An assessment of the biodistribution, biopersistence and toxicity of gold nanoparticles

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“What can be asserted without evidence can be dismissed without evidence”

Christopher Hitchens

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Preface

This thesis is submitted in fulfillment of the requirements of a Doctor of Philosophy in Pharmaceutics using the article format in accordance with the General Academic Rules (A.7.5.7.4) of the North-West University. Each experimental chapter (3-5) was written in accordance with specific guidelines as stipulated by the journals intended for publication. I Clinton Rambanapasi, the student did the following in the work presented in this thesis;

- Planned and designed the experiments.
- Carried out and participated in all the experiments with the exception of analysis done at independent laboratories
- Interpreted the results and discussed them with various co-authors.
- Drafted the manuscripts.

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All the co-authors have given permission that the manuscripts may be submitted for degree purposes as stipulated in the Manual for Post Graduate Students of the North-West University.

Abstract

The interest in biomedical applications of gold nanoparticles (AuNPs) has increased dramatically in the last decade due to their ease of synthesis, unique surface and optical properties. The main driver of this surge in research on potential biomedical applications which include *inter alia*; to photothermal therapy, diagnostic aids and drug delivery vehicles was their biocompatibility. Questions on the safety of AuNPs have resurfaced and justifiably due to the increase in the number of reports on their toxicity potential and toxicity. This whole debate on safety must be put to rest before biomedical applications of AuNPs can reach the clinic. Studies were designed to investigate the acute biodistribution, biopersistence, and bioaccumulation of AuNPs using a rodent model using male Sprague Dawley rats. In all the studies, toxicity endpoints were monitored. To fully understand the determinants of toxicity of AuNPs which are a multi-component system, the acute biodistribution of the gold core was determined simultaneously with that of the citrate coating using a novel dual radiolabeled technique. The amount of Au core and citrate surface coating was quantified using gamma spectroscopy and liquid scintillation respectively. The biopersistence was determined after a single intravenous injection over 56 days. The bioaccumulation was assessed over 56 days as well after intravenous administration of multiple (7) doses of AuNPs at 3 different dose levels. In both the biopersistence and bioaccumulation studies, toxicity endpoints were monitored using histopathological analysis of organs and assessment of markers of kidney (creatinine and blood nitrogen urea) and liver (alkaline phosphatase, alanine transferase and total bilirubin) damage. The amount of Au in the tissues was quantified using neutron activation analysis (NAA) in the biopersistence and bioaccumulation studies. The acute

biodistribution pattern of the Au core was found to be different to that of the citrate surface coating. In the acute study, Au widely distributed to all the tissues with the highest amount in the liver, spleen, lungs and bones in that descending order. After 56 days, there were considerable amounts of Au in the liver, spleen, lungs and bone. The biopersistence studies revealed that Au does not get cleared completely over eight weeks. The bioaccumulation study results showed that Au accumulates in the liver, spleen, lungs and bones albeit in a non-dose dependent fashion. In all the studies reported in this work, there was no peracute and acute toxicity as a result of exposure to AuNPs. In the biopersistence and bioaccumulation studies no peracute, acute, subacute and subchronic toxicity was observed. There were no differences in the levels of markers of liver and kidney damage. No abnormalities were detected during the histopathological analysis of the heart, kidneys, liver, lungs and spleen during the biopersistence and bioaccumulation studies. The acute biodistribution pattern of the Au core was different to that of the citrate surface coating and the Au core distributed widely in the body. The clearance of Au is low after a single intravenous injection over 56 days and Au has a high bioaccumulation propensity which is not dose dependent. Exposure to AuNPs did not result in peracute, acute, subacute and subchronic toxicity in a rodent model.

Keywords: gold nanoparticles, Sprague Dawley rats, biodistribution, biopersistence, bioaccumulation, acute, subchronic, toxicity, dual radiolabeling, neutron activation analysis, gamma spectroscopy

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Chapter 1: Problem Statement

1.1 Background

The potential biomedical applications of gold nanoparticles (AuNPs) had a notable expansion due to recent advances in their wet chemical synthesis and biomolecular functionalization (Khlebtsov & Dykman, 2011). The biomedical applications include inter alia drug and gene delivery (Bergen *et al.*, 2006; Craig *et al.*, 2012; Dobrovolskaia & McNeil, 2007; Donnelly *et al.*, 2005; Ghosh *et al.*, 2008; Kumar *et al.*, 2013; Libutti *et al.*, 2010; Paciotti *et al.*, 2004; Pissuwan *et al.*, 2011; Rana *et al.*, 2012), cancer therapy (Bhattacharya & Mukherjee, 2008; Cai *et al.*, 2008; Jain *et al.*, 2012) and diagnostics (Curry *et al.*, 2014; Huang & El-Sayed, 2010; Lu *et al.*, 2012; Mieszawska *et al.*, 2013). However, despite all the proof of concept studies there is insufficient information (both qualitative and quantitative) with regards to the safety of AuNPs based biomedical applications. Given the current situation (paucity of safety information) it is advisable or generally recommended to have more information to avoid being sorry in the future (Fadeel & Garcia-Bennett, 2010).

The notion and idea that AuNPs were biocompatible, and thus safe, was based on conclusions in early publications stating that they are safe (Connor *et al.*, 2005; Esther *et al.*, 2005; Goodman *et al.*, 2004; Hainfeld *et al.*, 2006; Merchant, 1998; Mukherjee *et al.*, 2007; Mukherjee *et al.*, 2005; Shukla *et al.*, 2005). These studies were mainly executed *in vitro*. However, more recent studies, both *in vitro* and *in vivo* have reported on the potential toxicity of AuNPs (Abdelhalim & Jarrar, 2011; Alkilany & Murphy, 2010;

Balasubramanian *et al.*, 2010; Choi *et al.*, 2012; Fraga *et al.*, 2014; Zhang *et al.*, 2011a; Zhang *et al.*, 2010). Since bulk gold is considered inert, the toxicity observed is thought to be due to the nanoscale, the form in which the gold was administered (Aillon *et al.*, 2009). Due to the conflicting research evidence, a need for more research clearly exists to answer the question of the safety of AuNPs.

Nanotoxicity, the study of the toxicity of nanomaterials, requires a paradigm shift in the approach and manner in which the potential toxicity is evaluated. Indicators have suggested that traditional screening approaches might not be appropriate to nanoscale structures (Oberdörster *et al.*, 2005). Measuring risk (which is a product of hazard, susceptibility and exposure) is the cornerstone of nanotoxicity studies. The hazard of a product/compound is a material property while the susceptibility is a property of the organism investigated. Rodent models have been used to determine susceptibility (Wang *et al.*, 2015). Properties of nanoparticle such as size distribution, shape, agglomeration state, surface area, surface chemistry and surface charge have an influence on the potential toxicity and controlling these properties is critical. The majority of risk assessment studies of AuNPs have varied the exposure and hazard by altering the properties (hazard) of the nanoparticles and route of administration (exposure) (Balogh *et al.*, 2007; De Jong *et al.*, 2008b; Hirn *et al.*, 2011; Lipka *et al.*, 2010; Morais *et al.*, 2012; Schleh *et al.*, 2012; Semmler-Behnke *et al.*, 2008; Wang *et al.*, 2015; Zhang *et al.*, 2009; Zhang *et al.*, 2011a), followed by determination of the biodistribution in rodent models.

In biodistribution and toxicity studies, accurately determining the amount of gold in various tissues and organs is absolutely necessary. The quantification of the other

components of an AuNP drug delivery vehicle such as the surface coating and surface attachments or the cargo, can assist in the elucidation of potential toxicity mechanisms. Several techniques have been used to measure the content of gold in rodents, namely inductively coupled plasma mass spectroscopy (ICP-MS) (Balasubramanian *et al.*, 2010; Cho *et al.*, 2009; De Jong *et al.*, 2008a; Sadauskas *et al.*, 2009; Simpson *et al.*, 2013; Sonavane *et al.*, 2008), atomic absorption spectroscopy (AAS) (Lasagna-Reeves *et al.*, 2010), radioactive analysis (RA) using gamma spectroscopy (Hirn *et al.*, 2011; Lipka *et al.*, 2010; Schleh *et al.*, 2012; Semmler-Behnke *et al.*, 2008) and neutron activation analysis (NAA) (Balogh *et al.*, 2007; Hillyer & Albrecht, 2001). Gamma spectroscopy and NAA are the preferred analytical techniques in biodistribution studies due to the lower limits of detection compared to AAS and ICP-MS. Gamma spectroscopy offers the added advantage of a quick and relatively simple sample preparation which only requires noting the mass of the sample and its activity. However, all these quantification methods lack the ability to simultaneously track and quantify the other components and surface attachments of AuNPs *in vivo*.

Biopersistence refers to the length of time that a substance, in this case the engineered nanomaterial (AuNPs) remains in a biological system such as a rodent and is a function of the system's ability to clear the material, in this case gold from the AuNPs. The clearance mechanisms have not been fully elucidated and remain unknown. The few studies reporting on the biopersistence of AuNPs after the administration of a single dose in rodents (Balasubramanian *et al.*, 2010; Fraga *et al.*, 2014; Sadauskas *et al.*, 2009; Zhang *et al.*, 2011b) differ widely in the dosages (mass concentration) of the AuNPs used. Thus the

results of these studies cannot easily be generalized. Further the different time points used in these studies further complicates any attempt at generalizing the results. The rationale used for the selection of dosages, time points and organs to be analyzed for their gold content is also not always clear. The dosages used in nanotoxicity studies tend to mimic accidental exposure (Balasubramanian *et al.*, 2010) or high toxic doses (4 mg Au/kg) are used (Zhang *et al.*, 2011a). It is imperative to have information on the biopersistence of AuNPs when dose levels that resemble intentional use are administered to determine the toxicity at several time points post administration and to assess the quantities of gold in organs that are chosen systematically based on experimental results.

Bioaccumulation occurs when an organism takes up or absorbs any material, chemical, or nanomaterial at a rate higher than its clearance rate. The bioaccumulation propensity of any nanomaterial is dependent on its biopersistence in the organ or tissue; biopersistent materials will have a higher bioaccumulation propensity. The route of exposure or administration has an influence on the organs exposed to the material and thus its eventual clearance and for systemic drug delivery purposes using AuNPs the intravenous route is the most important to study. Few studies report on the biopersistence (Fraga *et al.*, 2014; Sadauskas *et al.*, 2009) or on the bioaccumulation (Buzulukov *et al.*, 2014; Lasagna-Reeves *et al.*, 2010) of AuNPs after intravenous administration. The exposure of an organ to a metal or nanomaterial will increase when bioaccumulation occurs; thus there is a clear need to have more information on the bioaccumulation of AuNPs after repeated intravenous administrations.

Safety assessments of AuNPs include end organ toxicity that can result from acute and subchronic exposure. The influence of bioaccumulation on end organ toxicity must also be investigated in order to gather safety data. Serum enzymes and metabolites serve as good markers for hepatotoxicity and nephrotoxicity. Histopathological examination is a good indicator to assess structural damage. This approach has been used in studies assessing the safety of AuNPs albeit with different results (Abdelhalim & Abdelmottaleb Moussa, 2013; Lasagna-Reeves *et al.*, 2010).

Despite all the unanswered questions with regards to the safety issues surrounding AuNPs, a phase I and pharmacokinetic trial testing the delivering of recombinant human tumor necrosis factor alpha (rhTNF) by AuNPs has been conducted (Libutti *et al.*, 2010). Innovative pharmaceutical companies are also showing interest in AuNP based delivery systems (AstraZeneca, 2012). These developments illustrates a clear need to conduct the research to allow regulators to come up with evidence based positions in assessing any use of gold nanoparticles in humans that will be proposed.

1.2 Research questions

The research presented in this thesis addressed in a systematic manner the biodistribution, biopersistence and bioaccumulation of AuNPs in a rodent model. The basic question that the research sought to answer was: How safe are AuNPs in a rodent model at concentrations which may be used for biomedical applications? This was done through answering the following questions;

1. What is the biodistribution profile of the components of AuNPs (i.e. the gold core and the citrate surface coating)?
2. What is the biopersistence and toxicity of gold after a single dose has been administered to a rodent model?
3. What is the bioaccumulation propensity and toxicity of gold after multiple doses have been administered to a rodent model?

Synthetic methods for preparation of AuNPs are many and varied (Brust *et al.*, 1994; Fent *et al.*, 2009; Frens, 1973; Turkevich *et al.*, 1951; Zhao *et al.*, 2013). They all have one thing in common, the reduction of a salt of gold in solution that with AuNPs being the product. In general AuNPs refers to all structures of gold in the nanosize range, but in this work it only refers to spherical AuNPs. Due to the ease of synthesis and nontoxic nature of their precursors (Connor *et al.*, 2005), citrate coated AuNPs present the simplest form of AuNPs that can easily be functionalized and used in many biomedical applications. Therefore in this study we only used citrate coated AuNPs to answer the research questions using 3 aims (each addressed in the different experimental chapters).

1.3 Aims and objectives

The following aims and objectives were chosen to answer the questions;

1. Determination of the biodistribution profiles the two components of AuNPs, the gold core and the citrated surface, using citrate coated AuNPs after intravenous administration to a rodent model by:
    a. Synthesizing and characterizing dual radiolabeled AuNPs

   b. Determining and comparing of the acute biodistribution profiles of gold and the citrate coating

2. Determination of the biopersistence and toxicity of AuNPs after administration of a single intravenous dose by:
   a. Quantifying the amount of gold in organs using NAA
   b. Monitoring the markers of kidney and liver damage
3. Determination and assessment of the influence of the dose on the bioaccumulation and toxicity of AuNPs after multiple intravenous doses by:
   a. Quantifying of the amount of gold in the organs using NAA
   b. Monitoring the markers of kidney and liver damage

1.4 References

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Chapter 2: Literature Review

2.1 Colloidal gold: A brief history

In ancient times gold was the only metal that did not corrode: this made it valuable and it symbolized immortality. Solutions of gold were recommended for medical use as what was referred to as potable gold or aurum potabile. The origins of the idea that liquid gold could be the "elixir of life" are thought to have originated in China (Kauffman, 1985). Theophrastus Bombastus von Hohenheim or Paracelsus, the father of iatrochemistry, also made some of the earliest forms of colloidal gold in the 16th century for the cure of ailments (Kauffman, 1985). Most preparations of potable gold made during the time of Paracelsus seem to have been colloidal gold which methods for their preparation were well known (Kauffman, 1985). Today it is still common to encounter potable gold preparations being marketed for vitality. These ancient ideas still exist with red colloidal gold being used in Ayurvedic medicine for rejuvenation and revitalization (Mahdihassan, 1971).

Michael Faraday published a paper reporting on the preparation of colloidal gold in the middle of the nineteenth century when a more scientific interest in colloidal systems arose (Faraday, 1857). This paper is now regarded as the foundation of modern colloidal science. Gustav Mie gave the first theoretical description of the formation of colloidal gold (Mie, 1908). While studying the properties of gold sols, Richard Zsigmondy invented the ultra-microscope and also won a Nobel Prize in chemistry.

The invention of the electron microscope at the start of World War II opened up the detailed study of colloidal gold since their particle size was below the resolution of the

optical microscope (Turkevich, 1985). In 1951, John Turkevich and colleagues published a paper reporting on the reduction of a gold salt using sodium citrate. The electron microscope was used as the main tool for characterization of the formed colloidal gold(Turkevich *et al.*, 1951). Frens in 1971 published a paper reporting on controlling the of size of colloidal gold particles by varying the concentration of sodium citrate used in the reaction (Frens, 1973). To this date, a combination of these two methods commonly referred to as the Turkevich-Frens method, is used to prepare colloidal gold. The last milestone in the preparation of colloidal gold was the preparation of thiol stabilized gold nanoparticles by the reduction of chloroauric acid using sodium borohydride in the presence of alkane thiols (Brust *et al.*, 1994). The colloidal gold prepared using this method were different from those prepared before as they were stable over long periods of time and could be precipitated, re-dissolved and separated according to size by fractional crystallization.

2.2 Preparation of gold nanoparticles

Gold nanoparticles also known as colloidal gold refers to all nano structures of gold of various shapes. In this work it will only refer to spherical gold nanoparticles. The most common preparation methods are *in situ* by the chemical reduction of chloroauric acid by reducing agents (Zhao *et al.*, 2013).

2.2.1 Turkevich-Frens method

The Turkevich-Frens method uses trisodium citrate, both as the reducing and stabilizing agent, (Frens, 1973; Turkevich *et al.*, 1951) with a third role; pH mediator being suggested

(Ji *et al.*, 2007). Figure 1 shows a schematic of the Turkevich-Frens method. A solution of chloroauric acid ($HAuCl_4$) is boiled under reflux whilst vigorously stirring and the trisodium citrate (Na_3-Cit) is added. A wine red colour signifies the formation of the gold nanoparticles. In this method the size of the gold nanoparticles can be controlled by altering the molar ratio of the chloroauric acid to the trisodium citrate. This method has been extensively researched and shown to be a multi-step process (Kumar *et al.*, 2007). A reversed addition method was also developed and it can yield monodisperse sub 10 nm particles (Sivaraman *et al.*, 2011). Control of temperature and pH has also been shown to give monodisperse particles compared to those without controls (Li *et al.*, 2011). The citrate reduction method also known as the Turkevich-Frens method remains as an important method for preparing gold nanoparticles.

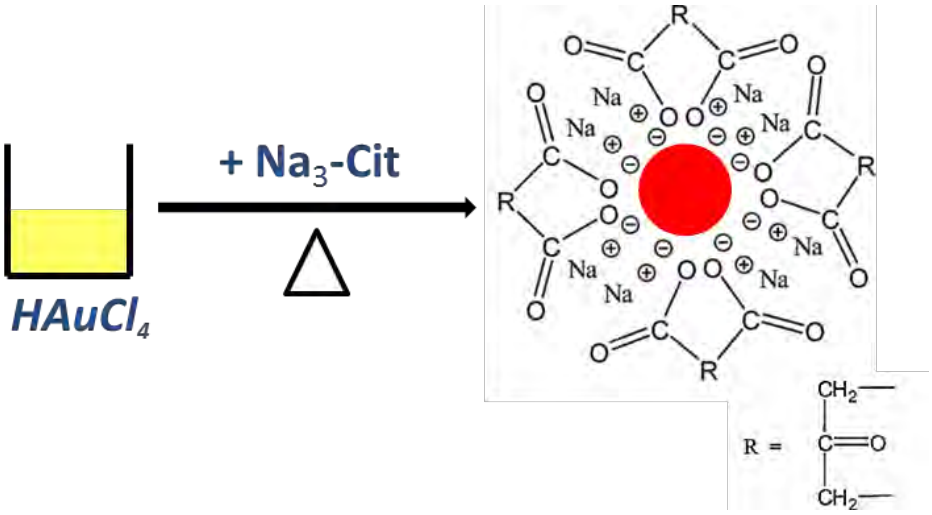

Figure 1 Turkevich-Frens method for the preparation of gold nanoparticles

2.2.2 Brust Method

The Brust method is one of the major preparation methods for gold nanoparticles (Zhao *et al.*, 2013). The method was developed by Mathias Brust and colleagues (Brust *et al.*, 1994) and is a two phase method (Figure 2). This was the first method to describe the preparation of thiol-stabilized gold nanoparticles via an *in situ* synthetic process (Zhao *et al.*, 2013). The shapes of the prepared gold nanoparticles are cuboctahedral and icosahedral with a size range of 2 - 5 nm. This method has several advantages over the Turkevich-Frens method namely: easy synthesis under ambient conditions, relatively higher thermal and air stability of the gold nanoparticles, better stability with regards to aggregation and decomposition after repeated isolation and re-dissolution, smaller size yields; 5 nm with narrow dispersity and easier functionalization and modification by ligand substitution. The biggest drawback of this method is the cytotoxicity of tetraoctylammonium bromide, a starting material in the synthesis (Connor *et al.*, 2005). Brust and colleagues improved their method to a procedure that yielded *p*-mercaptophenol-stabilized gold nanoparticles which were synthesized in a methanol solution without using the cytotoxic phase transfer agent, tetraoctylammonium bromide (Brust *et al.*, 1995). Any thiol that is soluble in the same solvent as chloroauric acid, such as methanol, ethanol, or water, allows the use of a single-phase system for the preparation of gold nanoparticles (Zhao *et al.*, 2013).

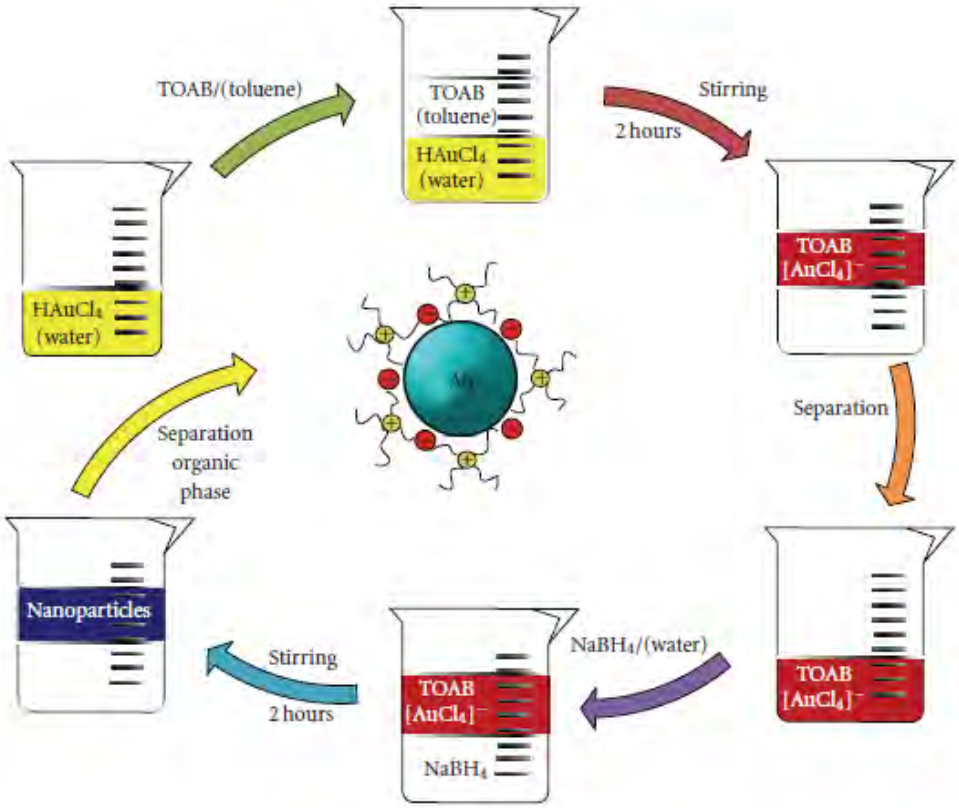

Figure 2 Schematic showing the synthetic steps of the Brust method (Calandra *et al.*, 2010)

Modified methods on the synthesis of thiolate-stabilized gold nanoparticles using a single-phase method have also been published (Di Pasqua *et al.*, 2009; Leontowich *et al.*, 2010; Sardar & Shumaker-Parry, 2009). These modifications widened the range and scope of the applications of gold nanoparticles prepared using the Brust method. The gold nanoparticles prepared using the Brust method are smaller due to the higher strength of the reducing agent (Zhao *et al.*, 2013).

2.2.3 Other methods

In the case of the citrate reducing method, the citrate also serves as the stabilizing agent in addition to being the reducing agent and pH modifier. A wide variety of stabilizing agents

have been reported in literature. Natural materials such as starch and gum arabic have been used as stabilizers (Chanda *et al.*, 2010; Fent *et al.*, 2009; Kannan *et al.*, 2012; Katti *et al.*, 2006). Vitamin C has been used as well in the preparation of gold nanoparticles (Khan *et al.*, 2013). Macromolecules (Thanh & Green, 2010), polymers, microbes and dendrimers have also been used to stabilize gold nanoparticles successfully (Zhao *et al.*, 2013). The seed growth method is also a popular method used in the preparation of gold nanoparticles and usually consists of two steps. The first step involves the preparation of small sized gold nanoparticle seeds followed by the addition of the seeds to a growth solution containing chloroauric acid, a reducing agent and the stabilizer (Jana *et al.*, 2001; Sau & Murphy, 2004). This method enables the synthesis of particles that have a specific shape and size. Like other nanoparticles, bottom up and top down approaches have been used to synthesis the gold nanoparticles(Zhao *et al.*, 2013).

2.3 Characterization of gold nanoparticles

Similar to other nanoparticles the following physico-chemical properties of gold nanoparticles are important: size distribution, agglomeration state, shape, surface area, surface chemistry, and surface charge. Characterization techniques have been developed to give information on the physico-chemical characteristics mentioned.

Due to the plasmon resonance phenomena, UV/Vis spectroscopy is one of the most powerful techniques to use to characterize gold nanoparticles. It gives information on the size and agglomeration state of dispersions of gold nanoparticles. With surface plasmon absorption, a strong absorption band in the visible region is present when the frequency of the electromagnetic field is resonant with the coherent electron motion (El-Sayed *et al.*,

2005; Eustis & El-Sayed, 2006; Huang & El-Sayed, 2010). Polarization of the electrons with respect to and relative to the ionic core occurs when the nanoparticles interacts with an electric field (Figure 3). The so-called plasmon absorption is because of the dipole oscillations of the free electrons (Link & El-Sayed, 2003).

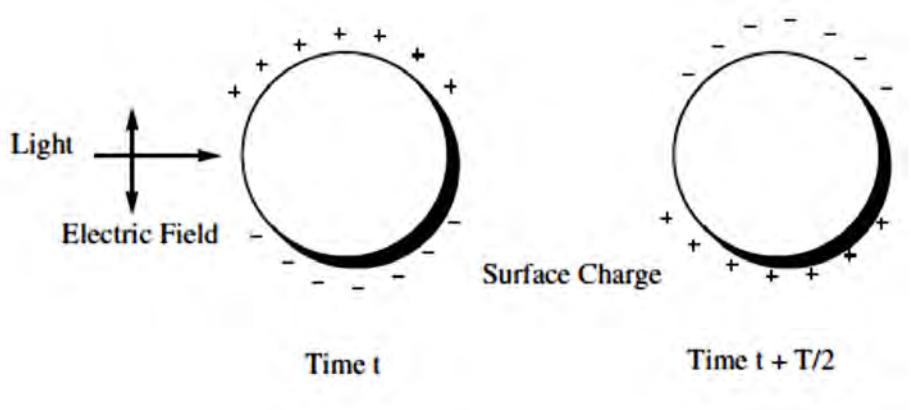

Figure 3 A schematic illustrating the excitation of the dipole surface plasmon oscillation called the surface plasmon absorption of spherical nanoparticles (Alanazi *et al.*, 2010)

The peak intensity and position of the surface plasmon absorption bands are dependent on the size, concentration and shape of the nanoparticles; a right shift of the peak is observed as the size increases (Figure 4) (Young *et al.*, 2012). It is also used to determine size and concentration of gold nanoparticles (Amendola & Meneghetti, 2009; Haiss *et al.*, 2007). The absorption spectra also give information on the agglomeration state albeit qualitative; absence of secondary peaks is normally indicative of monodispersity.

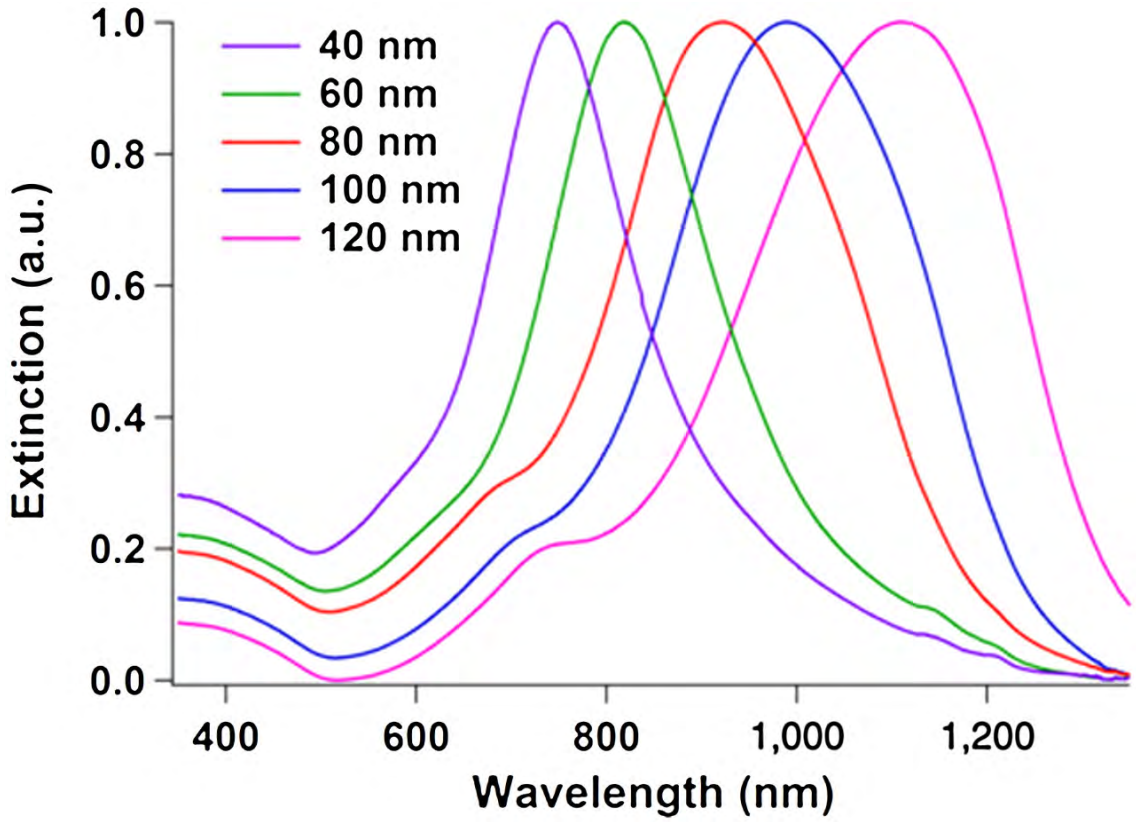

Figure 4 Right shifting of the surface plasmon resonance peak with an increase in particle size(Young *et al.*, 2012)

Information on the shape and size distributions (primary size) can be obtained using electron microscopy. Transmission electron microscopy is the gold standard as it has the highest resolution but scanning electron microscopy is still valuable in some instances. Size distributions can be obtained using ImageJ, free software of the NIH using at least 200 particles. Another technique that can be used to determine the size distribution of gold nanoparticles is size exclusion chromatography in the case of thiol-stabilized gold nanoparticles.

Dynamic light scattering (DLS) is an analytical technique also used for measuring the size and size distribution of particles in the nanometer size range (Philip, 2008). To obtain the measurement, a suspension of the particle is illuminated by a laser beam, and the

fluctuation of the scattered light is monitored and analyzed, to acquire the velocity of the particles' Brownian motion which is then used to infer their size.

DLS measures the hydrodynamic size of particles, which includes not only the physical size of the nanoparticle core, but also the surface coating and solvent layer associated with the particle. Aggregation of gold nanoparticles can also be measured with DLS. While non-aggregated monodispersed gold nanoparticles are measured with DLS as a single size population, aggregation of the particles can present a broadening of the peak, increase in the hydrodynamic size, and even multiple populations. The DLS measurement of gold nanoparticles is a very sensitive technique and can be applied to measure the size of the particles, characterizing their surface modification, and monitor the stability of the gold nanoparticles over a period of time.

Surface charge can be determined by measuring the zeta potential of the particles in various media and the charge is usually a property of the surface chemistry. Concentration can be expressed as a mass or number concentration and is usually calculated using the mass of gold used in the synthesis and making assuming that all the gold is reduced to nanoparticles (Liu *et al.*, 2007). For spherical gold nanoparticles the surface area can be calculated using the total number of nanoparticles and the primary size by calculating the surface area of the spheres.

2.4 Functionalization of gold nanoparticles

Gold nanoparticles prepared by the citrate reduction method must be functionalized in order to make use of them in various applications. This is possible due to the weakness of

the Au-citrate bonds. Functionalization occurs via substitution of the citrate ligands by stronger ligands usually functional thiols (Gao *et al.*, 2013; Shenoy *et al.*, 2006; Zhang *et al.*, 2012). The substitution is experimentally very simple and involves reaction of citrate coated gold nanoparticles and the corresponding functional thiols under ambient conditions. This property of citrate coated gold nanoparticles allows them to be versatile compared to thiol-stabilized gold nanoparticles from the Brust method, which are already prepared functionalized. Functionalized citrate coated gold nanoparticles have many biomedical applications. Other non-biomedical applications of functionalized gold nanoparticles include *inter alia* catalysis, electronics, sensors and probes.

2.5 Biomedical applications

The biomedical applications of gold nanoparticles can broadly be categorized into three classes, drug and gene delivery vehicles, diagnostics and therapy (Figure 5).

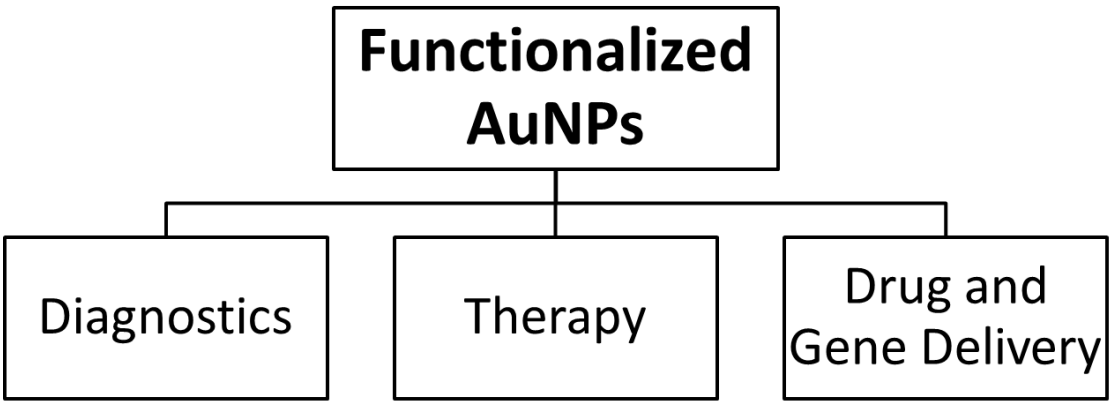

Figure 5 Biomedical applications of functionalized gold nanoparticles

2.5.1 Diagnostics

It has been proposed to use gold nanoparticles as biomarkers in the detection and diagnosis of a number of diseases and conditions. They are used as sensors for probing and imaging tumour cells because of their ability to interact strongly with visible light in what is known as the surface plasmon resonance (Figure 3). Tumour cells are often cancerous. Cancer is a global problem that transcends socio-economic classes and needs to be addressed as a matter of urgency. Early diagnosis is one of the cornerstones of successful therapy and in some cases prognosis. When used in cancer diagnosis, gold nanoparticles target and accumulate at sites of interest. Based on their optical scattering properties, they can be visualized thus allowing the region to be studied (Lim *et al.*, 2011). Gold nanoparticles must be conjugated with specific antibodies for antigens that are overexpressed in tumour cells thus allowing targeting and accumulation in the region. Surface-enhanced Raman spectroscopy has been used in imaging human epidermal growth factor receptor 2 (HER2) cancer cells (Lee *et al.*, 2009). However this approach only works when the tumour is close to the skin surface because optical signals have limited tissue penetration abilities (Cai *et al.*, 2008).

Currently there are a number of limitations to contrasting agents for X-ray usage and gold nanoparticles have been proposed as a suitable agent to replace the tri-iodobenzene platform (Hainfeld *et al.*, 2006). The main advantage of gold nanoparticles is that better contrast can be achieved with lower x-ray doses due to gold's higher absorption and thus less bone and tissue interference compared to the tri-iodobenzene platform (Hainfeld *et al.*, 2006).

Gold nanoparticles have also been incorporated in electrochemical immunosensors. They play a crucial dual role of enhancing the electrochemical signal transducing the binding

reaction of antigens at antibody immobilized surfaces and increasing the amount of immunoreagents in a stable mode (Tang *et al.*, 2006; Wang *et al.*, 2004). Immunosensors using gold nanoparticles have been constructed for the detection of the hepatitis B virus (Tang *et al.*, 2006), diphtheria antigen and diphtherotoxin (Tang *et al.*, 2005), and *Schistosoma japonicum* (Sj) antigen (Chu *et al.*, 2005; Lei *et al.*, 2003). More recently a rapid dual channel lateral flow assay for the detection of *Mycobacterium Tuberculosis* antibodies in human blood was developed (Mdluli *et al.*, 2014).

2.5.2 Therapy

The therapeutic properties of gold nanoparticles are mainly applicable in cancer therapy via two main mechanisms: photothermal therapy (Curry *et al.*, 2014; Huang & El-Sayed, 2010; Jain *et al.*, 2012; Melancon *et al.*, 2008) and radiotherapy (Chanda *et al.*, 2010; Fent *et al.*, 2009; Kannan *et al.*, 2012; Katti *et al.*, 2006).

Photothermal therapy is a cancer treatment method in which photon energies are converted to thermal energy to induce cell death. It is a highly selective form of cancer treatment since only the light irradiated areas can be affected and the photosensitizer ideally is nontoxic in the absence of light. Gold nanoparticles can be highly potent photothermal therapeutic agents, due to their strong light absorption and efficient heat conversion characteristics. They can provide sufficient thermal energy to kill cancer cells. Near Infrared light is used in photothermal therapy because it can penetrate deep into live tissue (beyond a few mm below the skin surface) and is relatively not affected by absorption and scattering by biomolecules and water. Gold nanoparticles cause local heating when they are irradiated with light in what is called the water window (800 - 1200 nm). Citrate coated

gold nanoparticles functionalized with an anti-epidermal growth factor receptor to target human oral squamous cell carcinoma cells were studied and the results showed that use of gold nanoparticles enhance photothermal therapy by 20 times (El-Sayed *et al.*, 2005). It was also reported that gold nanoparticles are efficacious in photothermal therapy as well (Rengan *et al.*, 2015; Shao *et al.*, 2013).

The goal of radiation therapy in cancer treatment is to selectively achieve maximum dose intensity at the tumour site so as to minimize side effects (Kannan *et al.*, 2012). This is the biggest drawback for most radiotherapeutic agents. Radioactive gold (^{198}Au) decays via the beta and gamma emission. The range of beta particles in tissue is short enough (11 mm) to allow the delivery of the maximum dose intensity intratumourly. The half-life of ^{198}Au of 2.7 days is also ideal if practical considerations such as preparation times and delivery are taken into account. Use of radioactive gum arabic gold nanoparticles was shown to be possible for radiotherapy because of their high affinity for tumour vasculature (Kannan *et al.*, 2012; Katti *et al.*, 2006). Laminin receptor specific AuNPs have also been used intratumourly to deliver ^{198}Au and showed efficacy in treating prostate cancer (Shukla *et al.*, 2012).

2.5.3 Delivery vehicles

Gold nanoparticles provide an attractive vehicle for delivering drugs, genetic material, proteins and small molecules (Figure 6) due to their ease of synthesis and surface properties. Using gold nanoparticles is ideal since the doses can be reduced and thus also the side effects due to better targeting, uptake into the cells and stability of the cargo

(Bergen *et al.*, 2006; Duncan *et al.*, 2010; Ghosh *et al.*, 2008; Kumar *et al.*, 2013; Papasani *et al.*, 2012; Rana *et al.*, 2012; Vigderman & Zubarev, 2013).

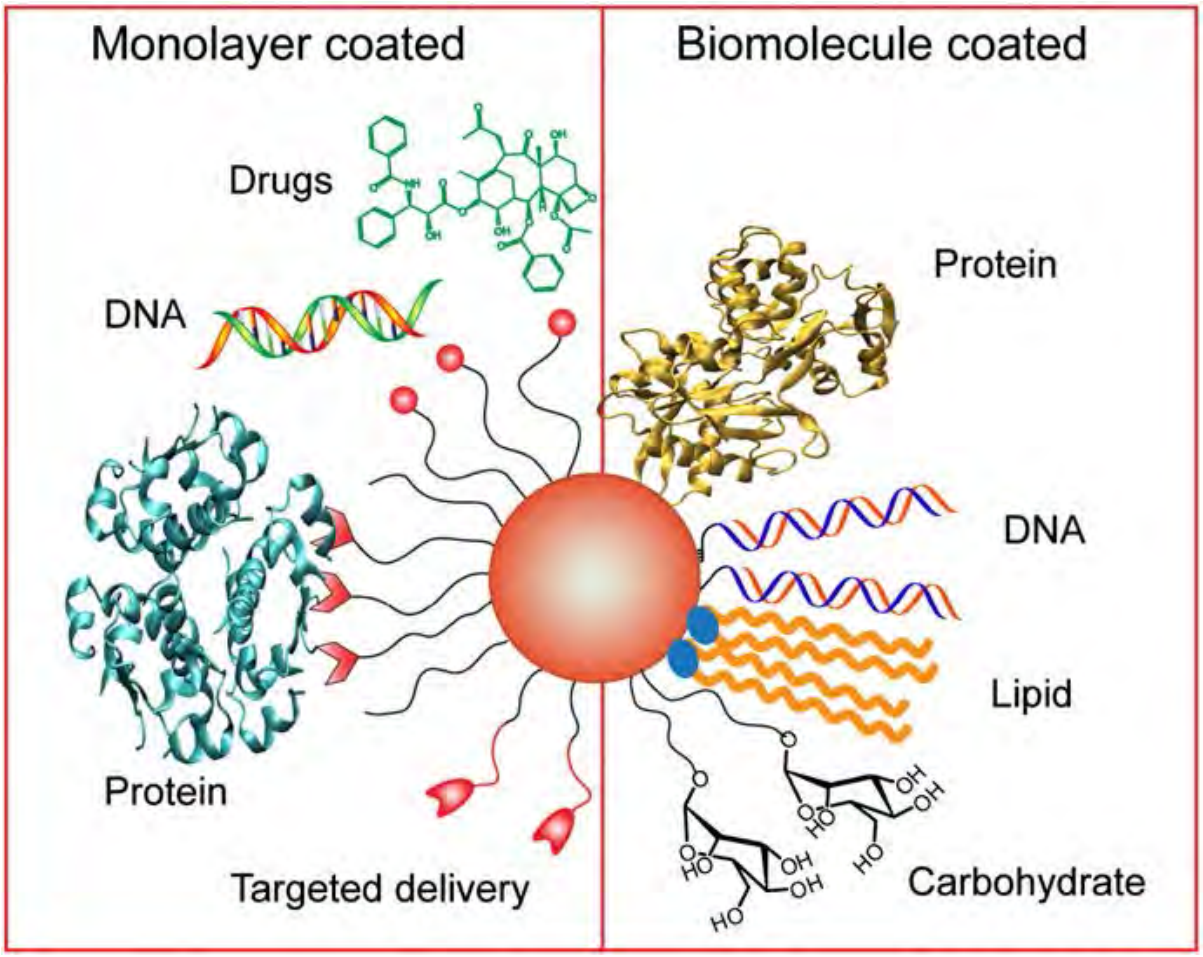

Figure 6 Schematic showing the diversity of cargo that can be delivered using gold nanoparticles (Rana *et al.*, 2012)

A number of strategies have been employed to attach materials to the surfaces of gold nanoparticles with the different covalent bonds being more popular due to their stability (Vigderman & Zubarev, 2013). Gold nanoparticles have also been used in both passive and active targeting and a number of cancer drugs can be conjugated to gold nanoparticles (Figure 7).

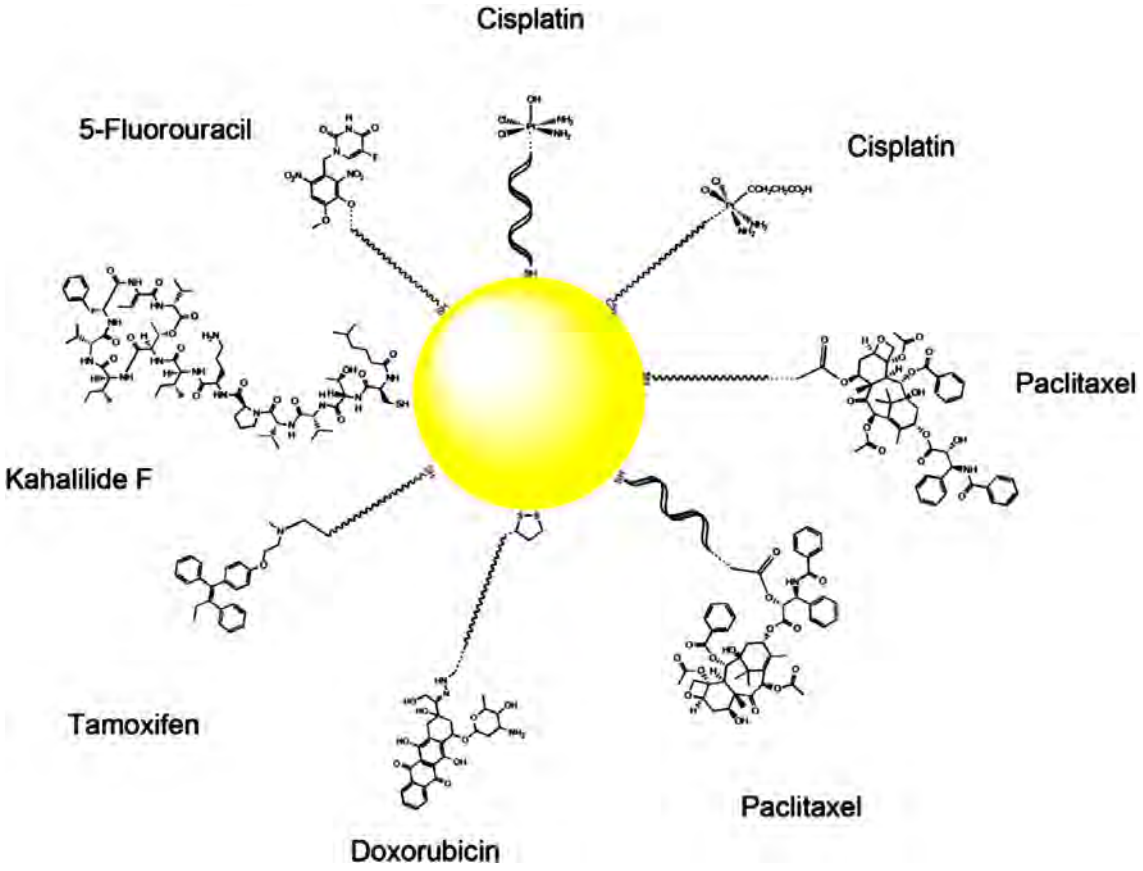

Figure 7 Anticancer drugs conjugated to gold nanoparticles (Vigderman & Zubarev, 2013)

Cisplatin has been conjugated to gold nanoparticles with enhanced reproducibility, drug loading and stability (Craig *et al.*, 2012). Better delivery for Oxaliplatin was shown after conjugation to gold nanoparticles (Brown *et al.*, 2010). A phase I and pharmacokinetic study has been conducted for a nanomedicine using gold nanoparticles to deliver human recombinant tumour necrosis alpha (Libutti *et al.*, 2010).

2.6 Nanotoxicity

Use of nanoscale materials, gold nanoparticles included, has led to a number of questions being asked about safely issues. Assessment of the risk associated with the use of gold

nanoparticles uses methods that are being developed in the new discipline of nanotoxicity, that is the study of the toxicity of nanomaterials. This is important because test methods used for bulk materials have been shown to be insufficient. Governmental organisations such as the National Institute of Standards and Technology and National Cancer Institute: Nanotechnology Characterization Laboratory in the United States of America and supranational organizations such as the European Union's Organization for Economic Cooperation and Development (OECD) have taken the initiative to standardize or attempt to standardize safety assessment of nanomaterials.

Apart from the scientific aspects of nanomaterial aspects there is a real need for regulatory agencies such as the United States Food and Drugs Agency (USFDA) European Commission's European Medicines Agency (EMA), World Health Organization (WHO) and the signatories of the International Conference on Harmonization (ICH) to come up with a position on how to regulate nanomedicines. This will create an enabling environment for development of the discipline of nanomedicine (Fatehi *et al.*, 2012). Like most new technologies public acceptance is key and this can be gained through the use of evidence based data to make decisions and (Malsch *et al.*, 2015).

Safety assessment of nanomaterials in general is being done in a number of ways: high-throughput screening, *in silico* (modelling) approaches, *in vitro* and *in vivo* testing (Fadeel *et al.*, 2013). Considering the interesting biomedical applications of gold nanoparticles, the next logical questions are: Are they safe and when are they going to reach the clinic?

2.6.1 Are gold nanoparticles safe?

This question remains unanswered; this is mainly due to the discordance between the *in vitro* (Connor *et al.*, 2005; Esther *et al.*, 2005; Goodman *et al.*, 2004; Hainfeld *et al.*, 2006; Mukherjee *et al.*, 2007; Mukherjee *et al.*, 2005) and *in vivo* reports (Abdelhalim & Jarrar, 2011; Balasubramanian *et al.*, 2010; Cho *et al.*, 2009; Zhang *et al.*, 2011; Zhang *et al.*, 2010). Currently what is known is that physico-chemical parameters such as size distribution and surface charge which is a function of surface functionalization and shape, are important determinants of toxicity as they are influence the exposure patterns of gold nanoparticles to tissues (Fadeel & Garcia-Bennett, 2010; Oberdörster, 2010; Oberdörster *et al.*, 2005). A huge challenge associated with attempts to generalize results of studies investigating the toxicity of gold nanoparticles is the different experimental designs. This is a big factor causing delays in answering the question of the safety of gold nanoparticles. Numerous efforts have been made to correlate physico-chemical properties and their interaction with biological systems (Fadeel *et al.*, 2013) but science still has a long way before the toxicity of gold nanoparticles can be assessed in an unquestionable manner (Fratoddi *et al.*, 2015). In the meantime it might be prudent to exercise a bit of caution to avoid being sorry in the future (Fadeel & Garcia-Bennett, 2010).

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Chapter 3: Dual Radiolabeling as a Technique to Track Nanocarriers: The case of Gold Nanoparticles

This manuscript describes the synthesis of dual radiolabeled ([^{14}C] Citrate coated-[^{198}Au]AuNPs) and the study to compare the biodistribution profile of the Au core and the citrate surface coating in male Sprague Dawley rats. The manuscript has been published in the Journal Molecules- Special Issue "Preparation of Radiopharmaceuticals and Their Use in Drug Development". The instructions to the authors are attached in Appendix 1: Guide to Authors.

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Article

Dual Radiolabeling as a Technique to Track Nanocarriers: The Case of Gold Nanoparticles

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Abstract: Gold nanoparticles (AuNPs) have shown great potential for use in nanomedicine and nanotechnologies due to their ease of synthesis and functionalization. However, their apparent biocompatibility and biodistribution is still a matter of intense debate due to the lack of clear safety data. To investigate the biodistribution of AuNPs, monodisperse 14-nm dual-radiolabeled [^{14}C]citrate-coated [^{198}Au]AuNPs were synthesized and their physico-chemical

characteristics compared to those of non-radiolabeled AuNPs synthesized by the same method. The dual-radiolabeled AuNPs were administered to rats by oral or intravenous routes. After 24 h, the amounts of Au core and citrate surface coating were quantified using gamma spectroscopy for ^{198}Au and liquid scintillation for the ^{14}C. The Au core and citrate surface coating had different biodistribution profiles in the organs/tissues analyzed, and no oral absorption was observed. We conclude that the different components of the AuNPs system, in this case the Au core and citrate surface coating, did not remain intact, resulting in the different distribution profiles observed. A better understanding of the biodistribution profiles of other surface attachments or cargo of AuNPs in relation to the Au core is required to successfully use AuNPs as drug delivery vehicles.

Keywords: gold nanoparticles; dual radiolabeling; biodistribution profiles; Sprague Dawley rats

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3.1. Introduction

The use of engineered nanomaterials, such as gold (Au) nanoparticles (AuNPs), promises to have a great impact on the field of nanomedicine and nanotechnologies. As a result, AuNPs have become an on-going area of research for a wide range of biomedical applications, such as plasmon-based labeling and imaging, diagnostics and therapeutics [1-3]. AuNPs' unique surface, electronic and optical properties, as well as their apparent biocompatibility [4] make them ideal drug delivery vectors [5-8]. However, their biocompatibility and toxicity have recently been questioned [9,10], and currently, there is no consensus on their biodistribution [4,11-13]. This can be attributed to the use of different methodologies with a diversity of objectives that do not collate easily into a single general conclusion. The lack of correlation between *in vitro* and *in vivo* toxicity results further complicates matters.

In biodistribution and toxicity studies, it is necessary to accurately determine the amount of Au in various tissues/organs. The quantification of the other components of an AuNP drug delivery vesicle, the surface coating and surface attachments or the cargo, can assist in the elucidation of potential toxicity mechanisms. Several techniques have been used to measure the content of Au in rodents, for example; inductively-coupled plasma mass spectroscopy (ICP-MS) [10,14-18], atomic absorption spectroscopy (AAS) [19], radioactive analysis (RA) using gamma spectroscopy [20-23] and instrumental neutron activation analysis (INAA) [24,25]. Gamma spectroscopy and INAA are preferred analytical techniques in biodistribution studies due to the lower limits of detection compared to AAS and ICP-MS. Gamma spectroscopy offers the added advantage of a

quick and relatively simple sample preparation. However, all of these quantification methods mentioned lack the ability to track and quantify the other components/surface attachments of AuNPs simultaneously *in vivo*.

Whilst the use of a single radiolabel is common [13,20,23], to the best of our knowledge, there are no published studies using dual radiolabeling to determine the biodistribution profiles of the different components in a multi-component systems for AuNPs. However, dual radiolabeling has been reported before to study the biodistribution of the components of a vaccine system (both adjuvant and antigen) [26] and for AuNPs with two radiolabels for use in single-photon emission computed tomography (SPECT) for bioimaging applications in diagnostics [27]. An approach similar to the one we are taking in this study was done for superparamagnetic iron using ^{59}Fe for the nanoparticle core and labeled surface attachments [28,29].

An understanding of the biodistribution profile of each component (Au core and any surface attachments) would be ideal, as this will enable any observed end organ toxicity to be attributed to the whole system or a part thereof. This can be achieved by radiolabeling each of the desired components; in this case, the Au core and the citrate surface coating. The methods used by Hirn *et al.* of radiolabeling AuNPs by irradiation of a pellet of AuNPs (^{197}Au (n,γ) ^{198}Au) [20,23] cannot be used for dual radiolabeling of the Au core and the surface coating, as neutron activation only produces ^{198}Au. In this study, the Au was radiolabeled using ^{198}Au, while [^{14}C]citrate was used for the citrate surface coating.

The aim of the present study was to synthesize dual-radiolabeled AuNPs and to determine the biodistribution profiles of the Au core and citrate surface coating, while investigating the influence of the route of administration and the dose level. Well-characterized 14-nm AuNPs that were dual-radiolabeled were administered to healthy male Sprague Dawley rats intravenously and orally. The study served as a proof of principle that dual radiolabeling can be used to determine the biodistribution profiles of the different components of a multi-component system. Therefore, in this study, the acute biodistribution profiles of the Au core and citrate surface coating after oral and intravenous (*i.v.*) administrations are presented. Future studies must investigate the biodistribution of Au after multiple doses and assess its biopersistence, while focusing more on toxicity endpoints.

3.2. Results

3.2.1. Synthesis and Characterization of AuNPs

AuNPs were synthesized from both radioactive and non-radioactive precursors using the citrate reduction method. The UV spectra peaks in Figure 1 were similar for both radioactive and non-radioactive AuNPs.

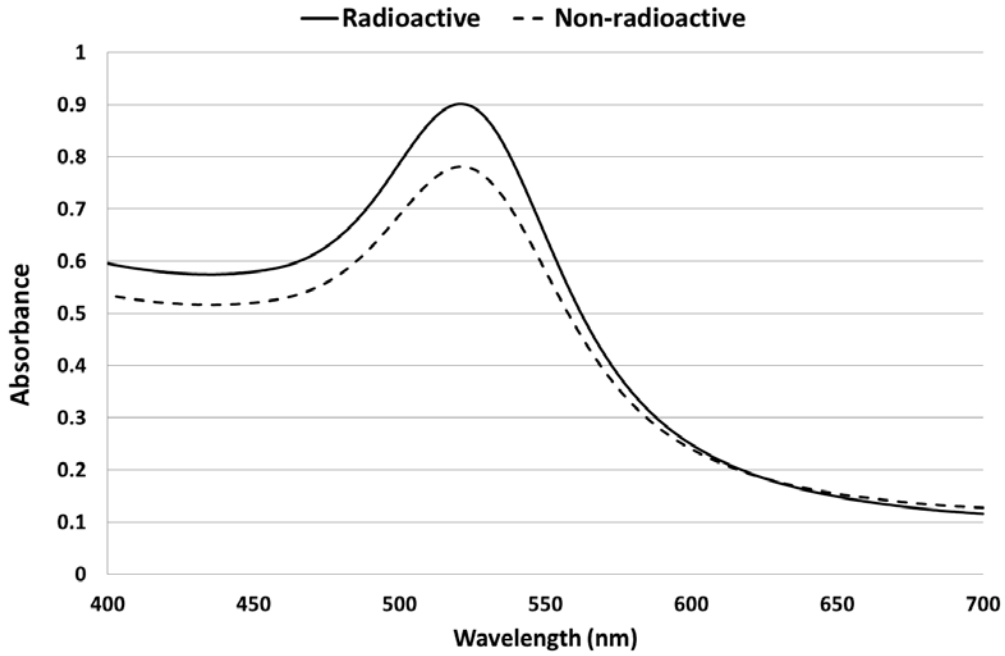

Figure 1. UV-Vis spectra of radioactive (continuous line) and non-radioactive (dashed line) AuNPs. The measurements were done after the synthesis of both samples.

The UV peak was around 520 nm as expected for this particle size range, whilst the dispersion quality was confirmed by the absence of absorbance at wavelengths greater than 600 nm [30].

From the TEM images and the particle size distribution plots (see Figure 2), it can be seen that the morphology and primary particles size distribution of 14 ± 1.2 nm and 14 ± 1.5 nm for radioactive and nonradioactive AuNPs, respectively, are similar/comparable. Hydrodynamic sizes of 25 nm for the non-radioactive preparation and 23 nm for the radioactive samples with zeta potentials of −50.9 mV and −48.9 mV were found, respectively.

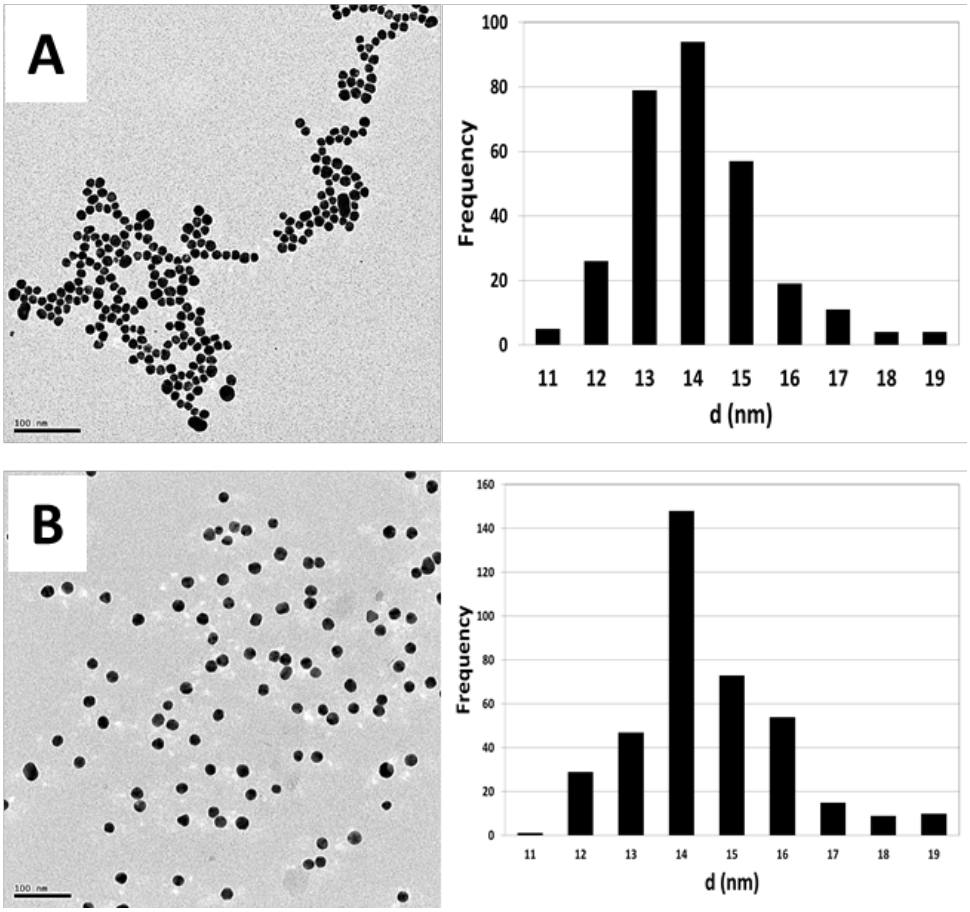

Figure 2. Morphology and size distribution profiles of the synthesized AuNPs fabricated from natural gold (**A**) and radioactive gold (**B**). On the left are TEM images, with the particle size distribution plots on the right.

The specific activity of the ^{198}Au was 108 GBq/g, with an activity concentration of 25.9 MBq/mL and an isotope ratio (^{198}Au:^{197}Au) of 1.45×10^{-5}. The specific activity of the ^{14}C was 52.43 MBq/g, with an activity concentration of 0.054 MBq/mL and an isotope ratio (^{14}C:^{12}C) of 0.8.

3.2.2. Biodistribution of Gold vs. *Citrate in the Rat*

3.2.2.1. Dosimetry

Table 1 summarizes the main dosimetric features of the [^{14}C]citrate-[^{198}Au]AuNPs used. The surface area and number of nanoparticles were calculated using the initial mass of Au used in the synthesis.

Table 1. Characteristics of the dual-radiolabeled AuNPs used in the study at the 2 dose levels used (high and low). The surface area of the AuNPs was calculated using the primary size determined using TEM.

		Dose	
		High	**Low**
Administered radioactivity per rat (MBq)	^{198}Au	12.95	1.22
	^{14}C	0.027	0.0027
Administered mass per rat (μg)	Au	90	9
	Citrate	520	52
Administered number of AuNPs per rat		3.27×10^{12}	3.327×10^{11}
Administered surface area (cm^2) of AuNPs per rat		20.16	2.02

3.2.2.2. Biodistribution Profiles

In order to ensure that the signal measured was only from gold and that there were no interferences that may impede the reliability of the results, a gamma spectrum was measured (see Figure 3). The peak at 411 keV is for ^{198}Au; the absence of other peaks shows that the gold used in the experiments did not have impurities, and only gold was quantified in the work.

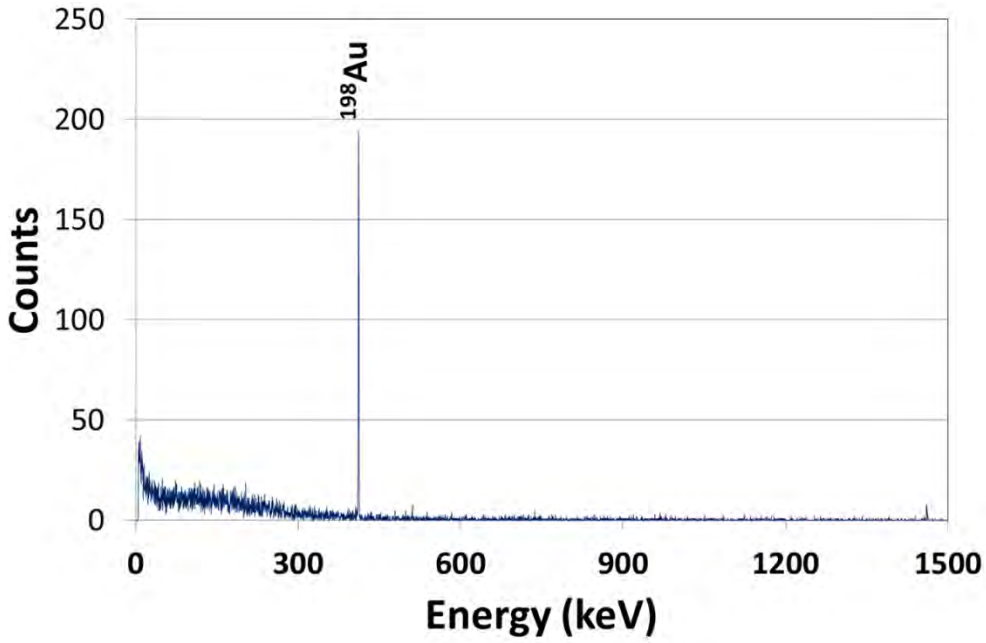

Figure 3. Gamma spectrum of the radioactive gold nanoparticles used in the animal study.

The biodistribution profiles of both the nanoparticle core and surface coating were investigated, with the route of administration and dose level as variables. The amount of Au and citrate was determined using γ-spectroscopy for ^{198}Au and liquid scintillation for ^{14}C at 24 h post a single dose administered intravenously and orally. The amounts of ^{198}Au

and ^{14}C in the liver, spleen, lungs and blood were determined in the analysis. Oral administration of AuNPs resulted in no systemic uptake of ^{198}Au; thus, no ^{14}C was measured in the oral group. Activities of the ^{198}Au were only measured/detected in the stomach and other parts of the GI tract, with no measurable activity in all other organs with less than 0.001 % injected dose (ID)/g in the blood, liver, spleen and lungs (results not shown). The inclusion of the oral group was because some measurable systemic uptake was expected, as described in the literature [24]. However, our findings correspond well with the results reported in previous studies and are attributed to the size of the particles used in the study [23,24,31]. Therefore, only results from the two intravenous groups (see Figure 4) are reported here. The results are expressed as the percentage injected dose per gram of organ/tissue (%ID/g) for the Au and citrate. The amounts of ^{198}Au measured in the urine and feces were used to perform a mass balance for Au.

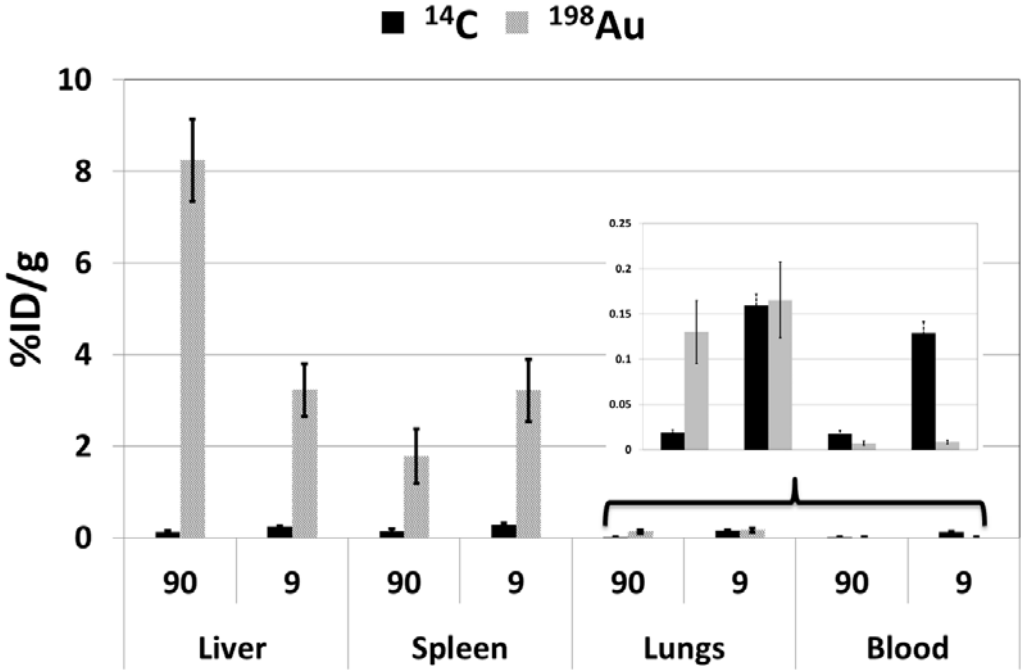

Figure 4. Amounts of gold and citrate expressed as a percentage of the injected dose per gram of organ/tissue (%ID/g) in the liver, spleen, lungs and blood, 24 h after intravenous administration of dual-radiolabeled AuNPs. Results are expressed as the mean ± SD.

Liver

The liver had the highest %ID/g of gold when administered in both 90-µg (high) and 9-µg (low) doses, with 8.2% and 3.2%, respectively. The %ID/g of citrate was less than 0.5% irrespective of the administered dose. The difference in the %ID/g of the Au was statistically significant using both the Mann–Whitney and Student *t*-tests; the *p*-values were calculated to be 0.03 and 0.0004, respectively, at the two dose levels used.

Spleen

The spleen had the second highest %ID/g of gold. However, contrary to the liver, the %ID/g was inverted relative to the administered doses, with 1.8% for ID of 90 µg and 3.2% when 9 µg were administered. The differences were not statistically significant. The values for the citrate were also determined to be under 0.5%.

Lungs

The %ID/g of both the Au and citrate were under 0.25% in the lung tissue. The biodistribution pattern was comparable between the Au and citrate only for the administered dose of 9 µg (low dose). The difference in the %ID/g of the Au was significant using both Mann–Whitney and Student *t*-tests; *p*-values of 0.03 and 0.002, respectively, at the two dose levels used.

Blood

The %ID/g of citrate was low (≤0.15%) and that of Au even lower (≤0.02%). There was not a statistically significant difference in the %ID/g of citrate at the two dose levels. The biodistribution profiles of the Au were independent of the dose quantity.

Summary of Biodistribution Profiles

In general, Au and citrate had unique biodistribution profiles, as shown by the differences in %ID/g portrayed in Figure 5 with the exception of the 9-µg administered dose in the lungs, where the %ID/g values were comparable.

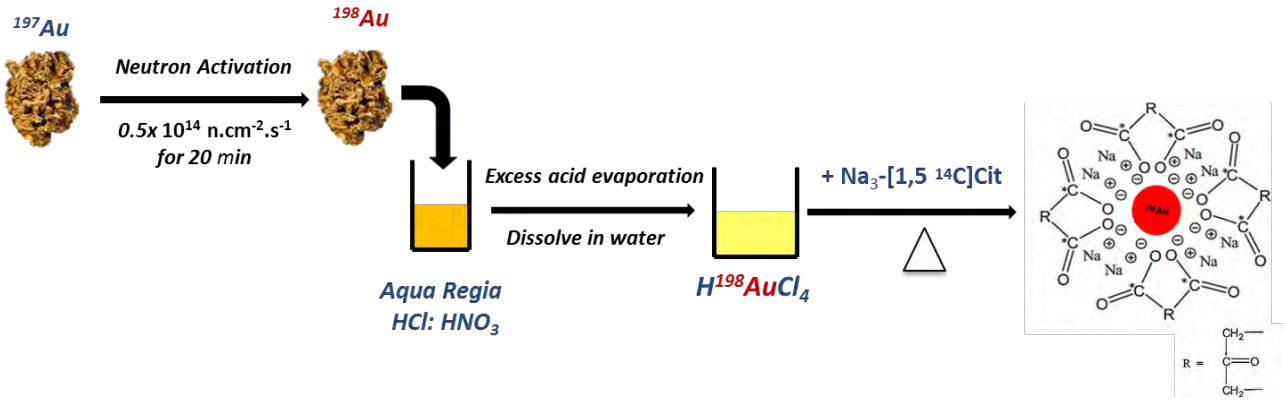

Figure 5. Schematic showing the synthesis of dual-radiolabeled AuNPs starting with the stable ^{197}Au isotope of gold. The neutron activation step is unique to the radioactive synthesis.

The biodistribution profile of the Au varied based on the dosing level, 90 µg *vs.* 9 µg. The ratios of %ID/g of Au between the 90 µg and 9 µg dose were: liver, 2.6; spleen, 0.6; lungs,

0.8; blood, 0.8; whilst for citrate, the ratios were: liver, 0.5; spleen, 0.5; lungs, 0.1; blood, 0.1.

3.3. Discussion

The need for extensive biodistribution studies to assess the safety of AuNPs can never be over emphasized, as this will ensure that AuNPs reach the clinic faster. With no consensus on the toxicity profile of AuNPs, a need to understand the biodistribution profile of each component of the AuNP system becomes apparent. It has been generally accepted that surface functionalization is an important determinant of the *in vivo* dynamics and toxicity [11,20,32]. In this study, we synthesized AuNPs, both non-radioactive and radioactive, and compared the two formulations to assess the impact of using radioactive precursors on the physico-chemical properties. The dual-radiolabeled AuNPs were used to determine the biodistribution profile of the Au core using ^{198}Au and surface coating using [^{14}C]citrate. The influences of the dose and route of administration were also investigated.

The use of radioactive precursors had no impact on the quality attributes of the synthesized dual-radiolabeled AuNPs. This was shown when the physico-chemical properties of the non-radioactive and radioactive AuNPs were compared. The UV/Vis spectra of the non-radioactive and radioactive batches were comparable and characteristic of the 14-nm size range, which has a defined plasmon resonance peak maxima around 520 nm [19]. The absence of secondary peaks at wavelengths higher than 600 nm also confirms the absence of agglomerates and/or aggregates in the suspensions [30]. The polydispersity index (PDI), a measure of monodispersity obtained with the Zetasizer Nano ZS, also showed that the suspensions were free of agglomerates/aggregates. The zeta potential was as expected: a high negative charge due to the negative charge of the citrate surface coating. This was comparable for both the non-radioactive and radioactive AuNPs. The molar ratio of the hydrogen chloroauric acid:citrate used in the synthesis of the AuNPs yielded nanoparticles with a core diameter around 14 nm, which is consistent with the sizes obtained by other researchers when similar molar ratios were used [15,16,33].

The radiotracers used in the synthesis of the dual-radiolabeled [^{14}C]citrate-[^{198}Au]AuNPs were well controlled and were adjusted to meet the varying requirements. The photons emitted during the decay of ^{198}Au have energies that can be detected by a gamma camera; thus, a change in the activity concentration of the Au during uptake in the various organs can be imaged. The method used in this work solves some challenges that are normally encountered when other ways of incorporating radiotracers into AuNPs are used. Agglomeration and/or aggregate formation when synthesized AuNPs are irradiated to neutrons activate the Au core [20-23]. The activity of both labels was homogenously distributed in the solution. This was shown experimentally when the doses were measured

using both volume and radioactivity. There was a correlation between the expected and determined value for each dose using ^{198}Au.

In this study, the biodistribution profiles observed for the Au core and surface coating were very different. Use of surface attachment as the radiotracer has been done [34] and suffers the disadvantage of misinterpretation of the biodistribution profiles. The radiotracers can be displaced from the core due to the formation of a bio-corona [35,36]. Usually, the biodistribution of the radiotracer is assumed to represent that of the Au core and the whole nanoparticle system. From our results, it is seen that surface attachments will not have to have the same biodistribution profile as that of the core or carrier molecule used to transport it. Caution must therefore be exercised when interpreting the results of biodistribution and toxicity studies of AuNPs with surface attachments that will not be present in those intended for biomedical applications. The addition of different surface attachments will most likely alter the biodistribution and toxicity profile of AuNPs *in vivo*, as surface chemistry plays an integral part in the toxicity and biodistribution of AuNPs and other nanomaterials [37].

The biodistribution profiles of the Au core and citrate surface coating were different in the organs/tissues used in the analysis. This can be explained by the formation of the bio-coronas around the nanoparticle core [38-43], which results in the dissociation of the surface attachment from the core. These results indicate that during the synthesis and design of therapeutic agents, the type of interaction between the Au surfaces and "cargo" should be carefully considered when surface modifications are made to AuNPs. This is especially important for the delivery of drug molecules to ensure that the cargo is not lost before the intended destination. Electrostatic interactions might be desirable, since covalent bonds require energy for the cargo to dissociate from the surface. A similar dissociation of surface attachments that had electrostatic interactions with the nanoparticle surface has been reported for superparamagnetic iron [28,29].

The effect of the dose was more prominent for the Au compared to the citrate surface coating with the exception of the liver, the %ID/g was higher in the lower dose level in all of the organs/tissues. For citrate, the opposite was observed: the %ID/g was lower in the higher dose level for blood and lung (a blood-rich organ). This can possibly be explained by isotopic exchange between the citrate (which predominates in blood) and its radiolabeled analogue. With higher dose, the amount of ^{14}C citrate will be the same in the blood as that in the lower dose, thus giving a lower %ID/g. The ratios of the %ID/g of the 90 μg:9 μg dosages for Au (liver: 2.6; spleen: 0.6; lungs: 0.8; blood: 0.8) may be an indication of a saturable transport mechanism of the Au into tissues/organs, with the liver taking up excess Au in the case of higher dosing levels. If this can be repeatedly shown, it may be a useful consideration when planning to use AuNPs as a drug delivery vector. To date, there is little evidence that AuNPs lead to histological changes and toxicity [33,44]. Whether this will be the case in an extensive treatment regime, with multiple doses administered over the course of weeks or months remains unknown. It is also not known

whether the systemic/tissue concentrations will be maintained by the prolonged exposure of the repeated doses, unlike in this acute study. The subchronic and chronic use of AuNPs presents another variable and so does the level of biopersistence. All of the above scenarios will need to be investigated.

3.4. Experimental Section

4.1. Preparation of AuNPs and Dual-Radiolabeled AuNPs

Elemental gold (24 carat) was purchased from Cape Precious Metals Holding Pvt. Ltd., Johannesburg, South Africa. 1,5-[^{14}C] citric acid (concentration: 3.7 GBq/mL; specific activity: 2.07 GBq/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Hydrochloric acid (HCl, 37%), nitric acid (HNO_3, 68%) (used to prepare aqua regia using an HCl:HNO_3 in a 3:1 ratio) and trisodium citrate ($Na_3C_6O_7H_5 \cdot H_2O$), were all of analytical grade and purchased from Merck (Billerica, MA, USA). Deionized water (resistance >18 MΩ) was prepared by an in-house ultrapure water system (Merck Millipore, Billerica, MA, USA). All chemicals, except for the 1,5-[^{14}C] citric acid (deprotonated using NaOH to make trisodium citrate), were used as received without purification. All radioactive materials were produced and handled at the South African Nuclear Energy Corporation (Necsa, Pelindaba, South Africa) facilities and laboratories.

Two 5-mg samples of natural gold (^{197}Au) metal were weighed using an analytical balance (5-decimal place Mettler Toledo). One sample was used as natural gold, while the other sample (target) was irradiated in the SAFARI 1 20 MW research reactor situated at Necsa in a hydraulic position with a neutron flux of 0.5×10^{14} n·cm^{-2}·s^{-1} for 20 min to obtain ^{198}Au. Both Au samples were dissolved in aqua regia (5 mL) dried down (using heat) and reconstituted in 0.5–1 mL 0.005 N HCl to yield $HAuCl_4 \cdot HAuCl_4$ and [^{198}Au]$HAuCl_4 \cdot$[^{198}Au]$HAuCl_4$ in 0.05 N HCl [45], the starting material in the synthesis of AuNPs. The activity of the [^{198}Au]$HAuCl_4 \cdot$[^{198}Au]$HAuCl_4$ was measured using a CRC-15R dose calibrator (Capintec Inc., Ramsey, NJ, USA). The radioactive $HAuCl_4$ sample was used to synthesize the dual-radiolabeled AuNPs. The activity concentration of 1,5-[^{14}C]trisodium citrate was determined by liquid scintillation. Three counting solutions were used to determine the activity concentrations of the 1,5-[^{14}C]trisodium citrate. These solutions were prepared using a standard containing 10 μL (37KBq) of 1,5-[^{14}C]trisodium citrate whose volume was made up to 1 mL (stock solution). Five, then and one hundred microliters of the stock solution were added to 20-mL glass vials containing 15 mL of the liquid scintillation cocktail (Bioscint). The activity measurements in the vials were 8491, 14,420 and 139,818 disintegrations per minute (DPM), respectively.

An adaptation of the method published by Turkevich, *et al.* [46] and Frens [47] was used to synthesize sterile radioactive and natural AuNPs. The volumes of the prepared solution of radioactive [^{198}Au]$HAuCl_4 \cdot$[^{198}Au]$HAuCl_4$ in 0.05 N HCl and the non-

radioactive $HAuCl_4 \cdot HAuCl_4$ in 0.05 N HCl were diluted to 25 mL using deionized water to make 1 mM solutions. Solutions of hydrogen chloroauric acid were heated to the boiling point with vigorous stirring, and the reducing agents were added to the solutions and boiled under reflux for a further 30 min. For the non-radioactive synthesis, 2.5 mL of 38.8 mM trisodium citrate were used as the reducing agent. For the dual radiolabel synthesis, 2.5 mL (38.8 mM) of solution containing 1,5-[^{14}C]trisodium citrate (1.52 MBq: 600 µL, 1.07×10^{-3} mmol) and non-labeled trisodium citrate (1.9 mL: 9.743×10^{-2} mM) were used as the reducing agent. Figure 4 shows the adapted method used to synthesize dual-radiolabeled [^{14}C]citrate-[^{198}Au]AuNPs.

4.2. Characterization of Dual-Radiolabeled AuNPs

Both the radioactive and non-radioactive AuNPs were characterized using the same techniques to assess the impact of using radioactive precursors in the quality attributes of AuNPs. With the exception of the UV/Vis spectra, the radioactive sample was analyzed after 10 half-lives (27 days), when the radioactivity of the samples was low enough to be safely cleared from Necsa laboratories and analyzed in non-radiological laboratories.

The hydrodynamic size (Z-average size) and polydispersity index (PDI) of the nanoparticles was acquired by dynamic light scattering with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) operated in backscattering mode at 173° with a He–Ne laser beam (λ = 632.8 nm). For the zeta potential measurements, which were performed at 25 °C with a scattering angle of 90°, the particles were dispersed in aqueous solution with an average pH of 6.2. The experiment was done in triplicate, and the results were averaged.

The morphology and primary size distributions of AuNPs were determined using transmission electron microscopy (TEM) (FEI Tecnai G2, Eindhoven, The Netherlands). Specimens were prepared by drop casting of a 10-µL aliquot of a dilute NP solution on an Athene® grid (Plano GmbH, Wetzlar, Germany). At least 250 particles were used to determine the primary size distributions using ImageJ software (Version 1.48; National Institutes of Health, Bethesda, MD, USA).

UV/Vis spectra were recorded for both the radioactive and non-radioactive AuNP suspensions using a PerkinElmer LAMBDA 1050 UV/Vis/NIR spectrophotometer (Waltham, MA, USA). The spectra were also used to determine the concentration [48].

4.3. In Vivo Study

4.3.1. Animals

The study was conducted in accordance with the South African National Standard for the Care and Use of Animals for Scientific Purpose. Ethical approval was sought and granted by the North-West University (NWU) Ethical Committee. Twelve (12) male

Sprague Dawley rats, 8–10 weeks old, weighing 200–250 g, were used in the study. The rats were bred and procured from the Department of Science and Technology (DST)/NWU/Preclinical Drug Development Platform (PCDDP) Vivarium (Potchefstroom, South Africa) and housed in stainless steel cages in groups of 4. The rats were kept under standard environmental conditions (23 ± 1 °C, 55% ± 5% humidity and 12/12 h light/dark cycle) with water and food provided *ad libitum* throughout the study.

4.3.2. Experimental Design

The rats were randomly divided into 3 treatment groups (n = 4 per group; see Figure 6). The dual-radiolabeled [^{14}C]citrate-[^{198}Au]AuNPs suspensions were administered as a slow intravenous injection using the tail vein in Groups 1 and 2 and orally via gavage in Group 3. The administered doses were 90 µg (high dose) for Group 1 and 9 µg (low dose) for Group 2. The administered doses were within the ranges found in the literature [13]. The volume of all of the administrations was 500 µL. The accuracy of the dose was controlled using both the volume injected and the radioactivity of ^{198}Au. Any activity remaining in the syringe was measured and used to calculate the exact dose injected.

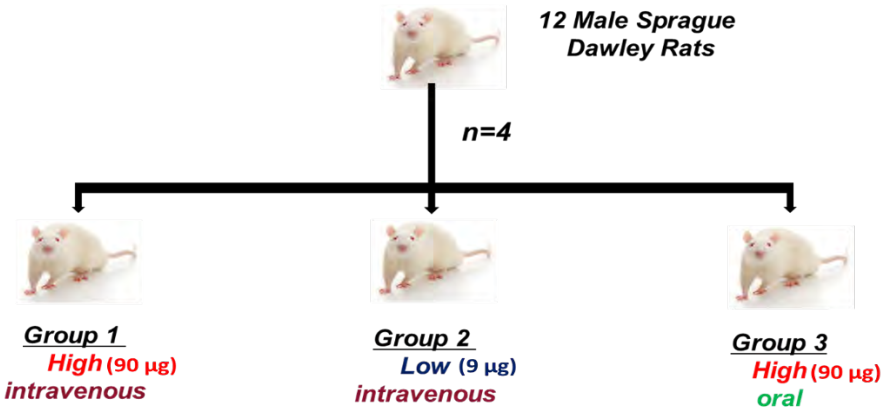

Figure 6. Study design of the animal experiment. Groups 1 and 2 received intravenous doses, while Group 3 got an oral dose.

After each administration, the rats were individually placed in metabolic cages to collect the total amount of urine and feces. All of the administrations were well tolerated with no apparent adverse events being observed during the 24-h study. The 24-h time point was selected based on acute biodistribution studies found in the literature [20,22]. At the termination of the study, the rats were euthanized using an overdose of Euthapent® (sodium pentobarbitone 200 mg/mL; Kyron laboratories, Johannesburg, South Africa), administered intravenously. Blood was collected using the cardiac puncture technique and stored without further processing before cutting open the chest cavity and abdomen of the rat to collect the liver, spleen and lungs. Together with the blood, these were used to

determine whether the gold core of the AuNPs is distributed in a similar pattern as the citrate surface coating, which is an indication of whether the NPs remain intact in physiological conditions. The mass of each sample, including the carcass, was measured and used in calculating the percentage of the injected dose per gram of the organ/tissue.

4.4. Quantification of Gold and Citrate in Samples

The quantification of citrate was done after at least 30 days (10 half-lives) of ^{198}Au, to avoid measuring the *beta* decay of ^{198}Au, as well. The quantities of citrate were determined only in the intravenous groups, since no absorption of Au was seen, thus negligible (≥0.001 %ID/g) amounts in the blood, liver, lungs and spleen (results not shown).

4.4.1. Gold

The ^{198}Au radioactivity of the blood, liver, lungs, spleen and the remainder (total remaining carcass) was measured without further sample preparation by γ-spectroscopy using a CRC-15R dose calibrator (Capintec Inc., Ramsey, NJ, USA) and a lead-shielded well-type NaI (TI) scintillation detector using the winTMCA32 software (FLIR Radiation, GmbH, Solingen, German). The counts were corrected for physical decay from the time of injection and any background radiation. A ^{198}Au standard prepared in the laboratory was used to correlate ^{198}Au radioactivities to the masses, numbers and surface areas of the AuNP suspension used in the study. To ensure that the entire administered dose was accounted for, the amounts of ^{198}Au in the total urine and feces and the total remaining carcass was measured.
A gamma spectrum of the ^{198}Au was also measured to give evidence that the signal measured was only from gold, and there were no interferences that may impede the reliability of the results.

4.4.2. Citrate

To measure the ^{14}C radioactivity in liver, lungs, spleen and blood, a known mass of approximately 200 mg of the liver, lungs and spleen and 500 μL of whole blood were added to a 20-mL glass scintillation vial. To each sample, 1–2 mL of the solubilizer (Biosol, National Diagnostics, Atlanta, GA, USA) were added, and the samples were incubated between 55 and 60 °C until the samples were completely solubilized or had a brown/green color in the case of the blood. The digestion times varied depending on the tissues (up to 5 h for liver). Two hundred microliters of 30% hydrogen peroxide (H_2O_2) were added in 2 aliquots to discolor the dissolved tissues. The samples were allowed to stand for 24 h. Scintillation cocktail (Bioscint, National Diagnostics, Atlanta, GA, USA) was added to fill up the vial to 20 mL. The samples were stored in a cool dark place and counted for 10 min using a Perkin-Elmer Tri-Carb 3100 TR scintillation spectrophotometer (Waltham, MA, USA). All measurements were done in triplicate.

4.5. Statistics

The statistical significance of the differences between the mean %ID/g values in the different groups was assessed by use of the non-parametric Mann–Whitney test and a Student *t*-test. Statistical probability (*p*) values less than 0.05 were considered significantly different.

3.5. Conclusions

With the present study, we have shown that the use of radioactive precursors does not have a negative impact on the physico-chemical properties of AuNPs, and dual radiolabeling is a good technique for studying the biodistribution of a multi-component nano-particulate system. The biodistribution profile of the Au core and citrate surface coating are different, and for the Au component, the biodistribution is dose dependent. At both dose levels, the majority of the Au accumulates in the liver and spleen, and an unexpected deposition in the lungs occurs after intravenous administration.

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Author Contributions

C.R. was involved in all aspects of the experiments and drafted the manuscript. A.G. and J.R.Z. were involved in the design of all of the experiments and reviewed the manuscript. N.B. was involved in the optimization of the transfer of the synthetic method to the radiochemical method and reviewed the manuscript. M.S. and D.J. were involved the handling of the radioisotopes, ^{14}C and ^{198}Au, respectively, during the preparation of dual-radiolabeled AuNPs and reviewed the manuscript. H.B. was involved in the design and conducting of the animal experiments. A.J. did the TEM analysis. H.S. was involved in the statistical considerations during the design of the animal studies and did the statistical analysis.

Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 4: Acute biodistribution, biopersistence and toxicity of 14nm gold nanoparticles after a single intravenous administration

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This manuscript reports on the acute biodistribution and biopersistence of AuNPs after a single dose over 56 days in male Sprague Dawley rats. The amount of gold in the tissues was quantified using neutron activation analysis at Necsa, the only facility with a nuclear reactor and a licence to handle radio isotopes. I did the sample preparation and part of this analysis for this technique. We plan to submit this work to the journal NANOMEDICINE: NANOTECHNOLOGY, BIOLOGY AND MEDICINE whose instructions to authors are included in Appendix 1.

Abstract

The safety of gold nanoparticles when intentionally administered for drug and vaccine delivery is not yet established and is a requirement for any clinical applications. In this study we synthesized and characterized 14 nm spherical citrated gold nanoparticles and used them to determine their acute biodistribution patterns and biopersistence in male Sprague Dawley rats. The endpoint of the acute biodistribution study was determined experimentally using imaging to reduce the number of rats. Serum metabolites and enzymes were monitored to assess the impact of exposure on organ toxicity together with general health assessments. After a single dose, gold nanoparticles distributed widely in the tissues with the highest percentage injected dose per gram found in the liver, spleen, lungs and femur respectively. In the biopersistence study after a single dose, the clearance from the liver, spleen, lungs and skeletal system was low indicating that gold nanoparticles might be biopersistent. No differences were noticed in the levels of markers of liver and kidney damage in the serum. No overt signs of toxicity were noticed in all the experimental groups. We conclude that gold nanoparticles widely distribute after an acute exposure and are biopersistent while not showing any signs of overt toxicity.

Keywords: Gold nanoparticles, acute biodistribution, Biopersistence, Sprague Dawley rats

4.1 Introduction

The real or perceived risk of engineered nanomaterials when intentionally administered as drug delivery vehicles is not yet established and as such the safety concerns are real. This is mainly because engineered nanomaterials have novel properties in terms of function and form and complex interactions with biological systems (Grainger, 2009). Gold nanoparticles (AuNPs) are versatile drug delivery vehicles (Kumar *et al.*, 2013; Paciotti *et al.*, 2004; Papasani *et al.*, 2012; Rana *et al.*, 2012; Vigderman & Zubarev, 2012) being able to deliver both chemical and biological drugs. Various *in vitro* studies showed consensus regarding the biocompatibility (and thus safety) of AuNPs (Connor *et al.*, 2005; Esther *et al.*, 2005; Goodman *et al.*, 2004; Hainfeld *et al.*, 2006; Merchant, 1998; Shukla *et al.*, 2005) but more recent evidence from *in vivo* studies suggests that AuNPs are toxic (Chen *et al.*, 2009; Cho *et al.*, 2009; Zhang *et al.*, 2010). This demonstrates that *in vitro* results do not always tally with *in vivo* so there is a clear need for more *in vivo* studies investigating the safety of AuNPs with clear dosimetry and methodologies that can be replicated.

AuNPs with a number of surface coatings have been used in biodistribution studies (Hirn *et al.*, 2011; Morais *et al.*, 2012; Semmler-Behnke *et al.*, 2008) and it is agreed that the type and charge of the coating have an influence on the biodistribution profile. Particle size also has an influence on the biodistribution profile and toxicity (Semmler-Behnke *et al.*, 2008; Sonavane *et al.*, 2008; Yang *et al.*, 2014; Zhang *et al.*, 2009; Zhang *et al.*, 2011b). The size range 10-20 nm is considered ideal for use as drug delivery vehicles and with regards to the biodistribution of the AuNPs the liver and splenic uptake is typically high (Hirn *et al.*, 2011; Sonavane *et al.*, 2008; Zhang *et al.*, 2011a). There is however a lack of information when one

considers the number of organs being reported in acute biodistribution studies which are relatively simple and much cheaper compared to the chronic and subchronic studies. A number of studies report on less than 12 organs (Cho *et al.*, 2009; Morais *et al.*, 2012; Semmler-Behnke *et al.*, 2008; Sonavane *et al.*, 2008; Yang *et al.*, 2014; Zhang *et al.*, 2011b) and there is limited information when it comes to more than 15 organs (Balasubramanian *et al.*, 2010). Thus there is a clear demand for further data on the acute biodistribution of AuNPs to be able to generalize the findings of these studies. Citrate coated AuNPs due to the ease of synthesis and nontoxic nature of the precursors (Connor *et al.*, 2005) make an ideal candidate to investigate the biodistribution of AuNPs.

Biopersistence refers to the length of time that an engineered nanomaterial (such as AuNPs) remains in a biological system. It is a function of the system's ability to clear the AuNPs. The few studies reporting on the biopersistence of AuNPs administered as a single dose in rodents (Balasubramanian *et al.*, 2010; Fraga *et al.*, 2014; Sadauskas *et al.*, 2009; Zhang *et al.*, 2011b) differ widely in the dosages of AuNPs used thus the results of these studies are not easily generalized. The different time points further complicate any attempt at generalizing the results. The rationale used during the selection of dosages, time points and organs to be analyzed for the content of Au is also not always clear. The doses used in the nanotoxicity studies also tend to mimic accidental exposure (Balasubramanian *et al.*, 2010) when stated or high toxic dosages (4mg of Au per kg) are used (Zhang *et al.*, 2011a). A clear need thus exists to use dose levels that resemble intentional use and several time points and assessment of quantities of Au in organs that are chosen systematically based on experimental results.

In this study we aimed to determine the endpoint of acute biodistribution using qualitative gamma imaging. This approach eliminated the need for multiple time points in the acute biodistribution study thus reducing the number of rats being used. In addition we aimed to determine the acute biodistribution profiles and biopersistence of AuNPs after a single intravenous injection. The Au content in the acute biodistribution study was quantified using gamma spectroscopy while instrumental neutron activation analysis was used for the biopersistence study. Furthermore we also aimed to assess any potential toxicity of AuNPs by monitoring physiological and behavioural indicators together with markers of kidney and liver damage. Histopathological assessment on organs of interests will also be done.

4.2 Materials and Methods

4.2.1 Preparation and Characterization of AuNPs

All chemicals used were of analytical grade. Spherical citrate coated non-radiolabeled and radiolabeled AuNPs, 14 nm in size were synthesized under sterile conditions using adapted modified Turkevich-Frens method (Frens, 1973; Turkevich *et al.*, 1953). Briefly a known mass (5 mg and 20 mg) of 24 Carat Au with natural isotopic composition was directly irradiated in a SAFARI 1 20 MW research reactor in a hydraulic position with a neutron flux of 0.5×10^{14} n.cm^{-2} s^{-1} for 20 min to obtain ^{198}Au. The radioactive gold (^{198}Au) was dissolved in aqua regia, dried and reconstituted in 0.5-1 mL 0.005 N HCl to yield H^{198}AuCl4.H^{198A}uCl4 in 0.05 N HCl (Katti *et al.*, 2006), the starting material in the synthesis of [198Au]AuNPs. For the non-radioactive AuNPs, gold (III) chloride- (≥99.9 % trace metal basis) ($HAuCl_4.3H_20$) (Sigma, St. Louis, MO, USA) was used as the starting material. A

100 mL solution of hydrogen chloroauric acid (1 mM) was brought to boil and 10 mL solution of trisodium citrate (38.8 mM) was added and the solution was boiled under reflux for 30 min.

The average particle size and morphology of the colloidal suspensions were determined using transmission electron microscopy (TEM) (FEI Tecnai G2, Eindhoven, The Netherlands). At least 250 particles were used to determine the primary size distributions using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA). The radioactive samples were analyzed after the activity had decayed to non-radiological levels. The UV-vis absorption spectra were obtained using a LAMBDA 1050 UV/Vis/NIR spectrophotometer (PerkinElmer, Massachusetts, USA). Concentrations of the prepared AuNPs; molar, number, mass and surface area were calculated using size determined by TEM, mass of gold salt used and making the assumptions that the reaction goes to completion and the particles are spherical (Liu *et al.*, 2007). The hydrodynamic size and zeta potential were determined using dynamic light scattering (DLS) using a Zetasizer Nano (Malvern Instruments, Worcestershire, UK) at 25°C. The average pH of the colloidal suspensions was 6.2.

4.2.2 *In vivo* Studies

4.2.2.1 Animals

The studies were conducted in accordance with the South African National Standard for the Care and Use of Animals for Scientific Purpose. Ethical approval was granted by the NWU Animal Ethics Committee and a total 46 male Sprague Dawley rats 8-10 weeks old weighing 250-300g were used. The rats were bred and procured from the DST/NWU/PCDDP

Vivarium (Potchefstroom, South Africa) and housed in stainless steel cages in groups of four. The rats were kept under standard environmental conditions with access to water and food *ad libitum*.

4.2.2.1.1 Experimental Design

Three studies were conducted; an imaging study (48 h) that served as a pilot study to determine the end point of the acute (24 h) study and the biopersistence (56 days) study. In all the studies the 500 µL of AuNPs was administered using a slow intravenous (*i.v*) injection via the tail vein. The volume is within the acceptable volume range for slow *i.v* injections for rats (Diehl *et al.*, 2001) and the administration was well tolerated with no adverse events being observed during the study.

4.2.2.1.2 Pilot: Imaging study

In the first study, [^{198}Au]AuNPs synthesized using 5 mg of Au (higher activity concentration: 3.92 GBq/mL) were administered to 2 rats as a single *i.v* dose with a mass concentration of 90µg of Au. A Siemens pinhole collimator (GE Healthcare, Little Chalfont, UK), was used to acquire dynamic and static images. During image acquisition the rats were lightly sedated using Isoflurane administered via inhalation and imaged from the prone position. During the 1st hour, dynamic images were taken followed by static images taken at 1, 2, 4, 6, 24 and 48 hours post administration. Image analysis to obtain the qualitative biodistribution data was done by manual delineation of regions of interests (ROIs). The pilot study was conducted to reduce the number of rats used in the acute biodistribution study. During the experiment the

rats were housed in steel cages and kept under a 12 h night and day cycle with food and water available *ad libitum*.

4.2.2.1.3 Acute biodistribution study

In the second study [^{198}Au]AuNPs synthesized using 20 mg of Au (lower activity concentration: 0.98 GBq/mL) were administered *i.v* to 8 rats divided into 2 treatment groups (n=4). Each rat received either 90 µg or 9 µg dose of Au (mass concentration) as [^{198}Au]AuNPs (radiolabeled). After administration of the formulations all rats were housed in metabolic cages where all the urine and feces were collected with food and water available *ad libitum*. General health assessments were performed by monitoring physiological and behavioral indicators (Clark *et al.*, 1997) on all the rats during the study and after 24 h the rats were heavily anesthetized using pentabarbitone (Euthapent®). Through the skin blood was collected using the cardiac puncture technique and this lead to the death of the rat. The following organs/tissues were collected and their respective masses noted; esophagus, stomach, small intestines, large intestines, cecum, and rectum, liver spleen, heart, lungs, brain, muscle, bone, tail(site of injection), and skin. The amount of Au in the tissues and organs was determined using gamma spectroscopy using CRC-15R dose calibrator (Capintec Inc., New Jersey, USA) without further sample preparation

4.2.2.1.4 Biopersistence study

In the third study AuNPs synthesized using non-radioactive $HAuCl_4$ were used to the test rats while the controls received normal saline (see Figure 1). Non-radiolabeled AuNPs were administered in the biopersistence study because the half-life of ^{198}Au is 2.7 days. A total of

36 rats were used; 24 received the test formulation: 90 µg of Au (mass concentration) as AuNPs in 500 µL and 12 received the control: 500 µL of normal saline, both groups received the dose *i.v* via the tail vein. After administration of the injections the rats were housed in groups of 4 in steel cages and kept under a 12 h night and day cycle with food and water available *ad libitum*.

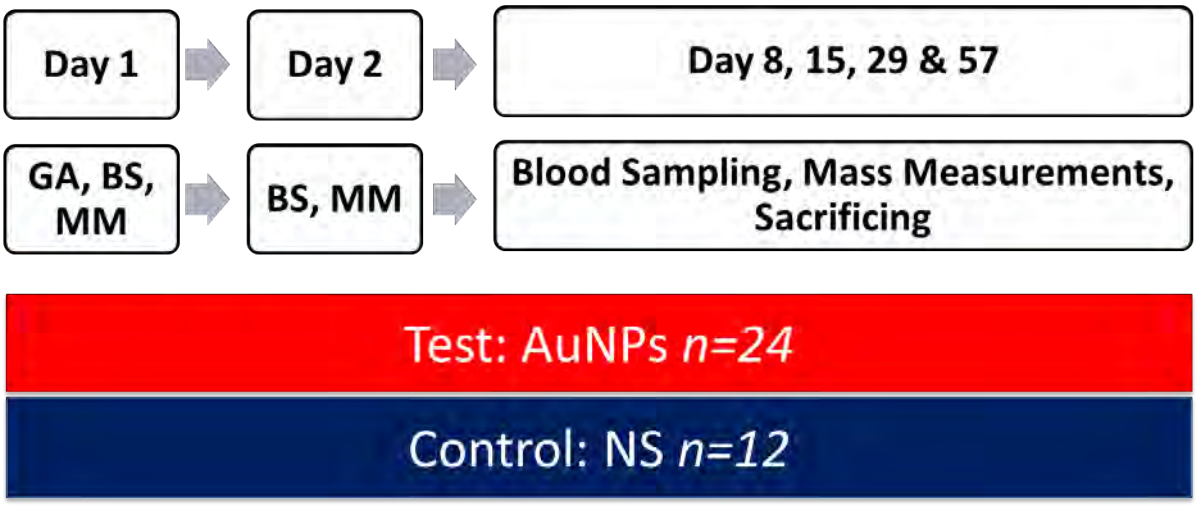

Figure 1 Biopersistence study design showing activities done in the study. GA-group assignment; BS-blood sampling and MM-mass measurements

General health assessments were done by monitoring physiological and behavioural indicators twice weekly (Clark *et al.*, 1997) and their masses measured on days 1, 2, 8, 15, 29 and 57. A total of 9 rats were sacrificed (6 tests and 3 control) using an overdose of pentobarbitone on days 8, 15, 29 and 57 and used as described in the sections below.

4.2.2.1.5 Markers of liver and kidney damage

On day 1 before administration of either; the test or control formulation and days, 2, 8, 15, 29 and 57 blood samples were collected. The blood was drawn into tubes with a clot activator and serum separation gel (BD Vacutainer®, New Jersey, USA). The tubes were centrifuged at 2 800 g for 15 min and stored at -80 °C until analysis Levels of liver enzymes alanine transferase (ALT) and alkaline phosphatase (AST), metabolites creatinine (Creat) and total bilirubin (BIL T) and blood urea (Urea) were measured using the Cobas 6000 (Roche Diagnostics, Basel Switzerland) to assess liver and kidney damage. The long duration of the study (56 days) and frequency of the blood sampling limited the volumes of blood that could be drawn and the tests performed, thus only hepatocellular evaluation was performed. Kidney damage was assessed using the serum metabolites only due to the long duration of the study as well. Glomerular filtrations rates were not possible to measure because use of metabolic cages was not possible.

4.2.2.1.6 Histopathological assessment

Tissues were collected from 3 test and 3 control rats on days 8, 15, 29 and 57; six rats from the test group and three from the control were fixed in 10% formalin and stored at room temperature until histopathological analysis was done by an independent laboratory (IDEXX, South Africa). The heart, kidneys, liver lungs and spleen were used in the analysis and images were taken using an Olympus Light Microscope.

4.2.2.1.7 Neutron Activation Analysis

The liver, lungs, skeleton, spleen and carcass of 3 rats in the test group were collected and weighed and used to quantify the amount of Au using neutron activation analysis (NAA)(Buzulukov *et al.*, 2014; Hillyer & Albrecht, 1998; Hillyer & Albrecht, 2001). Briefly, all the samples were dried in an oven at 65 °C for 48 h, and then ashed over 6 h at 650 °C. The final mass of the total ash was noted for each sample and approximately 200 mg was weighed and placed in a trace element free polyethylene flip-top vial. The vials were sealed by friction welding and placed in bigger vials which were also sealed by friction welding. The samples were irradiated in a SAFARI 1 20 MW research reactor in a RINGAS pneumatic system for 8 s using a neutron flux of 10^{14} $n.s^{-1}.cm^{-2}$ triggering the reaction ^{197}Au n, γ ^{198}Au. Standards with 0.1, 0.5, 1 and 2 μg of Au in containers with the same geometry as the sample holders were run with the experimental samples and used to calculate the amount of Au present in the samples. Blank samples were also irradiated for background correction. The γ decay energies of the samples were counted using a well-type high purity germanium (HPGe) (NATS, Middletown, USA) detector coupled to Genie 2000 program software. Using the activities measured in the standard samples the amount of Au in each sample was calculated.

4.2.3 Calculations and statistical analysis

The statistical significance of the differences between the mean values in the different groups was assessed using mixed linear models which took into account repeated measures (Wang & L. A. Goonewardene, 2004). Statistical probability (p) values less than 0.05 were considered significantly different.

4.4.3 Results

4.3.1 Synthesis and characterization AuNPs

The morphology and size distribution of the prepared AuNPs is shown in Figure 2 which is a representative of both the radioactive and non-radioactive preparations. The UV/Vis spectra showed the characteristic peak around 530 nm with no secondary peaks and low absorbance in the 600-700 nm wavelength region which indicates that the formulation was well dispersed with no agglomerates (Shim & Gupta, 2007). The zeta potential, which was negative because of the citrate surface coating and hydrodynamic size, is shown in Table 1 together with other dosimetric parameters.

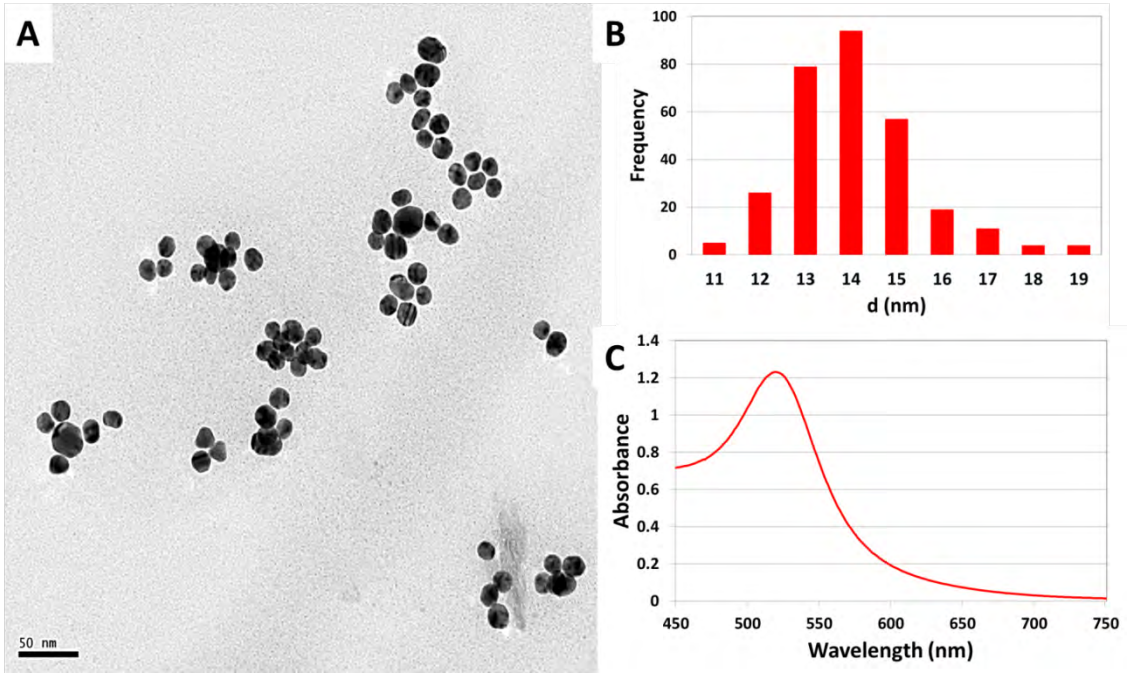

Figure 2 TEM image (A), particle size distribution (B) and UV/Vis Spectra (C) of AuNPs representing all the AuNPs used in the studies, both radioactive and non-radioactive

4.3.1.1 Dosimetry

Table 1 Physicochemical properties representative of both the radioactive and non-radioactive AuNPs used measured using the non-radioactive AuNPs

Primary particle size	14 nm	
Hydrodynamic particle size	25 nm	
Zeta potential	-47 mV	
Administered mass of AuNPs (μg) per rat	90	9
Administered number of AuNPs per rat	3.27×10^{12}	3.27×10^{11}
Administered surface area (cm^2)	20.155	2.0155

All AuNPs used in the studies reported in this paper were synthesized using the same method. The characteristics shown in Table 1 are representative of all the formulations used with regards to the mass and nanoparticle number concentrations as well as the surface area. The particle sizes both primary and hydrodynamic were comparable and can be considered to be 14 nm and 25 nm respectively.

4.3.2 Imaging study

The dynamic images (taken over 1 h) showed rapid translocation of the AuNPs to the liver and spleen area in both test subjects. After manual delineation of the ROIs in the static images (see Figure 3) the qualitative biodistribution data of AuNPs over 48 hours in male Sprague Dawley rats was obtained. The images taken at 24 and 48 h (see Figure 3) show no differences indicating that there were no further translocations of the AuNPs after 24 h. These results were used to select 24 h as the end point of the acute biodistribution study.

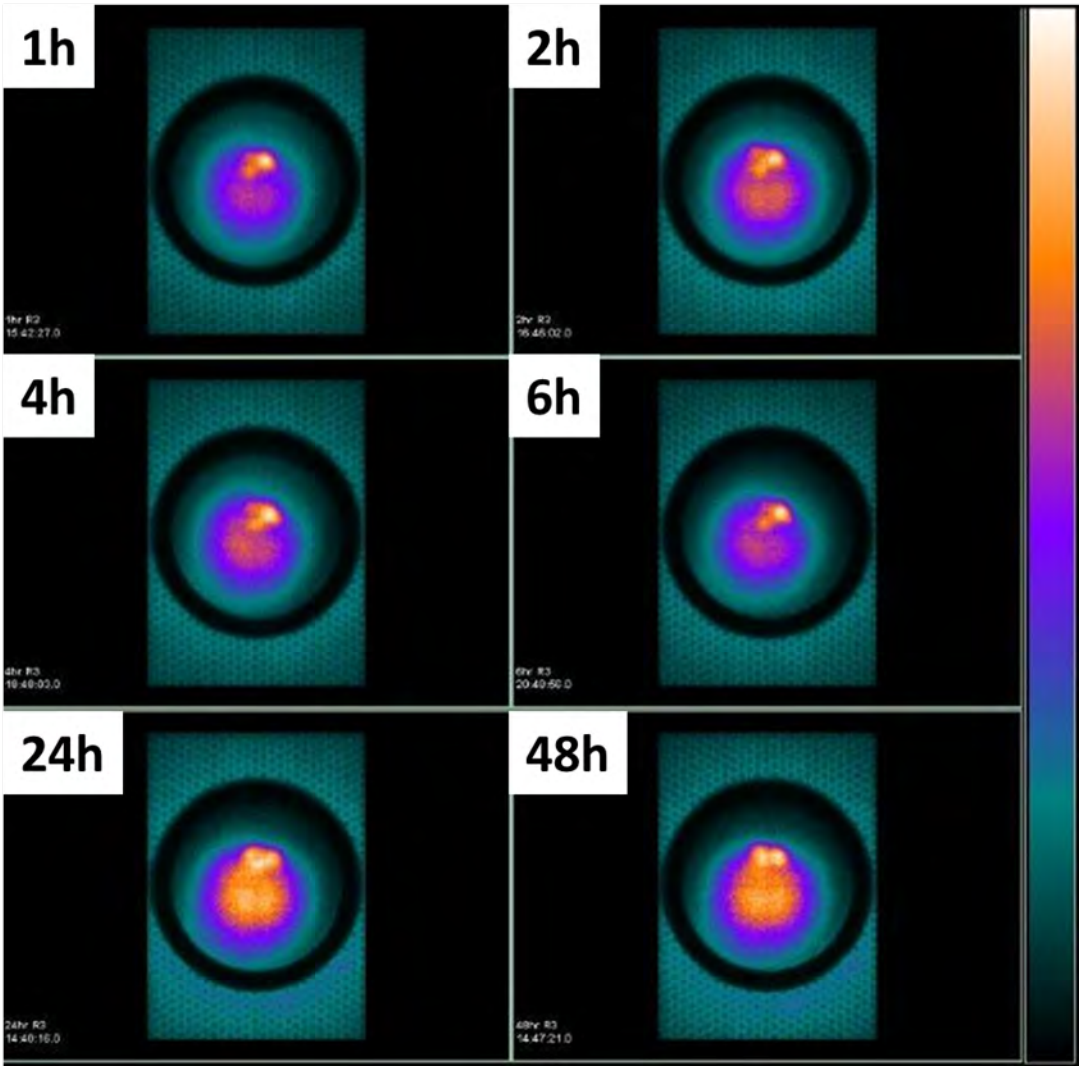

Figure 3 Static images showing qualitative biodistribution of AuNPs after intravenous administration over 48 h. The band that ranges from black to orange on the right side of the photo indicates the activity concentration of the Au with orange being more concentrated.

4.3.3 Acute biodistribution of AuNPs

4.3.3.1Biodistribution profiles

The acute biodistribution profiles of AuNPs after a single dose show wide systemic distribution of the Au at the 2 dosing levels used (see Table 2). However there were

differences in the biodistribution profiles based on the administered dose, (90 µg versus 9 µg) especially in the liver and spleen. The percentage injected dose per gram (%ID/g) was higher at the low dosage (9µg) in all organs/tissues except the liver. Bone and lung deposition at both dose levels was observed.

Table 2 Amounts of Au in tissues/organs 24 h after a single intravenous injection. The amounts are shown as percentage of the injected dose per gram (%ID/g) and mass of Au (µg) per gram of the respective organ/tissue. Each value is a mean of 4 rats. L. Int: Large intestine, S. Int: Small Intestine

	90 µg		9µg	
	%ID/g ± Std	**Au µg/g**	**%ID/g ± Std**	**Au µg/g**
Liver	8.24 ± 0.91	7.4	3.22 ± 0.58	2.9E-01
Spleen	1.78 ± 0.61	1.6	3.21 ± 0.68	2.9E-01
Lungs	0.13 ± 0.03	1.2E-01	0.17 ± 0.04	1.5E-02
Bone	0.08 ± 0.02	7.1E-02	0.10 ± 0.03	9.1E-03
Faeces	0.01 ± 0	1.0E-02	0.05 ± 0.02	4.7E-03
Stomach	0.00	2.7E-03	0.04 ± 0.06	3.6E-03
Tail	0.02 ± 0	1.4E-02	0.03 ± 0.03	2.7E-03
Oesophagus	0.01 ± 0	5.3E-03	0.02 ± 0.01	2.1E-03
Kidneys	0.01 ± 0	1.3E-02	0.02 ± 0.01	1.9E-03
Heart	0.01 ± 0	6.9E-03	0.01 ± 0	1.1E-03
Cecum	0.01 ± 0	5.2E-03	0.01 ± 0	7.7E-04
Blood	0.01 ± 0	5.9E-03	0.01 ± 0	7.5E-04
S.int	0.00%	1.3E-03	0.01 ± 0	5.1E-04
L.int	0.00%	2.1E-03	0.00%	4.3E-04
Muscle	0.00%	2.0E-03	0.00%	3.9E-04
Skin	0.00%	3.6E-03	0.00%	2.7E-04
Brain	0.00%	8.5E-04	0.00%	2.6E-04
Testes	0.00%	8.7E-04	0.00%	2.4E-04
Bladder	0.00%	7.8E-04	0.00%	2.1E-04

4.3.4 Biopersistence study

4.3.4.1 Amount of Au in tissues

The amount of Au in the tissues was quantified using NAA. Compared to the amount after 24 h there was a steady decrease of the amount of Au in the tissues/organs analyzed (see Figure 4). The Au was cleared from the tissues/organs albeit at different rates with the liver showing the fastest clearance. After 56 days the amount in the liver in terms of mass of Au per gram was still 1.2 µg/g of liver despite the fastest clearance, indicating that Au is biopersistent. The skeleton has a higher amount of Au on day 57 but within the same range and comparable to the preceding time points.

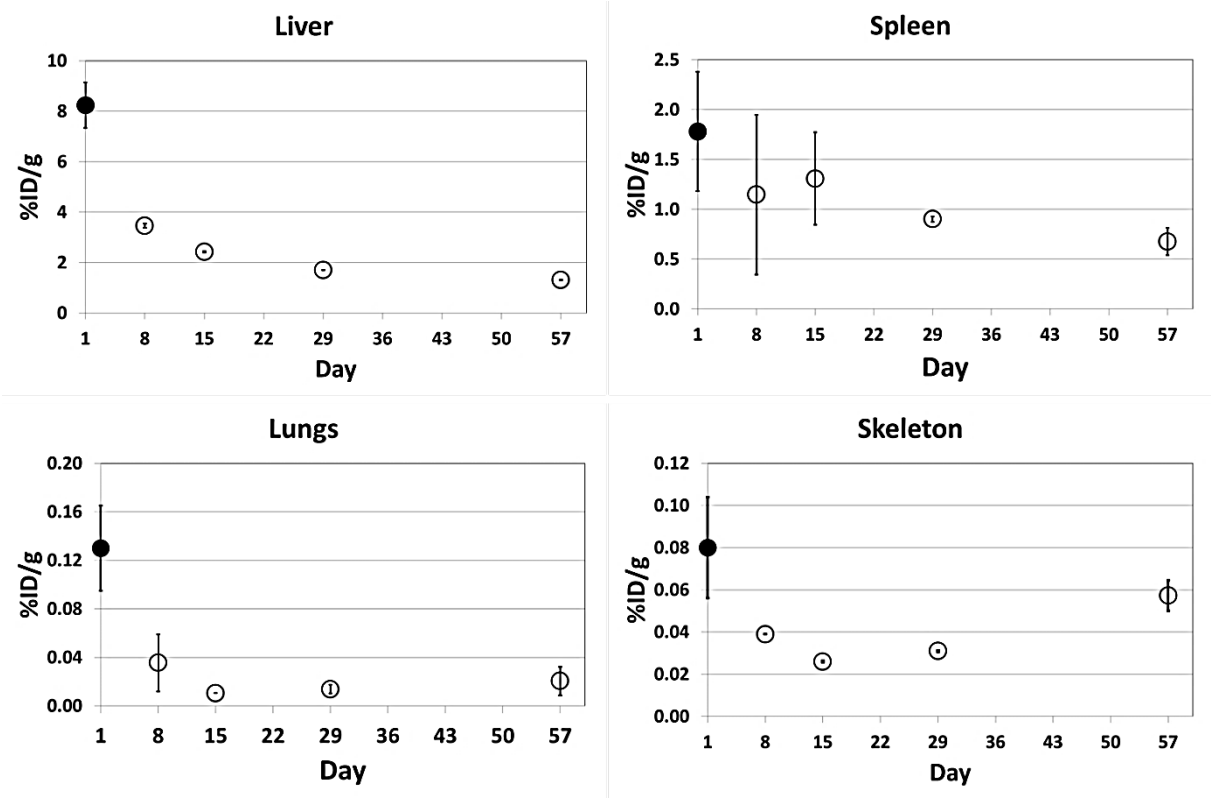

Figure 4 %ID/g of Au in the liver, spleen, lungs and bones after a single intravenous injection over 56 days. The amount on day 1 for each organ/tissues was quantified using γ-spectroscopy (acute biodistribution study) whilst the rest was quantified using NAA.

4.3.4.2 Toxicological Studies

During the study all the rats' monitored physiological and behavioural indicators did not reveal any signs of overt toxicity. The rats in both groups were alert and feeding normally and no rat had to be prematurely sacrificed due to distress or other ethical considerations. All rats showed a steady weight gain (see Figure 5).

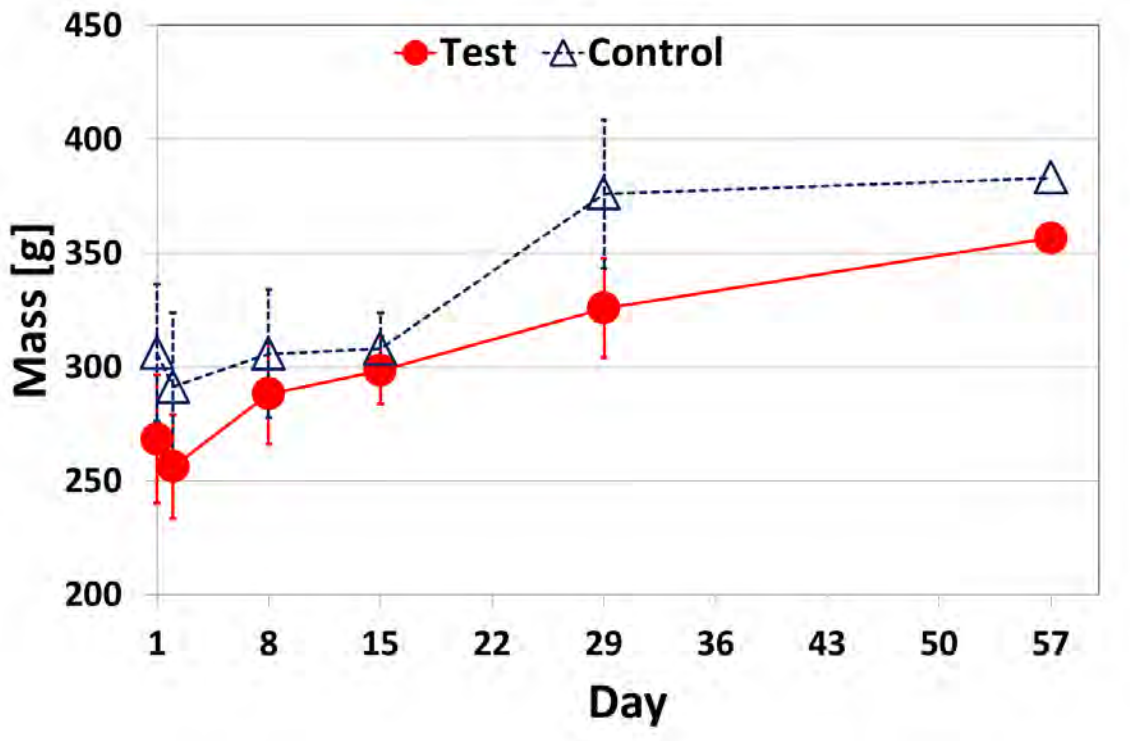

Figure 5 A comparison of the change in mass over time of the test and control groups. The general trend shows a steady increase in mass which is normal for the species used.

4.3.4.2.1 Markers of liver and kidney damage

Alanine transferase (ALT), alkaline phosphatase (ALP) and total bilirubin (BIL T) monitoring showed no differences between the tests and control groups (see Figure 6). Levels of ALP were consistently higher in the test group even though this difference was not statistically significant. Similar trends were observed when markers of kidney damage were monitored; no differences were noted between the test and control groups.

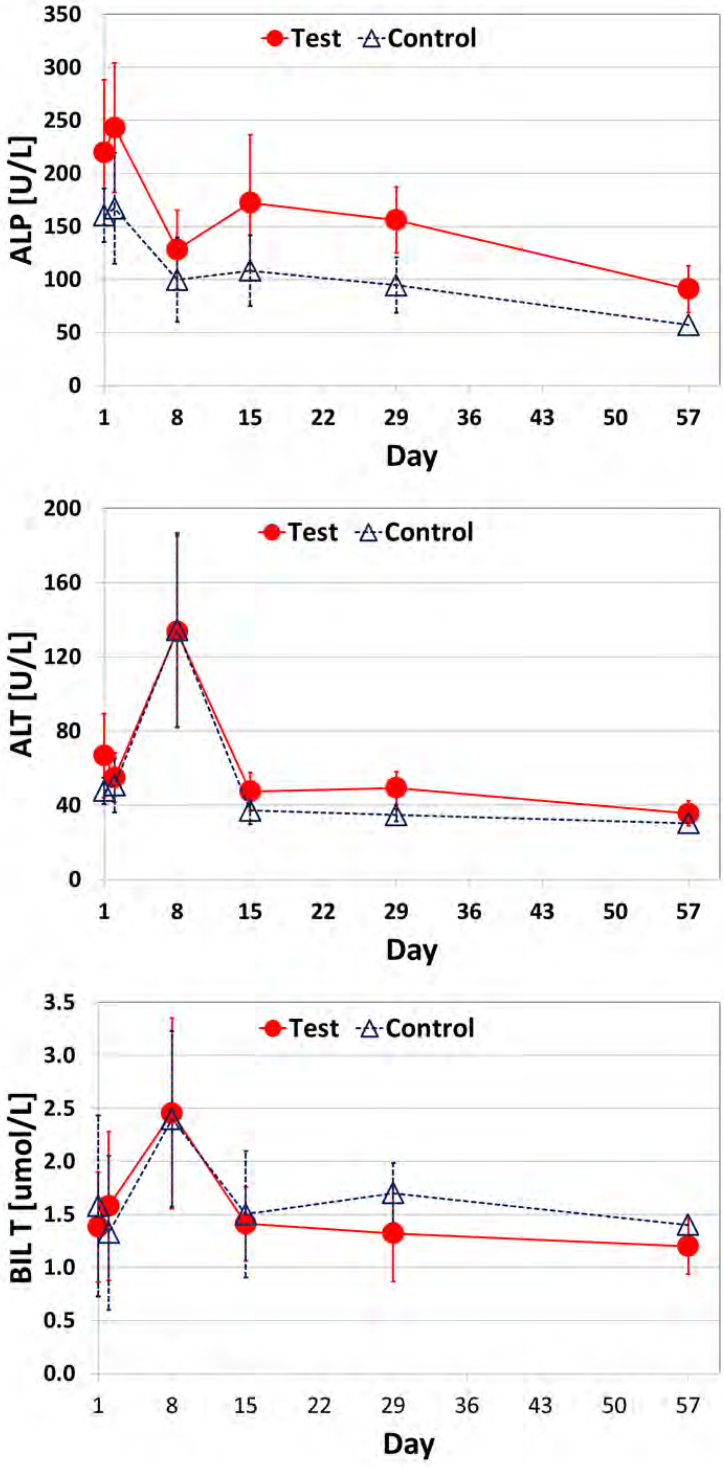

Figure 6 Time dependent serum analysis monitoring makers of liver damage over 56 days. ALP= alkaline phosphatase, ALP= alanine transferase, BIL T= total bilirubin

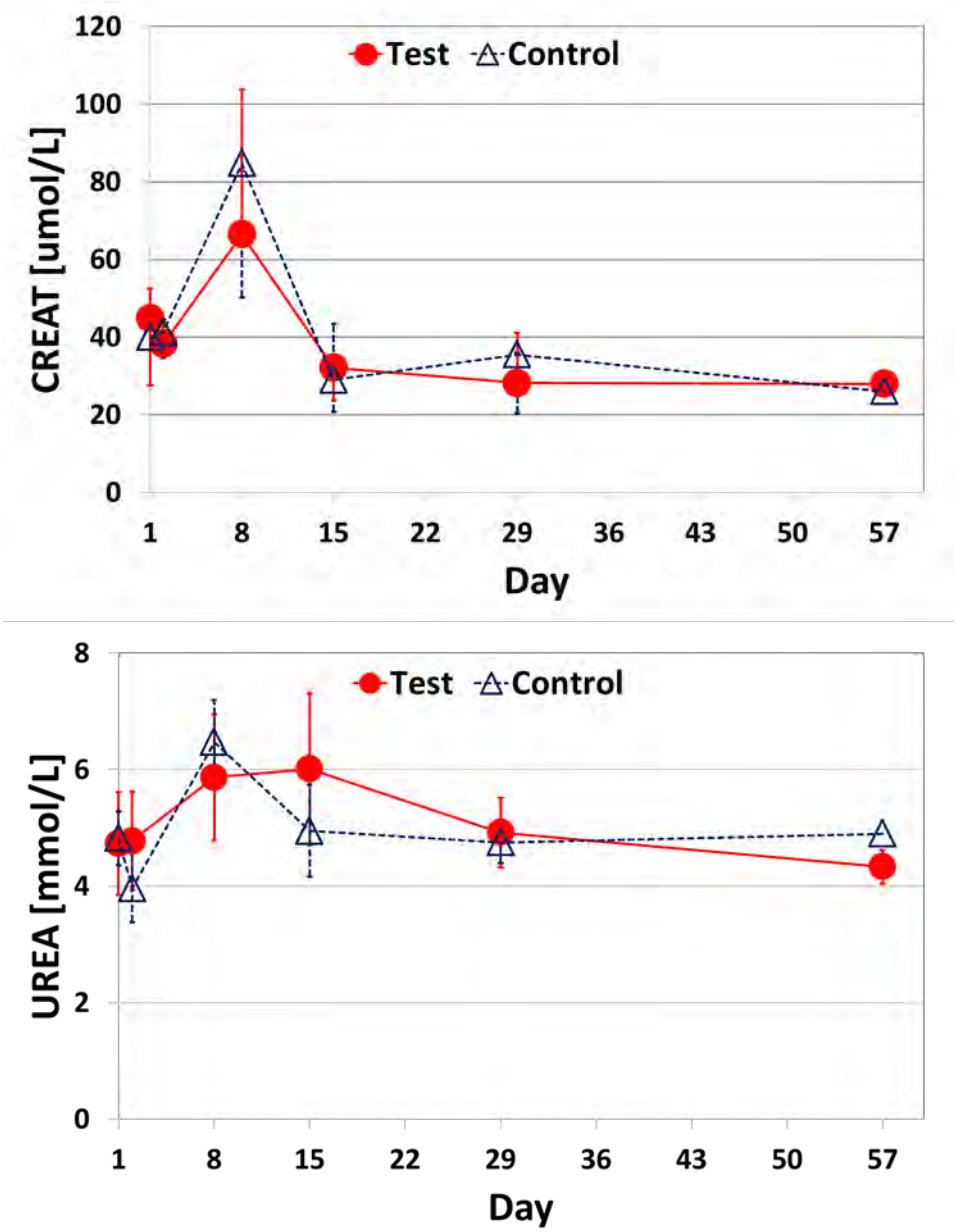

Figure 7 Time dependent serum analysis monitoring markers of kidney damage over 56 days. CREAT=creatinine and UREA=urea nitrogen.

4.3.4.2.2 Histopathological analysis

No abnormalities were detected in both the test and control groups at the time points used in the study. See Figure 8 for representative images of the liver, spleen, lungs and kidneys used in the study.

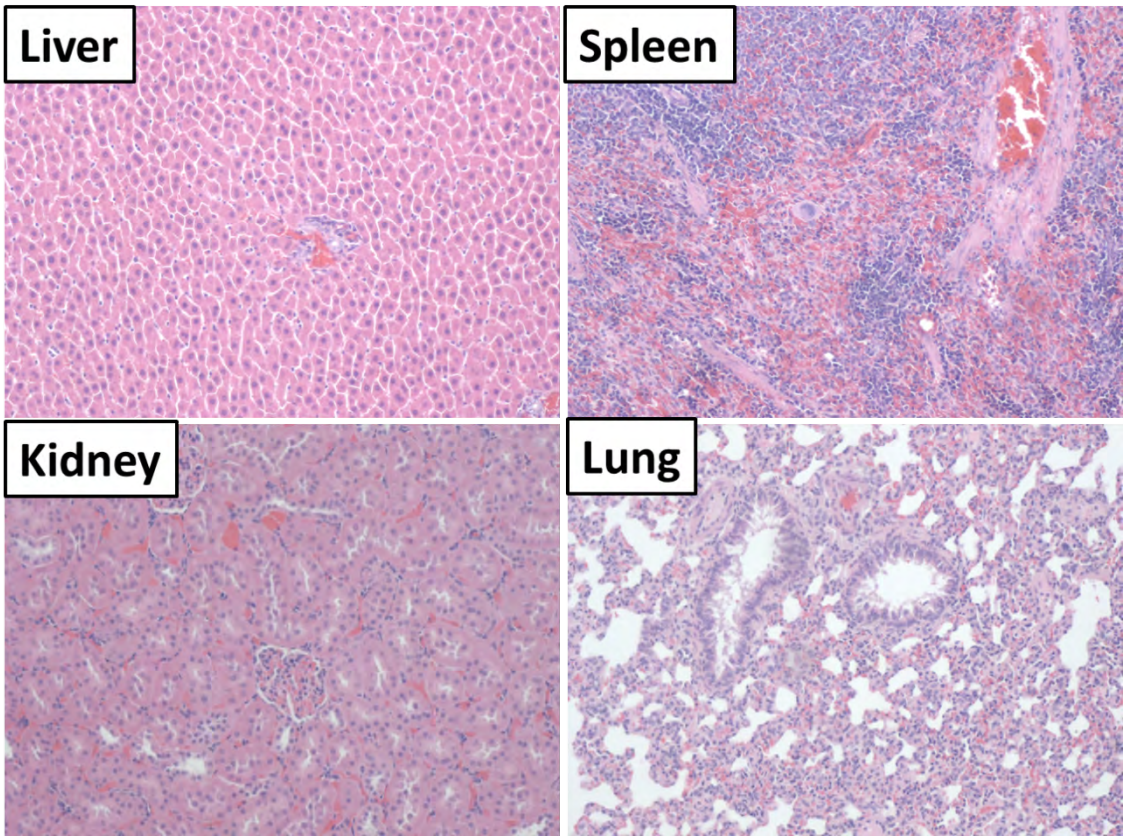

Figure 8 Images taken for histopathological analysis of the liver, spleen, lungs and kidneys.

4.4 Discussion

Progress in the use AuNPs for biomedical applications has been slow due to safety concerns. The paucity of information that currently exists with regards to the safety issue, tissue retention and slow elimination have been cited as the major obstacles (Fraga *et al.,* 2014). It is now generally accepted that physicochemical properties of certain engineered nanomaterials (Au included) have an impact on their biological outcomes (Fadeel *et al.*, 2013; Tay *et al.,* 2014; Verma & Stellacci, 2010). A thorough understanding of citrate coated AuNPs, which are extensively used in biomedical applications, will form a good basis of comparison with other AuNPs stabilized or coated with different ligands/materials. This will enable a better understanding of the changes, if any, that will ensue after a change in surface coating or stabilizing material. In this paper we present the qualitative and quantitative biodistribution profiles of Au obtained using synthesized radiolabeled AuNPs ([^{198A}u]AuNPs). The biopersistence patterns over 56 days of Au after a single dose are also presented together with results of toxicological endpoints.

The endpoint of the acute biodistribution study of AuNPs was determined using an imaging study. This was done to reduce the number of rats in the experiments while refining the protocol as well in line with the principle of three Rs (Forni, 2007). Synthesizing [^{198}Au]AuNPs starting with ^{198}Au enabled us to manipulate the activity of concentration of the colloidal dispersion while maintaining a good quality dispersion with no agglomerates/aggregates. The higher activity concentration used in the imaging study compared to the one in the acute biodistribution study allowed images to be obtained thus the determination of the acute biodistribution endpoint. The radioactive doses used in all the

experiments did not have any adverse effects to the rats' health in the studies as evidenced by the general health indicators monitored.

The acute biodistribution results showed a wide systemic distribution at the 2 dose levels used. This corresponds well with previous studies using AuNPs in the same size range (Hirn *et al.*, 2011; Lipka *et al.*, 2010; Semmler-Behnke *et al.*, 2008; Sonavane *et al.*, 2008; Terentyuk *et al.*, 2009). The liver, spleen, lungs and bones had the highest amount of Au deposition in that order. Translocation of Au to the lungs has previously been reported (Balasubramanian *et al.*, 2010; Cho *et al.*, 2009) and can be explained by the huge number of alveolar macrophages that clear all the foreign materials. Use of AuNPs to passively target the lungs can be further investigated based on these observations however it is important to determine the exact location in the lungs. Determination of the exact location can be difficult considering that the state of the AuNPs (discrete particles versus aggregates) is not known after intravenous administration. Attempts which appear futile have been made to find tissue localizations of Au after *i.v* administration (Cho *et al.*, 2009). We, as other researchers before (Balasubramanian *et al.*, 2010; Lipka *et al.*, 2010; Saterborg, 1973; Zhang *et al.*, 2011a) noted that there was bone deposition of Au after *i.v* administration however our results indicate that higher amounts are deposited compared to the previously published data (Balasubramanian *et al.*, 2010). The skeletal system in a mammal accounts for between 35 and 50 % body weight and is the place where stem cells are made. The exact location of the Au in the bone tissue (marrow, cancellous, cortical or poriosteum) is not known and it is crucial that a proper risk assessment be done. Deposition in the different parts will mean exposure to different cell types for instance the impact of bone deposition during periods of

rapid growth is also vital as AuNPs have been proposed for use in the delivery of vaccines which are often given to children. Au was detected in the feces in the acute biodistribution study, this supports the clearance mechanism via biliary route which has been proposed (Fraga *et al.*, 2014; Hirn *et al.*, 2011). The renal system has also been reported as a common pathway for the clearance of AuNPs after intravenous administration (Lipka *et al.*, 2010) even though we did not measure any significant amount in the renal system during the acute biodistribution study.

To assess the biopersistence of AuNPs in tissues/organs after a single dose, the amount of Au in the liver, spleen, lungs and skeletal system was measured over 56 days on day 8, 15, 29 and 57 after administration of a single dose. The amounts in the same tissue/organ were compared to that found 1 day after administration from the acute biodistribution study. There was a steady decrease in the amounts measured and the results indicate that Au is biopersistent in the tissues/organs studied at the dose level used. Other studies that investigated the fate of AuNPs after a single dose, also showed that Au is biopersistent (Balasubramanian *et al.*, 2010; Fraga *et al.*, 2014; Sadauskas *et al.*, 2009; Zhang *et al.*, 2011a) but the analytical methods used to quantify the amount of Au were not as sensitive as NAA and the durations vary. Our selection of the tissues/organs was based on the results of the acute biodistribution study (see Table 2). From Figure 4 the liver had the highest clearance due to the reported hepatobiliary system (Fraga *et al.*, 2014; Hirn *et al.*, 2011) rate compared to other organs/tissues. Faster clearance in the liver has been reported previously (Balasubramanian *et al.*, 2010) and it is important to note that accidental exposure doses were used in the study whilst we used a dose that was mimicking intentional exposure. The several

time points and length of our study give a good indication of the retention times of Au after a single intravenous dose which is within the nontoxic range. As is the case with most nanotoxicity studies of AuNPs our results are not easily comparable to those reported (Fraga *et al.*, 2014) due to limited time points they used, and the low sensitivity of their method of detection.

Histopathology analysis did not reveal any changes in the morphology of the tissues examined (heart, kidney, liver, lungs and spleen). There was no peracute or acute toxicity observed in the study. The control and test groups showed a similar steady rise in body weight with no abnormal physiologic or behavioural activities. Similar trends have been previously observed (Fraga *et al.*, 2014). Indicators of liver and kidney damage were comparable between the 2 cohorts this giving inconclusive information with regards to the organ specific toxicity.

4.5 Conclusions

AuNPs have the potential to be versatile drug delivery vehicles; however they demonstrated prolonged retention in liver, spleen, lungs and skeletal system. Use of citrate coating on AuNPs might limit their use in drug delivery unless the liver is the target organ. At this size range and doses used AuNPs did not cause any overt acute or delayed toxicity and kidney and liver damage. The histopathological results indicated that AuNPs did not cause any alterations in normal function. Gamma spectroscopy and NAA are good sensitive methods to study the biodistribution of Au *in vivo* as they have high sensitivity.

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Chapter 5: Bioaccumulation and subchronic toxicity of 14 nm gold nanoparticles

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This manuscript reports on the bioaccumulation and subchronic toxicity of AuNPs after multiple doses over 56 days in male Sprague Dawley rats. The amount of gold in tissues was quantified using neutron activation analysis at Necsa, the only facility which has a nuclear reactor and is licensed to do the analysis in the South Africa. I did all the sample preparation and part of the data analysis for this procedure. This work has been submitted to the International Journal of Nanomedicine (Submission ID: 91512). The guidelines to authors are attached in Appendix 1.

Abstract

Colloidal suspensions of 14 nm gold nanoparticles (AuNPs) were repeatedly administered intravenously at 3 dose levels (0.9, 9 and 90μg) to male Sprague Dawley rats weekly for 7 weeks, followed by a 14 day washout period. After sacrificing, the amount of gold was quantified in the liver, lungs, spleen, skeleton and carcass using Neutron Activation Analysis. During the study, pre- and post (24 h) administration blood samples were collected from both the test and control groups, the latter which received an equal injection volume of normal saline. General health indicators were monitored together with markers of kidney and liver damage for acute and subchronic toxicity assessment. Histopathological assessments were done on the heart, kidneys, liver, lungs and spleen to assess any morphological changes as a result of the exposure to AuNPs. The mass measurements of all the groups showed a steady increase with no signs of overt toxicity. The liver had the highest amount of gold (μg) per gram of tissue after 56 days followed by the spleen, lungs, skeleton and carcass. Markers of kidney and liver damage showed similar trends between the pre and post samples within each group and across groups. The histopathological examination also showed no hepatotoxicity and nephrotoxicity. There was accumulation of Au in tissues after repeated dosing albeit with no observable overt toxicity and kidney and liver damage.

KEYWORDS: gold nanoparticles, bioaccumulation, liver and kidney damage, Sprague Dawley rats

5.1 Introduction

Gold nanoparticles (AuNPs) present a good strategy for drug and gene delivery as they can deliver a wide variety of cargo as described in reviews and proof of concept studies[1-4]. In addition, a nanomedicine using AuNPs as a delivery vehicle has been assessed in a phase I and pharmacokinetic study for cancer[5]. Several features of AuNPs make them well suited for drug delivery and the recent advances in the synthesis and characterization techniques (ENMs)[6] enable the biomedical applications to be exploited easily.

Risk is a function of the product of hazard (a material property), susceptibility of the organism and the exposure. Different models have been used to assess susceptibility[7,8], however, the bulk of toxicity assessment studies for AuNPs varied mainly the exposure by altering the nanomaterial properties. Nanomaterial properties that can be altered include but are not limited to; shape, size, surface area, surface charge and concentration. The influence of particle size on acute biodistribution and toxicity has been extensively studied[7,9-13], however, there is a dearth of information when it comes to longer studies. Likewise, surface properties have been investigated mainly in short term studies[8,11,14-16]. From the published acute studies, there is a general agreement that liver and spleen uptake is high[12,16-18]. The influence of route of administration has been investigated in acute studies as well[19]. Considering what has been done, the information available is still insufficient to draw general conclusions due to differences in the study designs. This is not surprising considering that nanotoxicity is a fairly young discipline [20,21] with principles of characterization of nanomaterials from the leading voices only a decade old [22].

Bioaccumulation occurs when an organism takes up substances in this case nanomaterials at a rate higher than the clearance rate. The bioaccumulation propensity of any nanomaterial is dependent on its biopersistence in the organ/tissue. The route of exposure/administration has an influence on the organs that come into contact with the nanomaterial [22]. For systemic drug delivery purposes using AuNPs the intravenous route is the most important to study since there is limited oral absorption [14]. The number of studies reporting on the biopersistence of AuNPs after intravenous administration are relatively few[17,23] and even fewer studies on their bioaccumulation[24,25]. The exposure an organ will have to a metal or nanomaterial will increase due to bioaccumulation, thus there is a clear need to have more information on the bioaccumulation of AuNPs after repeated intravenous administrations.

Safety assessments of AuNPs include end organ toxicity that can result from acute and/or subchronic exposure. The influence of bioaccumulation on end organ toxicity must be investigated to gather safety data. Serum enzymes and metabolites serve as good markers for hepatotoxicity and nephrotoxicity. In addition, histopathological examination is a good indicator to assess structural damage. This approach has previously been used in studies assessing the safety of AuNPs albeit with different results[25,26].

The aim of this study was to assess and quantify the bioaccumulation of Au in male Sprague Dawley rats after multiple intravenously administered doses of AuNPs. Little is known about the influence of dose (concentration of AuNPs) on the bioaccumulation of AuNPs after repeated administrations over weeks, thus different doses were used in the study. Levels of serum enzymes and metabolites were measured to assess if repeated dosing caused any

kidney or liver damage. General health assessments were routinely done and histopathological assessment of tissues were done to assess for both overt and organ toxicity.

5.2 Materials and Methods

5.2.1 Preparation and Characterization of AuNPs

All chemicals used were of high purity or analytical grade. Spherical citrate coated 14 nm AuNPs were synthesized under sterile conditions using adapted modified Turkevich-Frens method [27,28]. Briefly 1 mM solution of hydrogen chloroauric acid ($HAuCl_4.3H_20$) (Sigma) was brought to boil and 10 mL 38.8 mM of trisodium citrate was added until the solution turned wine red., the solution was refluxed for 30 minutes.

The average particle size and morphology of the colloidal suspensions were determined using transmission electron microscopy (TEM) (FEI Tecnai G2, Eindhoven, the Netherlands). At least 250 particles were used to determine the primary size distributions using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA). The UV-vis absorption spectra were obtained using a LAMBDA 1050 UV/Vis/NIR spectrophotometer (PerkinElmer, Massachusetts, USA). Concentrations of the prepared AuNPs; molar, number and mass and surface area were calculated using size determined by TEM, mass of gold salt used and making the assumptions that the reaction goes to completion and the particles are spherical [29]. The hydrodynamic size and zeta potential were determined by dynamic light scattering (DLS) and electrophoretic potential respectively using a Zetasizer Nano (Malvern Instruments, Worcestershire, UK) at 25°C. The average pH of the colloidal suspensions was 6.2.

5.2.2 Animals and AuNPs treatment

Male Sprague Dawley rats, age: 8-10 weeks, weighing 240-300 g were used in the study. The rats were bred and procured from the Vivarium of the DST/NWU/Preclinical Drug Development Platform (Potchefstroom, South Africa). Animals were housed in stainless steel cages in groups of four under standard environmental conditions (22±2° C, 55±15 % RH) with access to water and food *ad libitum*. The study was conducted in accordance with the South African National Standard for the Care and Use of Animals for Scientific Purpose. Ethical approval was sought from and granted by the ethics committee of AnimCare of the North-West University (NWU-00029-14-A5). The rats were divided into 4 groups ($n=9$) with each group receiving 90 µg, 9 µg, 0.9 µg or 0µg of Au (mass concentration) in the form of AuNPs, intravenously (see Figure 1). The injection was administered once weekly for seven weeks via the tail vein and had a volume of 500 µL which is within the acceptable range of intravenous injection in rats[30].

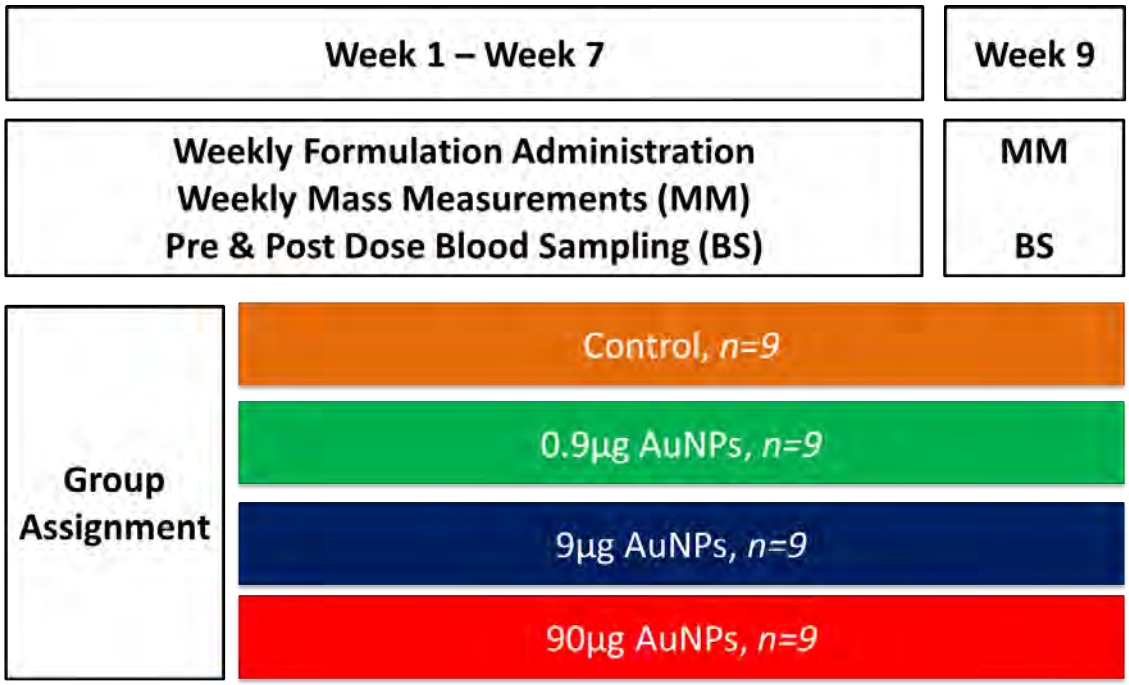

Figure 1 Study design for the bioaccumulation and subchronic toxicity of AuNPs

During the study, physiologic (mass gain over time) and behavioural (feeding habits and cage side behaviour) indicators were monitored [31] twice weekly to assess the general health. After the last dose there was a 14 d washout period before the termination of the study. On the last day of the study, all rats were sacrificed using an overdose of pentobarbitone (Euthapent®). In each group, the rats were further divided into the 2 groups; biodistribution group (n=4) and the group used to in the histopathological analysis to assess end organ toxicity (n=5).

5.2.3 Determination of Au in tissues: Neutron Activation Analysis

The liver, spleen, lungs, bones and remaining carcass of the rats in the biodistribution group were collected and weighed for the quantification of Au using neutron activation analysis (NAA) [24,32,33]. Briefly, all the samples were dried in an oven at 65 °C for 48 h, and then ashed

at 650 °C over a 6h period. The final mass of the total ash was noted for each sample and approximately 200 mg of it weighed and placed in a trace element free polyethylene flip-top vial which were sealed by friction welding. The vials were then placed in larger vials making the sample doubly encapsulated. The samples were irradiated in a SAFARI-1 20 MW research reactor in a RINGAS pneumatic system for 8 s using a neutron flux of 10^{14} $n.s^{-1}.cm^{-2}$ triggering the ^{197}Au (n, γ) ^{198}Au nuclear reaction. Standards with 0.1, 0.5, 1 and 2 μg of Au in containers with the same geometry as the sample holders were run with the experimental samples as reference standards. The reference standards were used to calculate the amount of Au present in the samples. Blank samples were also irradiated for background correction. The γ decay energies of the samples were recorded using a well-type high purity germanium (HPGe) (NATS, Middletown, USA) detector coupled to Genie 2000 program software. The 411 keV (95.6%) line was used to count the ^{198}Au content and used to calculate the amount of Au in each sample.

5.2.4 Toxicological studies

In the toxicity assessment group, pre- and post-dosing blood samples were collected in tubes with a clot activator and gel for serum separation (BD Vacutainer®) and immediately mixed as per manufacturer's instructions. The tubes were centrifuged at 3000 *g* for 10 min and the prepared serum samples were stored at -80° C. Analysis to measure levels of alanine transferase (ALT), alkaline phosphatase (ALP) and total bilirubin (BIL T) was performed to assess liver damage. ALT and ALP are enzymes are found intracellularly or anchored on the cell membrane and are induced, leaked and/or shed from hepatocytes in liver injury[34,35]. Bilirubin, a normal product of *heme* catabolism, is excreted via conjugation in the liver and its

levels in serum are increased in liver injury and inhibition of its conjugation and transport[36,37]. Kidney damage was assessed by measurement of the levels of creatinine (CREAT) and blood urea nitrogen (UREA). Both urea and creatinine are products of protein metabolism, which are cleared almost entirely by the kidneys. The serum levels of the enzymes and metabolites were done using the Cobas 6000 (Roche Diagnostics, Basel Switzerland). The kidneys, heart, lungs, liver and spleen were collected and fixed with 10 % formalin. All samples were stored at room temperature until histopathological analysis was done by an independent laboratory (IDEXX, South Africa). Micrographic images were captured using an Olympus light microscope (Tokyo Japan).

5.2.4 Calculations and statistical analysis

The statistical significance of the differences between the mean values in the different groups was assessed using mixed linear models which took into account repeated measures [38]. Statistical probability (p) values less than 0.05 were considered significantly different.

5.3 Results

5.3.1 Synthesis and characterization AuNPs

The citrate reduction method was used to synthesize 14 nm AuNPs using the Turkevich-Frens method [27,28]. The morphology and primary size (14 ±1.2 nm) distribution as determined by TEM (see Figure 2) was as expected due to the careful selection of the ratio of the gold salt to the reducing agent. The AuNPs had the characteristic resonance peak in the 520-530 nm wavelength range attributed to the 14 nm particles. The absence of secondary peaks confirmed the monodispersity of the colloidal suspension[39].

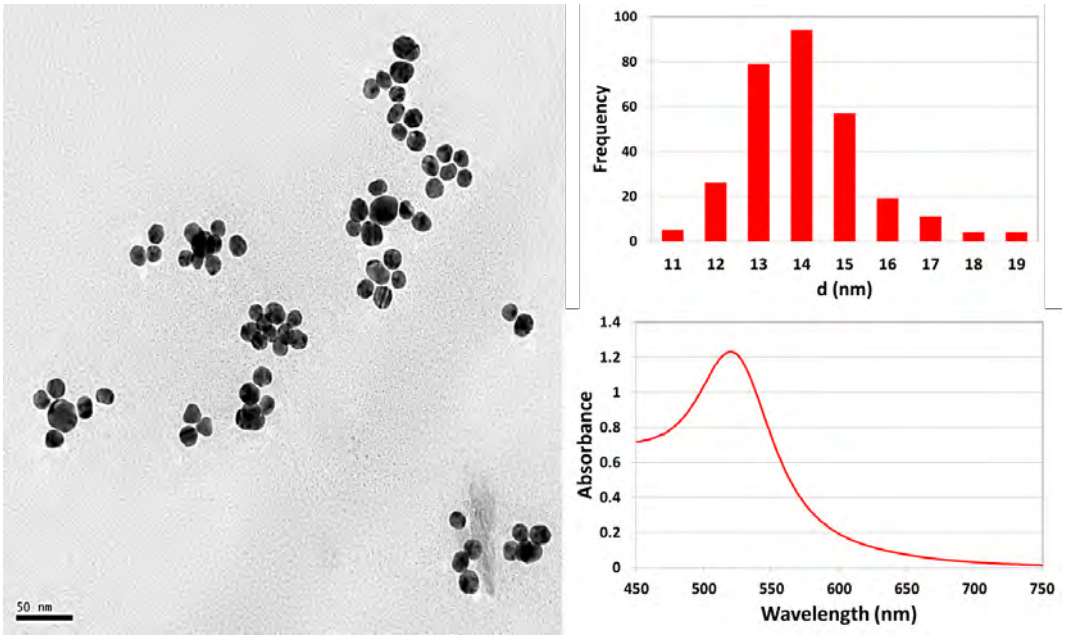

Figure 2 Characterization of AuNPs synthesized using the citrated reduction method. The TEM image shows the morphology and the bar graph shows the size distribution and the UV/Vis spectra

5.3.2 Bioaccumulation of Au in tissues after repeated dosing

The amount of Au was determined in the liver, lungs, skeleton, spleen and carcass using NAA. The organs were chosen based on results from an acute biodistribution study we conducted (data not shown). The liver and spleen had the highest amount of Au across all dose levels used in the study (see Figure 3). The values are expressed as micrograms of Au per gram of organ. The bioaccumulation in all tissues/organs did not exhibit any dose dependence patterns unlike in a previous report [25], there was no correlation between the amount in the organs and the dose administered. If the absolute amounts of Au are considered, the bioaccumulation will be in the following order skeleton>carcass>liver>spleen>lungs.

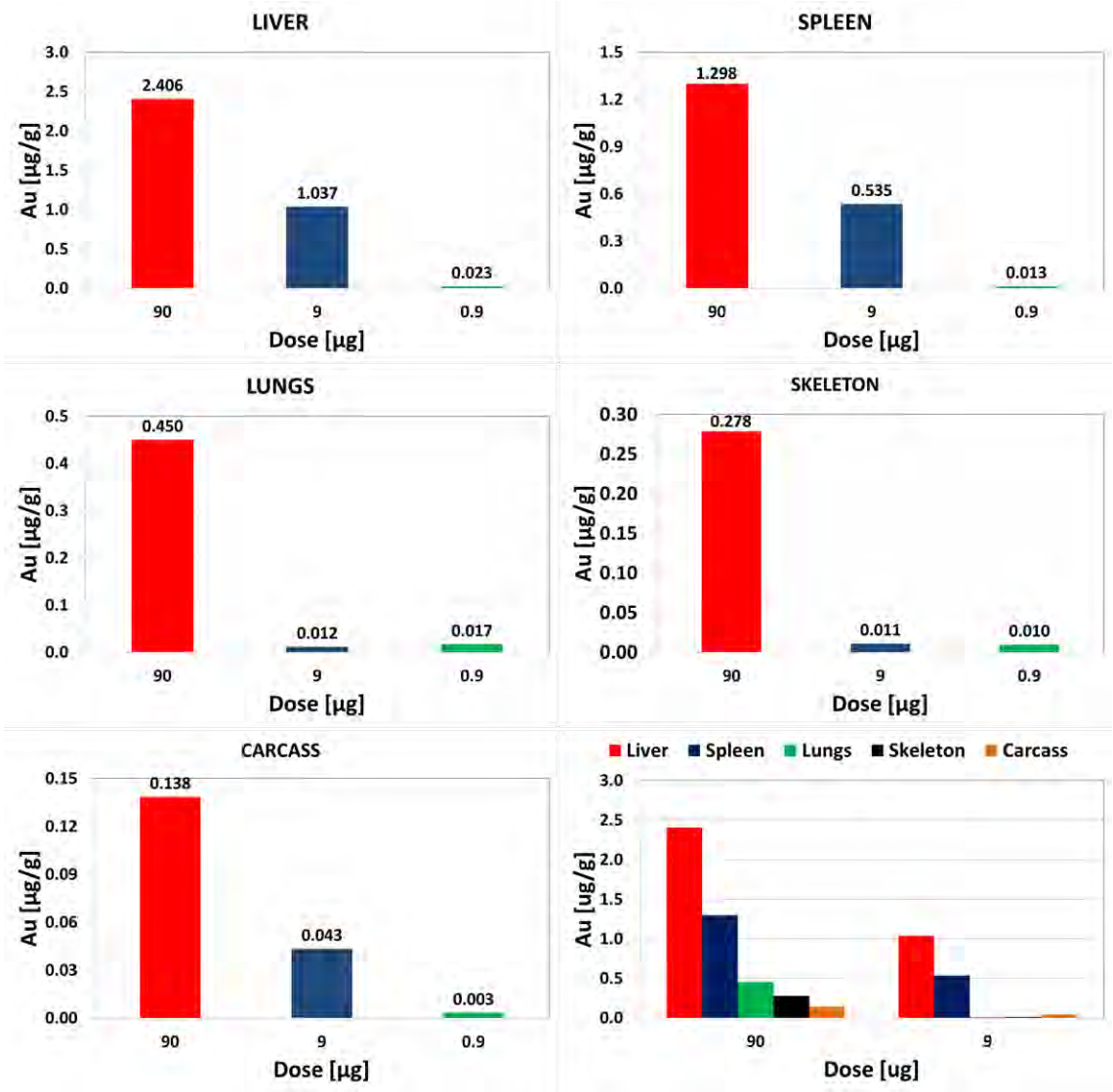

Figure 3 Bioaccumulation of Au in tissues. The rats received 7 intravenous doses weekly at 3 dosing levels of; 0.9, 9 and 90 µg. The Au was quantified using NAA in the liver, spleen, lungs, skeleton and carcass 2 weeks (washout period) after the last dose was administered. The bottom right graph shows a comparison of the levels in the liver, spleen, lungs, skeleton and carcass at the 90 and 9 µg dose levels. Please note that the Y scale is different in all the organs with the liver having the most amount of Au per gram and the carcass having the least.

5.3.3 Toxicological Studies

During the study all the rats monitored physiological and behavioural indicators and the observations did not reveal any signs of overt toxicity. All injections were well tolerated with none of the rats having to be sacrificed before the end of the study due to ethical considerations or distress. All the rats had a comparable steady mass gain (see Figure 4) with no differences between different groups being observed due to the exposure to AuNPs or the dose level.

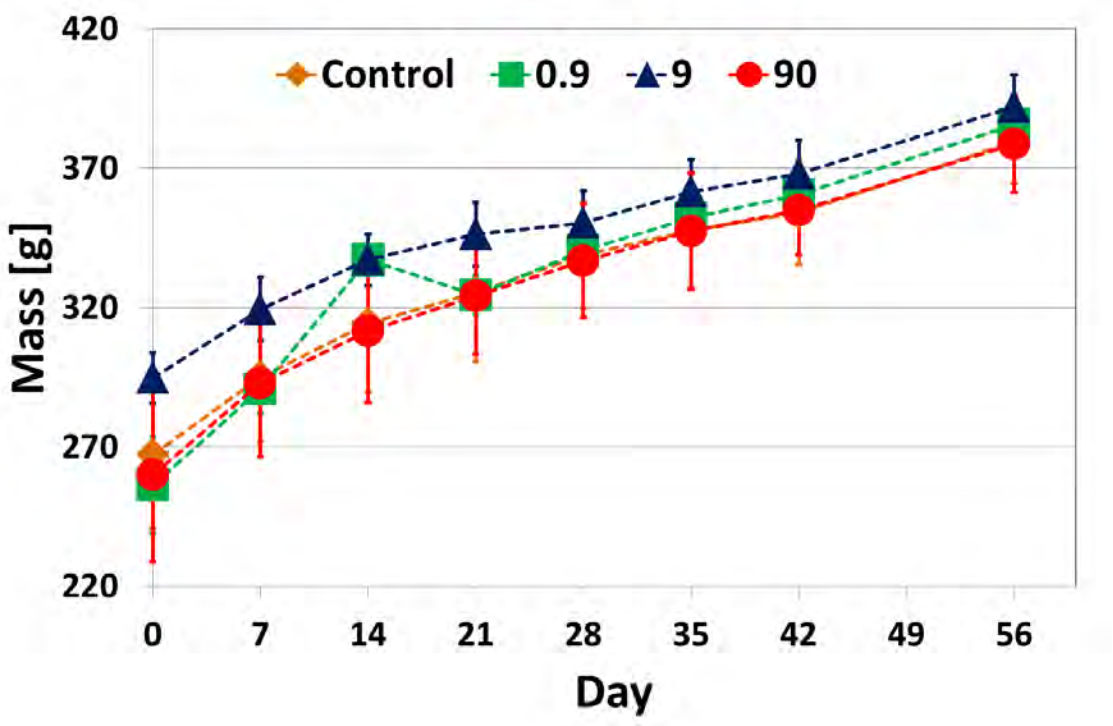

Figure 4 Comparison of masses of rats in the 4 treatment groups in the study over the 56 days. All the rats gained mass over time in a similar trend as expected for the species.

5.3.3.1 Markers of liver and kidney damage

The first pre-dose sample served as the baseline measurement and the other 6 samples were obtained before administration of the next dose which was also 7 days after the preceding dose. The post dose samples were obtained 1 day after each dose with the exception of the last one obtained 14 days after the last dose (washout period). The levels of ALT, ALP and BIL T which give an indication of liver damage showed similar trends in all groups in the study (see **Error! Reference source not found.**). No differences were noticed between the pre- and post-dose levels. Analysis of the levels of creatinine and urea nitrogen, the metabolites that give an indication of kidney damage, showed similar trends in all the groups (see Figure 6). No differences were detected between the pre and post dose levels of these two metabolites.

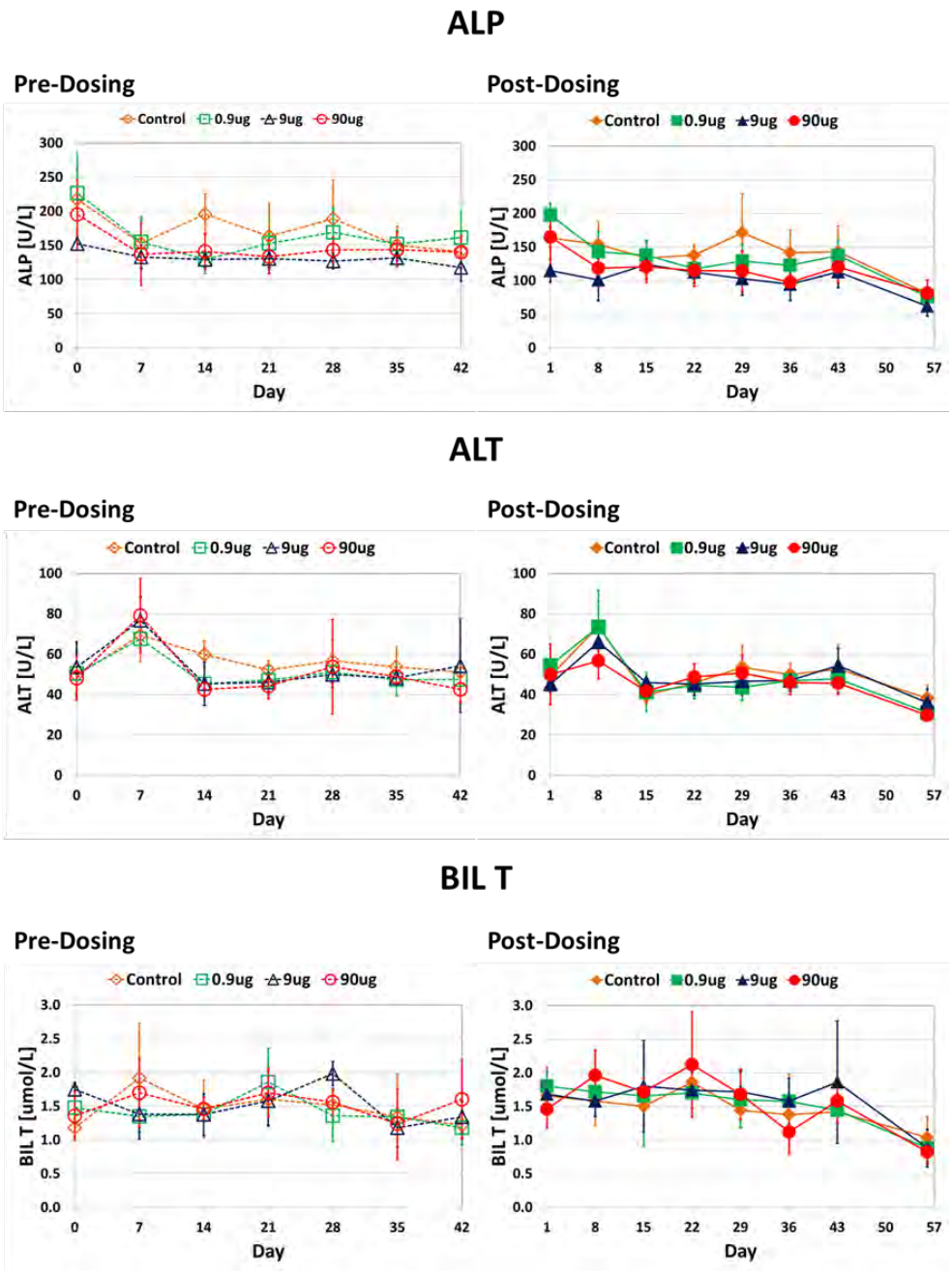

Figure 5 Similar trend were observed across all groups both for the pre- and post-dose measurements for markers of liver damage. The pre-dose measurements (on the left side) were done at baseline (T=0) and 7 days after the preceding dose. The post dose measurements were done 1 day after dosing and the last one 14 days (washout period) after the last dose. ALP= Alkaline phosphatase, ALT= Alanine Phosphatase and BIL T = Total Bilirubin.

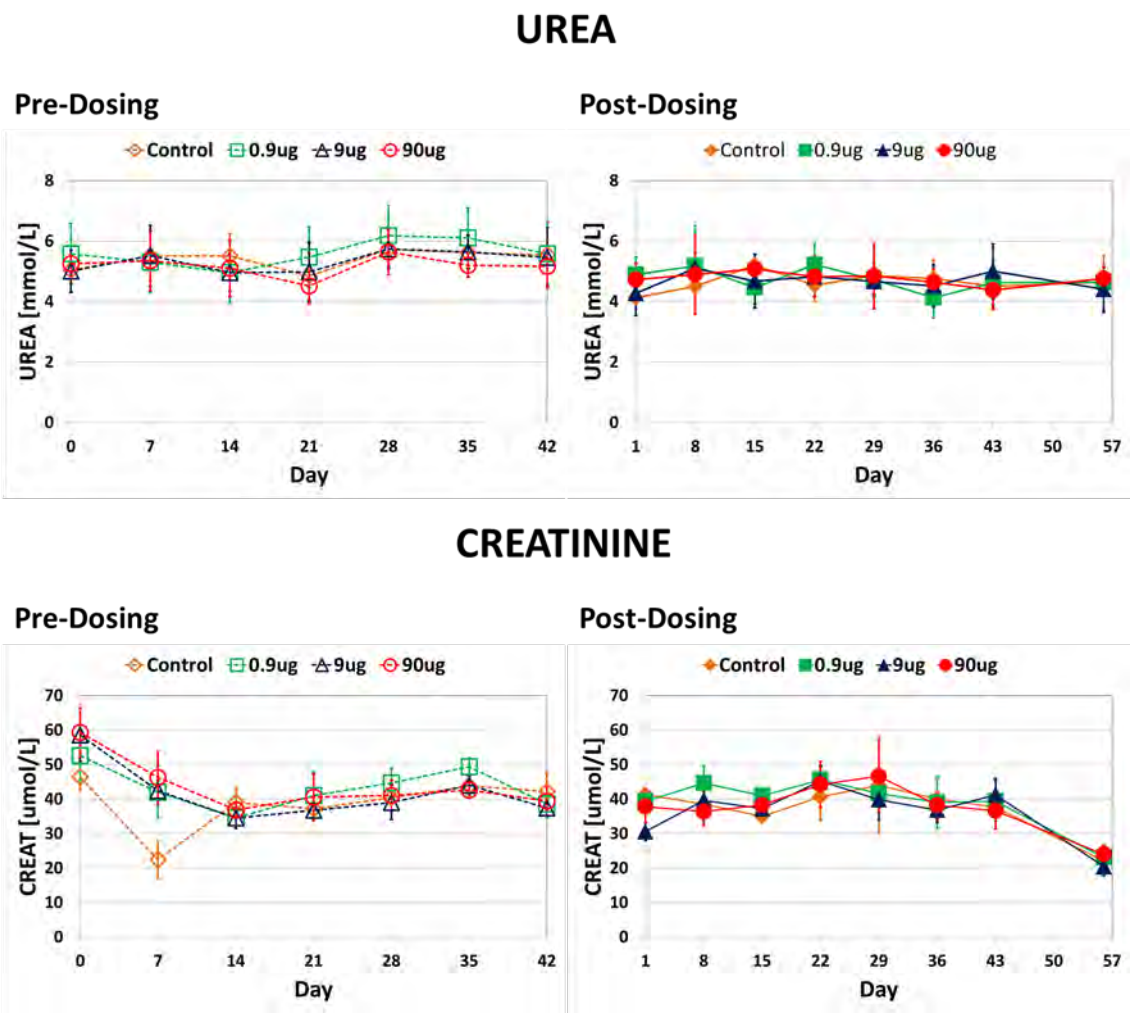

Figure 6 Similar trends were observed across all groups both for pre- and post-dosing measurements for the levels of markers of kidney damage. The pre-dose measurements (on the left side) were done at baseline (T=0) and 7 days after the preceding dose. The post dose measurements were done 1 day after dosing and the last one 14 days (washout period) after the last dose. Urea = Blood Nitrogen Urea, CREAT = Creatinine.

5.3.3.2 Histopathology

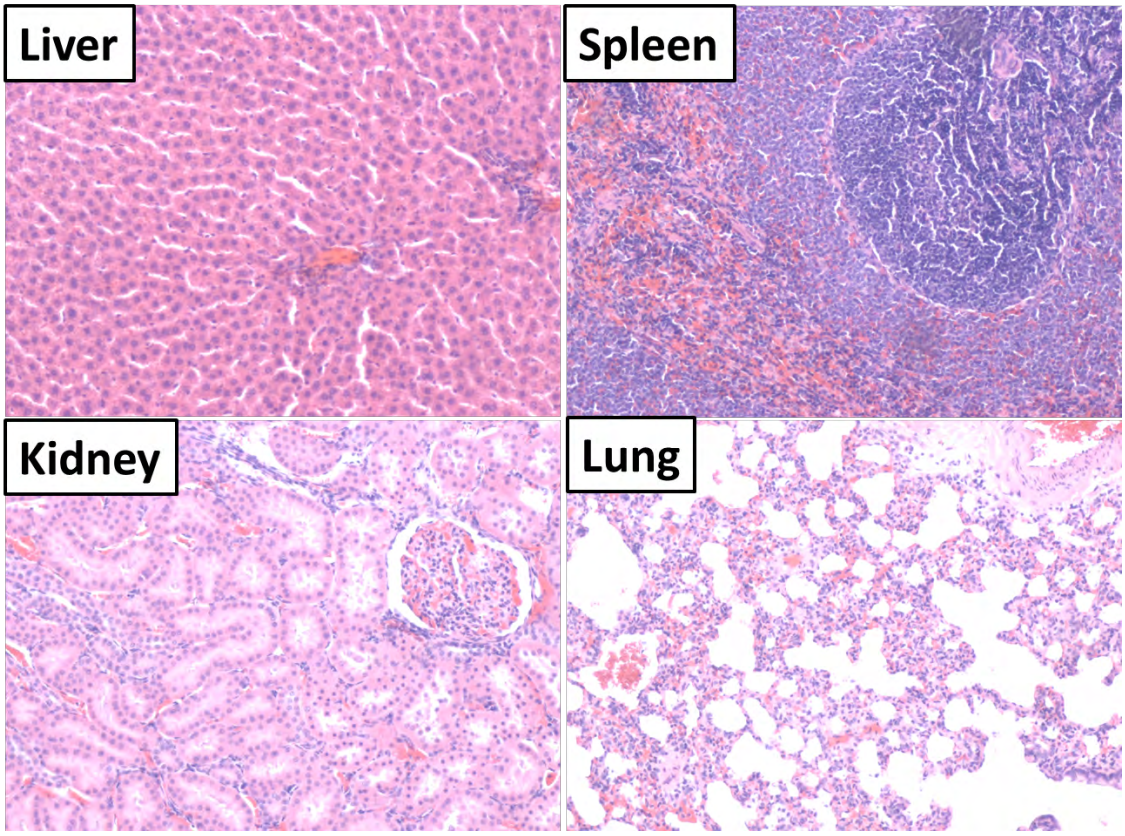

Figure 7 Histopathological analysis of the liver, spleen, kidneys and lungs after exposure to AuNPs at different dose levels. The organs represent all the treatment groups and the control as no abnormalities were detected in all the groups due to the exposure to AuNPs

Exposure to AuNPs at the different dose levels did not result in any tissue damage as revealed by histopathological assessment of the; heart, kidneys, liver, lungs and spleen (see Figure 7). The assessment showed comparable results between all test groups and the control.

5.4 Discussion

Use of AuNPs for drug delivery can only successfully reach the clinic when all questions with regards to the safety have been satisfactorily answered. The question on bioaccumulation of Au when administered as AuNPs for drug delivery purposes has not been extensively investigated and thus remains unanswered. There is a clear need for multifunctional nanocarriers (which AuNPs can be) with low bioaccumulation propensity [40]. Bioaccumulation is influenced by the biopersistence of a material which is a result of a biological system's failure to clear foreign material. In the study described here, the acute and subchronic toxicity and bioaccumulation of AuNPs after repeated dosing and a washout period in Sprague Dawley rats. The amount of Au was quantified using NAA and the toxicity endpoints were general health assessments and the monitoring of markers of kidney and liver damage in serum. The influence of dose on the bioaccumulation and acute and subchronic toxicity of AuNPs was investigated and no correlation was found.

The bioaccumulation patterns followed the order liver>spleen>lungs>skeleton>carcass with the liver having the highest amount in micrograms per gram of the organ/tissue. Contrary to an earlier report [25], there was no proportional increase in the amount of Au in all the organs/tissues examined with an increase in the dose administered. It must be noted that the earlier in earlier report the study duration was only 8 days. The amount of Au found in the organs was manifold higher than the background. The lowest amount detected (in all the samples analyzed) was a few times above the limit of detection of our quantification technique, this shows that all the Au was from the administered dose. A control group was not used in the study for quantifying Au because probability of finding natural Au in biological

samples is low as described in literature [24]. Excretion of Au is mainly via the hepatobiliary system and rats are known to eat their feces, therefore the amounts detected in tissues can only be attributed to the administered doses because of the little or no oral absorption we witnessed in our own studies see chapter 3 of this thesis which is also corroborated in literature [14].

There is a paucity of data on the bioaccumulation of Au [24,25] and other engineered nanomaterials (ENMs) [41] in general. The studies reporting on bioaccumulation could not be easily generalized due to the vast differences in the doses, study designs and organs used to quantify the ENMs. However in all the studies, the liver and spleen had the highest accumulation levels indicating that the hepatobiliary system is the main clearance mechanism[12,17,23]. Just as for biodistribution, bioaccumulation is also thought to be influenced by surface properties of the ENMs [42].

Based on the general health assessments which focused on monitoring behavioural (cage side behaviour and feeding patterns) and physiological (monitoring of mass gain and alertness) indicators there were no differences between the control and test groups in this study. This was comparable to the results observed when the masses of rats were monitored when magnetite an ENM was administered at different doses in a rodent model[41]. The markers of liver damage, ALP, ALT and BIL T together with histopathological examination of liver tissues showed no differences between the control and tests groups. The same trend was observed for the markers of kidney damage; CREAT and UREA together with histopathological assessment of kidneys which is regarded as the gold standard for the assessment of nephrotoxicity[43]. Histopathological examination of the heart, lungs and spleen

did not show any differences between the different groups. No influence of the dose level or the actual treatment with AuNPs was detected. The markers of liver and kidney damage were also similar between the pre and post dose samples indicating that there was no acute and/or subchronic damage.

Serum enzymes and metabolites are used as biomarkers for the assessment of drug induced liver injury (DILI) and nephrotoxicity, both acute and subchronic. If one considers the bioaccumulation levels of Au in the liver for instance, the lack of alteration in the levels of the markers assessed can be interpreted as a sign of safety or alternatively, that they are not the appropriate indicators with regards to nanomaterial safety. As with ENMs characterization techniques, there might be a need to come up with specific markers that can be used to assess nanomaterial induced liver injury (NILI) in the routine safety assessment of ENMs. The results of our study however are not in agreement with those of a previous study where they showed dose dependent detrimental effects on tissue histology changes [44]. The duration of this study was however short compared to ours but this highlights the lack of agreement on the issue of toxicity of AuNPs after repeated dosing.

5.5 Conclusion

There was accumulation of Au in the order liver>spleen> lungs>skeleton>carcass after 7 weekly doses (three dose levels) and a 2 week washout period in Sprague-Dawley rats. There was no observable acute or subchronic toxicity that can be attributed to the use of AuNPs after the repeated dosing despite the accumulation in organs/tissues. The absence of indicators

of toxicity maybe an indication sign that that the appropriate parameters with regards to nanomaterial safety testing are not being applied to investigate the safety of AuNPs.

5.6 Acknowledgements

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Chapter 6: General conclusions and future perspectives

Conclusions

The studies presented in this thesis give further evidence on the question of the safety of gold nanoparticles when intentional intravenously administered for biomedical applications. The conclusions drawn from this work are generally applicable to citrate coated gold nanoparticles in the size of 10-20 nm. There was wide systemic acute distribution of gold. Citrate, a surface attachment with an electrostatic interaction with the gold surface had different biodistribution profile compared to that of the gold core. After a single dose, gold as 14 nm gold nanoparticles were biopersistent over 56 days with the highest amount in the liver, spleen, lungs and bones in that descending order. The biopersistence raised some eyebrows in terms of the impact the Au will have on the tissues/organs over longer periods of time. Gold nanoparticles showed a non-dose dependent bioaccumulation propensity when multiple doses were administered. Despite the biopersistence and bioaccumulation there was no evidence of peracute, acute, subacute or subchronic toxicity due to the exposure of gold nanoparticles at different doses. Histopathological assessments of the heart, liver, spleen, lungs and kidneys showed no structural damage to the organs as a result of exposure to gold nanoparticles. There were no differences in the levels of alkaline phosphatase, alanine transferase and total bilirubin (makers of liver damage) in all groups in the biopersistence and bioaccumulation studies. The same trend was observed when creatinine and blood urea nitrogen (markers of kidney damage) were monitored. Gold nanoparticles do not cause any end organ damage, acute or subchronic toxicity despite being biopersistent and having a high bioaccumulation propensity.

Future perspectives

Our findings add more information on the question: How safe are AuNPs in a rodent model at concentrations which may be used for biomedical applications? As with any research, more questions arose from this inquiry. The exact location of AuNPs in the tissues (intracellular vs. intercellular) after intravenous administrations remains unknown or a matter of intense debate. From our acute biodistribution and biopersistence results, bone deposition was observed. Since adult male Sprague-Dawley rats were used in studies, the question that arose from the findings is: What is the impact of bone deposition on young developing bones and the cellular processes at that stage of development? Depending on the exact location of the gold in the bone tissue, bone deposition can have influences on the bone density and/or maturation of blood cells. When one considers the bioaccumulation propensity of gold when administered as AuNPs, the question on the levels of gold in tissues after chronic administration appears pertinent and with great merit to investigate. These questions can be answered in the future and will add weight to the debate on the safety of AuNPs.

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1. Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

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The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

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Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX , institute, city, country).

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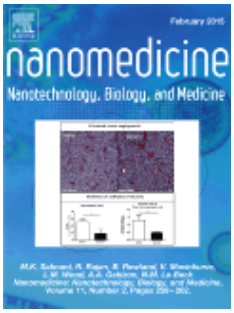

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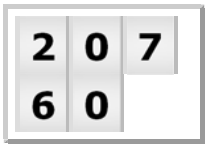

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