In vivo Stability and Biodistribution of Superparamagnetic Iron Oxide Nanoparticles Radiolabeled with Indium-111

Master’s Thesis

By

Haotian Wang

Advisor: Dr. Samuel John Gatley

to

The Bouvé Graduate School of Health Sciences

In Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmaceutical Science with Specialization in Pharmaceutics and Drug Delivery

NORTHEASTERN UNIVERSITY

BOSTON, MASSACHUSETTS

January, 2014
ABSTRACT

Nanoparticles of various kinds have generated considerable excitement as vehicles for drug delivery, and as imaging agents (Yen, 2013). Magnetic nanoparticles are of particular interest since their distribution in the body can be manipulated using magnetic fields, and they can also act as highly sensitive contrast agents in magnetic resonance imaging (MRI) (Bonnemain, 1998; Wang, Hussain, & Krestin, 2001). For some applications, radionuclide imaging (Positron emission tomography (PET) or Single-photon emission computed tomography (SPECT)) offers advantages when used in conjunction with MRI, since the strengths of the two modalities, higher spatial resolution for MRI, but better quantification for PET or SPECT, are complementary (Bouziotis et al., 2012). On the other hand, the validity of dual modality imaging requires that the association between radioactive label and magnetic label remains intact in the human or animal body during the period of study. Evaluation of the extent to which this requirement is true for PEGylated nanoparticles containing a core of superparamagnetic iron oxide (SPION), and also labeled with a radioactive metal which is commonly used in nuclear medicine, indium-111, is a major component of this proposal.

In the present study, we prepared SPIONs using co-precipitation of iron oxide, complexation with oleic acid, and final coating of the particles with DSPE-PEG plus DMPE-DTPA using the lipid rehydration technique. We radiolabeled the particles by introducing iron-59 ferric chloride or carbon-14 oleic acid during their preparation, and by forming a chelate between the anchored DTPA and indium-111. We conducted biodistribution studies in mice with particles labeled with each radionuclide, and also with indium-111 labeled indium citrate, indium-DTPA, and DMPE-DTPA-indium, and with
carbon-14 oleic acid. We found that the radioactivity from radioindium labeled SPIONs was, as hypothesized, preferentially localized in liver, spleen and bone associated with trapping of colloids by reticuloendothelial cells. However, radioactivity from SPIONs labeled with iron-59 was accumulated in liver, spleen and bone to a greater extent than from indium-111 labeled SPIONs, showing that the association of indium with the particles is not completely maintained in vivo. Radioactivity from SPIONs labeled with carbon-14 oleic acid exhibited a quite different distribution pattern to iron-59 or indium-111, and so does not remain with the iron oxide core after injection into mice.
NORTHEASTERN UNIVERSITY
Graduate School of Bouvé College of Health Sciences

Thesis Approval

Thesis title: *In vivo* Stability and Biodistribution of Superparamagnetic Iron Oxide Nanoparticles Radiolabeled with Indium-111

Author: Haotian Wang

Program: Pharmaceutical Sciences

Approval for thesis requirements for the Master of Science Degree in Pharmaceutical Sciences

Thesis Committee

(Chairman) ____________________ Date_____

Other committee members:

_________________________ Date_____

_________________________ Date_____

_________________________ Date_____

Dean of the Bouvé College Graduate School of Health Sciences:

_________________________ Date_____


ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude and deepest appreciation to my supervisor, Dr. John Gatley, for his invaluable advice and support throughout my project. His brilliant ideas, guidance and mentoring helped me overcome many hurdles throughout my Master’s research. Without your understanding and patience none of this would have been possible.

I would like to thank my thesis committee members, Dr. Richard Duclos, Dr. Rajiv Kumar and Dr. Ban-an Khaw, for their time, invaluable advice on my thesis project and moral support.

I would particularly like to thank Dr. Dattatri Nagesha for his help and support in the initial setting up of the project and for guiding me throughout.

Special thanks to Dr. Srinivas Sridhar for allowing me to learn the synthesis of SPIONs.

My sincere thanks to Sarom, Kun, Shilpa, Nidhi, Yu, Codi, Jodi, Rita and all my friends who have given their time, their knowledge and their resources to help me.

Last but most importantly, I want to thank my parents, who have sacrificed much to ensure that I have the best in life. And finally, I would like to thank my wonderful wife, Lu, who has been there for me through the toughest of times and completely supporting my dreams.
TABLE OF CONTENTS

ABSTRACT .................................................................................................................. 2

ACKNOWLEDGEMENTS ............................................................................................. 5

TABLE OF CONTENTS ............................................................................................... 6

LIST OF TABLES .......................................................................................................... 9

LIST OF FIGURES ....................................................................................................... 10

1. INTRODUCTION .................................................................................................... 12

1.1 Brief Introduction to the History of Nanotechnology ......................................... 12

1.2 Nanoparticles Platform ....................................................................................... 15

1.2.1 Liposomes ...................................................................................................... 15

1.2.2 Polymeric micelles ......................................................................................... 16

1.2.3 Superparamagnetic Iron Oxide Nanoparticles (SPIONs) ............................... 17

1.3 Rationale for each Specific Aim ......................................................................... 18

2. SPECIFIC AIMS .................................................................................................... 22

2.1 Objectives ........................................................................................................... 22

2.2 Hypothesis ........................................................................................................... 23
3. MATERIAL AND METHODS ............................................................................................................. 25

3.1 Animals ........................................................................................................................................ 25

3.2 Chemical Reagents ..................................................................................................................... 25

3.3 Preparation of Superparamagnetic Iron Oxide Nanoparticles (SPIONs)........... 26

3.4 Preparation of DSPE-PEGylated SPIONs ............................................................................... 27

3.5 Characterization of PEGylated SPIONs................................................................................... 27

   3.5.1 Particle Size and Surface Charge Analysis ................................................................. 27

   3.5.2 Transmission Electron Microscopy (TEM) ................................................................. 28

3.6 Radiolabeling PEGylated SPIONs with Indium-111 ............................................................. 28

3.7 Biodistribution Studies of indium-111 Radiolabeled PEGylated SPIONs....... 29

3.8 Biodistribution Studies of indium-111 Radiolabeled indium citrate, indium-
    DTPA and DMPE-DTPA-indium ................................................................................................. 29

   3.8.1 Preparation of indium-111 radiolabeled indium citrate solution .................. 29

   3.8.2 Preparation of indium-111 radiolabeled indium-DTPA solution .................. 30

   3.8.3 Preparation of indium-111 radiolabeled DMPE-DTPA-indium solution 30

   3.8.4 In vivo Biodistribution Studies ......................................................................................... 30
3.9 Biodistribution studies of iron-59 and carbon-14 oleic acid radiolabeled PEGylated SPIONs ..............................................................31

3.9.1 Preparation of iron-59 or carbon-14 oleic acid radiolabeled SPIONs ....31

3.9.2 In vivo biodistribution studies ..............................................................32

4. RESULTS AND DISCUSSIONS ................................................................33

4.1 Preparation and Characterization of PEGylated SPIONs ..................33

4.2 Radiolabeling of PEGylated SPIONs with Indium-111 ......................35

4.3 Biodistribution of indium-111 radiolabeled PEGylated SPIONs ..........35

4.4 Biodistribution of indium-111 radiolabeled indium citrate, indium-DTPA and DMPE-DTPA-indium ..........................................................38

4.5 Biodistribution of iron-59 or carbon-14 oleic acid radiolabeled PEGylated SPIONs ...........................................................................43

4.6 OVERALL CONCLUSIONS .................................................................49

5. LIMITATIONS AND FUTURE DIRECTIONS ....................................51

6. BIBLIOGRAPHY ..................................................................................52
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Radioactivity recovered of $^{111}\text{indium}$ with SPIONs in successive filtration.</td>
<td>35</td>
</tr>
<tr>
<td>Table 2</td>
<td>Biodistribution data of indium-111 labeled PEGylated SPIONs at different time points.</td>
<td>36</td>
</tr>
<tr>
<td>Table 3</td>
<td>Biodistribution data of indium-111 labeled DMPE-DTPA-indium, indium citrate and indium-DTPA.</td>
<td>42</td>
</tr>
<tr>
<td>Table 4</td>
<td>Biodistribution data of iron-59 labeled PEGylated SPIONs</td>
<td>44</td>
</tr>
<tr>
<td>Table 5</td>
<td>Biodistribution data of carbon-14 oleic acid labeled PEGylated SPIONs.</td>
<td>45</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1:</td>
<td>Photographs of (a) the Lycurgus cup; and (b) Church window in the Cathédrale Notre-Dame de Chartres.</td>
<td>14</td>
</tr>
<tr>
<td>Figure 2:</td>
<td>Schematic structure of (a) non-modified liposomes; (b) PEGylated liposomes with PEG coated on the surface; (c) immune-liposomes with antibodies attached to the surface.</td>
<td>16</td>
</tr>
<tr>
<td>Figure 3:</td>
<td>Schematic structure of self-assembled micelles.</td>
<td>17</td>
</tr>
<tr>
<td>Figure 4:</td>
<td>Schematic diagram of oleic acid stabilized SPIONs.</td>
<td>22</td>
</tr>
<tr>
<td>Figure 5:</td>
<td>Schematic structure of water dispersible polyethylene glycol SPIONs.</td>
<td>23</td>
</tr>
<tr>
<td>Figure 6:</td>
<td>Particles size of PEGylated SPIONs measured using dynamic light scattering in 90Plus particle size analyzer</td>
<td>33</td>
</tr>
<tr>
<td>Figure 7:</td>
<td>Stability Measurement of PEGylated SPIONs for consistent 7 days.</td>
<td>34</td>
</tr>
<tr>
<td>Figure 8:</td>
<td>TEM micrograph of the iron core of PEGylated SPIONs</td>
<td>34</td>
</tr>
<tr>
<td>Figure 9:</td>
<td>Graph of biodistribution of $^{111}$indium labeled PEGylated SPIONs at different time points.</td>
<td>37</td>
</tr>
<tr>
<td>Figure 10:</td>
<td>Indium-111 in urine after administration of $^{111}$indium labeled PEGylated SPIONs.</td>
<td>37</td>
</tr>
<tr>
<td>Figure 11:</td>
<td>Graphs showing liver-to-blood (left) and brain-to-blood (right) ratios versus time.</td>
<td>38</td>
</tr>
</tbody>
</table>
**Figure 12:** Comparison of biodistribution between indium-SPIONs and DMPE-DTPA-indium at 30 min and 2 h.

**Figure 13:** Comparison of liver-to-blood ratios versus time between indium-SPIONs and DMPE-DTPA-indium.

**Figure 14:** Comparison of urine radioactivity between indium-SPIONs and DMPE-DTPA-indium.

**Figure 15:** Comparison of biodistribution of indium-SPIONs, DMPE-DTPA-indium and indium citrate at 30 minutes post injection.

**Figure 16:** Comparison of indium-111, iron-59, and carbon-14 oleic acid biodistribution patterns in liver, spleen, bone, blood and kidney at 10 min, 30 min, and 24 h time points.
1. INTRODUCTION

1.1 Brief Introduction to the History of Nanotechnology

According to the International Union of Pure and Applied Chemistry (IUPAC), International Organization for Standardization (ISO), American Society of Testing and Materials (ASTM) and National Institute of Occupational Safety and Health (NIOSH), nanoparticles are defined as particles of size between 1 and 100 nm (Vert et al., 2012; Horikoshi & Serpone, 2013). Although terms such as “nanoparticle” and “nanotechnology”, which was first coined by Professor Taniguchi (1974), are of fairly modern origin practical uses of nanoparticles predate recognition of their existence by many centuries (Colomban, 2009). In ancient Rome (4th Century A.D.) craftsmen knew how to produce colored glasses, sculptures and paintings that we now know owed their optical properties to gold and silver nanoparticles (Hougha, Noblea, & Reich, 2011). The Lycurgus Cup (Figure 1a) in the British Museum of London is an amazing example. Its color changes from green in daylight to red when illuminated from the inside (Heiligtag & Niederberger, 2013; Freestone et al., 2007). From the Medieval Period to the Renaissance Period, stained glass church windows (Figure 1b) of breathtaking beauty were made that owed their ruby red color to gold nanoparticles and their deep yellow color to silver nanoparticles (Jin et al., 2001) and the size of metal nanoparticles is critical to producing particular colors. The astonishing discovery in nanoparticle research was made by Michael Faraday approximately 157 years ago (1857). Faraday prepared his deep red gold dispersions by reduction of an aqueous solution of
chloroaurate (Na[AuCl₄]) by phosphorus in carbon disulfide (a two-phase system). The red “solution” was due to the optical property of gold nanoparticles. Later, such “solutions” became known as colloidal systems, which are defined as particles of size between 1 and 1000 nm of one material or composition suspended in another material. Milk, blood and paint are colloidal systems. In fact, Faraday’s systematic studies on the interaction between light and metal nanoparticles is considered as the threshold of modern colloid chemistry and the beginning of Nanoscience and Nanotechnology (Edwards & Thomas, 2007). Thus there is considerable overlap in terminology between “nanoparticles” and “colloids”. An early medical application of a colloid was the X-ray contrast agent Thorotrast (Dickson 1932). This consisted of an injected preparation of particles of thorium-232 dioxide that were taken up by reticuloendothelial cells in the liver and, since thorium is opaque to X-rays, permitted medical imaging of this organ. It was used worldwide from 1930 to 1950 (Kaick. Et al., 1999; Jellinek, 2004). Unfortunately, thorium dioxide particles remained in the liver for the patients’ lifetime and is radioactive, and since thorium emits alpha particles, many people developed liver cancer many years later. This may be the first medical use of a nanoparticle. Another imaging application example is the utilization of sulfur colloid, labeled with Technetium-99m for liver and spleen scanning (Stern, McAfee, & Subramanian, 1966). This radiopharmaceutical is still used today in nuclear medicine (Bhalani et al., 2012).
In the 20th century, a much better understanding of colloidal systems developed due to advances in physical instrumentation. For example, conventional light microscopes are unable to visualize objects as small as 100 nm, but the development of the electron microscope by German scientists Max Knoll and Ernst Ruska in 1931 enabled researchers to detect and measure objects in this size-range.

Additionally, in the early 20th century, the idea of targeted delivery to enhance drug therapy was conceptualized by German scientist Dr. Paul Ehrlich. The word “Zauberkugeln” – “Magic Bullets” – in English was coined to describe selectively targeted chemotherapy (Kreuter, 2007; Yordanov, 2013). Ehrlich’s notion was the use of histological dyes, which had affinity for syphilis bacteria in tissues, to deliver arsenic, which was toxic to the bacteria. The dye was the vehicle and arsenic was the cargo (Ehrlich, 1877). Salvarsan was actually a low molecular weight compound, but the idea of drug delivery vehicles is now more commonly associated with nanoparticles.
Nowadays, the literature on this subject is very large. A MEDLINE search on “nanoparticle” and “drug delivery” on January 9th, 2014 yielded almost 17,000 articles.

1.2 Nanoparticles Platform

Nanotechnologies have the potential to revolutionize the drug development process and change the landscape of the pharmaceutical industry (Allen & Cullis, 2004; Shi et al., 2010). Many different nanoparticles platforms have shown promise in delivering drugs to desired tissue sites in the body such as liposomes, polymeric micelles, dendrimers, etc. (Farokhzad & Langer, 2009) Because these carriers have high loading efficiencies and protect drugs from undesired in vivo interference, nanotechnologies can improve the therapeutic effect of drugs by enhancing their efficacy and/or increasing their tolerability in the body (Swami et al., 2010; Zhang et al., 2007). Here are some examples:

1.2.1 Liposomes

Liposomes are multilayer vesicular platform made with interior aqueous phospholipid (such as phosphatidylcholine) bilayered membrane structures (Antimisiaris, Kallinteri, & Fatouros, 2007). Hydrophobic agents can be incorporated into the phospholipid membrane, while hydrophilic agents can be inserted into the aqueous interior. The vesicles size can range from several microns to 30 nm (Barenholz, 2001). The encapsulated drugs can be released in the body when the liposomes are broken down by enzymes or by the fusion of the liposomal bilayer with the cell membrane or
endosomal membrane (Farokhzad & Langer, 2006). Moreover, liposomes have properties including long circulation time in the body system, ease of surface modification, and fewer side effects that make them well suited as drug delivery carriers (Antimisiaris, Kallinteri, & Fatouros, 2007). Current research is focused on the development of multifunctional nanocarriers that combine the advantages of the basic types leading to improved efficacy (Torchilin, 2009). Major types of liposomes are shown in Figure 2. Liposomes were the first nano-based drug carriers to be approved by the Food and Drug Administration (FDA) for clinical use. For instance, DOXIL® (doxorubicin liposomes) was approved in 1995 for the treatment of patients with ovarian cancer whose disease has progressed or recurred after platinum-based chemotherapy (Barenholz, 2012).

![Figure 2. Schematic structure of (a) non-modified liposomes; (b) PEGylated liposomes with PEG coated on the surface make liposomes more stable and long circulating; (c) immune-liposomes with antibodies attached to the surface intended for recognizing specific target receptors and antigens. (Yordanov, 2013)](image)

1.2.2 Polymeric micelles

Polymeric micelles (Figure 3) are spontaneously self-assembling block copolymers with an inner hydrophobic core and hydrophilic shell, such as PEG, in aqueous solution at concentration above the critical micellar concentration (CMC) (Chan et al., 2010).
Polymeric micelles have proved that they have great potential modalities as therapeutic drug carriers (Matsumura & Kataoka, 2009; Nishiyama & Kataoka, 2006). As drug delivery carriers, polymeric micelles exhibit several unique properties including ease of preparation, long circulation in bloodstream, reduced adverse effects, ultra small particles size, as well as reaching target sites and controlled drug release, thus making them promising for effective tumor accumulation of therapeutic agents by the EPR-based mechanism (Bae et al., 2005; Nishiyama et al., 2005).

Figure 3. Schematic structure of self-assembled micelles (Yordanov, 2013).

1.2.3 Superparamagnetic Iron Oxide Nanoparticles (SPIONs)

Superparamagnetic iron oxide nanoparticles, magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃) with a small particle size of 5-25 nm, with appropriate surface coatings have higher capabilities as MRI contrast enhancers than conventional contrast agents such as gadolinium chelates (Shanehsazzadeh et al., 2013; Thomas, Park, & Jeong, 2013; Chen et al., 2010). SPIONs also have potential applications in drug delivery and in detoxification of biological fluids (Neuberger et al., 2005; Lodhia et al., 2010). The essential requirement for the use of SPIONs in these applications is a controlled particle size smaller than 100 nm with a narrow distribution and high magnetisation value
because the properties of the nanocrystals depend on their size and superparamagnetism (Gupta & Gupta, 2005). Uncoated iron nanoparticles tend to form large aggregates. Degradable nontoxic and biocompatible polymers such as PEG, poly(lactide-co-glycolide) (PLGA), and dextran are used to coat the iron oxide nanoparticles to prevent aggregation (Teja & Koh, 2009). Surfactants and surface coatings used can affect size, surface charge and physiological stability of nanoparticles, so that different pharmacokinetics and biodistributions can be obtained. A potential advantage of SPIONs is that they can be attracted to a tumor or organ by using an external magnet (Gupta and Gupta, 2005; Sawant, 2008).

Among all the techniques to synthesize SPIONs, the co-precipitation method is the easiest and cheapest (Qu et al., 1999; Gupta & Wells, 2004). The method has many advantages: the production scale can be easily amplified or reduced, and the particle size distribution ranges from 5 to 25 nm (Gupta and Curtis, 2004).

1.3 Rationale for each Specific Aim

**Rationale for Aim 1:** Expertise in making and characterizing this type of nanoparticle is available in the laboratory of Dr. Sridhar, which will greatly facilitate its preparation in our own hands. Dr. Sridhar’s group also has experience in using this kind of particles in MRI experiments. It will be necessary to transfer the methodology to our own laboratory, in order to work with radioactive materials.
**Rationale for Aim 2:** This method of radiolabeling has been used extensively with nanoparticles such as liposomes. The phospholipid hydrocarbon tails incorporate themselves into the lipid membrane, while the polar ethanolamine-phosphate head group is exposed on the surface of the particles. DTPA attached to the ethanolamine moiety via an amide bond chelates trivalent metal cations with very high affinity. One such metal is indium, and a radioisotope of this metal, indium-111, is commonly used in clinical nuclear medicine. It emits gamma-rays of energy 0.17 and 0.25 MeV, which combine the features of good ability to penetrate the human body, with efficient detection using nuclear medicine imaging instruments. The half-life is 2.8 days, which offers a reasonable “shelf-life” in the radiopharmacy with acceptably rapid clearance from the patient by physical decay after imaging studies have been completed. The electron-capture mode of decay avoids the higher radiation doses to tissues associated with nuclides that emit beta particles.

**Rationale for Aim 3:** Mice were chosen as test subjects for tissue distribution experiments following long established practice in experimental nuclear medicine. As fellow mammals, they are similar in terms of basic biochemistry and physiology to humans, and candidate radiopharmaceuticals can be conveniently administered via one of the prominent tail veins. Particles of sub-micron to micron size-range are well known to be accumulated from the blood by reticuloendothelial cells in the liver and spleen, and therefore if the radio-indium remains associated with the nanoparticles one should find the radioactivity predominantly in these organs following intravenous injection.
**Rationale for Aim 4:** Since indium is attached to DTPA via chelation, the DTPA is itself attached to phosphatidyl ethanolamine via an amide bond, and the DMPE-DTPA is attached to the nanoparticles via hydrophobic forces, it seems possible that any of these labeled species (indium citrate, indium-DTPA or DMPE-DTPA-indium) could be released from the particles. However, they are all expected to have biodistribution patterns that are distinct from that of indium labeled PEGylated SPIONs and distinct also from each other. A finding that none of these species of indium-111 (indium citrate, indium-DTPA or DMPE-DTPA-indium) selectively localizes in liver or spleen (as hypothesized for indium-111 administered as labeled nanoparticles) would therefore strengthen the argument that indium-111 remains firmly associated with the SPIONs *in vivo*.

One possible complication when using indium$^{3+}$ is the formation of a colloidal form of indium oxide that can also be accumulated by endothelial cells (Ebbe et al., 1996). For this reason, indium-111 is handled in dilute citric acid which forms a weak chelate compound, indium citrate. In the circulation, indium is strongly chelated by transferrin. A complication in administering the modified phospholipid DMPE-DTPA-indium is the possible formation of micelles, which could also be cleared by reticuloendothelial cells.
Rationale for Aim 5: If the nanoparticles remain intact after in the blood after administration, then all the components, iron oxide core, intermediate oleic acid layer and DMPE-DTPA-indium on the surface should be delivered to various organs to the same degree. If the core is labeled with a radioisotope of iron, and the oleic acid is labeled with a radioisotope of carbon, then the organ distributions of these nuclides after intravenous administration should be the same as that seen with indium-111 labeled nanoparticles. However, while our studies were in progress, Freund et al. (2012) reported that carbon-14 oleic acid label may be rapidly removed from the iron core after intravenous injection of similarly labeled SPIONs, so that carbon-14 labeled particles were unsuitable for in vivo studies. We sought to confirm or refute the findings of Freund et al (2012).
2. SPECIFIC AIMS

2.1 Objectives

1. Prepare and characterize SPIONs with an iron oxide core, an intermediate layer (the surfactant) of oleic acid and a surface coating of distearoyl phosphatidyl ethanolamine polyethylene glycol (DSPE-PEG) (Figure 4 and 5).

![Figure 4. Schematic diagram of oleic acid stabilized superparamagnetic iron oxide nanoparticles (Herranz, Pellico, & Ruiz-Cabello, 2012). As detailed in section 3.3, SPIONs nanoparticles are precipitated from a mixture of ferrous chloride and ferric chloride by addition of ammonium hydroxide. When oleic acid is added to the mixture, it induces the formation of oleic acid coated particles that can be handled as a dispersion in chloroform (Gupta & Wells, 2004).](image)

2. Evaluate the labeling of the SPIONs with radionuclide indium-111 by means of a lipid anchored chelating group of dimyristoyl phosphatidyl ethanolamine diethylenetriaminepentaacetic acid (DMPE-DTPA) (Figure 5).

3. Measure the concentration of indium-111 in major organs as a function of time after intravenous administration of indium-111 labeled SPIONs.

4. Compare the disposition of indium-111 from labeled SPIONs with the disposition of indium citrate, indium-DTPA and DMPE-DTPA-indium.
5. Compare *in vivo* disposition of indium-111 labeled SPIONs with the dispositions of iron-59 and carbon-14 oleic acid labeled SPIONs.

Figure 5. Schematic structure of water dispersible polyethylene glycol SPIONs formed by hydrophobic interaction between lipids. As detailed in section 3.4, addition of DSPE-mPEG(2000) to oleic acid coated SPIONs results in PEGylated particles that can be handled in aqueous media. (Yang et al., 2009)

### 2.2 Hypothesis

1. Indium-111 will become firmly attached to nanoparticles into whose surface coating DMPE-DTPA has been incorporated, as shown by recovery of indium-111 with nanoparticles when these are harvested by ultrafiltration.

2. Radioactivity will become relatively concentrated in liver and spleen at early times after intravenous administration because of trapping of particles by reticuloendothelial cells, and will remain located in these organs for many hours.

3. Following intravenous administration of indium-111 in the chemical forms of indium citrate, indium-DTPA or DMPE-DTPA-indium, the radioactivity concentrations in liver and spleen will be significantly lower than the liver and spleen radioactivity concentrations measured after administration of indium-111 labeled SPIONs.
4. Disposition patterns of radioactivity from nanoparticles labeled with iron-59 in the core, with carbon-14 in the oleic acid layer, or with indium-111 on the surface will be different because a very recent article comparing iron-59 and carbon-14 oleic acid labeling of similarly prepared particles reports that the carbon-14 label may not be stable \textit{in vivo} (Freund et al., 2012).
3. MATERIAL AND METHODS

3.1 Animals

Male Swiss Webster mice (Charles River laboratories, Cambridge, MA) weighed 25 ~ 30 g were used for all in vivo studies. Mice are maintained at the animal facility of Division of Laboratory Animal Medicine (DLAM) on 12 hour alternating light and dark period, with access to food and water ad libitum. Mice were treated in compliance with NIH guidelines for the use of laboratory animals and according to a protocol approved by Northeastern University Institutional Animal Care and Use Committee (IACUC).

3.2 Chemical Reagents

Iron (II) chloride tetrahydrate (99+%, Acros Organics) (FeCl$_2$·4H$_2$O), Iron (III) chloride hexahydrate (99+%, Acros Organics) (FeCl$_3$·6H$_2$O) and Ethanol (200 proof, USP/NF) were purchased from Fisher Scientific (NJ). Sodium Chloride (ACS Reagent, 99+%), Oleic acid (99+%), Ammonium hydroxide solution (ACS reagent, 28.0-30.0% NH$_3$ basis), Chloroform (ACS Reagent, 99.8+%), and HPLC grade water were purchased from Sigma-Aldrich (Saint Louis, MO). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-mPEG$_{2000}$) and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (DMPE-DTPA) were purchased from Avanti Polar Lipids (Alabaster, AL). Indium-111 Radionuclide indium citrate, Iron-59 Radionuclide ferric chloride, [1-$^{14}$C]-Oleic Acid, Solvable™ and Ultima Gold™ XR were purchased.
from Perkin Elmer (Waltham, MA).

3.3 Preparation of Superparamagnetic Iron Oxide Nanoparticles (SPIONs)

The SPIONs were prepared by using the co-precipitation method with slight modifications. Briefly, 15 mL of 0.1 M FeCl₂·4H₂O with 30 mL of 0.1 M FeCl₃·6H₂O were mixed in a round bottom flask equipped with a temperature probe. The molar ratio of Fe²⁺ and Fe³⁺ needs to be 1:2 (Schwertmann & Cornell, 1991). The solution was bubbled with argon and stirred for 20 minutes in the chemical hood before heating. When the temperature reached about 80 °C, 3 mL of 5 M NH₄OH was added drop-wise, the clear pale yellow-green solution immediately turned dark brown-black indicating the formation of iron oxide nanoparticles. At that point, 100 mg (111.7 µL) of oleic acid was added into the mixture. The heating continued for another 30 minutes while the temperature was maintained at 80 °C. The sample was then allowed to cool to room temperature (RT). The resulting SPIONs were separated from the solution using a strong magnet and the particles were washed with ethanol twice, dried under argon, and re-dispersed in chloroform. Using a 10 mL syringe (Fisher Scientific, NJ), the re-dispersed SPIONs were filtered through PVDF 0.45 µm filters (Millipore™ Millex™ Sterile Syringe Filters, Fisher Scientific, NJ) to remove large aggregates, dried under argon, carefully weighed and re-dispersed in chloroform to produce a 20 mg/mL SPIONs colloidal solution.
3.4 Preparation of DSPE-PEGylated SPIONs

PEGylated SPIONs were prepared by the rehydration method. In brief, 10 mg of DSPE-mPEG$_{2000}$ and 2.5 mg of SPIONs were mixed in chloroform. The organic solvent was removed by using a rotary evaporator to complete dryness. The film was then warmed in 80 °C water for 1 minute and rehydrated using 1 mL of HPLC grade water. The film was placed in a sonicating bath for 20 minutes to obtain PEGylated SPION. Uncoated particles were removed by applying an external magnet, and the supernatant was removed to a fresh vial.

3.5 Characterization of PEGylated SPIONs

3.5.1 Particle Size and Surface Charge Analysis

The particle size distribution and surface charge (zeta potential) of the PEGylated SPIONs were determined by dynamic light scattering (DLS) measurement with Brookhaven Instrument’s 90Plus particle size analyzer (Holtsville, NY). The particle size was determined at 90° angle and at 25 °C temperature and the average count rate was adjusted in the range of 100 - 500 kcps by proper dilution (10 μL of the PEGylated SPIONs solution was diluted in 2 mL deionized water). The mean number diameter of the particles was obtained on MSD distribution mode. The average zeta potential values were determined based on the electrophoretic mobility using the Smoluchowski-Helmholtz equation (Cho, Lee, & Frey, 2012). Each sample was performed five times with 10 cycles each time and an average value was obtained from the five
measurements. The PEGylated SPIONs were stored at 4 °C and their stability was assessed on day 2, day 3, day 4, day 5, day 6 and day 7.

3.5.2 Transmission Electron Microscopy (TEM)

A JEOL 100-X transmission electron microscope (Peabody, MA) was utilized for analyzing the structure of the iron core of the PEGylated SPIONs. 10 μL of the PEGylated SPIONs solution was properly diluted in HPLC grade water and placed on the carbon-coated copper grids and allowed to air-dry at RT.

3.6 Radiolabeling PEGylated SPIONs with Indium-111

To radiolabel PEGylated SPIONs with indium-111, the particles were synthesized as described above along with addition of 0.5 mol% of DMPE-DTPA to the lipid film. This was about 0.02 mg (Sawant, 2008). 1 mL of DMPE-DTPA containing SPIONs were incubated for 1 hour with 10 μL of 111-indium citrate solution (diluted to approximately 8 - 10 μCi by using 0.5 M HCl) at RT, to allow for the transchelation of indium-111 from a weak citrate complex into a strong DTPA complex. The radioactivity was measured in an ionization chamber (CAL/RAD MARK IV, Fluke Biomedical) with a setting of 676. The unbound indium-111 was removed by a 30 Kilo Dalton cut microcentrifuge filter (Amicon Ultra 0.5 mL centrifugal filters, Sigma-Aldrich, St. Louis, MO) at 14,000 rpm for 15 minutes at 4 °C for 3 times. The radiolabeled PEGylated SPIONs were recovered from the filter using 1.5 mL of 0.9% NaCl solutions (saline).
3.7 Biodistribution Studies of indium-111 Radiolabeled PEGylated SPIONs

Indium-111 labeled PEGylated SPIONs were administered to Male Swiss Webster mice (25-30 g) via a tail vein. 0.2 mL of indium-111 labeled SPIONs saline solution (containing about 0.3 mg SPIONs and 1 µCi radioactivity) was injected into each mouse. The mice were euthanized by cervical dislocation at times 10 minutes, 30 minutes, 1 hour, 2 hours and 24 hours post injection. Five mice were used for each time point. After euthanasia, blood, urine and other solid organs (brain, heart, liver, lung, spleen, kidney, testis, fat, bone, muscle and skin) were collected. Each sample was carefully weighed and placed in the bottom 1 cm of a 12 × 75 mm tube and counted for indium-111 radioactivity as count per minute (CPM) using a Cobra auto-gamma counter (Packard, a Canberra company). Lower and upper window thresholds in the counting protocol were 100 and 300 keV, respectively. From the data, the percentage of injected dose of radioactivity per gram (ID/g %) of each sample was calculated by 100 × (Tissue CPM – Background CPM) × Weight (g) / (Injected CPM – Background CPM).

3.8 Biodistribution Studies of indium-111 Radiolabeled indium citrate, indium-DTPA and DMPE-DTPA-indium

3.8.1 Preparation of indium-111 radiolabeled indium citrate solution

5 µL of ¹¹¹indium citrate solution (approximately 4 - 5 µCi) was dissolved in 1.25 mL of saline. 0.2 mL of ¹¹¹indium citrate saline solution was injected intravenously to each mouse (a group of 5) through tail vein. The mice were euthanized by cervical dislocation at time point 30 minutes post injection.
3.8.2 Preparation of indium-111 radiolabeled indium-DTPA solution

0.02 mg of DTPA was dissolved in 1.25 mL of saline. The solution was incubated with 5 μL of $^{111}$indium citrate solution (about 4 - 5 μCi) for 1 hour at RT, to allow for the transchelation of indium-111. 0.2 mL of $^{111}$indium-DTPA saline solution was injected intravenously to each mouse (a group of 5) through tail vein. The mice were euthanized by cervical dislocation at time point 1 hour post injection.

3.8.3 Preparation of indium-111 radiolabeled DMPE-DTPA-indium solution

0.02 mg of DMPE-DTPA was dissolved in 1.25 mL of ethanol-emulphor-saline (1:1:18). The solution was incubated with 5 μL of $^{111}$indium citrate solution (about 4 - 5 μCi) for 1 hour at RT, to allow for the transchelation of indium-111. 0.2 mL of DMPE-DTPA-$^{111}$indium solution was injected intravenously to each mouse (a group of 5) through tail vein. The mice were euthanized by cervical dislocation at time points 30 minutes and 2 hours post injection.

3.8.4 In vivo Biodistribution Studies

For all euthanized mice, blood, urine and other solid organs (brain, heart, liver, lung, spleen, kidney, testis, fat, bone, muscle and skin) were collected. Each sample was carefully weighed and counted for indium-111 radioactivity as CPM using the auto-gamma counter.
3.9 Biodistribution studies of iron-59 and carbon-14 oleic acid radiolabeled PEGylated SPIONs

3.9.1 Preparation of iron-59 or carbon-14 oleic acid radiolabeled SPIONs

Particles radiolabeled with iron-59 in the SPION core were prepared by adding a small volume (15 μL; 15 μCi) of radioactive $^{59}$Fe$^{3+}$ chloride solution to the mixture of ferrous and ferric chloride solutions before addition of ammonia (see procedure 3.3 for preparation of unlabeled particles, earlier in this section). Similarly, particles radiolabeled with carbon-14 oleic acid were prepared by adding a small volume (0.25 mL; 25 μCi) of carbon-14 oleic acid solution to the oleic acid that was added after addition of ammonia. In both cases, neither the additional volume nor the extra mass of material should have any effect on the formation and coating of the particles, since this is being done in a volume of 45 mL using approximately 250 mg of iron and 100 mg of oleic acid. The radioactivity in the preparation of iron-59 labeled SPIONs was estimated using the ionization chamber. The radioactivity in a 10 μL sample of the carbon-14 oleic acid labeled SPIONs was measured using a liquid scintillation counter (LS6500, Beckman Coulter) since carbon-14 emits no photonic radiation.

After the formulation of iron-59 and carbon-14 oleic acid labeled SPIONs, the PEGylated SPIONs were prepared as described above in section 3.4 with one difference. The PEGylated particles were rehydrated using 1.5 mL saline instead of HPLC grade water.
3.9.2 In vivo biodistribution studies

0.2 mL of iron-59 labeled PEGylated SPIONs saline solution or 0.2 mL of carbon-14 oleic acid labeled PEGylated SPIONs saline solution was injected intravenously to each mouse (a group of 5) through tail vein. The mice were euthanized by cervical dislocation at time points 10 minutes, 30 minutes and 24 hours post injection with five mice in a group for each time points. Besides, a comparison group of five mice was intravenously injected with 0.2 mL of ethanol-emulphor-saline (1:1:18) which only contained about 0.3 μCi carbon-14 oleic acid.

For mice administered with iron-59 labeled particles, blood, urine and other solid organs (brain, heart, liver, lung, spleen, kidney, testis, fat, bone, muscle and skin) were collected. Each sample was carefully weighed and counted for iron-59 radioactivity as count per minute (CPM) using the auto-gamma counter with appropriate energy windows (800-1600 keV).

For mice administered with carbon-14 oleic acid labeled particles, blood, urine and other solid organs (brain, heart, liver, lung, spleen, kidney, and testis) were collected. Tissues were completely dissolved in Solvable™ and bleached with 30% hydrogen peroxide before addition of UltimaGold™ XR liquid scintillation fluid. The samples were then assayed for carbon-14 using the liquid scintillation counter.
4. RESULTS AND DISCUSSIONS

4.1 Preparation and Characterization of PEGylated SPIONs

The PEGylated SPIONs were stable and did not show signs of aggregation after 1 week at 4 °C (Figure 7). The size of the PEGylated SPIONs, as well as DTPA anchored PEGylated SPIONs, was generally in the range of 30 - 50 nm (40.08 ± 6.75 nm, Figure 6). The surface zeta potential for the nanoparticles were always negative at about -36.6 ± 6.58 mV. TEM images of the iron core of PEGylated SPIONs are shown in figure 8. The size distribution of the iron core ranged from 5 nm to 25 nm. These results are in agreement with previous studies (Sawant, 2008; Plouffe et al., 2011).

Figure 6. Particles size of PEGylated SPIONs measured using dynamic light scattering in 90Plus particle size analyzer.
Figure 7. Stability Measurement of PEGylated SPIONs for consistent 7 days. Values are mean ± s. d. (n = 5)

Figure 8. TEM micrograph of the iron core of PEGylated SPIONs.
4.2 Radiolabeling of PEGylated SPIONs with Indium-111

The majority of the $^{111}$indium citrate used in the reaction mixtures was recovered in the DTPA modified PEGylated SPIONs. Table 1 shows counting data from filters and filtrates for successive centrifugations. The labeling efficiency percentage was higher than 70%.

**Table 1. Radioactivity recovered of $^{111}$indium with SPIONs in successive filtration. Values are mean ± s. d. (n = 5)**

<table>
<thead>
<tr>
<th></th>
<th>111Indium Radioactivity (μCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovered from membrane</td>
</tr>
<tr>
<td>First Centrifugation</td>
<td>7.7 ± 1.3</td>
</tr>
<tr>
<td>Second Centrifugation</td>
<td>7.6 ± 1.1</td>
</tr>
<tr>
<td>Third Centrifugation</td>
<td>7.6 ± 0.8</td>
</tr>
</tbody>
</table>

4.3 Biodistribution of indium-111 radiolabeled PEGylated SPIONs

Biodistribution studies were conducted at 5 time-points after administration of indium-111 labeled SPIONs: 10 minutes; 30 minutes; 1 hour; 2 hours; and 24 hours. The results (percent injected radioactivity per gram tissue wet weight) are shown in Table 2. Liver followed by spleen exhibited the highest radioactivity concentrations at all times. Radioactivity in the blood decreased with time after administration, as to a lesser degree did radioactivity in lung and heart, probably reflecting the large amounts of blood in these organs. In contrast, radioactivity in liver increased with time. This rise was significant ($p<0.05$) between 10 and 30 min. This is consistent with the notion that indium-111 remained bound to nanoparticles in blood during the period of our experiments, and that the particles continued to be accumulated by liver.
reticuloendothelial cells, and remained trapped in these cells. Similarly to the case with liver, the concentration of radioactivity in spleen and bone did not decrease over the course of our experiments, again consistent with trapping by reticuloendothelial cells in these organs (Martindale, Papadimitriou, & Turner, 1980) (Figure 9). Radioactivity concentrations decreased in the order skin > muscle > testes > fat > brain, and also fell between earlier and later time-points in these tissues. It is likely that all radioactivity measured in the brain was actually in the brain vasculature, since it was very low at all times, and its concentration was 3-5% of that in the blood at all time-points (Figure 11). Kidney had the third highest concentrations of radio-indium at all times, after liver and spleen; this could reflect trapping of smaller nanoparticles (HD < 10 nm) in the glomerulus (Zuckerman & Davis, 2013). Some radio-indium was recovered in urine, indicating that some loss of indium from the particles occurs (Figure 10).

**Table 2. Biodistribution data of indium-111 labeled PEGylated SPIONs at different time points (represented as the average percentage of injected activity per gram of each organ). Values are mean ± s. d. (n = 5)**

<table>
<thead>
<tr>
<th>Tissues/ Times</th>
<th>10 minutes</th>
<th>30 minutes</th>
<th>1 hour</th>
<th>2 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>27.04 ± 1.89</td>
<td>33.90 ± 1.44</td>
<td>33.35 ± 1.12</td>
<td>34.83 ± 0.57</td>
<td>37.28 ± 1.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>15.46 ± 0.41</td>
<td>15.71 ± 1.50</td>
<td>15.18 ± 1.06</td>
<td>13.43 ± 0.45</td>
<td>21.48 ± 2.41</td>
</tr>
<tr>
<td>Kidney</td>
<td>10.72 ± 1.57</td>
<td>7.71 ± 1.37</td>
<td>5.68 ± 0.91</td>
<td>9.57 ± 0.97</td>
<td>8.03 ± 1.00</td>
</tr>
<tr>
<td>Blood</td>
<td>9.59 ± 1.38</td>
<td>4.23 ± 0.81</td>
<td>4.22 ± 0.72</td>
<td>3.97 ± 0.58</td>
<td>0.55 ± 0.13</td>
</tr>
<tr>
<td>Lung</td>
<td>4.85 ± 1.01</td>
<td>2.99 ± 0.28</td>
<td>2.76 ± 0.50</td>
<td>2.65 ± 0.15</td>
<td>1.27 ± 0.31</td>
</tr>
<tr>
<td>Heart</td>
<td>2.32 ± 0.62</td>
<td>1.24 ± 0.16</td>
<td>1.08 ± 0.07</td>
<td>1.10 ± 0.18</td>
<td>0.62 ± 0.15</td>
</tr>
<tr>
<td>Bone</td>
<td>2.10 ± 0.19</td>
<td>3.14 ± 0.17</td>
<td>3.43 ± 0.40</td>
<td>3.66 ± 0.34</td>
<td>3.87 ± 0.25</td>
</tr>
<tr>
<td>Skin</td>
<td>1.36 ± 0.37</td>
<td>1.06 ± 0.07</td>
<td>0.73 ± 0.09</td>
<td>0.78 ± 0.20</td>
<td>1.00 ± 0.18</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.80 ± 0.24</td>
<td>0.65 ± 0.10</td>
<td>0.41 ± 0.07</td>
<td>0.58 ± 0.09</td>
<td>0.53 ± 0.12</td>
</tr>
<tr>
<td>Testis</td>
<td>0.40 ± 0.05</td>
<td>0.32 ± 0.09</td>
<td>0.34 ± 0.05</td>
<td>0.42 ± 0.04</td>
<td>0.45 ± 0.07</td>
</tr>
<tr>
<td>Fat</td>
<td>0.30 ± 0.03</td>
<td>0.29 ± 0.05</td>
<td>0.16 ± 0.01</td>
<td>0.26 ± 0.01</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>Brain</td>
<td>0.25 ± 0.09</td>
<td>0.13 ± 0.03</td>
<td>0.12 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Urine</td>
<td>11.17 ± 3.63</td>
<td>5.84 ± 0.97</td>
<td>2.47 ± 0.62</td>
<td>0.73 ± 0.34</td>
<td>7.83 ± 1.91</td>
</tr>
</tbody>
</table>
Figure 9. Graph of biodistribution of $^{111}$indium labeled PEGylated SPIONs at different time points (represented as the average percentage of injected activity per gram of each organ). Mice (25-30 g) were injected intravenously with indium-$^{111}$ labeled particles suspended in 0.2 mL of 0.9% NaCl solutions.

Figure 10. Indium-$^{111}$ in urine after administration of $^{111}$indium labeled PEGylated SPIONs.
Figure 11. Graphs showing liver-to-blood (left) and brain-to-blood (right) ratios versus time.

4.4 Biodistribution of indium-111 radiolabeled indium citrate, indium-DTPA and DMPE-DTPA-indium

Surprisingly, the distribution of label from DMPE-DTPA-indium at 2 h was similar to the distribution of label from $^{111}$indium labeled PEGylated SPIONs at that time, when tissues were ordered according to concentrations of label. However, liver, spleen and bone levels were lower for DMPE-DTPA-indium, while the blood, heart and lung concentrations were higher. At the earlier time-point of 30 min, these differences from the distribution seen for indium-SPIONs were more marked, and the concentrations in liver and spleen were only half for DMPE-DTPA-indium than for indium-SPIONs, while the blood level was 4 times higher for DMPE-DTPA-indium (Figure 12). At 30 min time point, the liver-to-blood ratio for DMPE-DTPA-Indium was <1, whereas for indium-SPIONs it was > 7 (Figure 13).
Figure 12. Comparison of biodistribution between indium-SPIONs and DMPE-DTPA-indium at 30 min and 2 h.

Figure 13. Comparison of liver-to-blood ratios versus time between indium-SPIONs and DMPE-DTPA-indium.

Together, the data at 30 min and 2 h for DMPE-DTPA-indium indicate a much slower transfer of label from blood to organs than for indium-SPIONs. It can therefore be inferred that our preparations of indium-SPIONs do not contain appreciable amounts
of free DMPE-DTPA-indium, and that the labeled phospholipid remains bound to the SPIONs during the distribution phase. Nevertheless, DMPE-DTPA-indium could dissociate from the SPIONs to some extent in the blood, and extensive dissociation could occur in tissues after uptake, since the distribution of DMPE-DTPA-indium at 2 h is similar to that of indium-SPIONs; presumably, then, labeled phospholipid released from indium-SPIONs would remain in the tissues where the nanoparticles were deposited.

Two hours after administration of labeled substance, urine contained about 10 times more radioindium for DMPE-DTPA-indium than for indium-SPIONs (Figure 14). Thus, indium incorporated into the PEGylated SPIONs is more resistant to loss of a radioactive species that can exit tissues and undergo clearance by the kidneys.

![Radioindium in Urine](image)

**Figure 14. Comparison of urine radioactivity between indium-SPIONs and DMPE-DTPA-indium.**

Thirty minutes after administration of $^{111}$indium citrate, blood and kidney, as well as bone, contained higher concentrations of radioactivity than after administration of
indium-SPIONs, while liver and spleen contained less (Figure 15). Indium is tightly complexed by the iron-transport protein transferrin, and the observed disposition of label may reflect a combination of the behaviors of Indium$^{3+}$ and indium-transferrin. Although this is speculative, one could suggest that the high blood level is due to indium-transferrin, while the high concentration in bone is due to incorporation of Indium$^{3+}$ into the bone marrow, or to formation of indium oxide colloid that is taken up by reticuloendothelial cells (Ebbe et al., 1996). Irrespective of the chemical forms present, this experiment supports the notion that the tissue dispositions seen with DMPE-DTPA-indium and indium-SPIONs are not predominantly due to Indium$^{3+}$ released from its chelated form. This notion is strongly supported by examination of the distribution of labeled form $^{111}$indium-DTPA at 1 h time point. Tissue concentrations of label were quite distinct from those seen when indium citrate was injected. As expected from the nuclear medicine literature on simple metal chelates, it was characterized by extensive urinary excretion (Corcoran et al., 2010; Fritsch et al., 2009). Tissue concentrations of radiolabeled $^{111}$indium-DTPA at 1 h were much lower than obtained with the other forms of radio-indium. Kidney had the highest level, probably reflecting indium chelate in the urinary collection system (Corcoran et al., 2010; Fritsch et al., 2009).
Figure 15. Comparison of biodistribution of indium-SPIONs, DMPE-DTPA-indium and indium citrate at 30 minutes post injection, showing distinct distribution patterns for each form of radioactive indium.

Table 3. Biodistribution data of indium-111 labeled DMPE-DTPA-indium, indium citrate and indium-DTPA (represented as the average percentage of injected activity per gram of each organ). Values are mean ± s. d. (n = 5)

<table>
<thead>
<tr>
<th>Tissues</th>
<th>DMPE-DTPA-(^{111})Indium (30 min)</th>
<th>DMPE-DTPA-(^{111})Indium (2 h)</th>
<th>(^{111})Indium citrate (30 min)</th>
<th>(^{111})Indium-DTPA (1 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>16.10 ± 1.19</td>
<td>25.27 ± 0.42</td>
<td>20.58 ± 2.79</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>Spleen</td>
<td>8.11 ± 1.21</td>
<td>7.03 ± 0.37</td>
<td>8.19 ± 1.60</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.79 ± 0.49</td>
<td>5.14 ± 0.60</td>
<td>27.33 ± 3.46</td>
<td>2.16 ± 0.65</td>
</tr>
<tr>
<td>Blood</td>
<td>18.60 ± 0.92</td>
<td>8.07 ± 0.91</td>
<td>14.58 ± 3.26</td>
<td>0.39 ± 0.17</td>
</tr>
<tr>
<td>Lung</td>
<td>11.19 ± 1.19</td>
<td>7.53 ± 0.77</td>
<td>10.73 ± 1.98</td>
<td>0.41 ± 0.06</td>
</tr>
<tr>
<td>Heart</td>
<td>4.57 ± 0.38</td>
<td>2.65 ± 0.33</td>
<td>4.22 ± 1.39</td>
<td>0.23 ± 0.13</td>
</tr>
<tr>
<td>Bone</td>
<td>1.79 ± 0.29</td>
<td>1.90 ± 0.37</td>
<td>6.72 ± 1.02</td>
<td>0.82 ± 0.35</td>
</tr>
<tr>
<td>Skin</td>
<td>0.55 ± 0.19</td>
<td>0.77 ± 0.12</td>
<td>2.35 ± 0.65</td>
<td>0.48 ± 0.09</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.61 ± 0.04</td>
<td>0.54 ± 0.07</td>
<td>2.22 ± 0.83</td>
<td>0.27 ± 0.16</td>
</tr>
<tr>
<td>Testis</td>
<td>0.50 ± 0.07</td>
<td>0.59 ± 0.06</td>
<td>1.05 ± 0.21</td>
<td>0.35 ± 0.33</td>
</tr>
<tr>
<td>Fat</td>
<td>0.16 ± 0.06</td>
<td>0.28 ± 0.08</td>
<td>1.20 ± 0.31</td>
<td>0.13 ± 0.07</td>
</tr>
<tr>
<td>Brain</td>
<td>0.53 ± 0.06</td>
<td>0.30 ± 0.17</td>
<td>0.51 ± 0.12</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Urine</td>
<td>7.74 ± 2.16</td>
<td>9.45 ± 2.37</td>
<td>16.53 ± 3.46</td>
<td>79.30 ± 37.75</td>
</tr>
</tbody>
</table>
4.5 Biodistribution of iron-59 or carbon-14 oleic acid radiolabeled PEGylated SPIONs

The incorporation efficiency for iron-59 was approximately 53% (8 μCi out of 15 μCi), assayed using the ionization chamber, while the carbon-14 oleic acid incorporation efficiency was about 8% (2 μCi out of 25 μCi), estimated by the liquid scintillation counter. The poor efficiency for labeling with carbon-14 is due to the large excess of oleic acid over iron salts used in the preparation of the SPIONs.

When the iron core of the SPIONs was labeled with iron-59, the distribution pattern at each time-point was marked by higher fractions of injected radioactivity in liver and spleen than for indium-111 (compare Table 2 with Table 4), and lower concentrations elsewhere. However, bone, kidney, blood and urine contained more radioactivity than other organs (except liver and spleen).

The fraction of injected iron-59 in liver decreased by about 15%, from 55% to 45% between 10 min and 24h (p < 0.05). This decrease, together with the finding of iron-59 in urine (as well as increased levels of radioactivity in kidney and bone) suggests that PEGylated SPIONs in the liver can be slowly broken down to one or more soluble iron species.
Table 4. Biodistribution data of iron-59 labeled PEGylated SPIONs (represented as the average percentage of injected activity per gram of each organ). Values are mean ± s. d. (n = 5)

<table>
<thead>
<tr>
<th>Tissues/Time</th>
<th>10 minutes</th>
<th>30 minutes</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>54.58 ± 0.87</td>
<td>51.16 ± 2.95</td>
<td>45.04 ± 2.10</td>
</tr>
<tr>
<td>Spleen</td>
<td>17.83 ± 0.65</td>
<td>17.78 ± 0.63</td>
<td>17.36 ± 1.45</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.40 ± 0.18</td>
<td>0.43 ± 0.14</td>
<td>0.72 ± 0.21</td>
</tr>
<tr>
<td>Blood</td>
<td>1.32 ± 0.19</td>
<td>0.44 ± 0.09</td>
<td>1.26 ± 0.43</td>
</tr>
<tr>
<td>Lung</td>
<td>0.52 ± 0.03</td>
<td>0.61 ± 0.07</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Heart</td>
<td>0.14 ± 0.09</td>
<td>0.29 ± 0.11</td>
<td>0.19 ± 0.09</td>
</tr>
<tr>
<td>Bone</td>
<td>0.95 ± 0.24</td>
<td>1.34 ± 0.47</td>
<td>1.79 ± 0.53</td>
</tr>
<tr>
<td>Skin</td>
<td>0.17 ± 0.10</td>
<td>0.04 ± 0.45</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.04 ± 0.03</td>
<td>0.16 ± 0.31</td>
<td>0.35 ± 0.14</td>
</tr>
<tr>
<td>Testis</td>
<td>0.00 ± 0.02</td>
<td>0.30 ± 0.08</td>
<td>0.07 ± 0.07</td>
</tr>
<tr>
<td>Fat</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.08</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>Brain</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.03</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Urine</td>
<td>0.27 ± 0.17</td>
<td>0.76 ± 0.29</td>
<td>0.73 ± 0.22</td>
</tr>
</tbody>
</table>

Together, the data for indium and iron labeled particles suggest that during the distribution phase about half of the radioactivity dissociates from the particles in the form of DMPE-DTPA-Indium. However, as demonstrated in the experiment where DMPE-DTPA-Indium itself was administered, much of this radioactivity can be subsequently taken up and retained by the liver and spleen. Thus, at 10 minutes post injection the radioiron/radioindium ratio in liver is 2:1, but due to recirculation of DMPE-DTPA-Indium at 24h it has decreased to 1.25 (Figure 16).

When particles labeled with carbon-14 oleic acid were injected into mice, a different pattern of distribution (Table 5) was observed than when the particles were labeled with iron-59. Although the liver contained the highest concentration of carbon-14 at 10 and 30 minutes post injection, this halved (18 % IA/g to 9 % IA/g) between the two early
time-points. Spleen, blood, kidney, heart and lung all contained approximately equal concentrations of carbon-14 (about 3% IA/g) at both 10 and 30 minutes, while the concentrations in testis and brain were much lower (about 1% IA/g). Carbon-14 levels in urine were higher than those in non-hepatic tissues at 10 and 30 minutes. By 24 hours after administration of labeled particles, radioactivity-concentrations in all tissues, including liver, were low (< 2% IA/g).

Table 5. Biodistribution data of carbon-14 oleic acid labeled PEGylated SPIONs (represented as the average percentage of injected activity per gram of each organ). Values are mean ± s. d. (n = 5)

<table>
<thead>
<tr>
<th>Tissues/Time</th>
<th>Carbon-14 oleic acid labeled PEGylated SPIONs</th>
<th>Carbon-14 oleic acid only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 minutes</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Liver</td>
<td>17.54 ± 1.71</td>
<td>9.47 ± 0.86</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.16 ± 0.40</td>
<td>2.53 ± 0.20</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.24 ± 0.65</td>
<td>3.17 ± 0.27</td>
</tr>
<tr>
<td>Blood</td>
<td>3.07 ± 0.75</td>
<td>3.47 ± 1.17</td>
</tr>
<tr>
<td>Lung</td>
<td>3.55 ± 0.56</td>
<td>3.29 ± 1.10</td>
</tr>
<tr>
<td>Heart</td>
<td>3.55 ± 1.31</td>
<td>2.73 ± 0.44</td>
</tr>
<tr>
<td>Testis</td>
<td>0.68 ± 0.08</td>
<td>0.64 ± 0.08</td>
</tr>
<tr>
<td>Brain</td>
<td>0.50 ± 0.05</td>
<td>0.40 ± 0.07</td>
</tr>
<tr>
<td>Urine</td>
<td>11.47 ± 1.05</td>
<td>10.03 ± 1.22</td>
</tr>
</tbody>
</table>

These observation, compared to those made using iron-59 labeled particles, demonstrate that oleic acid does not remain attached to the iron oxide core of the particles, in vivo. The failure to see high uptake in the spleen even at 10 minutes indicates that dissociation is quite rapid. The higher uptake in liver than other organs at 10 and 30 minutes may reflect initial storage in complex lipid pools in this organ, which is followed by incorporation into lipoproteins for export to other tissues via the blood.
Other processes such as partial oxidation to ketone bodies (acetoacetate and beta-hydroxybutyrate), again exported to other tissues, are also expected to occur.

**Figure 16.** Comparison of indium-111, iron-59, and carbon-14 oleic acid biodistribution patterns in liver, spleen, bone, blood and kidney at 10 min, 30 min, and 24 h time points.
Biodistribution data for SPIONs labeled with iron-59, indium-111 and carbon-14 which are discussed above for each radionuclide are re-displayed in Figure 16 for tissues, (a) liver, (b) spleen, (c) bone, (d) blood and (e) kidney, at 10 min, 30 min and 24h. This facilitates comparison of the three radionuclides in terms of their effectiveness in labeling SPIONs. (Panel (c) for bone shows data for iron-59 and indium-111 only since carbon-14 levels were too low to be measured in bone.) These comparisons illustrate the points made above that while indium-111 is not a perfect proxy for iron-59, it might be useful for imaging the distribution of SPIONs using SPECT. However, it is clear that the oleic acid component of the SPIONs does not remain with the iron oxide core after administration to mice.

As to the biodistribution of carbon-14 oleic acid alone, it is very different from the $^{14}$C-SPIONs at 30 minutes time point. Less radioactivity remained in tissues (except lung) after administration of oleic acid alone than after administration of carbon-14 oleic acid labeled SPIONs (Table 5). One explanation is that, in associating with and dissociating from the SPIONs, most or perhaps all of the carbon-14 oleic acid is converted to a chemical form that is cleared more slowly from the body. Oleic acid itself may be cleared rapidly because long chain fatty acid are a major metabolic fuel and are rapidly converted to labeled carbon dioxide, which leaves the body in exhaled breath. Additionally, there is more radioactivity in lungs than other tissues after injection of $^{14}$C-oleic acid alone, whereas there is more radioactivity in liver than other tissues after administration of carbon-14 oleic acid labeled SPIONs. Some compounds that are very
lipophilic are trapped in the lungs immediately after intravenous administration because they dissolve in the lung surfactant and oleic acid is one of these compounds (Koren et al., 1980). If this is the explanation for the higher uptake in lungs, we could argue that the failure to see higher lung uptake from oleic acid labeled SPIONs is consistent with the view that the carbon-14 released from the SPIONs is in a different chemical form. Thus, radiochromatographic studies on tissues will be necessary to confirm these speculations.

4.6 OVERALL CONCLUSIONS

After intravenous injection of radiolabeled particles, the radioactive indium was preferentially localized in those organs (liver, spleen and bone) associated with trapping of colloids by reticuloendothelial cells, with no reduction of radioactivity concentration up to the longest time-point examined (24h).

The tissue distributions obtained with indium-111 SPIONs at various times are encouraging for use of such labeled particles in imaging experiments. The differences in distribution of either indium-citrate or of indium-DTPA from those obtained with labeled SPIONs supports the view that a substantial fraction of the indium-111 remains associated with the iron oxide core. However, the somewhat similar distribution of DMPE-DTPA-indium to that of indium-111 labeled SPIONs, in terms of high uptake in organs containing reticuloendothelial cells, raises the question of whether some of the uptake seen with labeled SPIONs in liver and spleen is due to recirculation of dissociated DMPE-DTPA-indium to these organs. In other words, if this labeled
phospholipid itself preferentially distributes to liver and spleen, then the fact that indium-111 from the labeled SPIONs is found in liver and spleen does not absolutely prove that the labeled particles remain intact \textit{in vivo}.

In contrast to the situation with indium-111, it is clear that carbon-14 oleic acid incorporated into the SPIONs does not remain firmly associated with the iron oxide core. Carbon-14 appears to be released rapidly from the SPIONs in the blood since the spleen is not targeted by carbon-14 labeled SPIONs, though this organ is targeted by SPIONs labeled with indium-111 or iron-59. However, the carbon-14 released does not appear to be in the chemical form of oleic acid, since the biodistributions of radiolabel administered as oleic acid itself or oleic acid labeled SPIONs were quite distinct.
5. LIMITATIONS AND FUTURE DIRECTIONS

PEGylated SPIONs that were characterized by DLS and TEM were not radioactive, as we were not allowed to use radioactive materials in these analytical instruments.

The procedures used to prepare SPIONs radiolabeled with iron-59 and with carbon-14 oleic acid, addition of labeled reagent to the reaction mixture conducted on the usual scale, limited the radioactivity of carbon-14 or iron-59 that could be injected into a mouse. We were able to deal with this problem by using long counting times. However, if further studies with these radionuclides are warranted, one could improve radiochemical yields and specific activities by miniaturizing the mass scale of the syntheses but maintaining the same amounts of radioactive iron or oleic acid. However, it is very likely that the size and possibly other characteristics of the SPIONs may depend on factors such as heating rates that could be altered by the scale of the reaction, so that miniaturization might require extensive development work.

Further progress in understanding the behavior of the indium-111 labeled PEGylated SPIONs will require analysis of the chemical form(s) of indium present in tissues (including liver, spleen and especially kidney and urine) using radiochromatographic methods.
6. BIBLIOGRAPHY


Letters, 10(9): 3223–3230. doi:10.1021/nl102184c


doi:10.1038/sj.clpt.6100400