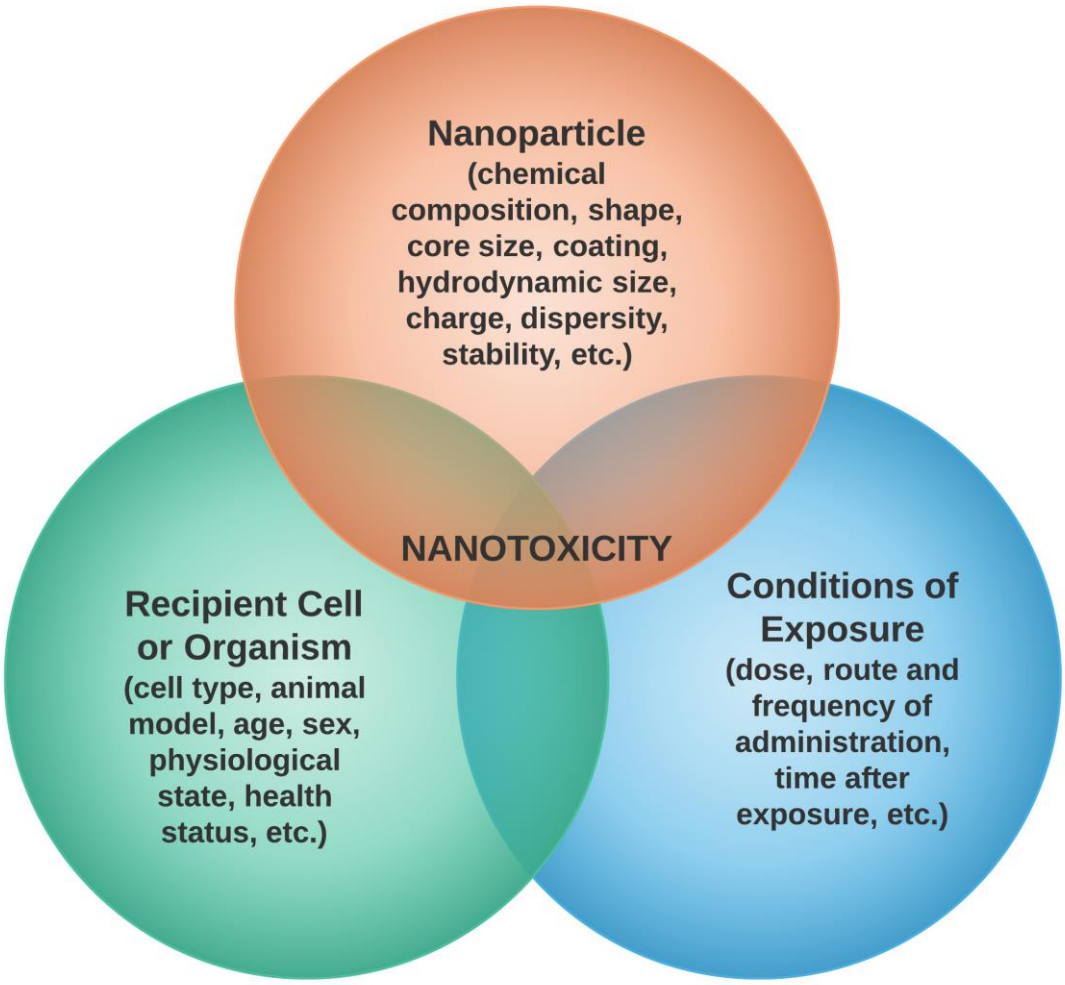

Figure 1. Factors influencing nanotoxicity.

CHAPTER II: Materials and Methods Utilized

All experiments were approved by the Institutional Animal Care and Use Committee and the Environmental Health and Safety Services at Virginia Tech.

A. Gold Nanoparticles

For all the experiments, 15 nm GNPs were purchased from Nanoprobes (AuroVist™-15 nm, Yaphank, NY). These GNPs were chosen because they are approved for *in vivo* use and were expected to be highly biocompatible. The product is described as having an LD50 (the dose that will kill half of the animals exposed) higher than 5000 mg/kg, low osmolality, and viscosity similar to water, even at high concentrations.[147]

As discussed in Chapter I, toxicity of nanomaterials appears intimately related with the material's physicochemical features.[23,28,32] Also, actual characteristics of nanomaterials may differ from the ones reported by the producer, due to variations in the fabrication process and/or changes transpired during storage.[36,47] For these reasons, it is of utmost importance to independently characterize the GNPs.[47] Collaborators in the department of Civil and Environmental Engineering of Virginia Tech characterized the GNPs used for these experiments by Raman spectroscopy (to identify the coating agent), dynamic light scattering (to determine the hydrodynamic size), transmission electron microscopy (to describe the gold core size and shape), and electrophoretic mobility (to identify the surface charge).

B. Animals and Gold Nanoparticle Exposure

Rodents are commonly used as models for general toxicity testing, and mice and rats are by far the most common animal models used for toxicity assessment of GNPs. Even though mice and rats are phylogenetically similar species, large differences in response to toxic compounds may occur.[148] The use of these two species in parallel experiments has the potential to detect species-specific sensitivities to GNP exposure, which could have great relevance when making use of conclusions from animal toxicity studies to recommend or regulate human exposure. At the same time, it allows comparison of the results and conclusions from the present study to previously published *in vivo* investigations on GNP toxicity.

The strains BALB/c (mice) and F344 (rats) were chosen for the first phase of this experiment (Chapter III) because both are inbred albino strains. The use of inbred strains guarantees a reliable and uniform animal model, decreasing variability in the response to GNP exposure and therefore increasing the probabilities of detecting post-exposure deviations from the control group. *In vivo* exposure to GNPs has been reported to cause darkening of skin and internal tissues.[79,111] The use of albino strains allows unobstructed macroscopic observation of changes in color of skin and other organs after exposure to GNPs. Also, on microscopic examination of tissues exposed to GNPs, these particles are seen as dark spots, which can be confounded with melanin in colored animals. The use of albino strains allows ruling out melanin pigment during histological examination of tissues.

The strain of mice C57BL/6 was selected for the long-term phase of this experiment (Chapter IV). Besides the benefits discussed above of being inbred animals,

this strain was primarily chosen because it is considered the most susceptible to atherosclerosis.[149,150] Other low-toxicity inorganic engineered nanoparticles have been shown to generate vasomotor dysfunction and exacerbate progression of atherosclerosis.[151] Nanoparticles used in biomedicine are likely to be injected in the blood stream, directly exposing endothelial cells to a much larger dose than other cell types.[40] Endothelial cells are able to internalize GNPs,[5,19,52] and since these particles are hardly eliminated, it is possible that they could exert chronic effects in blood vessels.[41]

For the study presented in Chapter III, 52 female BALB/c mice (Harlan, Dublin, VA) 5-6 weeks old and 40 female F344 rats (Charles River, Wilmington, MA) 5-6 weeks old were purchased and maintained with standard rodent diet (Harlan Teklad Global Diet 2018, Madison, WI) and water *ad libitum*. Following an acclimation period of 1 week, animals were randomly divided in two groups: "GNP" or "PBS". The GNP group received a single injection of GNPs in the tail vein at a dose of 1000 mg/kg, half the dose recommended by the manufacturer for diagnostic imaging purposes (volumes injected were 0.1 mL per mouse and 0.6 mL per rat approximately). The PBS group received an equivalent volume of phosphate buffered saline (PBS), the GNP vehicle, and is considered the control group. For all the following measurements and assessments, animals from the GNP group were compared to their PBS counterparts. All animals were sedated prior to GNP or PBS injection, using 5 mg/kg IP of diazepam. After the injections, they were closely monitored until fully recuperated. Following the exposure, groups of animals (5-6 mice and 4 rats per group) were euthanized via CO_2 asphyxiation, necropsied, and samples collected at 1, 7, 14, 21, and 28 days post-exposure. Additionally, behavior was monitored daily by veterinarians (subjective evaluation of

posture, locomotion, awareness of surroundings, reaction to stimulus, and stress indicators such as barbering and diarrhea) and body weight was recorded weekly for all the remaining animals. The samples collected at each time point were feces, urine, blood, heart, aorta, trachea, thyroid gland, esophagus, thymus, lungs, liver, spleen, gastrointestinal tract (stomach, duodenum, cecum), pancreas, kidneys, adrenal glands, bladder, uterus, ovaries, abdominal fat, lymph nodes (thoracic and abdominal), skin, hind limbs, and head.

For the study presented in Chapter IV, 12 C57BL/6 mice (offspring of mice obtained from Harlan, Dublin, VA) 6 weeks old (6 males and 6 females) were also maintained with free access to standard rodent diet and water and were randomly assigned to GNP or PBS groups (3 males and 3 females per group). These animals received 1000 mg/kg GNPs or PBS following the same procedure described above, and were monitored daily for behavioral changes and weekly for body weight changes until they reached 6 months of age (i.e., 20 weeks after GNP or PBS exposure). At that time, all the animals were euthanized via CO_2 asphyxiation, necropsied, and samples collected (blood, liver, spleen, kidneys, uterus, ovaries, testis, abdominal fat, lymph nodes, pancreas, gastrointestinal tract, lungs, heart, aorta, skin, hind limbs, and head).

It is important to mention that the objective of this study was to characterize the toxic effects of GNPs, as opposed to identify dose-response relationships. It is well known that generally humans are more vulnerable to toxic effects than experimental animals, and exposure of the latter to high doses is considered "a necessary and valid method of discovering possible hazards in humans".[148] Although the dose chosen for this study, 1000 mg/kg, may seem excessively high, it is considered appropriate by the federal

agencies for acute, subchronic, and chronic toxicity studies with rodents and nonrodents.[152] The specific GNPs used for this experiment are expected to be highly biocompatible, and the dose used is significantly lower than the reported LD50, so we did not anticipate high toxicity. Also, studies exposing animals to almost three-fold higher concentrations of GNPs from the same company, but 1.9 nm in size, reported no clinical signs of illness.[94,101] Overall, we selected a high dose in order to increase the probability of obtaining statistically valid results from a limited number of animals, but we are conscious that it may exceed the potential level of human exposure. This needs to be considered especially when extrapolating from high dose to low dose and across species, and results and conclusions of this study should be analyzed within their context.

C. Body Weight

Along with daily monitoring of behavior, body weight of mice and rats was measured and recorded weekly as an indicator of health and adequate nutrition. Before starting the experiment it was determined that if any animal loses more than 10% of its body weight, it would immediately be removed from the study and properly treated or euthanized.

D. Blood Glucose

Single non-fasted blood glucose levels were measured as an indicator of health and stress. Blood was drawn from mice via puncture of the lateral saphenous vein and glucose levels were measured using a hand-held glucometer (Accu-Check Compact,

Roche Diagnostics, Indianapolis, IN). Data from GNP animals was compared to their PBS counterparts.

E. Enzyme-Linked Immunosorbent Assays (ELISAs)

Given that previous studies have found GNP accumulation in cells of the mononuclear phagocyte system, we included assessment of markers related to macrophage activity. Commercially available ELISA kits were used to determine serum levels of malondialdehyde (MDA) in rats (Catalog number E90597Ge, Uscn Life Science Inc., Wuhan, China), interferon-gamma (IFN-γ) in mice (Catalog number EA100004, OriGene, Rockville, MD), and IL-18 in mice (Catalog number 7625, Medical & Biological Laboratories Co., Ltd., Woburn, MA) and rats (Catalog number KRC2341, Invitrogen Corporation, Camarillo, CA). Blood was collected from the animals via cardiac puncture under deep anesthesia with isoflurane, allowed to clot for 30 minutes to 1 hour at room temperature, centrifuged at 2000 rpm for 20 minutes, and then the serum collected in aliquots and stored at -80ºC. Once all samples at all time points were collected, serum was thawed and analyzed with the corresponding ELISA kit, following the manufacturer's instructions. All standards, samples, and controls were run in duplicate. The absorbance was measured at 450 nm immediately after finishing each protocol, using a spectrophotometer and accompanying software (SpectraMax 250 and SoftMax Pro software, Molecular Devices, Sunnyvale, CA). The intensity of the color generated was directly proportional to the amount of IL-18 and IFN-γ, and inversely proportional to the amount of MDA in the samples. Amount of protein was calculated using the best-fit curve equation for each ELISA, comparing the mean absorbance of

each sample to the standard curve. For all ELISAs, protein levels of GNP groups were compared to corresponding PBS groups.

Oxidative stress and inflammation have been pointed out as potential mechanisms of GNP toxicity.[20,24,128] Macrophages actively internalize and accumulate GNPs,[4,26,52] and there is extremely poor clearance, so evaluation of the activation (or inhibition)[129] of macrophages and chronic inflammation involving these immune cells are of high relevance when characterizing GNP toxicity. Malondialdehyde is a byproduct of lipid peroxidation by free radicals and ROS, and it is extremely reactive. It is known to form highly genotoxic complexes with macromolecules such as proteins and DNA[20] and it has been measured as a marker of oxidative stress in nanotoxicology research.[20,30] Interferon-gamma is a proinflammatory cytokine with significant immunostimulatory and immunomodulatory effects on both innate and acquired immunity and is especially important for activation of macrophages. Interleukin-18 (also known as IFN-γ Inducing Factor) is a proinflammatory cytokine produced almost exclusively by macrophages and other antigen presenting cells. It is involved in chronic inflammation, autoimmune diseases, cancer, and infectious diseases, contributing to host defenses (cell-mediated immunity) and inflammation.[153]

F. Biochemical Profile

Serum levels of glucose, urea nitrogen, creatinine, phosphorus, calcium, total protein, albumin, globulin, ALT, AST, ALP, GGT, total, direct, and indirect bilirubin, cholesterol, sodium, potassium, chloride, CO_2, and anion gap were measured as indicators of health and organ function, as well as presence or absence of lipemia, icterus, and

hemolysis. Fresh serum obtained as previously described for the ELISAs was submitted to the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) Clinical Pathology Laboratory for analysis with the AU480 chemical analyzer. This technique requires a minimum of 300 μL of serum, so some mice samples were pooled (2 mice per sample) in order to provide sufficient serum. At each time point, GNP and PBS groups were compared.

G. Histopathology

After euthanasia, each animal was subjected to macroscopic examination and tissue samples were collected and stored in 10% neutral buffered formalin. For microscopic morphologic analysis by light microscopy, tissues were trimmed and submitted to the VMRCVM Histology Laboratory for routine histologic processing (samples are embedded in paraffin wax, sectioned [4 μm thick], and stained with hematoxylin-eosin [H&E]) and special stains (Oil-Red O, periodic-acid-Schiff, Masson's trichrome, Prussian blue and bile for visualization of lipids, glycogen, fibrosis, iron hemosiderin, and bile, respectively). Tissue slides from C57BL/6 mice were also stained with Fontana-Masson stain to differentiate melanin from GNPs. Tissue samples containing bone were placed in decalcifying solution (Enhanced Decalcification Formulation, StatLab Medical Products Catalog number SL85-32, McKinney, TX) for 24 to 48 hours, prior to submission to the Histology Laboratory. Macroscopic and microscopic morphology of tissues from GNP animals was compared to corresponding PBS animals.

H. Immunohistochemistry – von Willebrand Factor

In order to assess GNP accumulation in endothelial cells as well as alterations in cell morphology, paraffin-embedded tissues were submitted to the Connecticut Veterinary Medical Diagnostic Laboratory for immunohistochemical staining with von Willebrand Factor Antibody (Catalog number A0082, Dako, Carpinteria, CA), a marker of endothelial cells. Marker immunoreactivity in blood vessels was evaluated via light microscopy.

I. Transmission Electron Microscopy (TEM)

In order to assess GNP intracellular location and cellular alterations, samples of mice liver were fixed and stored in a mixture of 5% glutaraldehyde, 4.4% formaldehyde, and 2.75% picric acid in 0.005 M sodium cacodylate, and submitted to the VMRCVM Morphology Laboratory for routine TEM processing (samples are post-fixed with osmium tetroxide, dehydrated, infiltrated with propylene oxide and Poly/Bed 812, embedded in molds, oven-cured, thick-sectioned [1 μm thick], observed under light microscope to select area of interest, and thin-sectioned [60-90 nm thick]). Processed samples were examined and pictures taken using the Zeiss 10CA Transmission Electron Microscope equipped with AMT Advantage GR/HR-B CCD Camera System. Samples from GNP and PBS groups were compared.

J. Scanning Electron Microscopy coupled with Energy Dispersive X-ray Spectroscopy (SEM-EDX)

In order to visualize and confirm GNP accumulation within tissues, samples of mice and rat liver and spleen were analyzed by SEM-EDX, comparing GNP and PBS groups. Samples were fixed and stored in a mixture of 5% glutaraldehyde, 4.4% formaldehyde, and 2.75% picric acid in 0.005 M sodium cacodylate and were submitted to the VMRCVM Morphology Laboratory for SEM processing (samples are post-fixed with osmium tetroxide, dehydrated, critical point dried, and mounted on stubs). For GNP visualization and elemental determination, processed samples were taken to the Institute of Critical Technology and Applied Science (ICTAS) Nanoscale Characterization and Fabrication Laboratory, where they were finely coated with carbon (carbon was used as an alternative coating to avoid interference of the normally used gold coating with the elemental determination of GNPs within tissues) and examined using the FEI Quanta 600 FEG environmental Scanning Electronic Microscope equipped with Bruker EDX with a Silicon Drifted Detector (FEI, Hillsboro, OR).

K. Atomic Absorption Spectrometry (AAS)

For quantitative assessment of GNP excretion and tissue accumulation, samples of mice and rat urine, feces, liver, and brain (frozen: urine and feces, or stored in 10% neutral buffered formalin: liver and brain) were submitted to the VMRCVM Toxicology Laboratory. Samples were homogenized, subjected to nitric acid/perchloric acid digestion, and analyzed with the Varian SpectrAA 220FS Atomic Absorption

Spectrometer for measurement of gold content. Gold concentrations in samples of GNP groups were compared to PBS groups, as well as different time points within each group.

L. Statistical Analysis

The program JMP® Pro 10.0.2 (SAS Institute Inc., Cary, NC) was used to analyze the results and identify differences between groups via one-way analysis of variance (ANOVA), student's t-test, Tukey HSD test, and logistic modeling. Data were considered statistically significant if $p < 0.05$.

CHAPTER III: Species Differences in Gold Nanoparticle Toxicity

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Running Title: Species differences in GNP toxicity

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A. Abstract

Nanotoxicity studies are greatly needed in order to advance nanomedical technologies into clinical practice. We assessed the toxic effects of a single intravenous exposure to commercially available gold nanoparticles (GNPs) in two *in vivo* models, mice and rats. Fifteen nm GNPs were purchased and independently characterized. All animals were exposed to either 1000 mg GNPs/kg body weight (GNP group) or an equivalent volume of phosphate buffered saline (PBS group) in the tail vein. Subsets of animals were euthanized at 1, 7, 14, 21, and 28 days post-exposure. At those times, samples of feces, urine, serum, and over 20 tissues were collected for biochemistry, histopathology, electron microscopy, and atomic absorption spectrometry analysis. Independent characterization demonstrated that the physicochemical properties of the purchased GNPs were in good agreement with the information provided by the supplier. Important species differences in immune response were seen when comparing mice and rats. Gold nanoparticles induced the formation of liver microgranulomas in mice, along with transiently increased serum levels of the proinflammatory cytokine interleukin-18. No such alterations were found in rats. Differences in GNP biodistribution and excretion were also detected between the two species, with higher relative accumulation of GNPs in spleen and greater fecal excretion in rats. In conclusion, GNPs are not innocuous and have the ability to incite a robust macrophage response in mice. However, after balancing their potential harmful effects with the potential benefits of their use in biomedicine, we believe GNPs are highly biocompatible and should continue to be considered among the top candidates for biomedical applications.

Key Words: gold nanoparticles; nanotoxicity; microgranulomas; mouse; rat

B. Introduction

Materials in the nanoscale acquire unique properties that are not present in bulk material (larger particles) of identical composition. These properties can be highly useful and carry the potential of having great impact on key areas of science and technology, including food science, materials science, electrical engineering, pharmacology, and medicine.[6,8,10] The rapid development of nanotechnology worldwide brings a subsequent increase in generation and usage of engineered nanoparticles, and therefore increased risk of exposure and toxicity.[6,13] Most nanotoxicity research has focused on accidental exposures,[35] but usage of nanomaterials in the medical field requires intentional exposure to significant doses by different routes, including intravenous (IV), subcutaneous (SC), or intramuscular (IM) injections.[24,36,40] Unfortunately, the safety of nanomaterials remains largely unknown or incompletely understood.[75] This is currently the most critical issue obstructing the advance of nanomedical technology into clinical practice.[71,73]

Gold nanoparticles (GNPs) in particular are one of the top candidates for biomedical uses, mainly due to their high stability and biocompatibility when compared to other nanomaterials.[154] They are easy to synthetize and characterize, and they can be conjugated with multiple chemicals and biological molecules to create biocompatible, targeted, and controlled diagnostic, treatment, and delivery systems.[32,75,77] Their use as carriers for targeted drug delivery, as contrast agents for advanced imaging, and as diagnostic and therapeutic agents for cancer treatment are currently being intensely investigated.[76,89]

In order to take full advantage of GNP's potential biomedical uses, we must understand their fate and potential adverse effects after they have fulfilled their intended purpose.[25] There is currently much controversy regarding their potential for toxicity, with some studies supporting their safety and others reporting toxicity *in vitro* and *in vivo.* The main source of disagreement in results and conclusions comes from the great diversity in experimental design among the different research groups, making genuine comparison rarely possible.[89,106] It is well known that the reactivity and toxicity of nanomaterials depends largely on their physicochemical characteristics, thus nanoparticle characterization is an essential portion of nanotoxicology research.[48,49,57] Research groups frequently synthesize their own GNPs and often do not report much information on characterization, resulting in assessment of particles with diverse physicochemical features. They also use different cell and animal models, doses, routes of administration, and endpoints, increasing the variability among studies.

The purpose of our research was to identify and describe the health effects of a single IV exposure to commercially available GNPs in two animal models in parallel: BALB/c mice and F344 rats. We decided to assess commercially available GNPs in order to mimic the real-life scenario of biomedical uses of these particles, in which they will be industrialized, as opposed to each user synthetizing their own. It has been reported that nanomaterials can vary with different production methods or post-production modifications, and can even change spontaneously during storage. They may also vary in separate lots from the same producer.[34,47] This variability is likely to persist in the nanomaterial industry, including those particles destined to the biomedical field, as it is expected that the manufacturing processes will function within a range of

physicochemical properties that ensures functionality without over-increasing the production cost.[34] Therefore, toxicity assessment of industrially produced GNPs is of high relevance before the advancement of these particles to widespread use. On the other hand, due to the same intrinsic variability of nanomaterials, actual physicochemical features of commercially available GNPs may vary from the information provided by the manufacturer. For that reason, independent characterization of the GNPs assessed is still an essential element in nanotoxicity studies,[47] and it was integrated in our research.

To our knowledge, the inclusion of two distinct animal species, for instance mice and rats, in parallel experiments to assess GNP toxicity has never been reported before. This fulfills two relevant objectives: firstly, it allows the detection of potential specie-specific reactions to GNP exposure. This is especially important when using conclusions from animal toxicity studies to write recommendations or regulations for human exposure. While mice and rats are phylogenetically similar, nonetheless large differences in response to toxic compounds may occur.[148] Mice and rats are by far the most common animal models used for toxicity assessment of GNPs. This is relevant to the second objective: to facilitate comparison of the results and conclusions from the present study to previously published *in vivo* investigations on GNP toxicity.

The results of this study demonstrate that there are differences in the effects of GNP exposure in mice and rats. For instance, mice develop liver microgranulomas, while rats do not. Differences in biodistribution also occur, with greater accumulation in rat spleen when compared to mouse spleen. Furthermore, acute death was observed in rats but not in mice, although the mechanism could not be ascertained.

C. Materials and Methods

1) Gold nanoparticles

Nominative 15 nm GNPs were purchased (AuroVist™-15 nm, Nanoprobes, Yaphank, NY) and characterized. AuroVist™-15 nm GNPs were chosen for this study due to their expected high biocompatibility. The product is described as having a LD_{50} > 5000 mg/kg, low osmolality, and viscosity similar to water, even at high concentrations.[147] A Philips EM420 Transmission Electron Microscope (TEM, Philips, Somerset, NJ) operating at 120 kV was used to determine the gold-core size distribution and shape. Micrographs were analyzed using Image-J (National Institutes of Health, Bethesda, MD). Dynamic light scattering (DLS) and electrophoretic mobility were performed to determine GNP hydrodynamic diameter and surface charge, as well as agglomeration state. For DLS measurements, a Zetasizer NanoZS (Malvern Instruments, Westborough, MA) particle analyzer was used. The method of cumulants was employed to determine the average hydrodynamic diameter of the particles (Z_{ave}). The Smoluchowski approximation was used to obtain zeta potential of the particles. Additionally, Raman spectroscopy was used to identify the GNP coating agent by comparing GNPs of known coating properties with the purchased GNPs. Briefly, citrate-coated GNPs were prepared as described by Turkevich et al.[155] Thiolated polyethylene glycol (PEG) with a molecular weight of 5000 Da was purchased from Nanocs (New York, NY). A 1 mM PEG solution was prepared and frozen until further use. PEG-coated GNPs (PEG-GNPs) were prepared by mixing 30 μL of the 1 mM PEG solution with 100 μL of the citrate-coated GNP suspension. A WITec Alpha500R Raman Spectrometer (WITec, Knoxville, TN) with a 10x microscope objective, 300 gr/mm grating, and 785-

nm laser was used for all Raman measurements. Sample volumes of 2 μL of PEG, PEG-GNPs, and the purchased GNPs were applied to glass slides, covered with aluminum foil, and allowed to dry in a fume hood. Raman spectra were collected from dried samples with a 5-mW laser power, 1-s integration time, and 10 accumulations. The graph background subtraction tool of the WITec Project Software was used to remove the fluorescent background signal from the collected spectra.

2) Animals and exposure

All experiments were performed in accordance with the Guiding Principles in the Use of Animals in Toxicology and were previously approved by the Institutional Animal Care and Use Committee and the Environmental Health and Safety Services at Virginia Tech. Female BALB/c mice (Harlan, Dublin, VA) 5-6 weeks old and female F344 rats (Charles River, Wilmington, MA) 5-6 weeks old were purchased and maintained at 22 ± 1ºC, 40-60% humidity, and 12:12-hour light:dark cycle, with standard rodent diet (Harlan Teklad Global Diet 2018, Madison, WI) and water *ad libitum*. Following an acclimation period of 1 week, animals were randomly divided in two groups: "GNP" or "PBS". The GNP group received a single injection of GNPs in the tail vein at a dose of 1000 mg/kg (volumes injected were 0.1 mL per mouse and 0.6 mL per rat approximately). The PBS group received an equivalent volume of phosphate buffered saline (PBS), the GNP vehicle, and is considered the control group. All animals were sedated prior to GNP or PBS injection, using 5 mg/kg IP of diazepam. After the injections, they were closely monitored until fully recuperated. Groups of animals (5-6 mice and 3-4 rats per group) were euthanized via CO_2 asphyxiation, necropsied, and samples collected at 1, 7, 14, 21,

and 28 days post-exposure. Additionally, behavior was monitored daily by veterinarians (subjective evaluation of posture, locomotion, awareness of surroundings, reaction to stimulus, and stress indicators such as barbering and diarrhea) and body weight was recorded weekly. The samples collected at each time point were feces, urine, blood, heart, aorta, trachea, thyroid gland, esophagus, thymus, lungs, liver, spleen, gastrointestinal tract (stomach, duodenum, cecum), pancreas, kidneys, adrenal glands, bladder, uterus, ovaries, abdominal fat, lymph nodes (thoracic and abdominal), skin, hind limbs, and head.

The dose selected for this study, 1000 mg/kg, is considered appropriate by the federal agencies for nonclinical toxicity studies in rodents.[152] The specific GNPs used for this experiment are described as highly biocompatible, and the dose used is significantly lower than the reported LD_{50}. Also, studies exposing animals to almost three-fold higher concentrations of GNPs from the same company, but 1.9 nm in size, reported no clinical signs of illness.[94,101] A high dose was selected in order to increase the probability of obtaining statistically valid results from a limited number of animals, although it may exceed the potential level of human exposure. This needs to be considered especially when extrapolating from high dose to low dose and across species, and results and conclusions of this study should be analyzed within this context.

3) Biochemical parameters

In order to evaluate biochemical parameters, blood was collected from the animals via cardiac puncture under deep anesthesia with isoflurane, allowed to clot for 30 minutes to 1 hour at room temperature, and centrifuged at 2000 rpm for 20 minutes. The

serum was collected in aliquots and analyzed immediately or stored at -80ºC for further use.

Malondialdehyde, interferon-gamma, and interleukin-18. Oxidative stress and inflammation have been suggested as potential mechanisms of GNP toxicity.[20,24,128] Macrophages actively internalize and accumulate GNPs,[4,26,52] and there is extremely poor clearance, so evaluation of the activation (or inhibition)[129] of macrophages and chronic inflammation involving these immune cells is of high relevance when characterizing GNP toxicity. Enzyme-linked immunosorbent assay (ELISA) kits were used to determine serum levels of malondialdehyde (MDA) in rats (Catalog number E90597Ge, Uscn Life Science Inc., Wuhan, China), interferon-gamma (IFN-γ) in mice (Catalog number EA100004, OriGene, Rockville, MD), and IL-18 in mice (Catalog number 7625, Medical & Biological Laboratories Co., Ltd., Woburn, MA). Malondialdehyde is an extremely reactive byproduct of lipid peroxidation by free radicals and ROS. It is known to form highly genotoxic complexes with macromolecules such as proteins and DNA[20] and it has been measured as a marker of oxidative stress in nanotoxicology.[20,30] Interferon-gamma is a proinflammatory cytokine with significant immunostimulatory and immunomodulatory effects on both innate and acquired immunity and is especially important for activation of macrophages. Interleukin-18 (also known as IFN-γ Inducing Factor) is a proinflammatory cytokine produced almost exclusively by macrophages and other antigen presenting cells. It is involved in chronic inflammation, autoimmune diseases, cancer, and infectious diseases, contributing to host defenses (cell-mediated immunity) and inflammation.[153]

Serum was thawed and analyzed with the corresponding ELISA kit, following the manufacturer's instructions. All standards, samples, and controls were run in duplicate. The absorbance was measured at 450 nm immediately after finishing each protocol, using a spectrophotometer and accompanying software (SpectraMax 250 and SoftMax Pro software, Molecular Devices, Sunnyvale, CA). Amount of protein was calculated using the best-fit curve equation for each ELISA, comparing the mean absorbance of each sample to the standard curve. For all ELISAs, protein levels of GNP groups were compared to corresponding PBS groups.

Biochemical Profiles. Serum levels of glucose, urea nitrogen, creatinine, phosphorus, calcium, total protein, albumin, globulin, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), total, direct, and indirect bilirubin, cholesterol, sodium, potassium, chloride, CO_2, and anion gap were measured as indicators of health and organ function. Presence or absence of lipemia, icterus, and hemolysis was also evaluated. Fresh serum was submitted to the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) Clinical Pathology Laboratory for analysis with an AU480 chemical analyzer. This technique requires a minimum of 300 μL of serum, so some mouse samples were pooled (2 mice per sample) in order to provide sufficient serum. At each time point, GNP and PBS groups were compared.

4) Histopathology

Tissue samples collected during necropsy were stored in 10% neutral buffered formalin. For microscopic morphologic analysis by light microscopy, tissues were trimmed and submitted to the VMRCVM Histology Laboratory for routine histologic processing (samples are embedded in paraffin wax, sectioned [4 μm thick], and stained with hematoxylin-eosin [H&E]) and special stains (Oil-Red O, periodic-acid-Schiff, Masson's trichrome, Prussian blue and bile for visualization of lipids, glycogen, fibrosis, iron hemosiderin, and bile, respectively). Tissue samples containing bone were placed in decalcifying solution (Enhanced Decalcification Formulation, Catalog number SL85-32, StatLab Medical Products, McKinney, TX) for 24 to 48 hours, prior to submission to the Histology Laboratory. Macroscopic and microscopic morphology of tissues from GNP animals was compared to corresponding PBS animals.

5) Immunohistochemistry – von Willebrand factor

In order to assess GNP accumulation in endothelial cells as well as alterations in cell morphology, paraffin-embedded tissues were submitted to the Connecticut Veterinary Medical Diagnostic Laboratory for immunohistochemical staining with von Willebrand Factor Antibody (Catalog number A0082, Dako, Carpinteria, CA), a marker of endothelial cells. Stained slides were evaluated via light microscopy.

6) Scanning electron microscopy with energy dispersive x-ray spectroscopy (SEM-EDX)

Samples of mice and rat liver and spleen were analyzed by SEM-EDX, to visualize and confirm GNP accumulation within tissues. Samples were fixed and stored in a mixture of 5% glutaraldehyde, 4.4% formaldehyde, and 2.75% picric acid in 0.005 M sodium cacodylate and were submitted to the VMRCVM Morphology Laboratory for SEM processing (samples are post-fixed with osmium tetroxide, dehydrated, critical point dried, and mounted on stubs). For GNP visualization and elemental determination, processed samples were taken to the Institute of Critical Technology and Applied Science (ICTAS) Nanoscale Characterization and Fabrication Laboratory, where they were sputter-coated with carbon (carbon was used as an alternative coating to avoid interference of a gold coating with elemental determination of GNPs within tissues) and examined using an FEI Quanta 600 FEG environmental Scanning Electronic Microscope equipped with Bruker EDX with a Silicon Drifted Detector (FEI, Hillsboro, OR).

7) Atomic absorption spectrometry

For quantitative assessment of GNP excretion and tissue accumulation, frozen samples of urine and feces, as well as samples of liver and brain fixed in 10% neutral buffered formalin were submitted to the VMRCVM Toxicology Laboratory. Samples were homogenized, subjected to nitric acid/perchloric acid digestion, and analyzed with a Varian SpectrAA 220FS Atomic Absorption Spectrometer for measurement of gold content (Gold Ultra Hollow Cathode lamp, 242.8 nm wavelength, 1.0 nm slit width,

background correction on). Gold concentrations in samples of GNP groups were compared to PBS groups.

8) Statistical analysis

Statistical analysis was performed only when data from at least 3 samples from different animals were available. Results were analyzed via one-way analysis of variance (ANOVA), student's t-test, Tukey HSD test, and logistic modeling as appropriate, using JMP® Pro 10.0.2 (SAS Institute Inc., Cary, NC). Data were considered statistically significant if $p < 0.05$ and are reported as mean ± standard error of the mean except when indicated, with *n* denoting the sample size.

D. Results

1) Characterization of GNPs

The mean diameter of the GNP's gold core was determined by TEM to be 13.09 nm with a standard deviation of 7.89 nm ($n = 300$). The GNP cores were roughly spherical and with diameters relatively polydispersed, as shown by the high standard deviation. Table 1 gives the summary statistics for TEM. Figure 1 shows the size distribution histogram determined by image analysis of collected micrographs, as well as one representative micrograph. Regarding DLS analysis, the average particle hydrodynamic diameter (Z_{ave}) determined via the method of cumulants is 42.64 nm with a standard deviation of 0.145 nm. The polydispersity index (PDI) is 0.154 with a standard deviation of 0.005. Detailed data and statistics of the DLS analysis are summarized in

Table 2. These results indicate that the GNP hydrodynamic diameters are relatively monodispersed and the particles are not agglomerated. The GNPs have a mean zeta potential of -34.43 mV with a standard deviation of 2.71 mV. However, the zeta potential distribution is very wide, with the mean zeta deviation measured to be 21.47 mV± 5.35 mV. Detailed data and statistics of the electrophoretic mobility analysis are summarized in Table 3. Raman spectroscopy was used to investigate the nature of the surface coating used to stabilize the purchased GNPs. PEG was suspected as a likely coating material because of its superior capacity for stabilizing GNPs and its common use in biomedicine.[156] The Raman spectra of PEG, PEG-GNPs (synthesized by our group), and the purchased GNPs are shown in Figure 2. Differences between PEG and PEG-GNP spectra (e.g., peak broadening and frequency shifts) are attributable to the less ordered structure of the PEG coating relative to pure PEG.[157,158] The purchased GNPs and PEG-GNPs exhibit close resemblances in the bands resulting from the CH_2 rocking (810 and 845 cm^{-1}), twisting (1240 and 1280 cm^{-1}), and bending (1440 and 1470 cm^{-1}) modes, which implies that the unknown coating material is either PEG or a polymer with a structure and conformation similar to the PEG coating. The peaks at 1060 and 1137 cm^{-1}, which are due to the C-O-C stretching mode with a contribution from the CH_2 rocking mode at 1060 cm^{-1}, are weak in the spectrum of the purchased GNPs. Also, an additional sharp peak appears at 1090 cm^{-1}. A decrease in Raman intensity in this region and a shift toward 1090 cm^{-1} was also evident in the Raman spectra of PEG-silicate nanocomposites recently reported by Chrissopoulou et al.;[158] therefore, the 1000-1170 cm^{-1} region of the Raman spectrum of the purchased GNPs is still consistent with the conclusion that the unknown coating is PEG. Finally, the core diameter is much smaller than the DLS-

determined hydrodynamic diameter. This is a good indication of the presence of a thick layer of PEG coating around the gold core.

2) Toxicological profile, excretion, and bioaccumulation

Three out of the 19 rats exposed to GNPs died unexpectedly within 24 hours of exposure. Necropsy of the deceased animals did not reveal probable causes of death. No deaths were registered in rats exposed to PBS or mice exposed to GNPs or PBS. No alterations in behavior, body weight (Figure 3), MDA, IFN-γ, or any of the parameters measured in biomedical profiles were found in any of the animals.

Grossly, a change in color was evident immediately after the injection (Figure 4). At first all blood vessels appeared highlighted in red (Figure 4-B). One day after the injection, the color change had expanded into the skin and internal tissues (Figure 4-C). By one week post-exposure the color had shifted from red to dark purple, color that persisted until the end of the study (Figure 4-D). Gold nanoparticles could also be directly observed in serum up to 7 days post-exposure (Figure 5).

Low concentrations of gold were detected in urine up to 7 days post-GNP exposure (Figure 6), with similar levels for mice and rats. In feces, concentrations of gold were higher than in urine, but still low overall. Gold could be measured in mice feces up to 14 days post-exposure and in rat feces up to 28 days post-exposure, with consistently higher levels in rats when compared to mice (Figure 7).

In liver, gold content significantly increased between 1 and 7 days post GNP-exposure in mice and rats, and remained high in both species until the end of the study (Figure 8). Low concentrations of gold were also detected in brain homogenate of GNP-

exposed mice and rats (Figure 9). Gold levels were higher in rat brain 1 and 7 days post-exposure compared to mouse brain, and decreased by day 14. After this time, concentrations of gold in mice and rat brain were similar, and stayed low until the end of the study (Figure 9).

From all the biochemical parameters assessed, the only significant difference between PBS- and GNP-exposed animals was found in mouse serum levels of IL-18. Seven days post-exposure, IL-18 was significantly higher in GNP-exposed mice when compared to their PBS-exposed counterparts ($p < 0.05$, Figure 10). However, serum levels of this cytokine decreased to similar levels as the control group by day 14, and registered no further increases until the end of the study.

3) Histological examination

Gold nanoparticles accumulated in all tissues examined, mainly in macrophages and endothelial cells. The greatest accumulations were observed in liver and spleen. In liver, GNPs accumulated mainly in Kupffer cells, with seemingly equivalent levels for mice (Figure 11) and rats (Figure 12). Nevertheless, by 14 days post-exposure Kupffer cells in mice liver could be observed forming microgranulomas (macrophage clusters containing multinucleated giant cells, arrowheads in Figure 11). This was not observed in rat liver, where most GNP-containing Kupffer cells stayed individualized (Figure 12). In spleen, GNP-containing macrophages were restricted, for the most part, to the red pulp, with markedly higher accumulation in rats when compared to mice (Figure 13).

Figure 14 illustrates GNP accumulation in other tissues and cell types, namely endothelial cells (Figure 14-A), brain (Figure 14-B), skin (Figure 14-C),

spindle/mesenchymal cells (Figure 14-D), lung (Figure 14-E and F), ovary (Figure 14-G), and bone marrow (Figure 14-H). It was determined that accumulation of GNPs in tissues was responsible for the observed change in color. In general, 1 day post-exposure GNPs could be observed in blood and some tissue macrophages. By day 7, most tissue macrophages showed GNP accumulation. Interestingly, trace amounts of particles could also be observed throughout the parenchyma of diverse tissues, including liver, uterus, adrenal gland, and lung. Figure 15 depicts GNP-containing hepatocytes and Figure 14-F illustrates GNPs throughout the alveolar interstitium, as examples. Upon subjective evaluation, it appears that the amount of GNPs distributed throughout tissue parenchyma decreased by days 21 and 28 post-exposure, while tissue macrophages appeared more packed with GNPs. Also, this transient localization of GNPs within tissue parenchyma was more evident in rats than in mice.

4) Elemental analysis of tissues

Gold nanoparticles were identified within tissues via elemental analysis with SEM-EDX. Gold is a dense material; therefore it is seen as bright spots in SEM imaging (Figure 16-B). Elemental analysis via EDX demonstrated that these bright agglomerates correspond to gold (Figure 16-C). Gold was absent in PBS-exposed tissues (Figure 16-A).

E. Discussion

Independent characterization of purchased GNPs demonstrated that, in general, physicochemical characteristics were in accordance with the information provided by the

producer. The shape was roughly spherical and the average size of the gold core was 13.09 nm, close to the 15 nm declared by the manufacturer. The lack of agglomeration and PEG coating are also concordant with the described high GNP stability and biocompatibility, as this coating decreases detection and phagocytosis by the mononuclear phagocyte system and is considered one of the most promising nanoparticle coatings to ensure high biocompatibility.[78,83] The relatively high variability detected in gold core size was somehow expected, as industrial production of nanomaterials is likely to aim for size ranges more than for a strict size, as soon as the product maintains its functionality. Hydrodynamic size, however, showed little variability, with an average of 42.64 nm and a PDI of 0.154. The average diameter measured by TEM is always smaller than the one measured by DLS, as TEM considers only the gold core size, while DLS considers the core, the coating, and any layer of solvent adhered to the particles.[159] Regarding the differences in dispersity, it is possible that the manufacturer's synthesis technique yields relatively polydispersed gold cores, but after PEGylation they might have applied a size-exclusion technique to ensure the final overall size of the GNPs is relatively monodispersed. This hypothesis is supported by the zeta potential results. The zeta potential deviation is rather wide, meaning that the individual zeta potential value for each particle in the GNP suspension varies significantly. This is consistent with high variability of gold:PEG ratio across the particles. Gold nanoparticles prior to PEGylation are generally negatively charged, due to the use of citrate as stabilizing agent during synthesis, which is negatively charged.[160] Polyethylene glycol, in contrast, is generally neutral.[161] Coating of GNPs with PEG has an insulating effect that lowers the zeta

potential magnitude. Therefore, if the gold:PEG ratio varies across the particles, it could give rise to corresponding variations in zeta potential.

Regarding the animal studies, our results indicate that there are similarities, but also important differences in response to GNP exposure between mice and rats. Although neither had obvious clinical deficits, mice had a superior ability to recognize the particles and mount an inflammatory response. This is supported by the formation of liver microgranulomas and the transient increase in IL-18 in mice, indicating a robust macrophage response. Cho et al.[26] exposed mice to a significantly lower dose (0.85 mg/kg IV) of 13 nm PEGylated GNPs, and reported a transient increase (1 and 7 days post-exposure) in indicators of oxidative stress and liver damage, even though they did not find treatment-related histological abnormalities. Interestingly, another study by the same group[7] reported acute toxicity in mice after IV exposure to 13 nm PEG-coated GNPs (0.85 and 4.26 mg/kg), with acute liver inflammation and apoptosis. No histological abnormalities were found by another group[94] after exposing CD1 mice to up to 700 mg/kg IV of GNPs with similar characteristics to the ones used in the present study and from the same provider, but 1.9 nm in size. This difference in size may be responsible for the differences in response, since the 1.9 nm particles were readily excreted in urine,[94] while the 15 nm GNPs used in the present study were very poorly eliminated. No studies were found that investigated the effects of IV exposure to PEGylated GNPs in rats, or that included histological examination of tissues after exposure to any GNP by any route. Khan et al.[138] evaluated changes in the expression of inflammatory cytokines in rat liver and kidney after single or repeated IP exposure to 10 and 50 nm citrate-coated GNPs (0.022 mg/kg daily for 1 or 5 days). They found a

significant increase in the expression of proinflammatory cytokines in both organs after a single GNP-exposure, but normal expression after repeated exposures. On the other hand, a study by Siddiqi et al.[74] demonstrated generation of oxidative stress and impairment of antioxidant enzymes in rat brain after repeated IP exposure to 20 nm citrate-coated GNPs (0.02 mg/kg daily for 3 days).

The observed change in color of GNP-exposed tissues can be explained by the accumulation and agglomeration of particles. Gold nanoparticle solutions are usually red, but this color shifts to blue/purple in response to close proximity of the particles,[82] i.e., agglomeration. This particularity can be used to track GNPs within tissues and to determine their agglomeration state. In our study, immediately after the GNP injection a red coloration was observed in blood and subsequently (1 day post-injection) in most tissues, which is consistent with the presence of non-agglomerated GNPs.[79] The subsequent change in color to dark purple by day 7 post-exposure is consistent with GNP destabilization and agglomeration within the tissues.[79] This was further confirmed by the observation of GNP agglomerates within tissues via SEM (Figure 16). The integrity of the PEG coating after cellular uptake of the GNPs and its role in the intracellular destabilization and agglomeration of particles remains to be determined.

Differences in biodistribution and excretion were also observed when comparing mice to rats. Rats showed higher GNP accumulation in spleen than mice, and higher gold concentration was measured in rat brain 1 and 7 days post-exposure as compared to mouse brain. This is consistent with an observation made by Khlebtsov and Dykman[106] when comparing the studies by De Jong et al.[29] and Sonavane et al.,[132] which investigated GNP biodistribution in rats and mice, respectively. They drew attention to the fact that

the proportion of GNPs accumulated in rat spleen[29] was notably higher than in mouse spleen.[132] They also observed that relative blood levels of GNPs 1 day post-exposure were higher in rats[29] than in mice.[132] We believe this explains the higher gold concentration measured in rat brain at earlier time-points in our study, when compared to mouse brain. Blood within tissue samples is known to contribute to the nanoparticle content of the tissue.[64] Since no attempts were made to remove the blood from tissues before gold measurements, and since the overall concentration of gold in brain was very low, it is possible that higher blood concentrations of gold in rats influenced the measurements in brain. This hypothesis is supported by the fact that GNPs were largely removed from the blood stream by 14 days post-exposure (Figure 5), which is coincident with the time gold concentration in rat brain decreased to similar levels with mouse brain (Figure 9). Regarding excretion, we detected very low concentration of GNPs in urine and slightly higher concentration in feces, with greater fecal excretion in rats compared to mice. Our results are in agreement with a previously published report by Cho et al.,[26] in which excretion of 13 nm PEGylated GNPs in mouse urine and bile was detected up to 7 days post-IV exposure to 0.85 mg/kg. Balasubramanian et al.[13] demonstrated urinary and fecal excretion of 20 nm citrate-coated GNPs only up to 7 days post-IV exposure in rats, at a dose of 0.01 mg/kg. The difference with our results could be due to the significantly higher dose we administered, as well as the potential influence of the coating agent in GNP fecal excretion.

Our results demonstrate that the main cell types that internalize and accumulate GNPs are primarily tissue macrophages and secondarily vascular endothelial cells, but GNPs were also visualized within other cell types. This includes spindle/mesenchymal

cells, hepatocytes, and cells of the alveolar interstitium. These results are in agreement with a study by Dragoni et al.,[52] in which they demonstrated GNP uptake by rat Kupffer cells, endothelial cells, and hepatocytes after IP exposure to 10 mg/kg of polyvinylpymolidone (PVP)-coated 5 nm GNPs. Contrarily, other studies[7,21,26] found PEG-coated GNP accumulation exclusively in mouse Kupffer cells and macrophages, using IV doses up to 5 mg/kg. The lower doses used in the latter studies are probably the reason of the restricted internalization of GNPs by cell types other than macrophages. One possibility is that the amount of GNPs internalized by these other cell types was too low and therefore it was not detected. A second option is that with the high dose used in our study, uptake by tissue macrophages was saturated and therefore uptake by other cell types was granted. Interestingly, the decreased amount of GNPs distributed throughout tissue parenchyma over time indicates redistribution of the GNPs from non-phagocytic cells to phagocytic cells.

Finally, the sudden deaths observed in GNP-exposed rats could be indicating a species-specific sensitivity to GNP acute toxicity. It has been stated that nanoparticle aggregation following systemic administration can lead to capillary occlusion and subsequent organ damage.[83] However, no publications supported this hypothesis, and the cause of death of the rats could not be determined. Further research is needed in order to elucidate the significance of this finding.

F. Conclusions

Our findings demonstrate that commercially available PEGylated GNPs accumulate in phagocytic cells of all tissues examined and that they are not innocuous,

having the potential to generate inflammatory and immune responses. Additionally, important differences between mice and rats were established regarding biodistribution, excretion, and immune reaction to GNP exposure. However, considering that the use of GNPs in biomedicine carries the potential for a tremendous impact in the medical field, and that the effects reported here were very mild in spite of the high dose administered, PEGylated GNPs can still be considered highly biocompatible nanomaterials.

G. Funding

This research was supported by the Institute of Critical Technology and Applied Science (ICTAS) at Virginia Tech and the Edward Via College of Osteopathic Medicine.

H. Acknowledgments

The authors thank Betsey Midkiff, Marlice Vonck, Jenn Rudd, Barbara Wheeler, Kathy Lowe, and Geraldine Magnin-Bissel for their technical assistance, Ana María Ortega for her statistical assistance, and Stephen McCartney from the ICTAS Nanoscale Characterization and Fabrication Laboratory for his assistance with SEM-EDX.

I. Tables

Table 1. Summary statistics of GNP size distribution by TEM

Mean	13.09 nm
Standard Error	0.46 nm
Median	11.48 nm
Mode	4.03 nm
Standard Deviation	7.89 nm
Sample Variance	62.22 nm^2
Kurtosis	0.12
Skewness	0.81
Range	39.28 nm
Minimum	1.96 nm
Maximum	41.24 nm
Sum	3928.03 nm
Count	300.00
Confidence Level (95.0%)	0.90

Table 2. Summary data and statistics of DLS analysis of GNPs

Sample	Z_{ave} (nm)	PDI	Number Mean (nm)	Volume Mean (nm)	Intensity Mean (nm)
1	42.64	0.157	26.53	34.44	50.51
2	42.49	0.157	25.69	33.41	50.49
3	42.78	0.148	26.47	34.59	50.24
Mean	42.64	0.154	26.23	34.15	50.41
Std Dev	0.145	0.005	0.4686	0.6424	0.1504
RSD %	0.34	3.37	1.79	1.88	0.298

Z_{ave}, average particle hydrodynamic diameter; PDI, polydispersity index; Std Dev, standard deviation; RSD, relative standard deviation.

Table 3. Summary data and statistics of GNP zeta potential

Sample	Conductivity (mS/cm)	Mobility (µmcm/V*s)	Mobility Deviation (µmcm/V*s)	Zeta Potential (mV)	Zeta Deviation (mV)
1	0.00786	-2.866	1.953	-36.6	24.9
2	0.00276	-2.766	1.897	-35.3	24.2
3	0.016	-2.465	1.198	-31.4	15.3
Mean	0.012	-2.699	1.683	-34.43	21.47
Std Dev	0.005	0.21	0.42	2.71	5.35
RSD %	45.3	7.73	25.00	7.86	24.93

Std Dev, standard deviation; RSD, relative standard deviation.

J. Figures

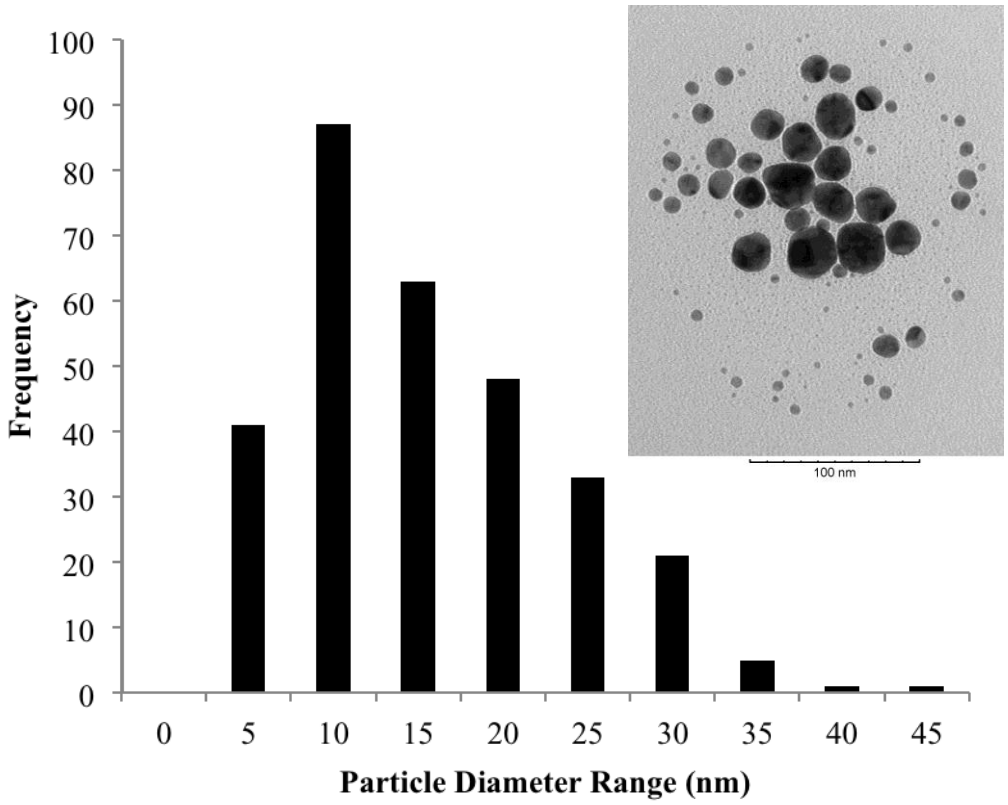

Figure 1. Gold nanoparticle size distribution histogram. Size distribution of the GNP's gold core was determined by analysis of TEM micrographs with Image-J software. The mean diameter was 13.09 ± 7.89 nm (standard deviation; n = 300). The inset depicts a representative TEM micrograph.

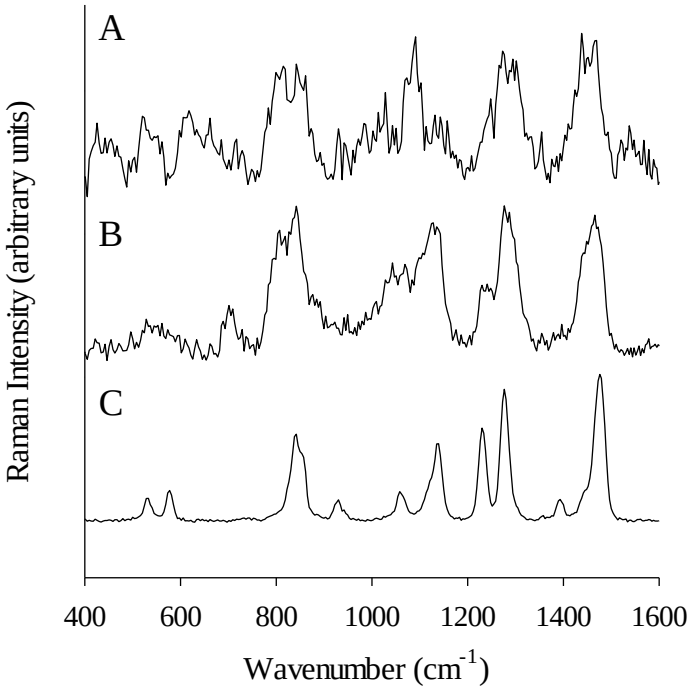

Figure 2. Raman spectra of GNPs and PEG. Raman spectra of (A) purchased GNPs, (B) PEG-GNPs, and (C) PEG. Spectra are normalized and offset for clarity.

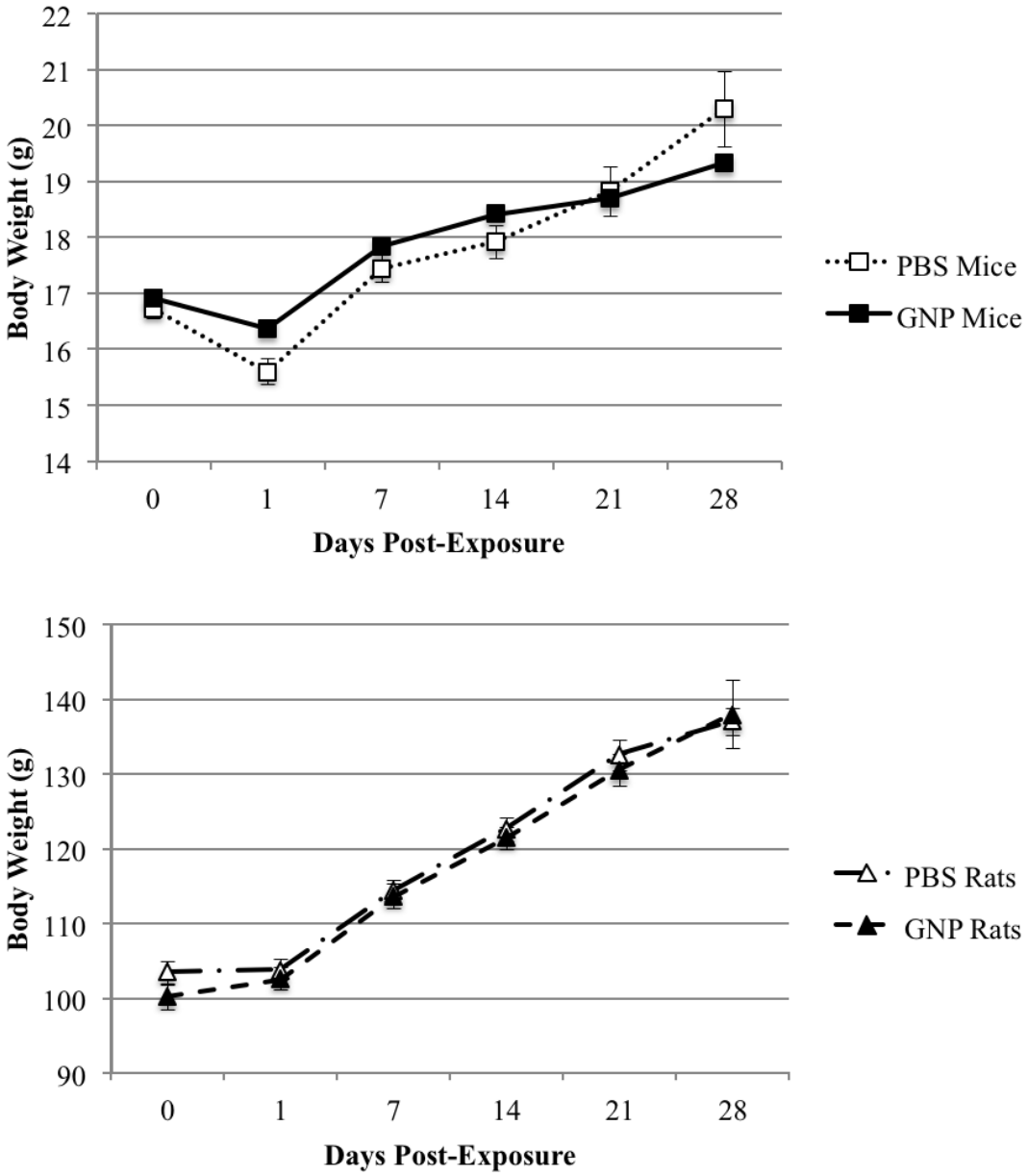

Figure 3. Body weight. Mouse and rat body weight before and after GNP or PBS exposure. Data are mean ± standard error of the mean (n = 5-27 for mice and 3-20 for rats).

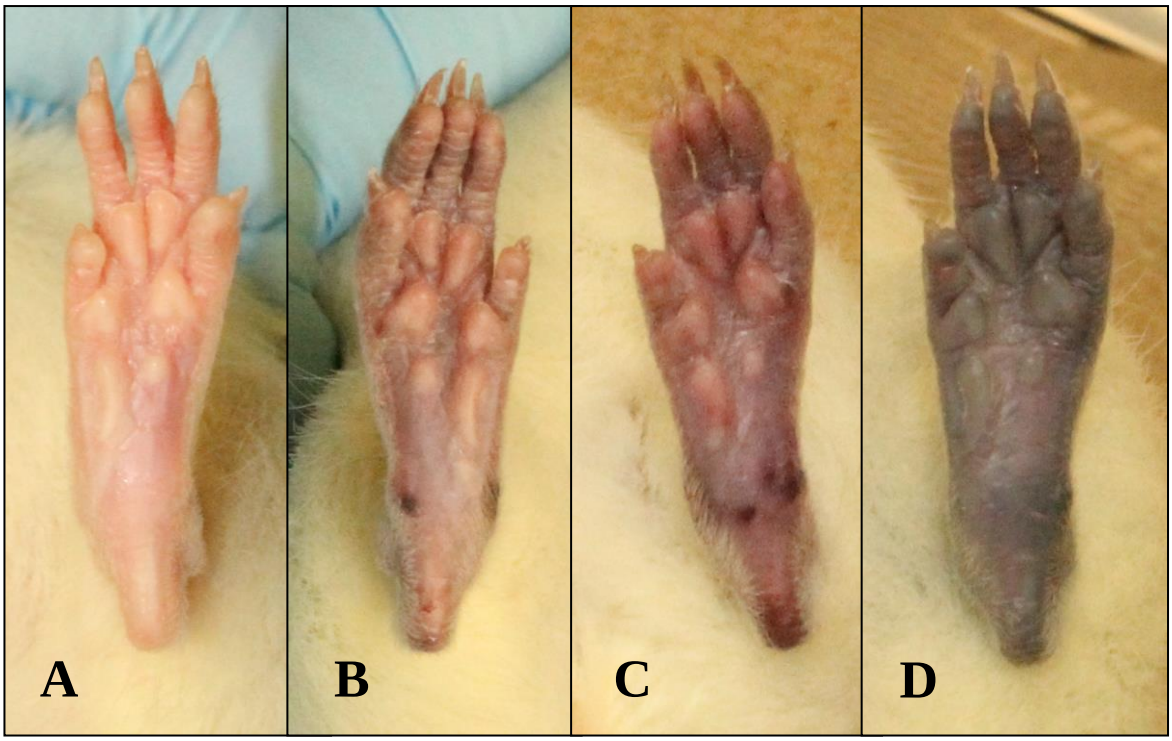

Figure 4. Color change post-GNP exposure. (A) PBS-exposed rat, (B) GNP-exposed rat immediately after the injection, (C) GNP-exposed rat 1 day post-injection, and (D) GNP-exposed rat 28 days post-injection.

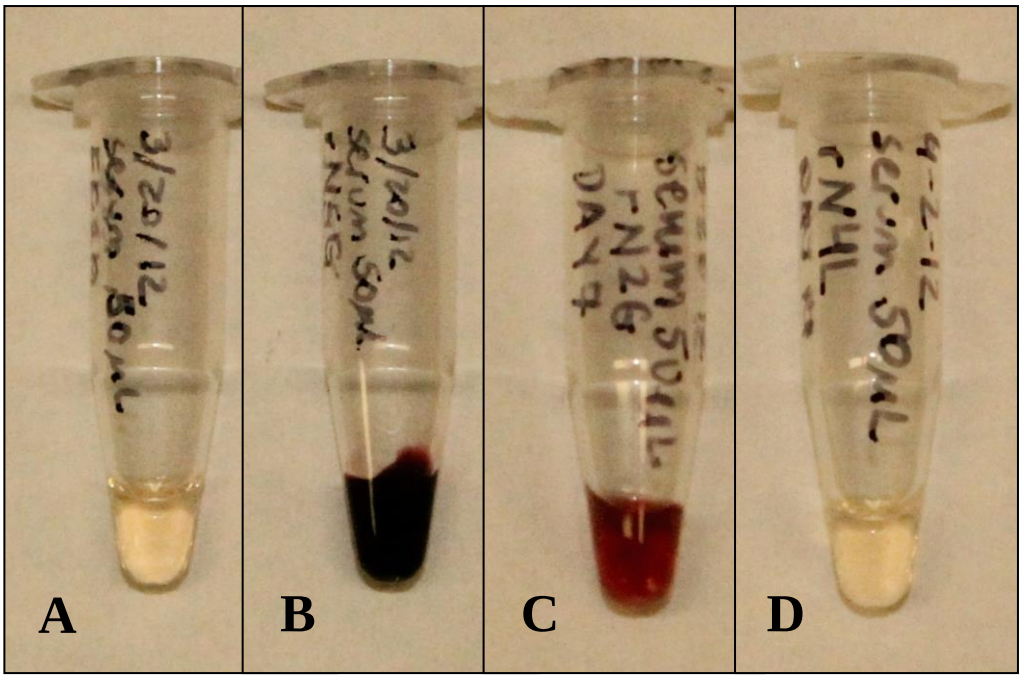

Figure 5. Gold nanoparticles in serum. Serum of (A) PBS-exposed rat, (B) GNP-exposed rat 1 day post-injection, (C) GNP-exposed rat 7 days post-injection, and (D) GNP-exposed rat 14 days post-injection.

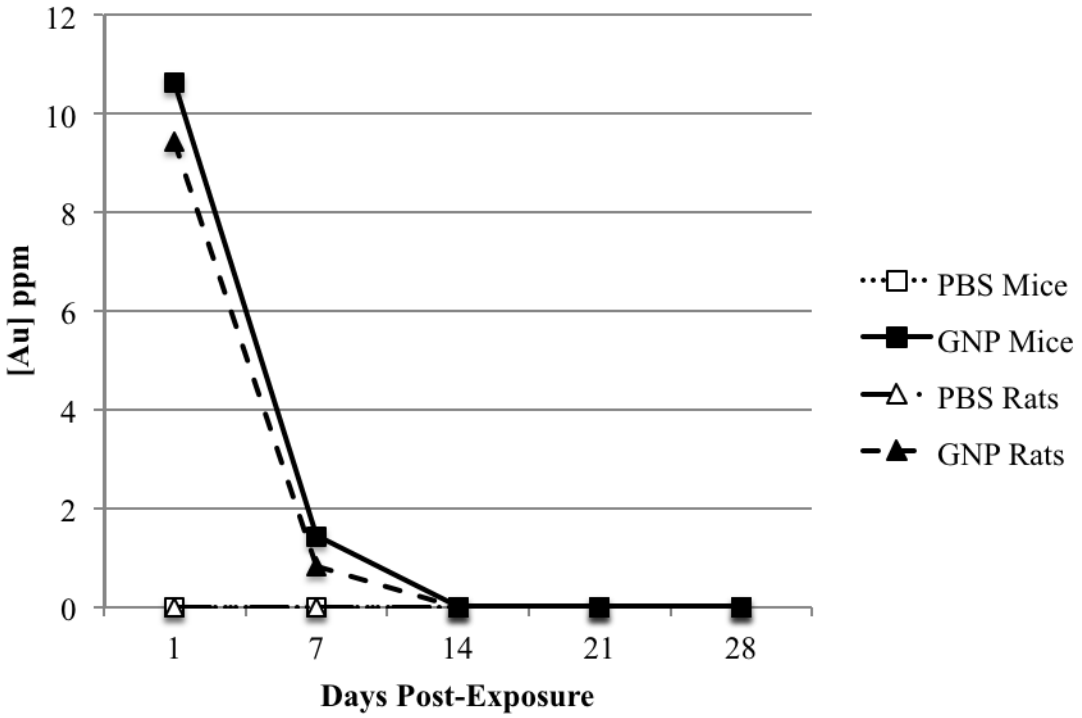

Figure 6. Gold concentration in urine. Gold concentration in urine of mice and rats exposed to PBS or GNPs was measured via atomic absorption spectrometry (n = 3 for mice and 2 for rats 1 day post exposure, and n = 1 for all other time points).

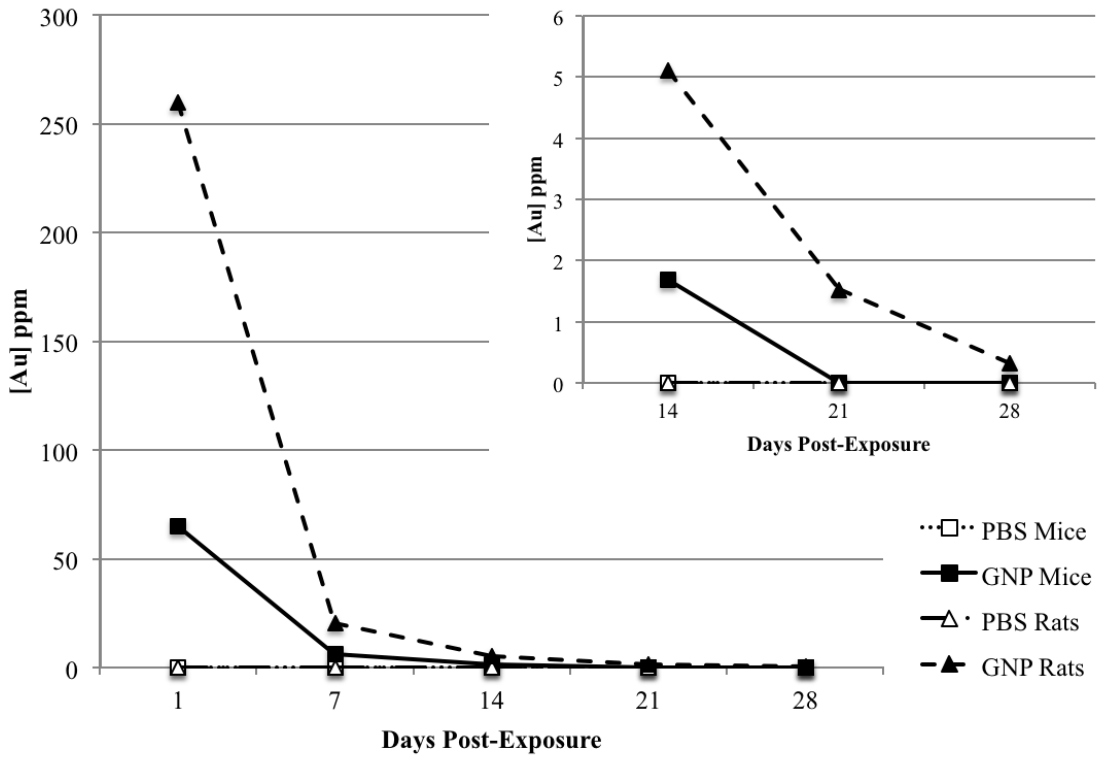

Figure 7. Gold concentration in feces. Gold concentration in feces of mice and rats exposed to PBS or GNPs was measured via atomic absorption spectrometry. The inset depicts concentrations of gold in feces between 14 to 28 days post-exposure, which are indistinguishable in the main graph (n = 3 for mice and rats 1 day post exposure, and n = 1 for all other time points).

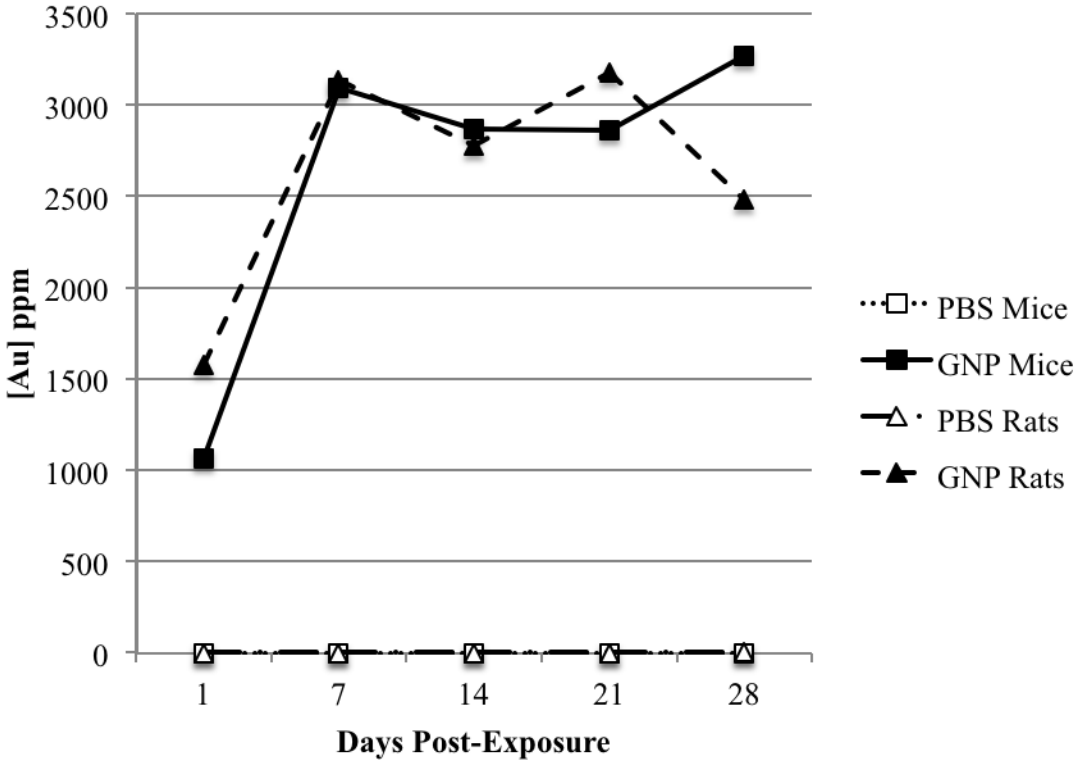

Figure 8. Gold concentration in liver. Gold concentration in liver of mice and rats exposed to PBS or GNPs was measured via atomic absorption spectrometry (n = 1).

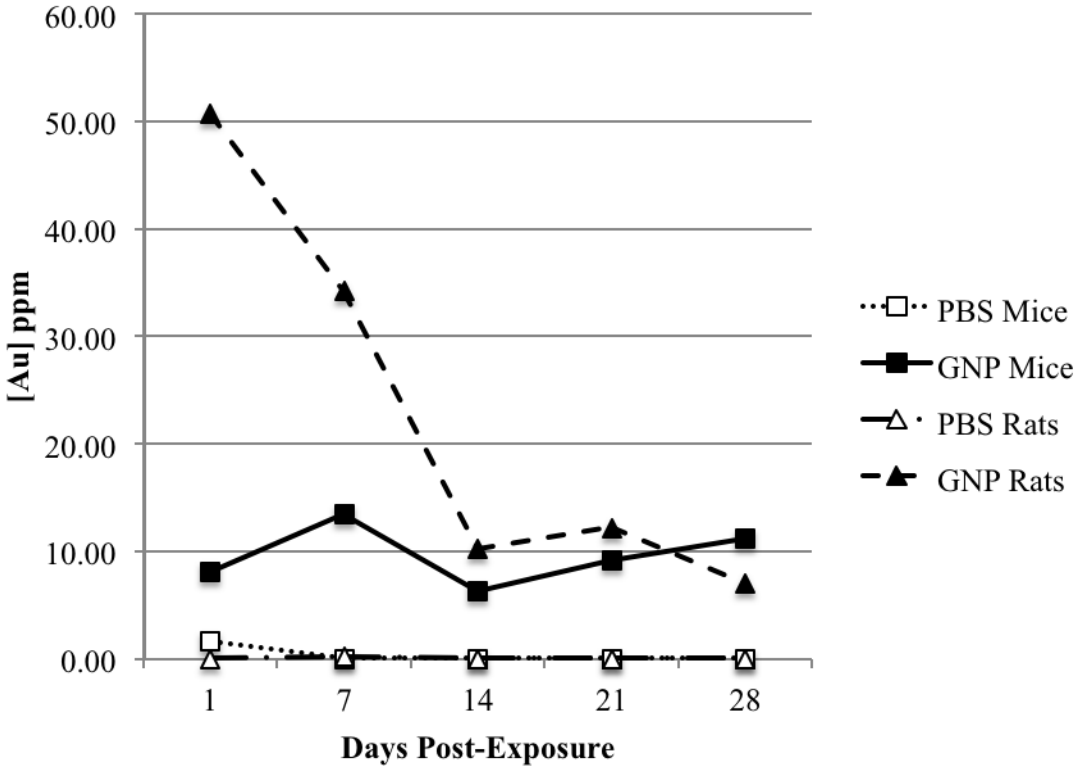

Figure 9. Gold concentration in brain. Gold concentration in brain of mice and rats exposed to PBS or GNPs was measured via atomic absorption spectrometry (n = 1).

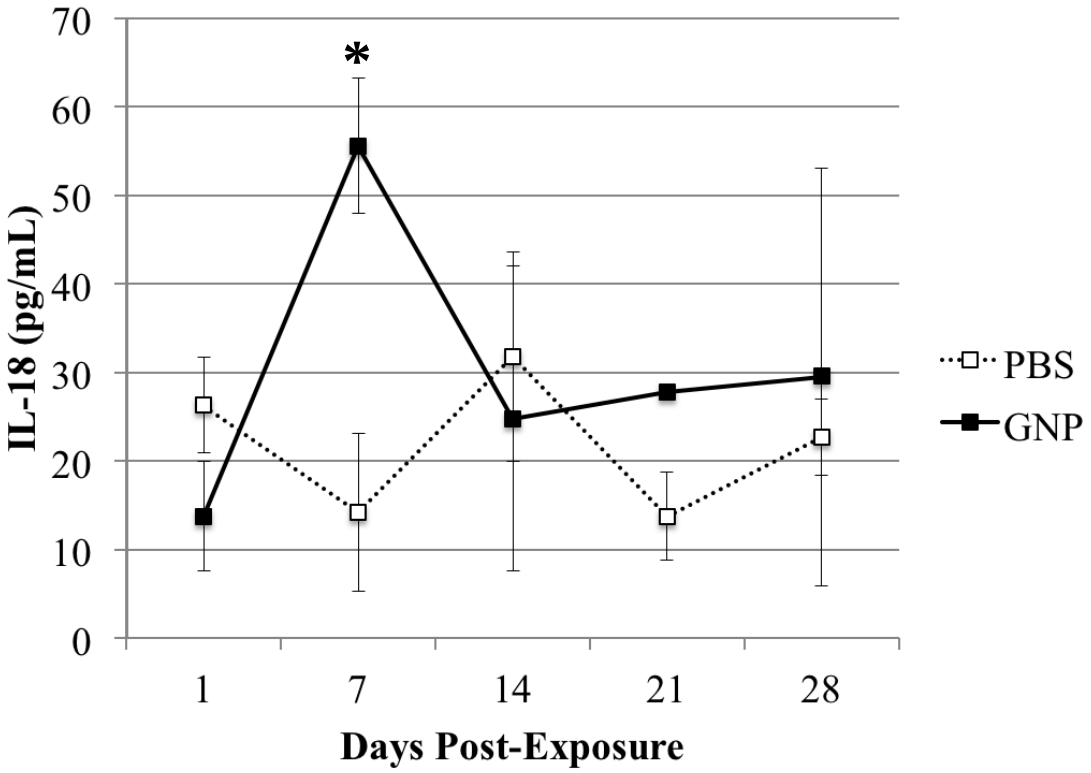

Figure 10. Mouse serum interleukin-18. Serum levels of IL-18 in PBS- and GNP-exposed mice were measured via ELISA. Data are mean ± standard error of the mean. * $p < 0.05$ when comparing GNP and PBS groups at equivalent time points ($n = 4$).

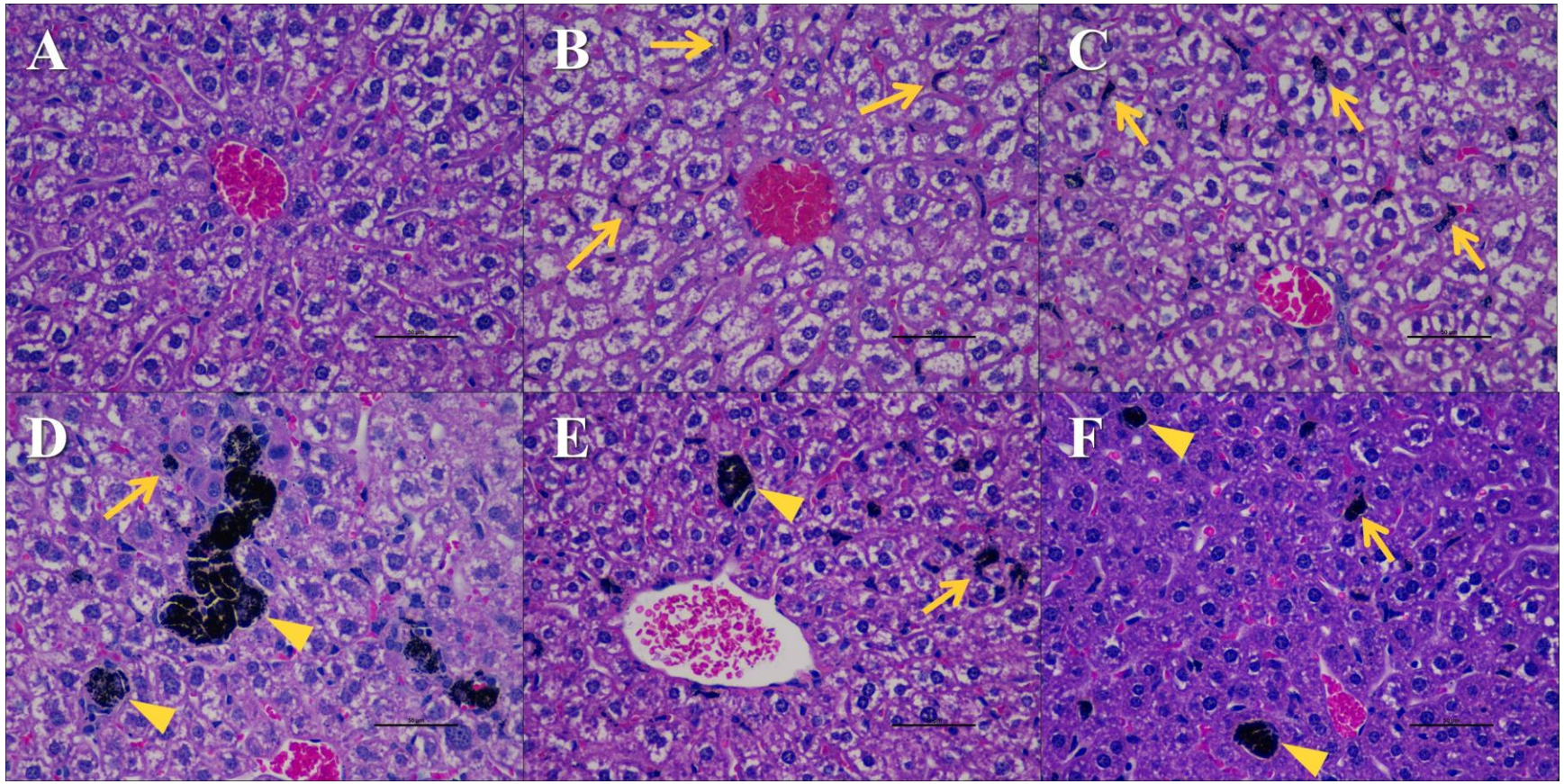

Figure 11. Gold nanoparticles in mouse liver. Representative images of mouse liver of (A) PBS-exposed and GNP-exposed animals (B) 1 day, (C) 7 days, (D) 14 days, (E) 21 days, and (F) 28 days post-exposure. Note GNP-containing cells (arrows), as well as microgranulomas with multinucleated giant cells (arrowheads). Hematoxylin and eosin stain, 40x magnification, scale bars = 50 μm.

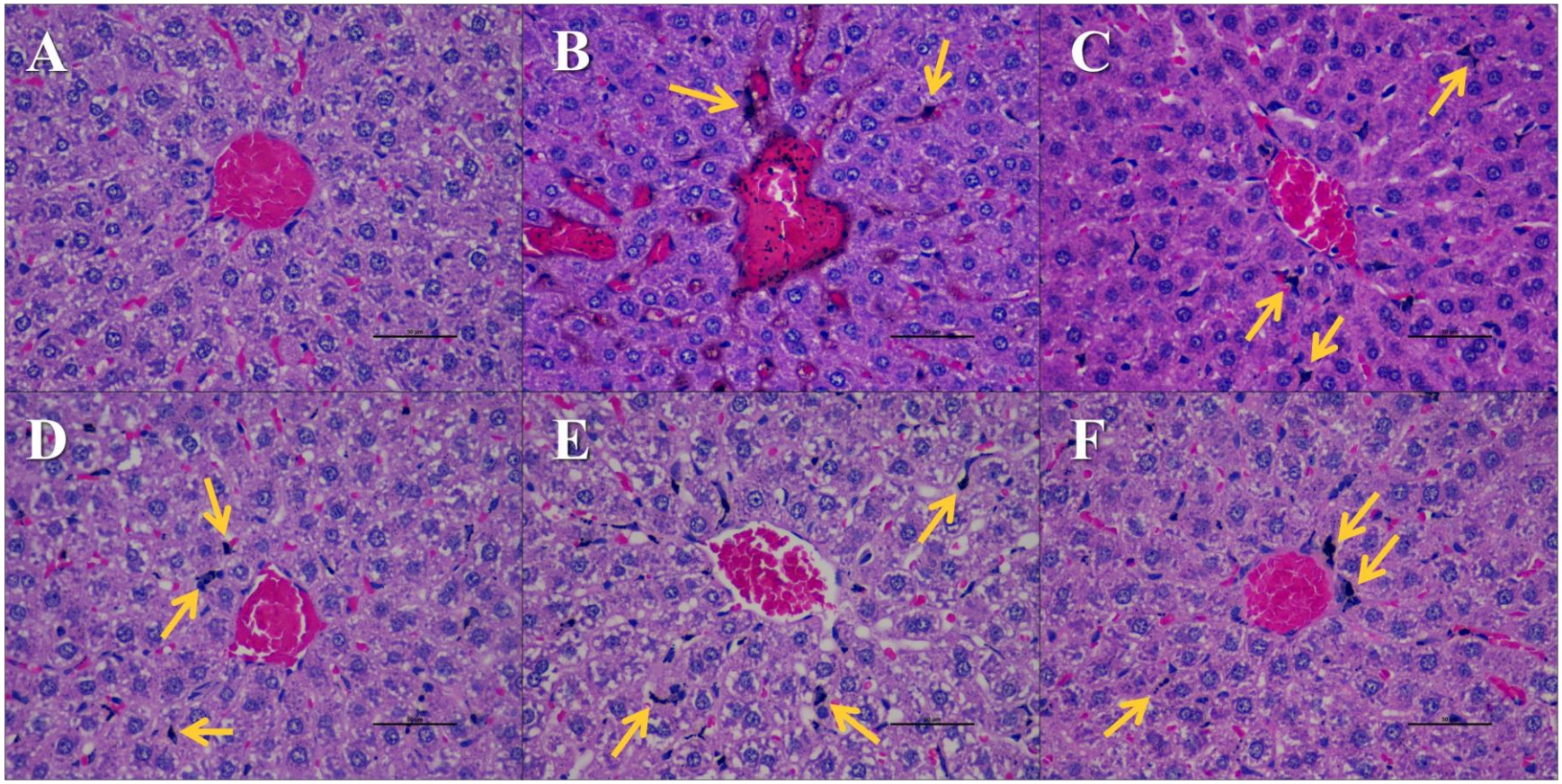

Figure 12. Gold nanoparticles in rat liver. Representative images of rat liver of (A) PBS-exposed and GNP-exposed animals (B) 1 day, (C) 7 days, (D) 14 days, (E) 21 days, and (F) 28 days post-exposure. Note GNPs within blood vessels 1 day post-GNP exposure (B) and GNP-containing cells (arrows). Hematoxylin and eosin stain, 40x magnification, scale bars = 50 μm.

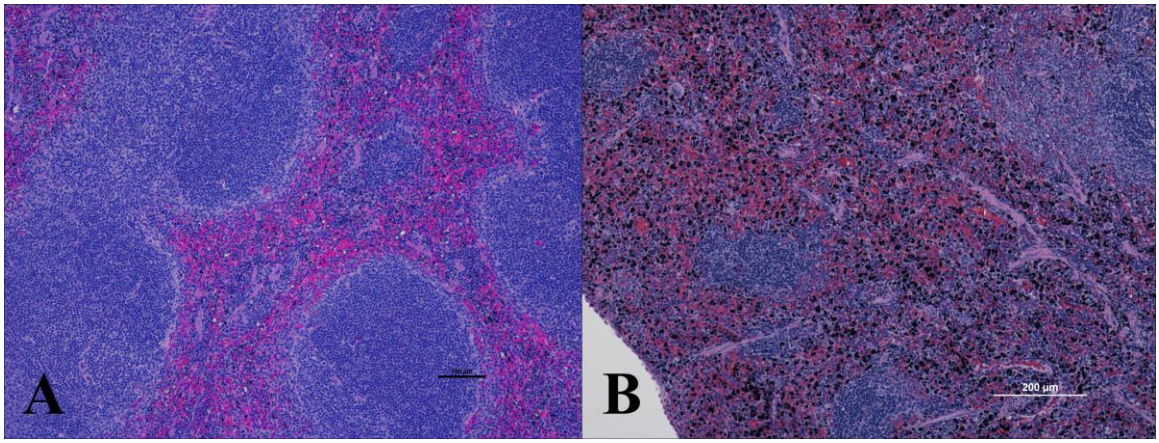

Figure 13. Gold nanoparticles in spleen. Representative images of (A) mouse spleen and (B) rat spleen 7 days post-GNP exposure. Note the higher number of GNP-containing cells in rat spleen versus mouse spleen. Hematoxylin and eosin stain, 10x magnification, scale bars = 100 μm (A) or 200 μm (B).

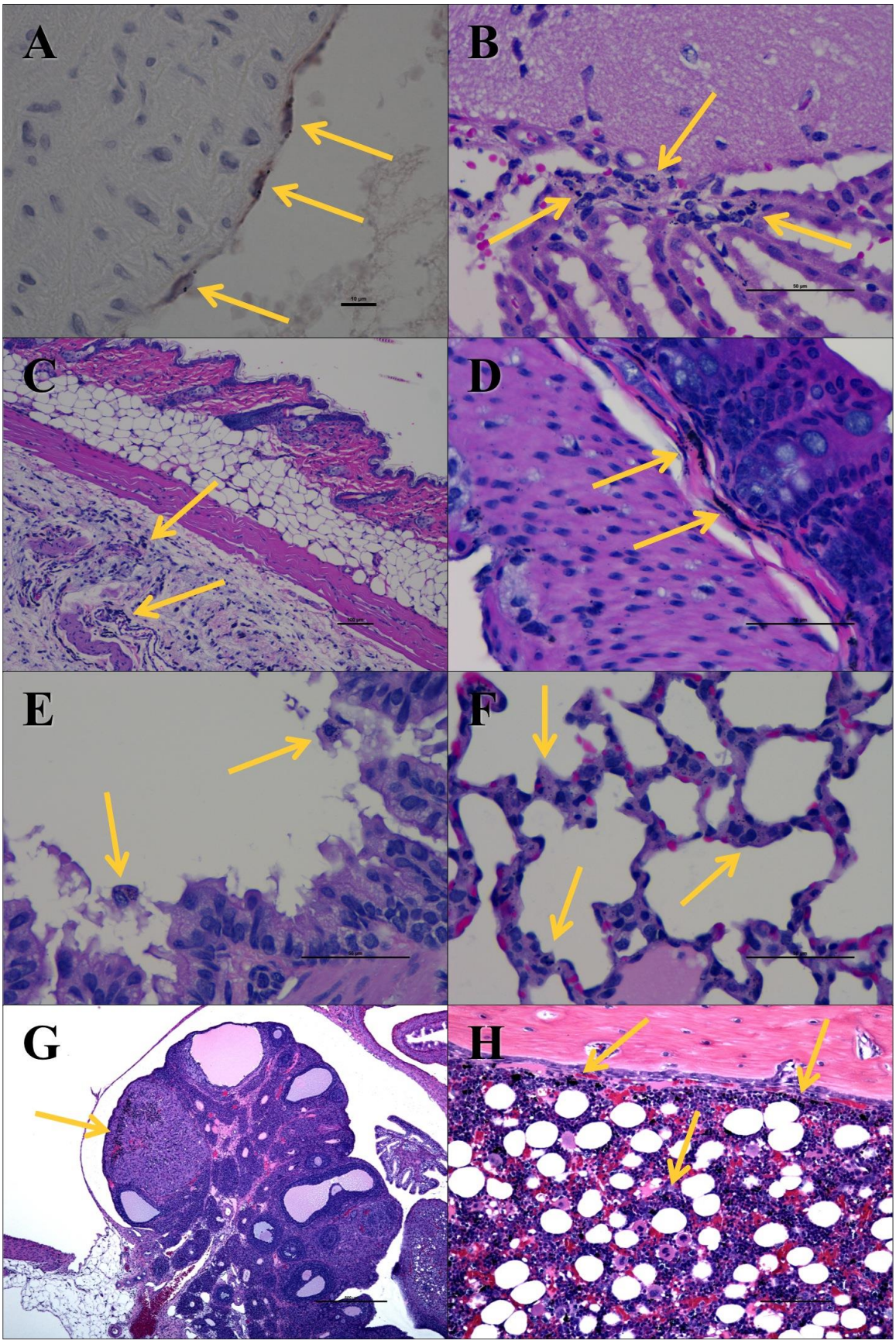
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Figure 14. Gold nanoparticles in various tissues. Representative images of tissue accumulation of GNPs. (A) Rat aorta 21 days post-GNP exposure. Note GNPs within endothelial cells (arrows). Immunohistochemical stain (von Willebrand Factor), 100x magnification, scale bar = 10 μm. (B) Mouse brain 28 days post-GNP exposure. Note GNPs within cells of the choroid plexus (arrows). Hematoxylin and eosin stain, 60x magnification, scale bar = 50 μm. (C) Mouse skin 21 days post-GNP exposure. Note mild granulomatous (histiocytic) panniculitis with GNP-containing macrophages (arrows). Hematoxylin and eosin stain, 10x magnification, scale bar = 100 μm. (D) Mouse cecum 21 days post-GNP exposure. Note GNPs within spindle/mesenchymal cells of the submucosa (arrows). Hematoxylin and eosin stain, 60x magnification, scale bar = 50 μm. (E) and (F) Rat lung 14 days post-GNP exposure. Note GNPs within bronchiolar macrophages (E, arrows) and throughout the alveolar interstitium (F, arrows). Hematoxylin and eosin stain, 60x magnification, scale bar = 50 μm. (G) Rat ovary 7 days post-GNP exposure. Note accumulation of GNPs within degenerating corpora lutea (arrow). Hematoxylin and eosin stain, 4x magnification, scale bar = 500 μm. (H) Rat bone marrow (right femur) 28 days post-GNP exposure. Note GNPs within macrophages of the bone marrow (arrows). Hematoxylin and eosin stain, 20x magnification, scale bar = 100 μm.

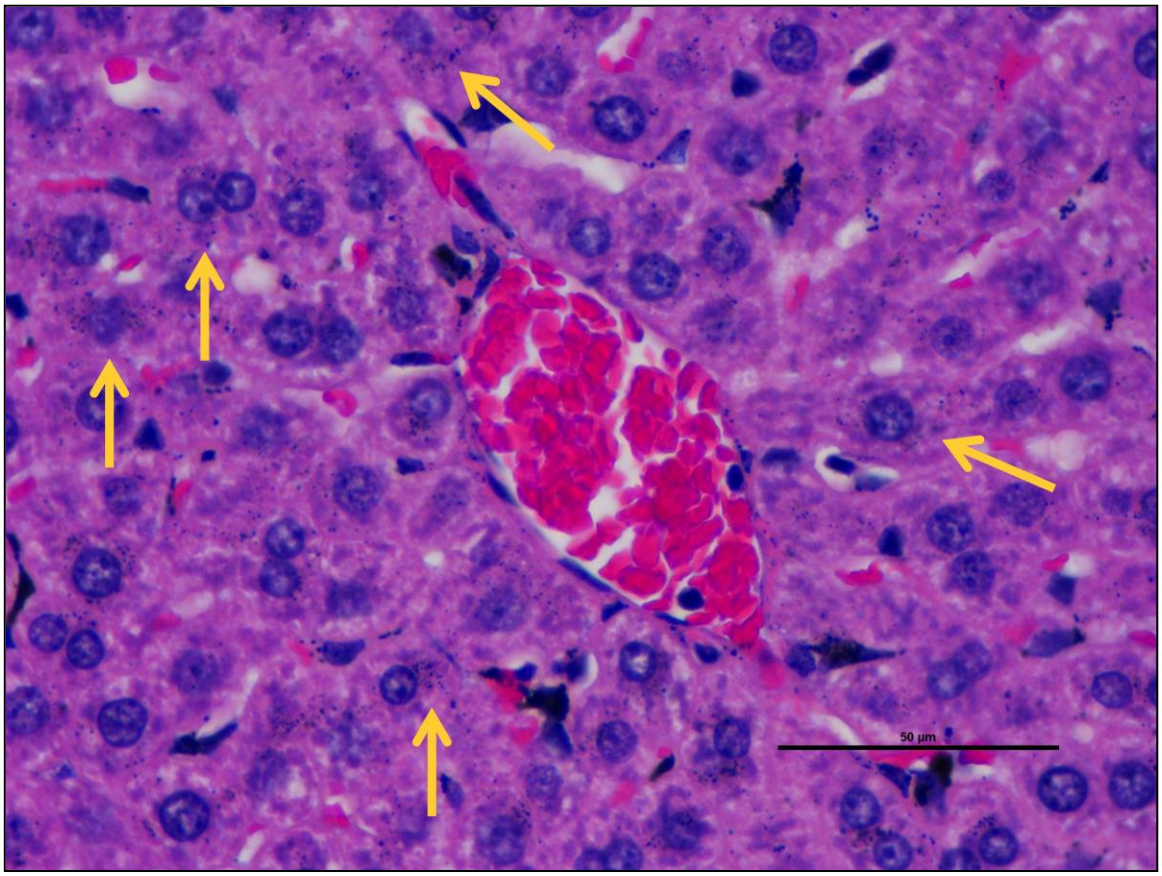

Figure 15. Gold nanoparticles in liver parenchyma. Higher magnification of rat liver 7 days post-GNP exposure. Note GNP-containing hepatocytes (arrows). Hematoxylin and eosin stain, 60x magnification, scale bar = 50 µm.

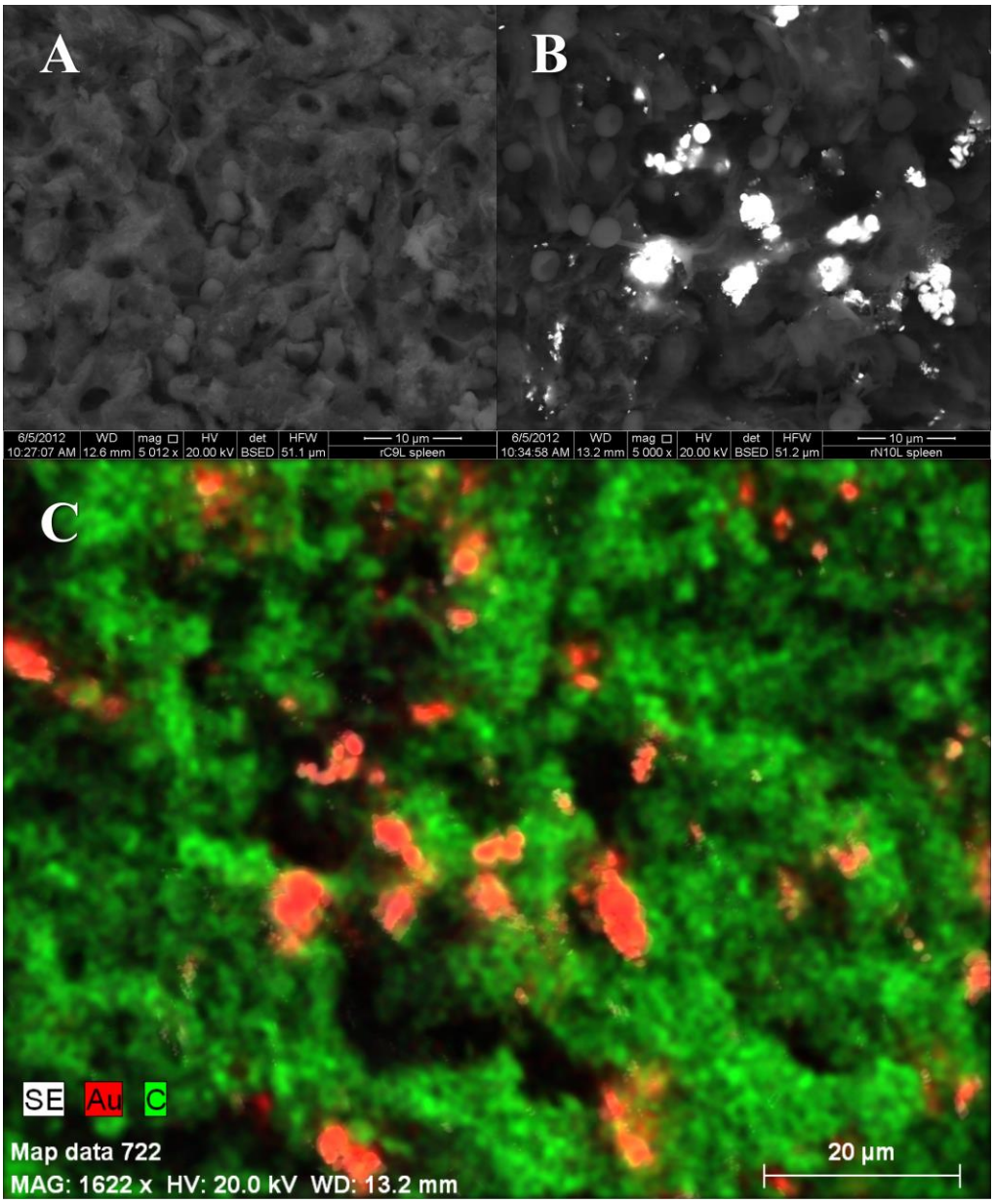

Figure 16. Elemental analysis of rat spleen. Representative SEM micrographs of rat spleen 28 days post- (A) PBS or (B) GNP exposure. Note agglomerates of GNPs, seen as bright spots in GNP-exposed spleen (B). 5000x magnification, scale bars = 10 μm. (C) Representative illustration of SEM-EDX elemental analysis of rat spleen 28 days post GNP-exposure. Gold is shown in red and carbon is shown in green, for reference. 1622x magnification, scale bar = 20 μm.

CHAPTER IV: Chronic Inflammation in Mice Following Single Administration of Gold Nanoparticles

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Running Title: Chronic Inflammation by Gold Nanoparticles

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Keywords: gold nanoparticles; nanotoxicity; chronic inflammation; microgranulomas; mice

Abbreviations: ANOVA, analysis of variance; BBB, blood-brain barrier; BTB, blood-testis barrier; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbent assay; GNPs, gold nanoparticles; H&E, hematoxylin and eosin; IL-18, interleukin-18; IV, intravenous; LD_{50}, lethal dose 50; PBS, phosphate buffered saline; PEG, polyethylene glycol; PEGylated, PEG-coated; ROS, reactive oxygen species; SEM, standard error of the mean; TEM, transmission electron microscopy; VMRCVM, Virginia-Maryland Regional College of Veterinary Medicine.

Declaration of Conflicts of Interest: The authors declare no potential conflicts of interest.

Declaration of Financial Disclosure: This work was funded by the Institute of Critical Technology and Applied Science (ICTAS) at Virginia Tech and the Edward Via College of Osteopathic Medicine (VCOM).

A. Abstract

Gold nanoparticles (GNPs) are currently being intensely investigated for their potential use in biomedical applications. Upon intentional exposure, they accumulate in liver and spleen with extremely poor clearance from the organism. The objective of this study was to identify and characterize the long-term effects of a single intravenous (IV) exposure to commercially available GNPs in mice. Gold nanoparticles were purchased and independently characterized. Male and female mice were injected with either 1000 mg/kg GNPs or an equivalent volume of phosphate buffered saline (PBS) in the tail vein, and euthanized 20 weeks post-exposure. Exposure to GNPs incited chronic inflammation, characterized by the formation of microgranulomas in liver, spleen, and lymph nodes, as well as increased serum levels of the proinflammatory cytokine interleukin-18. Impairment of body weight gain in the long-term was also observed in the GNP-exposed group. No sex differences were noted. Considering the mildness of the toxic effects identified in the long-term, despite the high dose selected for the study, GNPs continue to have great potential for biomedical uses. However, further studies are needed in order to determine specific mechanisms of toxicity and the role of chronic inflammation in the development of adverse effects after co- or post-exposures.

B. Introduction

Materials in the nanoscale (1-100 nm) do not have the same properties as larger particles of the same chemical composition. Nanomaterials have enormous potential for utilization in diverse areas, including pharmacology and medicine.[2,5,7,10] Unfortunately, the great nanotechnology advances accomplished during the last decade have not been accompanied by an equivalent extent of research on the potential harmful effects of nanomaterials.[13,16] Currently, the largest obstacle for the progress of biomedical nanotechnology to widespread use is the lack of knowledge on nanotoxicity.[71,73]

Gold nanoparticles (GNPs) are among the favorite candidates for biomedical applications due to their superior stability and biocompatibility, as well as their easy synthesis, manipulation, and conjugation with a variety of chemicals and biomolecules. This allows the creation of numerous specialized GNPs for applications in the medical field, including targeted drug delivery, advanced imaging, and cancer diagnosis and treatment.[75,76] These possibilities are being intensely investigated, with at least three clinical trials involving GNPs currently in progress and high hopes of prompt advancement into clinical practice.[83,100] Conversely, not much is known about the potential for toxicity of GNPs. They are generally considered biocompatible, but very few nanotoxicity studies are available to draw comprehensive conclusions, and most of these studies assess *in vitro* acute exposures.[81,89,106] Currently available information on GNP biodistribution and toxicity is very scarce and inconsistent, especially for chronic *in vivo* studies. After *in vivo* exposure, GNPs are known to be bio-persistent and have extremely poor clearance, accumulating primarily (and indefinitely) in cells of the

mononuclear phagocyte system.[8,26] Thus, it is of utmost importance to assess the long-term effects of GNPs in complex organisms, even after a single exposure event.

On top of the general lack of toxicity studies, there is great variability in experimental design among different groups, which leads to discrepancies in their results and conclusions.[89,106] Most research groups synthesize their own GNPs and many do not provide detailed information about nanoparticle characterization and experimental design, thus hindering reproducibility and comparison among studies, as well as the elaboration of general nanotoxicity conclusions. Physicochemical characteristics of nanomaterials can vary with changes in the production method, among batches of the same provider, and even with time, during storage.[47,48] These variations are expected in nanomaterial industry, as the manufacturing process will probably aim to produce particles with physicochemical characteristics within a certain range that assures functionality while maintaining profitability.[34] This may or may not have an impact in GNP toxicity profile, but it is very unlikely that each end-user will synthetize their own GNPs. Therefore, the future use of these particles in biomedicine implies intentional exposure to significant doses of industrially produced GNPs as intravenous (IV) injections.

These considerations prompted us to carry out the present study, which purpose was to identify and characterize the chronic effects after a single IV exposure to commercially available GNPs in male and female C57BL/6 mice.

C. Materials and Methods

1) Gold nanoparticles

Gold Nanoparticles of nominative 15 nm in size were purchased from Nanoprobes (AuroVist™-15 nm, Yaphank, NY) and independently characterized. These GNPs were chosen for the study because they are approved for *in vivo* use and were described as highly biocompatible. The producer reports an LD_{50} (lethal dose 50, the dose that would kill 50% of the exposed animals) higher than 5000 mg/kg, low osmolality, and viscosity similar to water, even at high concentrations.[147] Toxicity of nanomaterials appears intimately related with the material's physicochemical features,[23,28,32] hence it is of utmost importance to independently characterize the GNPs.[47] In order to determine the shape and size distribution of the gold-core, samples were analyzed by transmission electron microscopy (TEM) using a Philips EM420 Transmission Electron Microscope (Philips, Somerset, NJ) operating at 120 kV. The obtained micrographs were subsequently analyzed using Image-J (National Institutes of Health, Bethesda, MD). Gold nanoparticle hydrodynamic diameter and agglomeration state were determined via dynamic light scattering (DLS). Electrophoretic mobility was performed to determine surface charge. A Zetasizer NanoZS (Malvern Instruments, Westborough, MA) particle analyzer was used for all DLS measurements. The method of cumulants was employed to determine the average hydrodynamic diameter of the particles and the Smoluchowski approximation was used to elucidate the zeta potential of the particles. Raman spectroscopy was used to compare GNPs of known coating properties with the purchased GNPs, in order to identify the coating agent of the latter. Briefly, the method described by Turkevich et al.[155] was used to prepare citrate-coated GNPs. Thiolated polyethylene

glycol (PEG, 5000 Da) was purchased (Nanocs, New York, NY) and used to prepare a 1 mM PEG solution. Polyethylene glycol-coated GNPs (PEG-GNPs) were prepared by mixing 30 μL of PEG solution with 100 μL of citrate-coated GNP suspension. For all Raman measurements, a WITec Alpha500R Raman Spectrometer (WITec, Knoxville, TN) with a 10x microscope objective, 300 gr/mm grating, and 785-nm laser was used. Two μL samples of PEG, PEG-GNPs, and the purchased GNPs were applied to glass slides, covered with aluminum foil, and allowed to dry in a fume hood. Raman spectra were measured from dried samples with a 5-mW laser power, 1-s integration time, and 10 accumulations. In order to remove the fluorescent background signal from the collected spectra, the graph background subtraction tool of the WITec Project Software was used.

2) Animals and exposure

All experiments were approved by the Institutional Animal Care and Use Committee and the Environmental Health and Safety Services at Virginia Tech. Female and male C57BL/6 mice (offspring of mice obtained from Harlan, Dublin, VA) 6 weeks old (6 males and 6 females) were maintained at 22 ± 1ºC, 40-60% humidity, and 12:12-hour light:dark cycle and with free access to standard rodent diet (Harlan Teklad Global Diet 2018, Madison, WI) and water. For the experiment, animals were randomly assigned to GNP or PBS groups (3 males and 3 females per group). The GNP group received 1000 mg/kg GNPs as a single injection in the tail vein (volume injected was approximately 0.1 mL per mouse). The PBS group was used as a control group, and received an equivalent volume (0.1 mL) of phosphate buffered saline (PBS), the GNP vehicle. All animals were sedated with diazepam (5 mg/kg intraperitoneal) prior to GNP or PBS injection and were

closely monitored until fully recuperated. Behavior was observed daily by veterinarians (subjective evaluation of posture, locomotion, awareness of surroundings, reaction to stimulus, and stress indicators such as barbering and diarrhea) and body weight was measured and recorded weekly until animals reached 6 months of age (i.e., 20 weeks post-exposure). At that time, all mice were euthanized via CO_2 asphyxiation, necropsied, and samples were collected.

3) Blood glucose

Non-fasted blood glucose levels were measured as an indicator of health and stress. Blood was drawn via puncture of the lateral saphenous vein and glucose levels were measured using a hand-held glucometer (Accu-Check Compact, Roche Diagnostics, Indianapolis, IN).

4) Interleukin-18

Potential mechanisms of GNP toxicity are oxidative stress and inflammation.[20,24,128] Tissue macrophages actively internalize and accumulate GNPs[4,26,52] and there is extremely poor clearance, so evaluation of macrophage activation and chronic inflammation are of high relevance when characterizing GNP toxicity. Interleukin-18 (IL-18, also known as interferon-gamma inducing factor) is a proinflammatory cytokine produced almost exclusively by antigen presenting cells, including macrophages. It is involved in chronic inflammation, autoimmune diseases, cancer, and infectious diseases, contributing to cell-mediated immunity and inflammation.[153] An enzyme-linked immunosorbent assay (ELISA) kit was used to

determine mouse serum levels of IL-18 (Medical & Biological Laboratories Co., Ltd., Woburn, MA). Blood was collected via puncture of the lateral saphenous vein, allowed to clot for 30 minutes to 1 hour at room temperature, centrifuged at 2000 rpm for 20 minutes, and then the serum was collected and analyzed following the manufacturer's instructions.

5) Histopathology and immunohistochemistry

After euthanasia, each animal was subjected to macroscopic examination and tissue samples were collected and stored in 10% neutral buffered formalin (heart, aorta, trachea, thymus, lungs, liver, spleen, pancreas, kidneys, uterus, ovaries, testis, abdominal fat, thoracic and abdominal lymph nodes, skin, right hind limb, and head). For microscopic morphologic analysis by light microscopy, tissues were trimmed and submitted to the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) Histology Laboratory for routine histologic processing (samples are embedded in paraffin wax, sectioned [4 μm thick], and stained with hematoxylin-eosin [H&E] and special stains [Oil-Red O, periodic-acid-Schiff, Masson's trichrome, Prussian blue, bile, and Fontana-Masson for visualization of lipids, glycogen, fibrosis, iron hemosiderin, bile, and melanin respectively]). Prior to submission to the Histology Laboratory, tissue samples containing bone were placed in decalcifying solution (Enhanced Decalcification Formulation, StatLab Medical Products, McKinney, TX) for 24 to 48 hours.

Paraffin-embedded tissues were submitted to the Connecticut Veterinary Medical Diagnostic Laboratory for immunohistochemical staining with von Willebrand Factor

Antibody (Dako, Carpinteria, CA), a marker of endothelial cells. Stained slides were assessed via light microscopy in order to determine GNP accumulation in endothelial cells as well as alterations in cell morphology.

6) Transmission electron microscopy

In order to assess GNP intracellular location and cellular alterations, samples of mice liver were fixed in a mixture of 5% glutaraldehyde, 4.4% formaldehyde, and 2.75% picric acid in 0.005 M sodium cacodylate, and submitted to the VMRCVM Morphology Laboratory for routine TEM processing (samples are post-fixed with osmium tetroxide, dehydrated, infiltrated with propylene oxide and Poly/Bed 812, embedded in molds, oven-cured, thick-sectioned [1 μm thick], observed under light microscope to select area of interest, and thin-sectioned [60-90 nm thick]). Processed samples were examined and pictures taken using a Zeiss 10CA Transmission Electron Microscope equipped with AMT Advantage GR/HR-B CCD Camera System.

7) Atomic absorption spectrometry

For quantitative assessment of GNP tissue accumulation, samples of liver and brain fixed in 10% neutral buffered formalin were submitted to the VMRCVM Toxicology Laboratory. Tissues were homogenized, digested with nitric acid/perchloric acid, and analyzed with a Varian SpectrAA 220FS Atomic Absorption Spectrometer for measurement of gold content (Gold Ultra Hollow Cathode lamp, 242.8 nm wavelength, 1.0 nm slit width, background correction on).

8) Statistical analysis

The program JMP® Pro 10.0.2 (SAS Institute Inc., Cary, NC) was used to analyze the results and identify differences between groups via one-way analysis of variance (ANOVA), student's t-test, and Tukey HSD test. Data are reported as mean ± standard error of the mean (SEM) except when indicated, with *n* denoting the sample size. Results were considered statistically significant when $p < 0.05$.

D. Results

1) Characterization of GNPs

The results of GNP characterization are detailed and discussed in Chapter III. Briefly, the mean diameter of the GNP's gold core was 13.09 ± 7.89 nm (standard deviation, $n = 300$), as determined by TEM. No agglomeration was observed. The average particle hydrodynamic diameter determined via DLS analysis was 42.64 ± 0.145 nm (standard deviation). The polydispersity index was 0.154 ± 0.005 (standard deviation). The mean zeta potential of the GNPs was -34.43 ± 2.71 mV (standard deviation) and the mean zeta deviation was -21.47 ± 5.35 mV (standard deviation). The coating of the purchased GNPs was PEG, as determined by Raman spectroscopy.

2) Toxicological profile and bioaccumulation

Gold nanoparticle exposure was associated with a change in color of the skin and internal tissues, as shown in Figure 1. This color change was evident immediately after the GNP injection and persisted until the end of the study (20 weeks post-exposure). No

significant differences in body weight were detected when considering males and females separately (Figure 2). However, a consistent reduction in weight gain was detected in the GNP group as compared to the PBS group when males and females were combined, with significant differences starting 10 weeks post-exposure (Figure 3). Twenty weeks post-exposure, serum levels of IL-18 were significantly higher in GNP-exposed animals when compared to PBS-exposed counterparts (Figure 4). Gold was detected in liver and brain of GNP-exposed mice, with high concentration in liver and very low concentration in brain (Figure 5). No abnormal behavior or differences in blood glucose levels were detected in any of the animals, and no treatment-related differences between males and females were detected for any of the parameters studied.

3) Histological examination

Gold nanoparticles accumulated and persisted in all tissues examined, especially in liver and spleen. Individualized or clustered GNP-laden Kupffer cells and macrophages were observed in liver (Figure 6) and spleen (Figure 7), respectively. In liver, these Kupffer cells were located around blood vessels and scattered in the parenchyma. In spleen, GNP-filled macrophages were mostly restricted to the red pulp. Occasional multinucleated giant cells were present within the clusters, forming microgranulomas in liver, spleen, and to a lesser extent in lymph nodes.

On microscopic evaluation of blood-tissue barriers, gold nanoparticles could be distinguished within cells that conform the blood-brain barrier (BBB, Figure 8-A) and the blood-testis barrier (BTB, Figure 8-B), but they were not visualized within the brain parenchyma or seminiferous tubules, respectively.

Although less than in liver and spleen, cells containing various amounts of GNPs were observed in all tissues examined, which explains the color change after GNP-exposure. Most GNP-containing cells were macrophages/phagocytic cells, but trace to small amounts of GNPs could also be distinguished within vascular endothelial cells and spindle/mesenchymal cells of the supporting connective tissue.

4) Transmission electron microscopy

Microscopic examination of liver revealed that GNPs are located within cytoplasmic vesicles of Kupffer cells (Figure 9). No particles were observed free in the cytoplasm or inside cell organelles such as mitochondria or nucleus.

E. Discussion

It is important to mention that the objective of this study was to identify and characterize the long-term effects of GNPs, as opposed to identifying dose-response relationships. It is well known that humans are usually more vulnerable to toxic effects than experimental animals, and exposure of the latter to high doses of toxic compounds is considered "a necessary and valid method of discovering possible hazards in humans".[148] A high dose was selected for this study in order to increase the probability of detecting long-term effects of GNP exposure from a limited number of animals, although it may exceed the potential level of human exposure. The dose used, however, is considered appropriate by federal agencies for chronic toxicity studies in rodents.[152] AuroVist™-15 nm are defined as highly biocompatible, and the dose selected is considerably lower than the LD_{50}. Also, previous studies exposing mice to almost three-fold higher concentrations

of GNPs from the same company reported no clinical signs of illness.[94,101] These considerations need to be taken into account especially when extrapolating from high dose to low dose and across species, and results and conclusions of this study should be analyzed within their experimental context.

Our results demonstrate that single IV exposure to a high dose of commercially available GNPs causes chronic mild inflammation, characterized by the formation of microgranulomas in blood filtration organs (including liver, spleen, and lymph nodes), increased serum levels of the proinflammatory cytokine IL-18, and impaired long-term weight gain. These alterations were not accompanied by abnormal behavior or clinical signs of disease at any time during the study. Hainfeld et al.[94,101] exposed tumor-burdened mice to a significantly higher dose (2700 mg/kg IV) of 1.9 nm GNPs purchased from the same provider than the GNPs used in the present study. They reported no clinical signs of illness up to 1 year post-exposure, which is concordant with our findings. Additionally, they did not find alterations in hematology, blood chemistry, or histopathology up to 30 days post-exposure to doses between 7 and 800 mg/kg, but they did not assess these parameters in the long-term. Contrary to our findings, Cho et al.[26] exposed mice to 0.85 mg/kg IV of 13 nm PEG-coated (PEGylated) GNPs, and did not find treatment-related histopathological lesions up to 6 months post-exposure. Interestingly, another report from the same group[7] demonstrated acute toxicity in mice exposed to 0.85 or 4.26 mg/kg of similar GNPs, with hepatic inflammation and increased apoptosis. No long-term evaluation was done for the latter study, with the longer time-point examined being 7 days post-exposure.

In the present study, no sex differences were detected in relation to GNP treatment, which could be due to the small sample size when considering males and females separately. This is in disagreement with a study by Chen et al.[93] in which significant differences between male and female mice were detected 28 days after IP exposure to 4.4 to 36.1 nm PEGylated GNPs (4 mg/kg). These differences, however, were detected in parameters different than the ones considered in our study, specifically hematology and blood chemistry. They did not find GNP-related alterations in body weight or histopathology, as opposed to our findings, although we did not detect significant alterations in body weight until 10 weeks post-exposure. No other studies considering gender differences in GNP toxicity were found.

In the study by Cho et al.,[26] gold was detected in mouse brain, testis, and spinal cord up to 6 months post-IV exposure to 13 nm PEGylated GNPs (0.86 mg/kg). The authors considered this finding as an indicator of penetration of the BBB and BTB, even though the method used to measure gold content in tissues was inductively coupled plasma-mass spectroscopy, which requires tissue digestion previous to the measurement. Several other published reports[7,131,132] demonstrate gold accumulation in brain, and suggest that small GNPs are able to cross the BBB, but all of them utilize methods that require tissue homogenization. In a recent review by Yokel et al.[64] on the interactions of metal-based nanoparticles with the nervous system, no studies were found providing evidence of GNP distribution into the brain parenchyma. In all cases in which gold was detected in brain, it could be attributed to GNPs in blood or associated with cells of the BBB. In our study, GNPs were also present in detectable levels in digested brain long after the exposure, but microscopic evaluation of sections of brain and testis could not

demonstrate the ability of these particles to cross the BBB or the BTB. We believe there is still not sufficient evidence as to conclude that GNPs of any size are able to cross the blood-tissue barriers. It is important to keep in mind that direct access to the brain parenchyma is not a prerequisite for a substance to exert neurotoxic effects, as secondary compounds generated elsewhere or alterations to the BBB itself could be the mechanisms of indirect neurotoxicity.

Finally, it was determined that the observed change in color after GNP administration is persistent, and is explained by accumulation of GNPs within tissues. The long-term intracellular location of GNPs was found to be cytoplasmic vesicles, in agreement with the findings of Cho et al.[26] and Sadauskas et al.[6] No particles were visualized free in cytoplasm or within other organelles such as nucleus or mitochondria. This is relevant in terms of toxicity, as the particles are circumscribed by membranes and have lesser possibilities to interact with intracellular biomolecules such as proteins and DNA. This is probably also important when elucidating the mechanisms of toxicity, as it seems like these GNPs induce inflammation indirectly, by activating macrophages and other antigen-presenting cells. Even if they do not induce acute toxicity, the persistence of the GNPs for long periods of time within phagocytes can translate into constant stimulation of the immune system, therefore inducing the chronic mild inflammation seen in the exposed animals. It is known that inflammation, even if undamaging alone, can lead to adverse effects upon exposure to other toxicants at normally harmless doses.[162] Therefore, specific mechanisms of chronic inflammation as well as long-term co- or post-exposures to GNPs need to be investigated, in order to ensure a safe advancement of this technology to biomedical utilization. Accumulation of GNPs in tumors and subsequent

treatment with irradiation appears of special relevance, since this is one of the proposed uses of these particles for cancer treatment and irradiation of GNPs have been shown to modify their size and increase their cytotoxic potential,[119] as well as generate reactive oxygen species (ROS).[126] Also, accumulation of GNPs in dermis and subcutis and subsequent long-term exposure to UV light should be investigated, as UV light exposure has also been shown to modify nanomaterials and generate ROS.[126,127]

In conclusion, our study demonstrates that single IV exposure to commercially available PEGylated GNPs can lead to delayed toxic effects. Chronic mild inflammation was characterized by the formation of microgranulomas and increased serum levels of the proinflammatory cytokine IL-18 in GNP-exposed mice. Additionally, long-term body weight gain was impaired in the GNP group. These findings are of great significance regarding the future use of these particles in biomedicine. Considering the high dose selected for this study and the very mild toxicity observed, we believe that GNPs continue to have great potential for biomedical uses. Further studies are needed in order to elucidate specific mechanisms of toxicity as well as long-term effects of co- and post-exposures.

F. Acknowledgments

This study was supported by the Institute of Critical Technology and Applied Science at Virginia Tech and the Edward Via College of Osteopathic Medicine. The authors thank Betsey Midkiff, Marlice Vonck, Jenn Rudd, Barbara Wheeler, Kathy Lowe, and Geraldine Magnin-Bissel for their technical assistance, as well as Ana María Ortega for her statistical assistance.

G. Figures

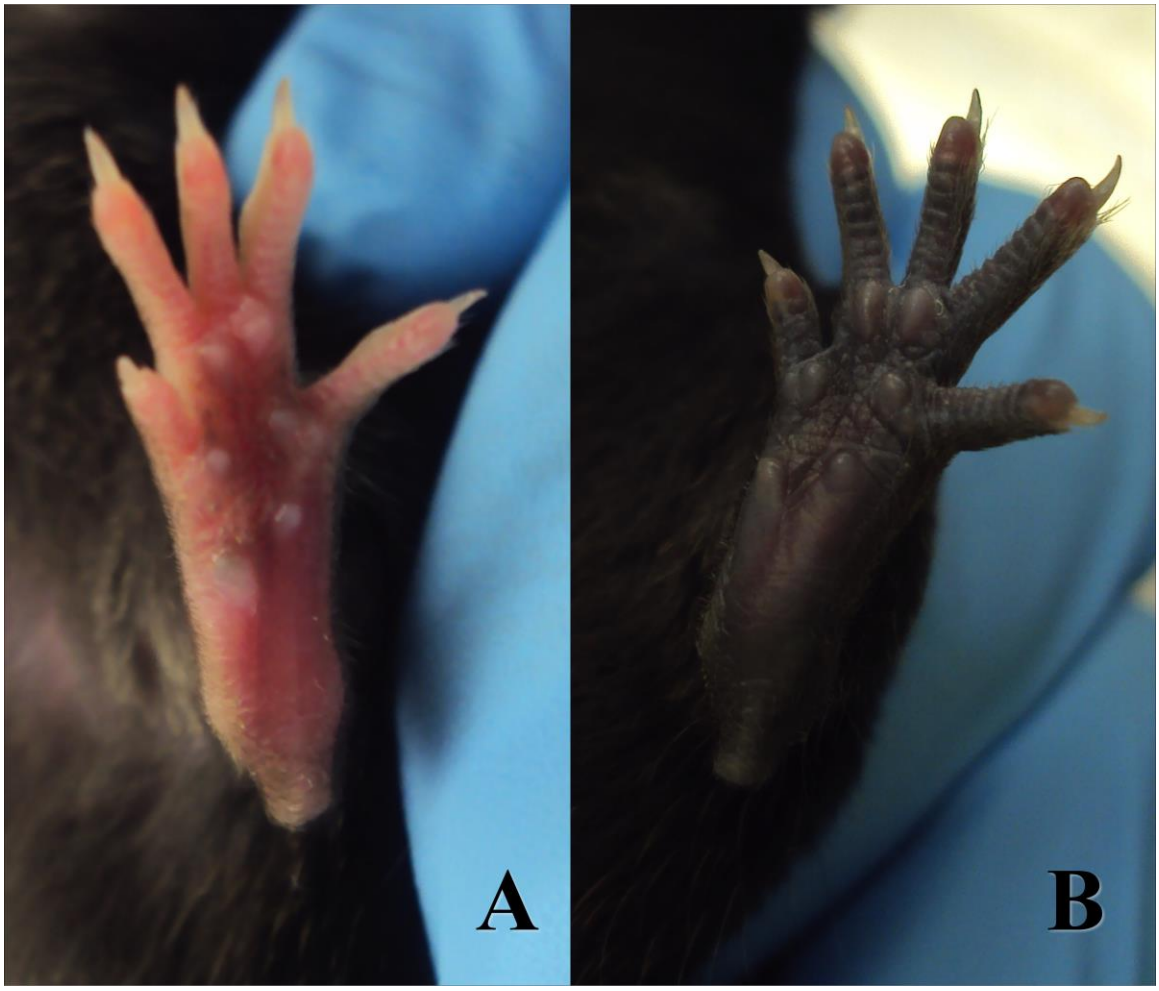

Figure 1. Color change post-GNP exposure. (A) PBS-exposed mouse and (B) GNP-exposed mouse 6 weeks post-exposure. The change in color was evident immediately after the GNP injection and persisted until the end of the study.

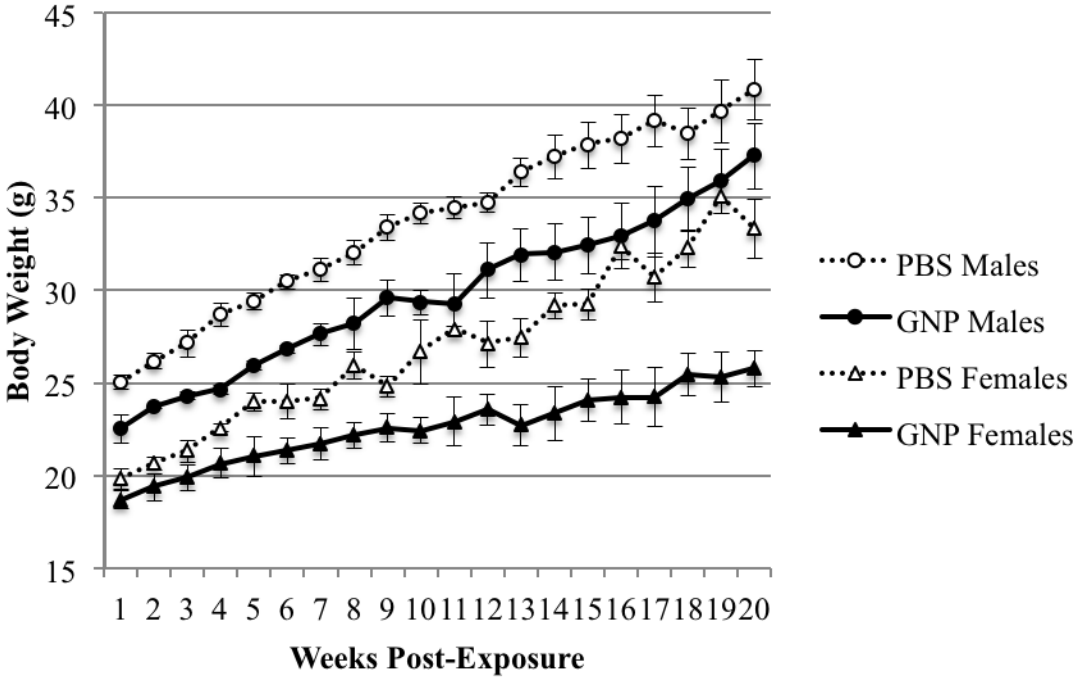

Figure 2. Body weight (separate males and females). Mouse body weight was recorded weekly for males and females after GNP or PBS exposure. Data are mean ± SEM (*n* = 3).

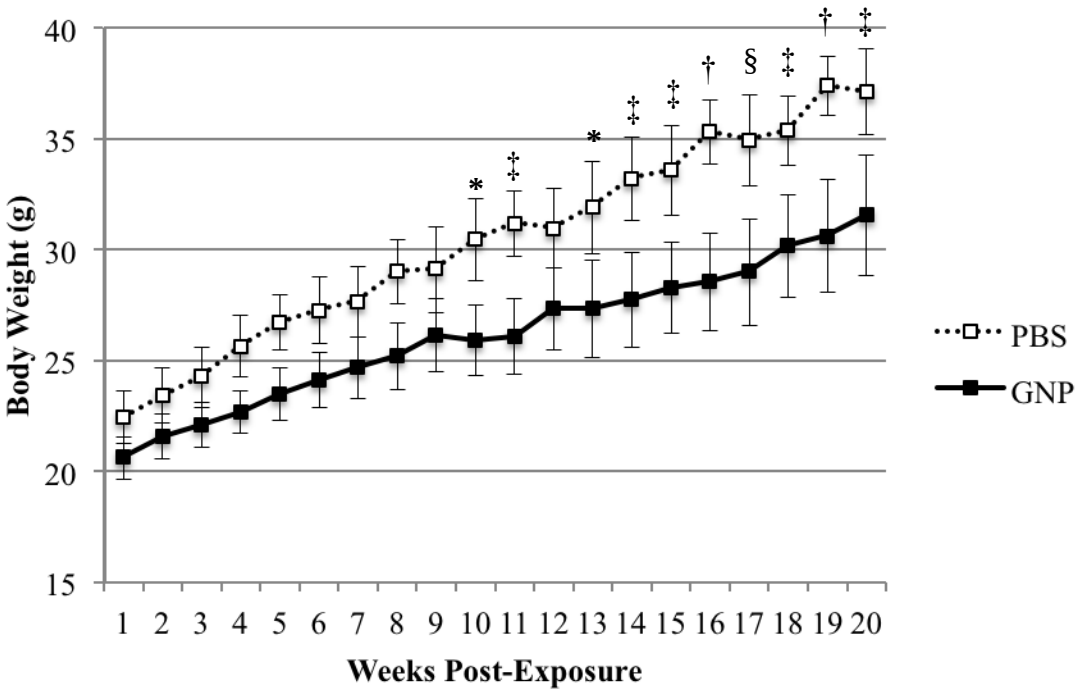

Figure 3. Body weight (males and females combined). Mouse body weight was recorded weekly after GNP or PBS exposure. Data are mean ± SEM (n = 6). Symbols represent significant differences when comparing GNP and PBS groups at equivalent time points: * $p < 0.05$, ‡ $p < 0.01$, § $p < 0.001$, † $p < 0.0001$.

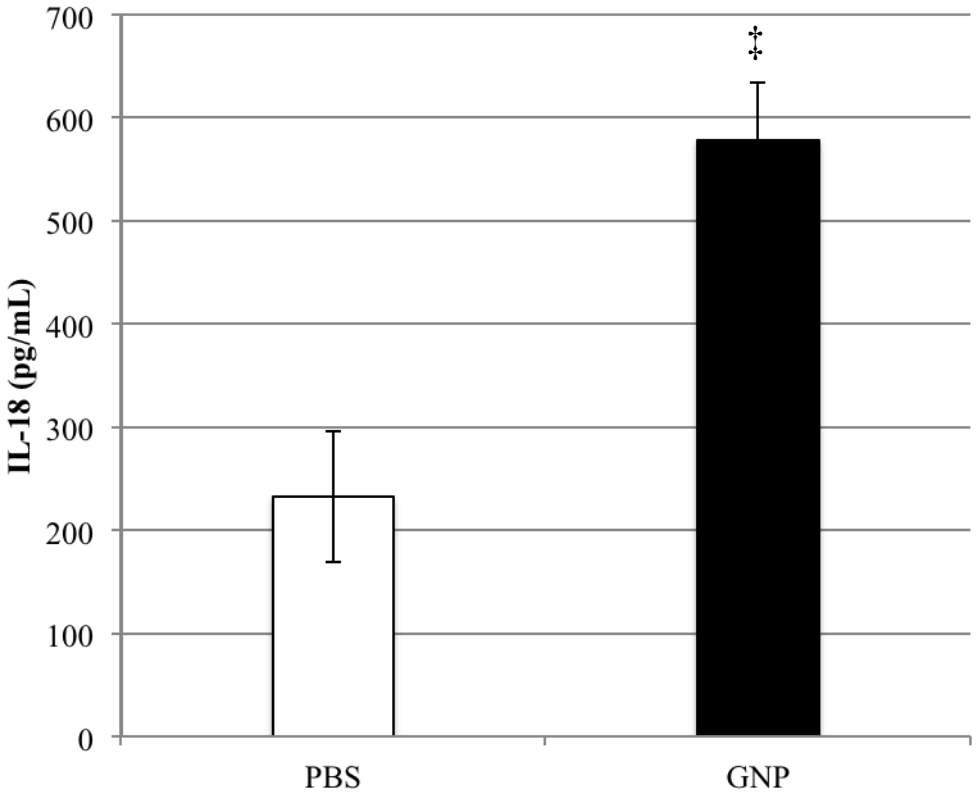

Figure 4. Serum IL-18 20 weeks post-exposure. Mouse serum IL-18 was measured 20 weeks post PBS or GNP exposure. Data are mean ± SEM (n = 6). ‡ $p < 0.01$ between GNP and PBS groups.

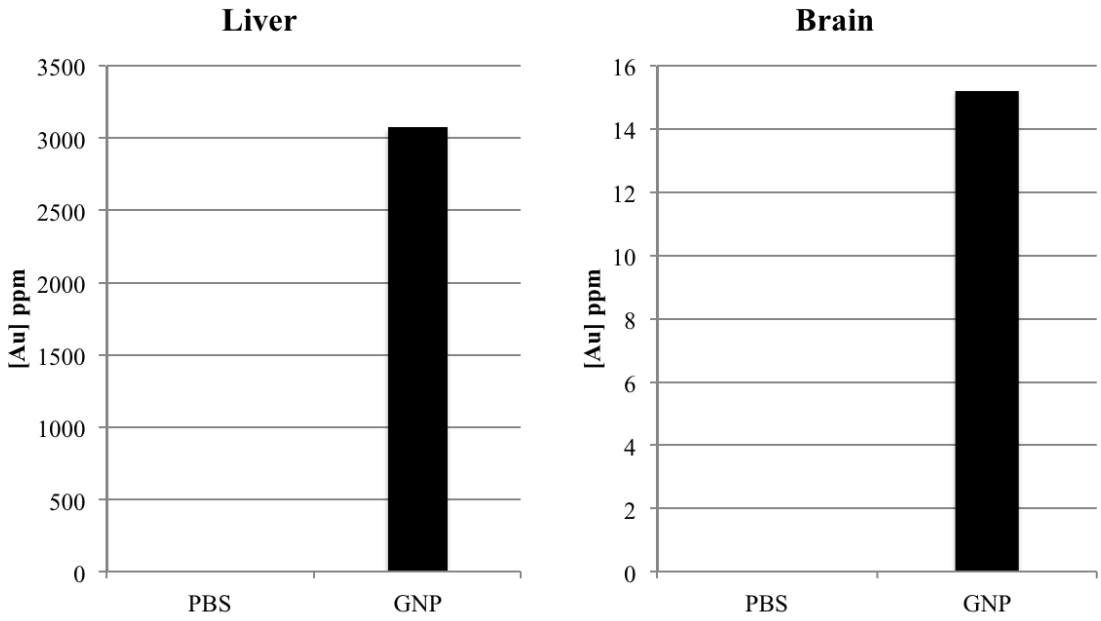

Figure 5. Gold concentration in liver and brain. Gold concentration in mouse liver and brain were measured 20 weeks post PBS or GNP exposure (n = 1). Note the difference in gold concentration values in the y-axis.

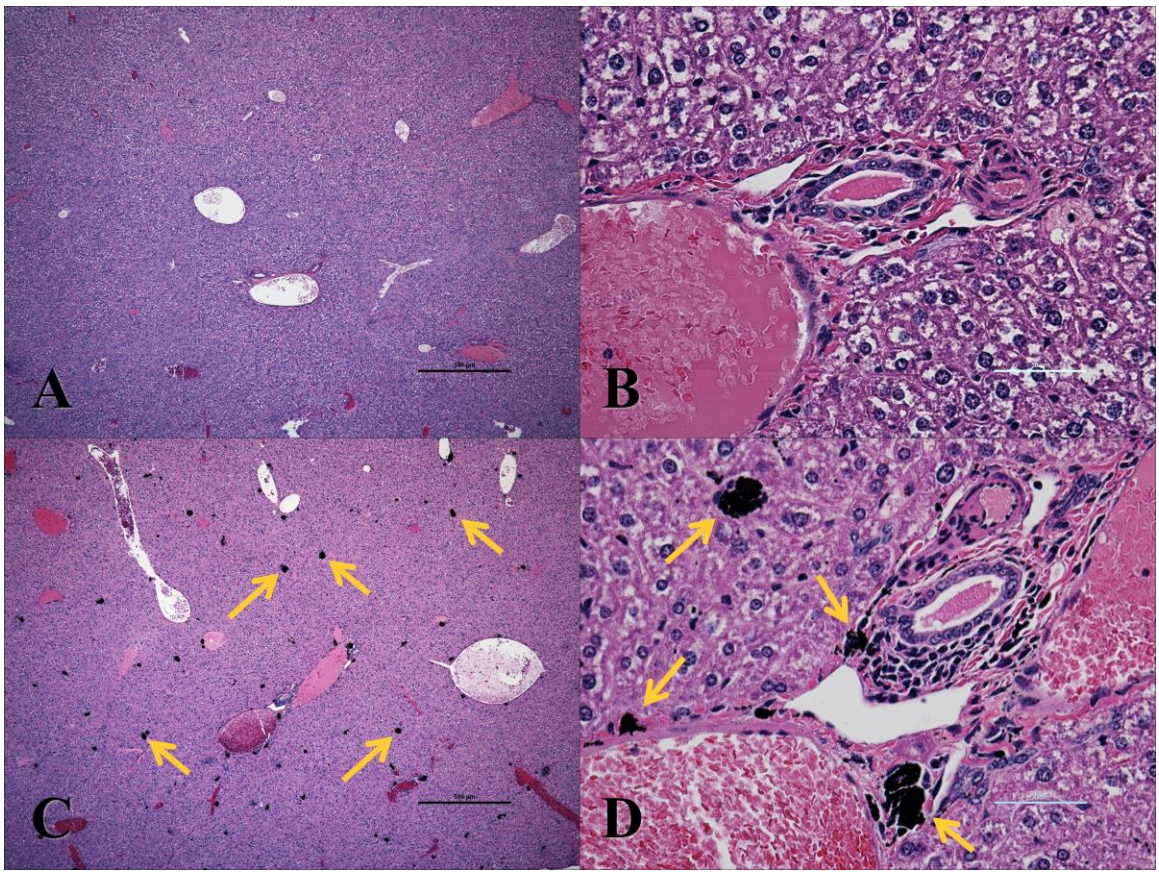

Figure 6. Gold nanoparticles in liver. Representative images of mouse liver 20 weeks post- (A, B) PBS or (C, D) GNP exposure. Note GNP-containing macrophages forming microgranulomas (arrows). Hematoxylin and eosin stain, (A, C) 4x magnification, scale bars = 500 μm, (B, D) 40x magnification, scale bars = 50 μm.

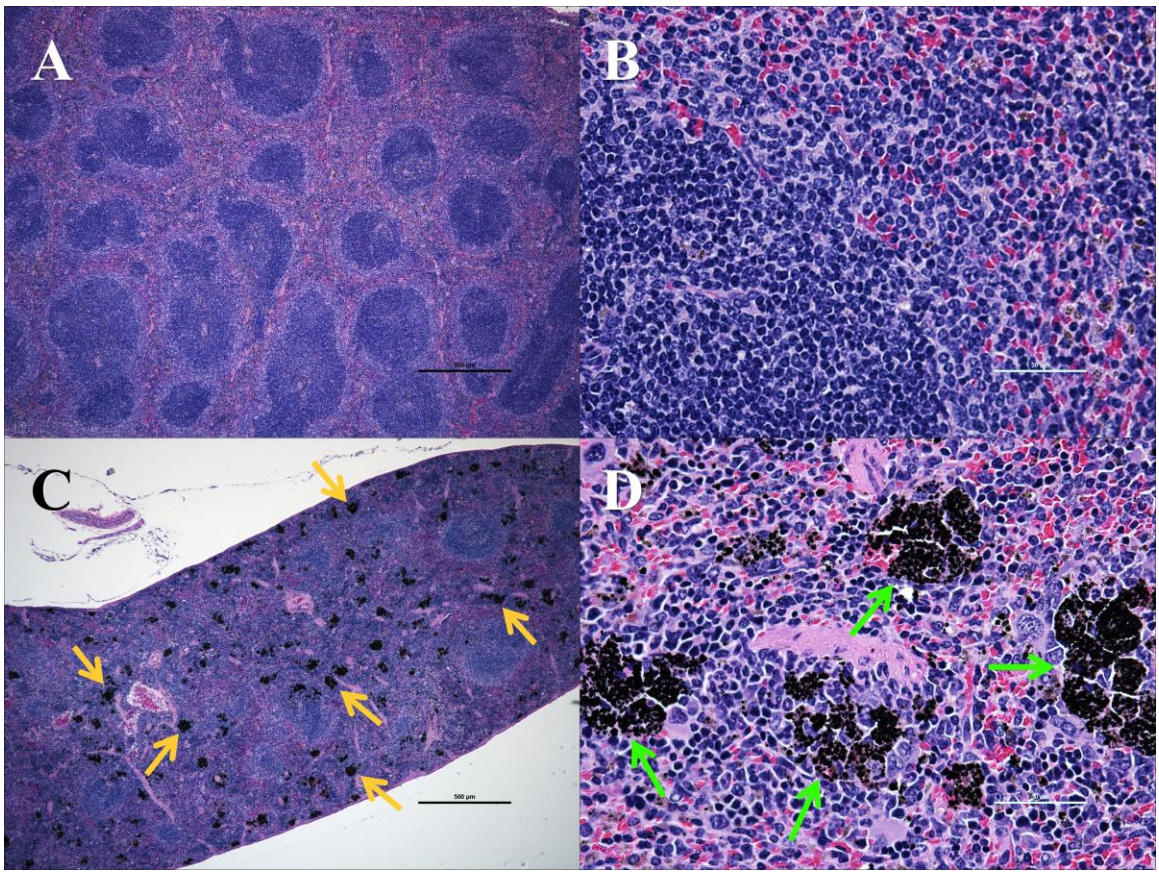

Figure 7. Gold nanoparticles in spleen. Representative images of mouse spleen 20 weeks post- (A, B) PBS or (C, D) GNP exposure. Note GNP-containing macrophages forming microgranulomas (arrows). Hematoxylin and eosin stain, (A, C) 4x magnification, scale bars = 500 μm, (B, D) 40x magnification, scale bars = 50 μm.

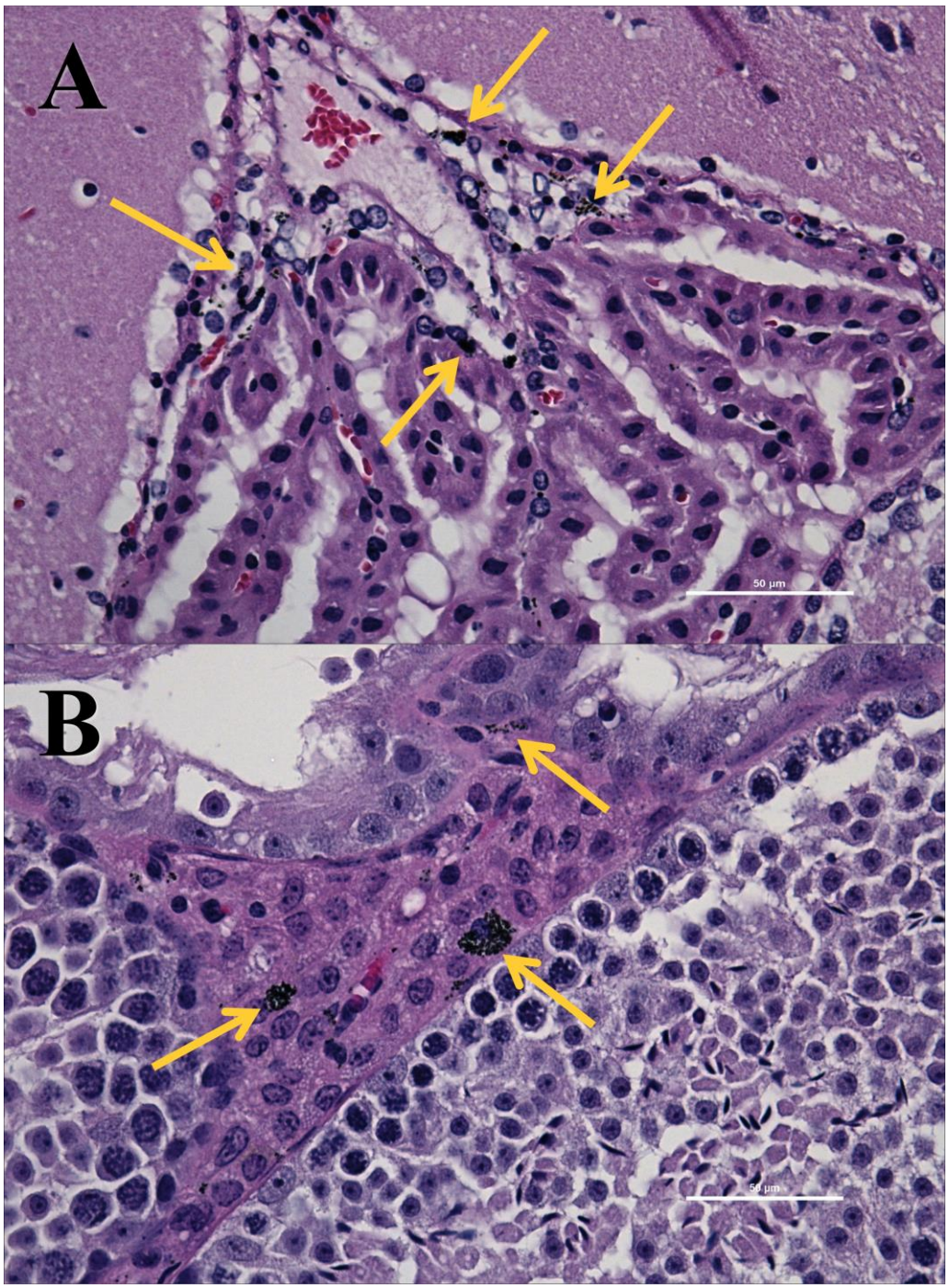

Figure 8. Gold nanoparticles in blood-tissue barriers. Representative images of mouse (A) brain and (B) testis 20 weeks post-GNP exposure. Note GNPs inside cells located within and around the (A) blood-brain barrier and (B) blood-testis barrier (arrows). Hematoxylin and eosin stain, 40x magnification, scale bars = 50 µm.

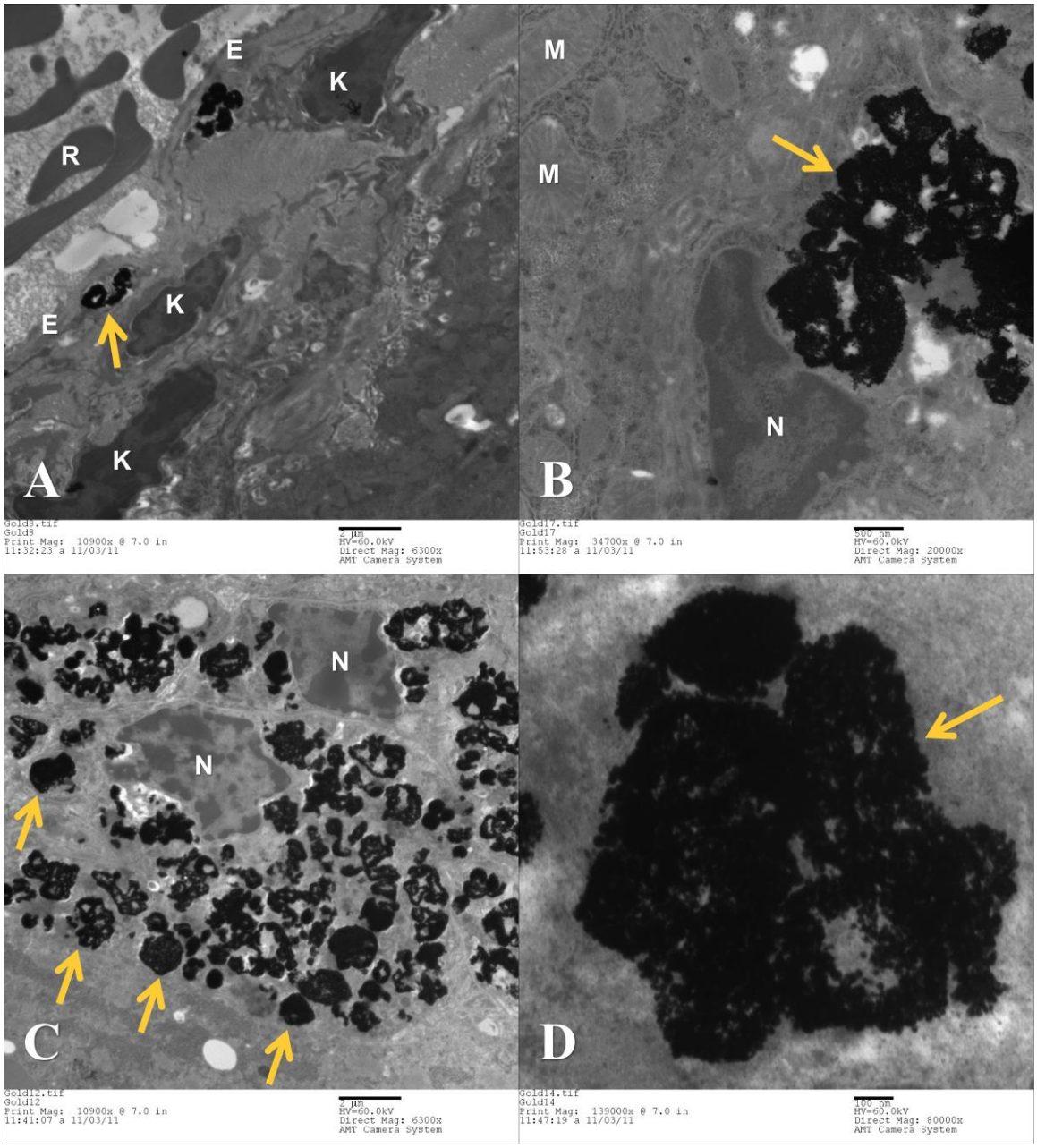

Figure 9. Intracellular location of GNPs in liver. Representative TEM micrographs of mouse liver 20 weeks post-GNP exposure. Note GNPs enclosed in cytoplasmic vesicles within Kupffer cells (arrows). R, red blood cell; E, endothelial cell; K, Kupffer cell; M, mitochondria; N, nucleus. (A, C) 6300x magnification, scale bar = 2 μm, (B) 20000x magnification, scale bar = 500 nm, (D) 80000x magnification, scale bar = 100 nm.

CHAPTER V: Summary, Conclusions, and Future Directions

A) Summary and Conclusions

Nanomaterials are being extensively investigated due to their enormous potential for varied and novel uses. Despite the growing incidence of human nanoparticle exposures, the health risks of nanomaterials are generally not understood and are poorly documented. Gold nanoparticles in particular are promising candidates for biomedical uses, such as targeted drug delivery, cancer therapy, and diagnostic contrast imaging, but little is known about their potential for toxicity. The main purpose of the research presented in this dissertation was to identify and characterize the acute, subacute, and chronic effects of a single exposure to commercially available GNPs in two *in vivo* models: mice and rats. Our overall hypotheses were that 1) GNP exposure will result in particle sequestration within macrophages of blood filtration organs and will negatively impact short-, medium-, and/or long-term health of mice and/or rats, 2) mice and rats will present differences in type and/or severity of adverse effects observed post-exposure, and 3) independent characterization of the purchased GNPs will demonstrate physicochemical characteristics in good agreement with the information provided by the supplier. Improved knowledge about potential adverse effects upon intentional exposure to GNPs will greatly contribute to the development of safe nanotechnology-based biomedical devices and techniques, with high hopes of prompt advances in early diagnosis and treatment of diverse pathologies.

Regarding our first hypothesis, our results demonstrate that PEGylated GNPs are not innocuous, having the potential to incite a robust macrophagic response. The particles

accumulate and persist in phagocytic cells of all tissues, but especially in liver and spleen. In these tissues, macrophages are seen individualized or clustered, forming microgranulomas that persist indefinitely. In mice, a transient increase in the proinflammatory cytokine IL-18 was detected 7 days post-exposure, and a greater increase was detected 20 weeks post-exposure. This indicates a rapid immune response to the initial GNP exposure, and a delayed inflammatory reaction to the constant stimulus of the immune system product of the persistence of GNPs within phagocytic cells. Even though these alterations were not reflected in clinical signs of disease at any time, a chronic impairment of weight gain was detected; supporting the fact that GNP exposure can negatively impact health, especially in the long-term. However, it is very important to take into account the magnitude of this effect in relation to the dose selected for this study. Considering the high dose administered and the mild toxicity detected, we consider that GNPs can continue to be considered highly biocompatible. Nevertheless, it is extremely important that further long-term studies are conducted in order to determine the role of GNP-induced chronic inflammation upon exposure to other toxicants, such as UV light, x-rays, laser irradiation, or hepatotoxic drugs.

With respect to our second hypothesis, it was demonstrated that there are similarities but also important differences in reaction to GNP exposure between mice and rats. It appears that GNPs in mice induce a stronger immune response, while rats seem to have better mechanisms of fecal excretion of this compound. Differences in biodistribution were also noticed, with rats having longer circulation times and a relatively greater accumulation of GNPs in spleen, as compared to mice. The striking and unforeseen acute death observed in rats exposed to GNPs could also be indicating a

species-specific sensitivity to acute toxicity by these particles, especially considering that all animals (rats and mice) from control groups and all mice from the GNP group survived, having been exposed to the same procedure and receiving the same dose of GNPs. Further research is needed in order to determine the reproducibility of this finding, especially at lower doses than the one used in this study, as well as specific mechanisms of acute toxicity.

Concerning our third hypothesis it was determined that, in general, the physicochemical characteristics of the purchased GNPs are in good agreement with the information provided by the seller. A relatively high variability was detected in some of the parameters measured, but this is expected from industrial nanomaterials and is likely to exist also for biomedical nanoparticles. The parameters that showed greater irregularity were the gold-core diameter and the zeta potential. The influence this could have on the potential for toxicity of GNPs is unknown.

In conclusion, the research presented in this body of work attempted to recognize and describe the short- and long-term effects of a single intentional exposure to commercially available GNPs in mice and rats. It was demonstrated that GNPs are not innocuous, having the potential to generate inflammatory responses even long after the initial exposure. Additionally, important species-related differences in biodistribution, excretion, and toxicity were detected when comparing mice and rats. Finally, it was determined that the purchased GNPs had physicochemical characteristics in concordance with the product's description. Considering the high dose selected for this study and the relatively small magnitude of the alterations identified, we believe that GNPs meet the requirement of biocompatibility required for biomedical applications. Further research is

needed to elucidate the role of chronic inflammation in co- or post-exposures, as well as species-specific mechanisms of toxicity.

B) Future Directions

Our research demonstrates that GNPs have the ability to trigger an immune response and incite chronic inflammation. A high dose was selected in order to increase the probability of detecting treatment-related alterations from a limited number of animals. In the future, it is essential to investigate if these findings are reproducible with lower doses or just occur at very high doses. It is also important to elucidate specific mechanisms of activation of the immune system and inflammation. Immunohistochemical stains for specific macrophage markers (such as F4/80, CD68, MAC2, or CD163) and for specific T-cells (CD4 or CD8) could define the cellular interactions triggered by GNP exposure. Phagocytic activity of tissue macrophages can be measured via uptake of ^{51}Cr-labeled sheep red blood cells, as described elsewhere.[163-165] Gold nanoparticles have been described to impair adipocyte differentiation *in vitro*[123] and decrease fat mass *in vivo* (72 hours post-exposure to 7.85 mg/kg IP of 21.3 nm citrate-coated GNPs).[77] In our study, a significant difference in body weight was detected between GNP and PBS groups starting 20 weeks post-exposure, but body composition was not investigated. In future studies, it would be important to incorporate an *in vivo* body composition analysis, to determine if the impairment of body weight gain was a consequence of loss of fat mass. This can be achieved using a nuclear magnetic resonance analyzer, as previously performed in our laboratory.

Other area of great interest to our group is the ability of GNPs to cross or alter biological barriers, such as BBB, BTB, or placenta. The present research demonstrated that low concentration of gold can be measured in brain homogenate long after GNP-exposure, and that GNPs accumulate inside cells that comprise the BBB and BTB. However, we could not determine conclusively if these particles are able to cross these barriers or if this accumulation generates alterations on these barriers. In the future, BBB *in vitro* systems[166] could be used to further investigate this interrogation. Regarding placenta, no research has been conducted, to our knowledge, to determine the developmental effects of GNP exposure. A preliminary study would be ideal to start elucidating this question, and it should include exposure to GNPs before pregnancy, during pregnancy, and during lactation, including low, medium, and high doses.

Lastly, future studies should evaluate the effect of GNP-induced chronic inflammation in co- or post-exposures. Biomedical applications of GNPs imply not just the direct administration of these particles to patients, but also post-exposure to energy sources such as x-rays and laser irradiation with therapeutic purposes. Long-term *in vivo* studies should be designed that mimic these applications, for example evaluating the long-term health of tumor-burdened experimental animals that have been treated with GNPs and posterior irradiation. Also, our research demonstrates that GNPs accumulate and persist indefinitely in macrophages of the skin. Since humans are exposed to UV light throughout their lives, it is of high relevance to investigate if this accumulation, even in small quantities, will carry delayed adverse effects.

In summary, our research demonstrates that GNP exposure can carry adverse effects, even if mild, long after the initial exposure. Important differences in immune

response were also determined between mice and rats after exposure to GNPs. Future directions of research in this area include the determination of specific mechanisms of immune stimulation and inflammation, ability of GNPs to cross or alter biological barriers, developmental effects of GNP exposure, and co- or post-exposures to GNPs and energy sources such as x-ray, laser, or UV irradiation.

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