

**Elucidating the effects of metabolic state on
nanoparticle distribution in mice and in vitro uptake**

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

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December 2016

Acknowledgements

Thank you to my parents, Jim and Joan Quigley for supporting me. Thank you to my wonderful wife, Lauren, who is going through graduate school herself. In addition, thank you to all of those who have helped me complete my graduate research in the Dalhaimer lab, especially Dr. Alex Meyers and Katherine Krouse.

Abstract

Since almost 70% of the U.S. population over 20 years old is overweight and 30% is obese, with much of the western world following suit, many patients that will potentially be administered circulating nanoparticles designed to localize to tumors and avoid non-target areas will have significant amounts of white adipose tissue (WAT), enlarged livers, and additional metabolic complications such as type 2 diabetes. However, studies on nanoparticle biodistribution and efficacy take place almost without exception in lean rodents with healthy metabolic states. In this work, I determined the biodistribution of model nanoparticles – neutral filomicelles and polystyrene spheres both carrying near infrared (NIR) dye - as a function of mouse diet, weight, and metabolic condition. The livers of diet-induced obese mice show increased uptake of nanoparticles, while mice with non-alcoholic fatty liver disease (NAFLD) do not see increased uptake despite livers that are four fold as massive. Macrophages and hepatocytes had increased particle internalization in vitro in media with increasing amounts of fatty acids. From these observations, I postulate that the metabolic condition of the patient will change the efficacy of current nanoparticle technologies.

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Chapter 1: Introduction

1-1 Nanoparticle Distribution and Metabolic State of the Patient

Obesity both increases the risk for certain cancers and worsens the chances of survival, while physical activity during treatment improves patient outcomes [1]. Overweight and obese women diagnosed with breast cancer saw decreased rates of survival; additionally, weight gain post-diagnosis has been shown to reduce survival rates along with adverse body composition changes including an increase in fat mass, while reductions in weight improved survivability [1]. Obesity not only effects the treatment of diseases but also increases the risk of developing cancers. Obesity is the second highest cause of cancer after tobacco [2]. Post-menopausal women with obesity see elevated levels of the hormone estradiol, which has been linked to breast cancer [3].

With over two thirds of Americans either overweight or obese, it is important to consider the metabolic state of the typical patient receiving drug delivery treatments in the form of nanoparticles [4]. Most studies of nanoparticle efficacy are carried out in mice with ideal metabolic states that do not represent realistic clinical scenarios. Many patients in the clinic with conditions that may require nanotechnology will be overweight and obese. Those receiving treatment will typically have high levels of white adipose tissue (WAT), may also suffer from other related conditions such as arteriolosclerosis, fatty liver, and diabetes. In this work, I explored a subset of specific factors that may affect the efficacy of nanoparticles. The goal of this work was to take an in-depth look at

nanoparticle patient interactions based on metabolic state to further the advancement of drug delivery vehicles in the treatment of cancer.

The elevated amount of WAT poses concerns that may impact nanoparticle biodistribution and thus efficacy. Triacylglycerol, the main component of WAT is hydrophobic as are many dyes and drugs carried by nanoparticles to identify and treat tumors. These hydrophobic drugs may localize to these fat deposits and diminish the impact on desired targets. Additionally, the number of macrophages increased from 10% of all cells in WAT of lean animals to more than 50% in cases of advanced obesity [5]. Though often poorly vascularized, nanoparticles circulating through WAT could be engulfed by the elevated level of macrophages and the T-cells that they regulate preventing particles from reaching targets [6]. My *in vitro* work using murine macrophages in conditions designed to induce the formation of lipid droplets further showed potential for a reduction in nanoparticle efficacy due to elevated fat levels. Macrophages with higher amounts of fat showed an increase in nanoparticle uptake.

One of the most challenging aspects of treating cancer in obese patients is the difficulty in determining the correct dose to administer for effective treatment. These considerations go far beyond the body mass index (BMI) and simple measures of body fat of patients [7]. These dosing considerations are often inconsistent between different types of cancers, or the appropriate measure, such as body surface area, may be difficult to collect accurately [7-8]. Circulating plasma proteins, such as those related to cholesterol and levels of inflammation both impact health, drug delivery and cancer treatment. From development of atherosclerosis, to interactions with injected drugs, understanding these proteins is an active health interest.

1-2 Current Cancer Treatment with Nanoparticles

The size, shape, charge, and surface chemistry all determine the fate and distribution of nanoparticles [9-11]. Most cancer drugs are designed to take advantage of the enhanced permeability and retention (EPR) effect where particles of diameters between 10 and 200 nm are able to utilize the leaky vasculature surrounding solid tumors and passively accumulate in the tissue surrounding the tumor. There are a number of currently approved drugs that are designed with advantages over free drug such as increased circulation, reduced negative side effects and improved efficacy. These drugs include protein drug complexes and a variety of liposomes. Nanoparticles that are currently available in the United States, Europe, or Asia for cancer treatment are listed in Table 1-1. While these drugs are a hopeful advancement in treatment, they are still subject to drawbacks that prevent widespread success [12].

Many polymer based nano carriers undergo accelerated blood clearance upon repeated injections, which can cause difficulties with extended treatment, drug resistant tumors, and recurring cancer [13-14]. Drugs in the typical size range for the EPR effect—10 to 200 nm—are able to accumulate in the interstitial space of the tumor vasculature but they often have trouble delivering drug homogeneously in the tumor tissue or are unable to effectively transport drug past the tumor cell surface [12]. Polyethylene glycol, (PEG), is often used to modify the surface of particles to minimize the immune response [9, 15]. Particles with a near neutral zeta potential typically see the longest circulation times and have increased delivery efficiency compared to negative and positively charged counterparts, which is related to both protein adsorption and detection by the immune system [16]. The binding of serum proteins to

Table 1-1. Nanoparticle Formulations Currently Approved for Cancer Treatment

<i>Drug Name</i>	<i>Description</i>
Abraxane	10 nm Albumin bound paclitaxel.
DaunoXome	50 nm Liposomal daunorubicin
Genexol-PM	20-50 nm Cremophor-free polymeric micelle paclitaxel
Lipusu	Liposomal paclitaxel.
MM-398	100 nm Liposomal irinotecan.
Myocet	150-180 nm non-PEGylated liposomal doxorubicin
PICN	100-110 nm Paclitaxel stabilized by polymer and lipids

nanoparticles in the blood does not necessarily correlate to reduced circulation time, as nonspecific binding of proteins such as albumin has been shown to prolong particle circulation times [12, 17]. Pre-incubation of particles with albumin has been shown to decrease binding of specific whole plasma proteins and reduce clearance by macrophages. These results on protein absorption and immune clearance show that not only is the protein corona crucial in determining nanoparticle fate, but also required for prolonged circulation.

Tumor Transport Properties

The tumor microenvironment presents challenges to drug transport and efficacy. Drug carriers must traverse a series of stages to deliver drug to the tumor cells: blood circulation, tumor vasculature, interstitial space, the tumor cell interior. Transvascular transport is controlled through the pressure difference between the tumor vasculature and the cells' interstitial fluid as well as interactions between the particle and the surface of the vessel walls. This pressure is determined by the permeability of the vessel walls, which is highly variable between different cancer morphologies, and a dysfunctional lymphatic system characterized by poor drainage and increased fluid flow to the vasculature. High interstitial pressure often limits transport to diffusion, which is more challenging for larger particles. Hindrance to transport can be size, charge, and hydrodynamic effects as the particle diameter approaches the size of the pore opening. While negatively charged and neutral particles often have increased circulation times, positively charged particles often see greater transvascular transport due to affinity towards the negatively charged vessel walls according to mathematical models [18-19]. Many nanocarriers are designed not to cross this barrier but rather to localize then

release drug to diffuse into the tumor. Elongated particles of an aspect ratio greater than four with the same hydrodynamic diameter of spherical particles experience greater flux into tumor tissue possibly due to increased interactions with vascular walls when tumbling and rolling.

Effective distribution in the tumor tissue is directly linked to improved patient outcomes. Once inside the interstitial tumor tissue, transport remains limited by diffusion. Due to the high interstitial pressure of a tumor, the drop to normal pressure at the periphery of tumor can cause flux away from the target cells. Excessive extracellular fibers may also hinder the distribution of particles with a diameter larger than 50 nm in contrast to particles 5-50 nm, which generally diffuse homogenously in the tissue. Positively and negatively charged particles both see decreased diffusivity due to charged interactions with negatively charged hyaluronic acid, of the intracellular matrix and positively charged collagen fibers. Flexible nanorods exhibit faster and more uniform distribution compared to comparable spheres and ridged elongated particles. While small particles diffuse rapidly, they are also cleared from the tumor tissue due to poor retention compared to larger particles.

Similar to tumor angiogenesis, accumulation of white adipose tissue (WAT) causes the growth of additional blood vessels. It is possible that some drug carriers designed to utilize the EPR effect may see reduced efficacy due to the added blood vessels in WAT. Some passive targeting of adipose vasculature has been seen in mice of the diet induced obesity model; while targeting adipose tissue via the growth in vascular architecture may be desirable in some applications, this may cause adverse effects during cancer treatment [20-21].

Transport to the cell surface, internalization, and intracellular transport is likewise a function of size, shape, charge and chemistry. Gold and silver nanoparticles undergo receptor-mediated endocytosis most readily in the range 40-50 nm while larger particles were unable to reach drug targets within the cell. Negatively charged particles, for example carboxylated spheres, have in some systems shown increased cellular uptake *in vitro* [18]. For particles under 100 nm in diameter, spherical particles are endocytosed more readily than rod shaped particles however high aspect ratio particles larger than 100 nm diameter see more efficient uptake compared to spheres. Cellular uptake of nanoparticles is less relevant in cases where drug delivery vehicles are designed to release in the tumor microenvironment, for example by hydrolysis [22]. Tumor penetrating peptides have been used successfully alongside Doxil, Abraxane, micelles, iron oxide particles, quantum dots and hydrogels [12, 23].

Challenges in Drug Delivery of Nanoparticles

Size, shape, charge, and surface chemistry all determine the fate and distribution of nanoparticles. Nanoparticles for drug delivery are typically limited to diameters from 10-200 nm. To take advantage of the larger, leaky vasculature of tumors, which causes increased solid tumor drug localization (i.e. EPR), particles should be larger than the healthy tissue pore diameters of 6-12 nm [24-25]. Blood circulation clearance from organs also limits particle size on the upper and lower ranges. Some drug targeting strategies utilize multi-stage drug carriers to combine the circulation properties of particles greater than 10 nm while releasing cargo capable of penetrating tumor tissue and reaching drug targets within cells. Strategies include external thermal ablation and reaction to the tumor microenvironment such as hydrolysis in lower pH environments

[22, 26]. The altered condition of patients with non-ideal metabolic states may affect how these particles react to the tumor environment with additional fatty acid present.

After intravenous injection, drug carriers experience a number of unique constraints from interactions with organs, blood, and the mononuclear phagocyte system. The size of blood vessels places an upper bound on particle size, which the capillaries in the lungs are among the smallest, between 2 and 13 μm , as they are meant to preferentially transport soft, deformable red blood cells over rigid white blood cells [11]. Nanoparticles approaching this range can become sequestered in the lungs where flexible particles or those of 3 to 6 μm are confined in the lungs temporarily, while rigid particles may be unable to re-enter circulation [27]. This can also be a problem with particle-particle interactions where smaller diameter carriers can aggregate and be captured in the lung vasculature. Particles with a hydrodynamic diameter less than 5-6 nm are cleared by the kidneys, which for proteins is typically a molecular weight less than 60 kDa [28]. Of the larger particles that are retained passing through the kidneys, those larger than 200 nm are removed by the mononuclear phagocyte system, the liver and the spleen [19]. For non-biodegradable carriers larger than 6 nm, the only excretion pathway is through bile [11]. The ease of phagocytosis is mediated but the size, shape, curvature and orientation of the particles; for non-spherical particles the angle of attack is an important factor, where the contact with the larger dimension first slows uptake [29-30]. The size limit for phagocytosis, determined *in vitro*, is 20 μm , which is larger than a particle that could circulate freely in the vasculature [31]. Positively charged particles are also cleared rapidly by the mononuclear phagocyte system. Proteins in the blood plasma that influence the signaling and subsequent clearance by the immune

system bind these positively charged nanoparticles [32]. Neutral charged particles, such as polystyrene spheres, typically show longer circulation times when compared to their charged analogous counterparts [32-33].

Macrophages make up a significant fraction of the immune response and were a focus of *in vitro* investigation. The liver and spleen are the primary targets of nanoparticle localization with macrophages contributing to particle capture in the liver. Kupffer Cells, the macrophages localized to the liver, comprise 80-90% of tissue-associated macrophages [34-35]. Kupffer cells are involved in the liver's response to infection, toxins, injury repair, and inflammation [36]. The spleen is comprised of distinct parts of red pulp containing macrophages and white pulp responsible for lymphocyte proliferation, of both B and T cells. The spleen's architecture is not consistent between species, leading to different areas contributing to particle retention in mice versus humans [11]. Nanoparticle localization to the spleen leads to increased nanoparticle clearance on repeated injections, known as accelerated blood clearance [37]. Interactions of drug carriers with B-cells in the spleen induce the production of circulating specific antibodies, which reduce the circulation times of PEG functionalized nanoparticles [38-39]. Drug carriers conjugated to PEG that see decreased liver uptake are retained by the spleen in greater quantities relative to non-PEG drug particles [40-41]. Non-PEGylated particles are not significantly affected by accelerated blood clearance on repeated injections mostly due to clearance by the liver before circulating antibodies affect their fate [11].

Macrophages can be polarized into different activation states involving particle uptake, inflammation, and debris clearance. Classically, macrophages are divided into

three groups: inactive M0 macrophages, classically activated M1 macrophages, and alternatively activated M2 macrophages. However it has been shown that these activations states are not a simple polarization; some macrophages show mixed M1/M2 activation markers and various levels of pro or anti-inflammatory behavior [42]. WAT macrophages show M2-like behavior as determined by metabolic activation rather than classical pathways [43]. It is possible the metabolic activation of macrophages could contribute to difficulties in treating diseases in patients with obesity. M2 macrophage polarization is associated with increased particle uptake in WAT [44]. The inflammation associated with this macrophage polarization is also associated with cancer development [45-46].

Cells that are in high fat environments such as WAT store free fatty acids in organelles called lipid droplets. Formation of lipid droplets in macrophages has been simulated in vitro with the addition of both arachidonic acid and oleic acid, with arachidonic acid showing increased droplet numbers and sizes [47]. Lipid droplets are present in all cell types in the body and are involved in the management of excess fatty acid that is typical of non-ideal metabolic states. The role of lipid content on the internalization of particles was a focus of this research.

Modeling Obesity in Mice

Mouse models for obesity include diet-induced obesity and genetic modifications. Mouse models can be used that not only alter metabolic state but also alter the levels of circulating plasma proteins, cause accelerated atherosclerosis, and model relevant diseases such as type 2 diabetes. The primary obesity model used in my experiments is *ob/ob* mice that lack leptin, which regulates satiety leading to increased food

consumption by the mice. A less severe obesity model, *A^y/J*, visibly characterized by yellow fur, has increased fat mass compared to control mice. Mice with a knockout of the LDLR liver receptor, have a reduced ability to remove low-density lipoproteins (LDLs) from the blood resulting in two- to four-fold more total cholesterol due to higher circulating LDL levels. High-density lipoprotein (HDL) levels remain relatively constant in these mice [48]. High levels of LDL cholesterol relative to HDL cholesterol is a risk factor for atherosclerosis and cardiovascular disease which is a leading cause of death in the United States [49]. LDLR mice develop arterial lesions and eventually atherosclerosis on a western diet, typically consisting of 17% kcal from fat. Alternatively, a knockout of the SCARB1 receptor in the liver can increase circulating HDL levels. Conversely, knockouts of required protein components of HDL, ApoA I and ApoA II, or ApoB-100 for LDL can be used to reduce these cholesterol levels.

Chapter 2: Investigating Filomicelles for Drug Delivery

Applications

2-1 Filomicelle Stability

Circulation times of days or weeks rather than hours are desirable for a variety of drug delivery applications for the goal of lowering doses and longer periods of bioavailability [50]. Filomicelles have diameters of ~50 nm and lengths up to 10 microns having the shapes of filoviruses and have circulation times in mice of at least two days [51-53].

Through various experiments we found the polybutadiene-polyethylene-glycol (PBD-PEG) filomicelles to be stable at physiological conditions (Figure 2-1). However, either the stability of various dyes or the ability of the micelles to hold cargo was compromised by some separation techniques relative to the cargo retention *in vivo* and in storage over two years at 4°C. The PBD-PEG filomicelles are stable in both length and cargo retention at times longer than the two day length of our *in vivo* experiments. Filomicelles after two weeks in physiological conditions - 37°C in phosphate buffered saline (PBS) at pH 7.4 – are shown in Figure 2-2. The filomicelles and dye were stable and visible through fluorescent microscopy up to 70°C. While using hydrophobic PKH26 dye either through spectroscopy or fluorescent microscopy I found it became necessary to add additional dye due to either a breakdown or loss of dye carried by the vehicles after ultracentrifugation. Although the loss of dye would be useful in determining the loading and delivery of drug, using only an initial addition of dye to the micelles proved

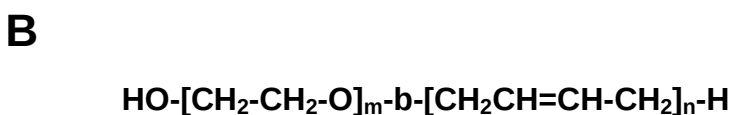
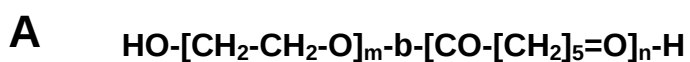
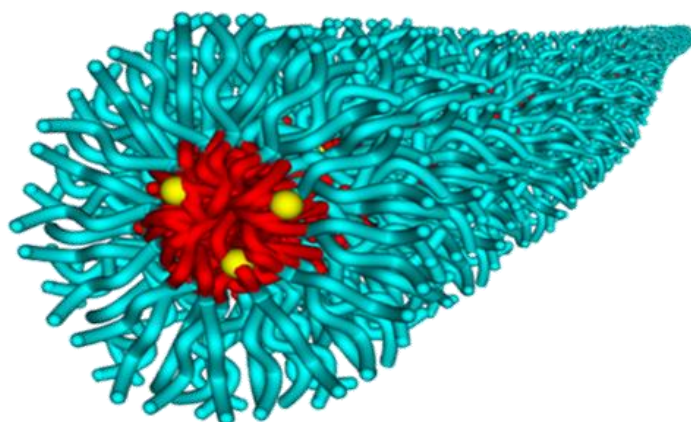


Figure 2-1. Filomicelle structure.

Polyethylene glycol (cyan) comprises the hydrophilic block of the copolymer whereas either polybutadiene or polycaprolactone (red) comprise the hydrophobic block of the copolymer. Hydrophobic dye molecules are represented by yellow spheres. The chemistries of the two copolymers are shown: PEG-PBD **(A)** and PEG-PCL **(B)**. (Filomicelle cross-section structure by P. Dalhaimer.)

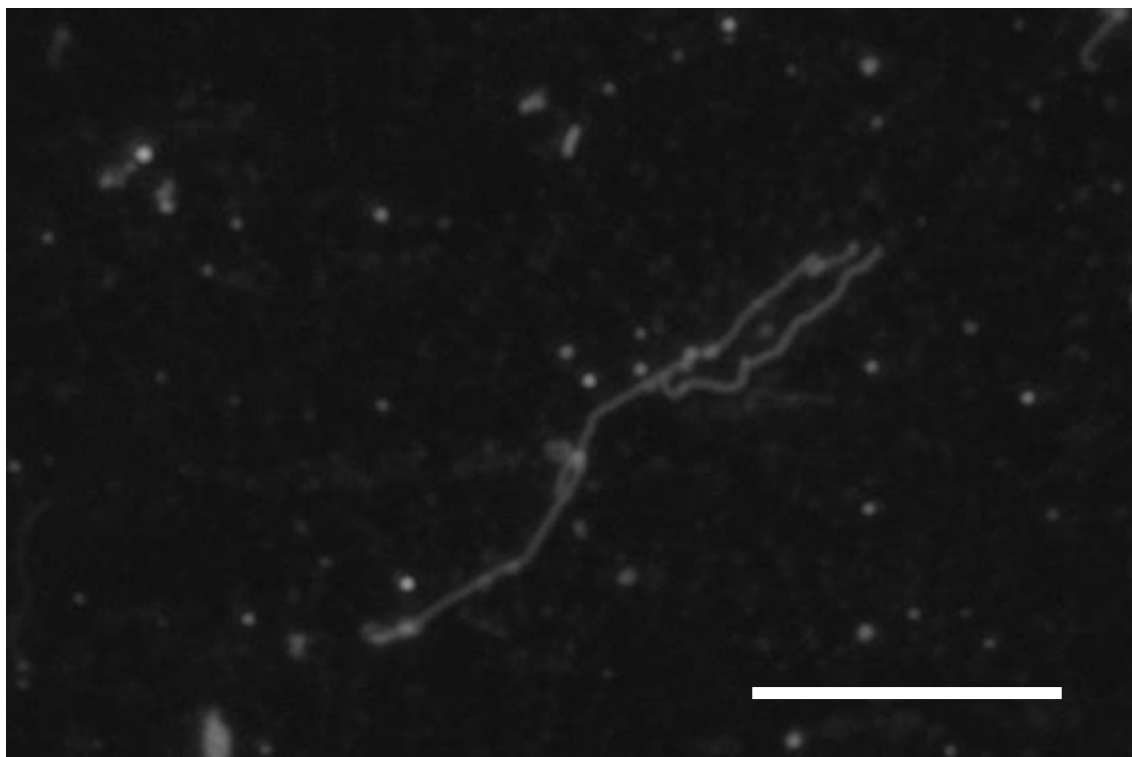


Figure 2-2 Filomicelle with PKH26 dye after two weeks at 37°C

Filomicelles loaded with PKH26 dye (yellow spheres in Figure 2-1) were incubated in PBS for two weeks at physiological temperature and pH retained dye and micelle length. Scale bar is 10 μm .

to only measure the stability of the dye, which was not as robust as the filomicelles during ultracentrifugation.

In my experiments, I investigated the stability of the filomicelles and the leakage of dye using the fluorescence of the loaded dye as a marker. Methods used to remove excess dye or isolate nanoparticles included dialysis, chromatography, ultracentrifugation of the nanoparticles, and the addition of 3 μm polystyrene spheres, which could be removed by centrifugation. Removing excess dye through dialysis is possible due to the favorable partitioning of the dye inside the filomicelles. The advantage of removal by 3 μm polystyrene beads is the use of a benchtop centrifuge at 17,000 $\times g$ rather than an ultracentrifuge.

Ultracentrifugation is often used to separate nanoparticles of various sizes, densities, and shapes and is used in binding affinity studies and in the characterization of the proteins bound to the surfaces of nanoparticles either in the protein corona, or in purification. Separating the filomicelles through centrifugation is desirable to measure protein or potentially separate the longer particles. Ultracentrifugation of the filomicelles proved difficult due to inconsistencies in the amount of particles left in the supernatant. The fraction of nanoparticles removed from solution was to be measured by a reduction in fluorescent PKH26 dye, via UV-vis. At speeds higher than 100,000 $\times g$, the micelles began to lose the initially loaded dye while only a small fraction of nanoparticles were in the pellet. While fluorescence in the solution diminished, the amount of filomicelles in the pellet was negligible. This was determined by supplementing additional PKH26 dye via fluorescent microscopy. Interestingly the fraction of filomicelles in the pellet does not seem dependent on length. The cylindrical micelles are subject to tangling and

clumping, especially in resuspended pelleted samples; however, many aggregated filomicelles remained in the supernatant.

2-2 Separation of Filomicelles by Length

As with most if not all self-assembling systems, the lengths of filomicelles vary. Longer filomicelles have been shown to be more effective in circulation and in targeting solid mass tumors than shorter filomicelles [52]. Thus, I wished to separate the filomicelles by length with the goal of keeping the longer filomicelles for *in vivo* experiments and discarding the shorter filomicelles. Filomicelles were conjugated to peptides to bind to various columns so they could be eluted by length. The idea was to chemically cleave off the peptide after separation by length. SourceS and SourceQ resins were used in combination with appropriate buffer, such as sodium phosphate, sodium citrate, or Tris-HCl, based on the isoelectric point of the peptide. The filomicelles showed weak column affinity.

Filomicelles have lengths ranging from 50 nm to ~10 microns. Given the wide distribution of sizes, I attempted to separate the filomicelles through centrifugation due to the differences in sedimentation rates. Despite large aggregates and other complexes present, there presented no clear difference in the nanoparticles between the pellet and supernatant even at speeds over 100,000xg.

2-3 Filomicelle surface modification

Many advances in nanoparticles as drug delivery vehicles require modification of the surface with various functional groups: peptides and proteins. Targeted surface moieties can increase localization, cell uptake, and circulation times. Peptides have been used to avoid detection of the immune system and to target cancer cell surface

markers which may be overexpressed [50]. To this end, I intended to use linker chemistry, with Sulfo-NHS and EDC, to bind the N-terminus of peptides to a carboxylic acid functionalized polymer. Commercially available COOH-PEG-PBD polymers were significantly shorter than the polymer used to form filomicelles. Attempts to conjugate peptides to filomicelle complexes with 0.1 wt% of COOH bound polymer were unsuccessful. This is possibly due to the incompatible length or poor incorporation of the COOH functionalized polymer into the micelles. Higher weight percentages of COOH-PEG-PBD led to shorter, lower concentration filomicelles.

To produce polymer of the same length as those used in filomicelle preparation, I used potassium permanganate to oxidize the hydroxy terminated PEG-PBD polymer to produce a carboxylic acid. The reaction required dissolving the polymer in pyridine and extraction with diethyl ether. In the future, this process could be avoided by collaboration with the polymer synthesis process as the anionic polymerization can easily substitute the reagent used to terminate the polymerization to produce COOH-PEG-PBD. Producing functionalized polymer in the same batch as the base polymer will remove variability in the use of differing polymer lengths or complex chemistries.

2-4 Materials and Methods

Filomicelle Preparation

Filomicelles were prepared with block copolymers through self-assembly using two methods utilizing the evaporation of chloroform. For micelles based on PEG (Polyethylene glycol) - PCL (Polycaprolactone) (Polymer Source Inc.), 50 mg of solid copolymer was dissolved in 5 mL of chloroform (Fisher Scientific). 30 mL of PBS was added to the chloroform solution and mixed on a stir plate at room temperature until the

chloroform evaporated and the solution became opaque, which took approximately two hours. All of the following steps were done at 4°C to reduce polymer degradation. The polymer solution was centrifuged twice for five minutes at 17,000xg with the pellet discarded each time [52].

PEG-PBD based filomicelles were produced through film evaporation. 100 mg of PEG-PBD was dissolved in 10 mL of chloroform in a glass vial. Chloroform was evaporated under flow of nitrogen while the vial was rotated to ensure even distribution of polymer film. Once all visible chloroform was evaporated, the vial was placed under flow of nitrogen for an additional 15 min. To promote self-assembly of micelles, 10 mL of PBS was added to the dried film and placed in an oven at 60°C overnight.

Dye was loaded into the filomicelles after formations of the micelles by hydrophobic association. Hydrophobic PKH26 dye (Sigma) was added at a ratio of 1 µL dye for 1 mL of filomicelle solution and distributed into the filomicelles via pipette mixing. Excess dye can be removed through dialysis, however very little excess dye is present at this concentration.

Affinity Chromatography

Separation of filomicelles was done in a manually controlled flow column and an FPLC. The advantage of the FPLC is the ability to use a controlled flow, up to 0.5 mL/min, and use a gradient of elution buffer. For the peptide A20.1, with an isoelectric point less than 7, SourceQ beads were used with Sodium phosphate buffer at pH 7.4. An elution buffer of sodium chloride was used as a gradient up to 1M and manually added as 1M. For the peptide A20.36, with an isoelectric point above 7, SourceS beads were used with Tris-HCl buffer pH 7.

2-5 Results and Discussion

The filomicelles proved to be robust in high shear, high temperature, and in the presence of plasma proteins. Difficulty purifying and separating exact amounts of filomicelles could prove challenging when these techniques are vital to their use as a drug delivery vehicle in a clinical setting. Theoretically long cylindrical particles should be able to cross through filtration membranes as long flexible polymers of similar dimensions are able to pass while stiff polymers do not [54]. However, the filomicelles do not pass through a 100KDa MW centrifugal filter. While this filter retention can be advantageous for the isolation and characterization of the filomicelles for examining protein-filomicelle affinity or quantifying the amount of filomicelles present in a blood sample, ability to filter sterilize is important in the use as a clinical drug carrier.

The idea of binding filomicelles to an affinity column through the attachment of peptides should be explored further. Adding a defined range of micelle lengths rather than the range of 40 nm spheres to cylinders tens of microns long, would allow for more control on particle circulation time and fate. These experiments were incomplete without the adequate polymer chemistry and remain useful beyond separation by size. Purification by affinity column ensures micelles that were not functionalized with targeting peptide are removed from the same.

It remains unclear why the PBD-PEG filomicelles are unable to be separated via ultracentrifugation while the PKH26 dye comes out of solution. Attempts to pellet the filomicelles at speeds greater than 100,000xg for one hour were unsuccessful. This is surprising as other micelles, including cylindrical micelles, have been pelleted via ultracentrifugation at lower speeds [55]. It is possible that these PBD-PEG filomicelles

are too similar or lower in density compared to water to see significant sedimentation. In further experiments investigating blood plasma protein filomicelle binding using ultracentrifugation, an increasing amount of protein is pelleted without any interactions with nanoparticles as higher centrifuge speeds were attempted. Control centrifugations with blood plasma alone showed no difference between samples with and without nanoparticles. As particle-protein interactions have become my primary use of ultracentrifugation, I have avoided using solvents other than water that may affect the particle-protein equilibrium. Using separation methods such as a sucrose gradient may be necessary in the future to isolate filomicelles via ultracentrifugation.

Chapter 3: Nanoparticle Studies in vitro and in vivo

3-1 Protein Corona

The protein corona is a key factor in determining the fate of nanoparticles [12]. Previously it was found that amine modified nanoparticles bound strongly to lipoproteins. Increased levels of lipoproteins in patients with non-ideal metabolic states, including LDL, may alter the immune response and fate of drug carriers. Specific binding of proteins, as opposed to nonspecific binding from plasma proteins such as albumin, determines the circulation time and biodistribution of nanoparticles. This effect is largely why the “stealth effect,” utilized by PEG coating, increases blood circulation time and the mechanism behind decreased clearance and specific binding after a pre-incubation in albumin before injection or incubation in whole plasma. Immunoglobulin proteins, IgG and IgM, have been shown to increase particle aggregation, which increases clearance [56-57].

3-2 Obesity Mouse Models

Mouse Models

The mouse strains, age, diet and average weight at the time of injection are shown on Table 3-1. I used C57BL/6J mice from Jackson Laboratory’s Diet-Induced Obesity program (Jackson Labs; #000664) for the first three weight sets and leptin-deficient mice (*ob/ob*) (Jackson Labs; #000632) for the obese weight set. Mice in the lean set were fed a diet with 10% fat (Research Diets, #D12450B), whereas mice in the other three sets were fed a diet with 60% fat (Research Diets, #D12492). Additionally

Table 3-1. Mouse strains and injection conditions

<i>Strain designation</i>	<i>Age (weeks)</i>	<i>Diet kcal fat %</i>	<i>Average weight (g)</i>
C57BL/6J “Lean”	10	10	26.6
C57BL/6J “Medium”	10	60	30.7
C57BL/6J “Heavy”	16	60	38.1
<i>A^y/J</i>	16	60	44.2
<i>ob/ob</i>	12	60	55.3

A^y/J mice were used as a model for less severe obesity compared to the *ob/ob* mice. Harvested organs were imaged for individual fluorescence and used to quantify biodistribution. Kupffer cells, macrophages from the liver, were also isolated to determine the macrophage contribution to the high amounts of liver fluorescence. Kupffer cells are a major factor in the liver's immune response to foreign particles and evading phagocytosis of these cells is a major challenge in drug delivery. Phagocytosis is mediated by recognition of the nanoparticle surface by the Kupffer cells, which interface with blood in the liver.

3-3 In Vitro Particle Uptake

Murine macrophages from bone marrow were used as a model for macrophage uptake. I investigated the *in vitro* cell uptake using 50 nm polystyrene spheres with three surface chemistries, pristine, carboxylate (negatively charged), and amine (positively charged). Electron microscopy of these particles can be seen in Figure 3-1. As with the *in vivo* experiments in mice, the spheres were loaded with DiR dye, a lipophilic near infrared dye. The cells were imaged and fluorescence was measured via fluorescent microscope and the whole cell fluorescence was quantified using ImageJ (NIH). Fluorescence was determined by a histogram of pixel grey values from 0, black, to 255, white, in the cell area and weighted by the multiplication of pixel count by the grey value. A threshold value of fluorescence was determined by quantifying fluorescence of both empty space between cells and measurements of cells without fluorescence. Pixels of grey values below 50 (on the 0 to 255, black to white scale) were not counted towards fluorescence via this threshold. Fluorescence in macrophages was averaged per cell; however, due to the confluence of HepG2 cells, fluorescence was averaged

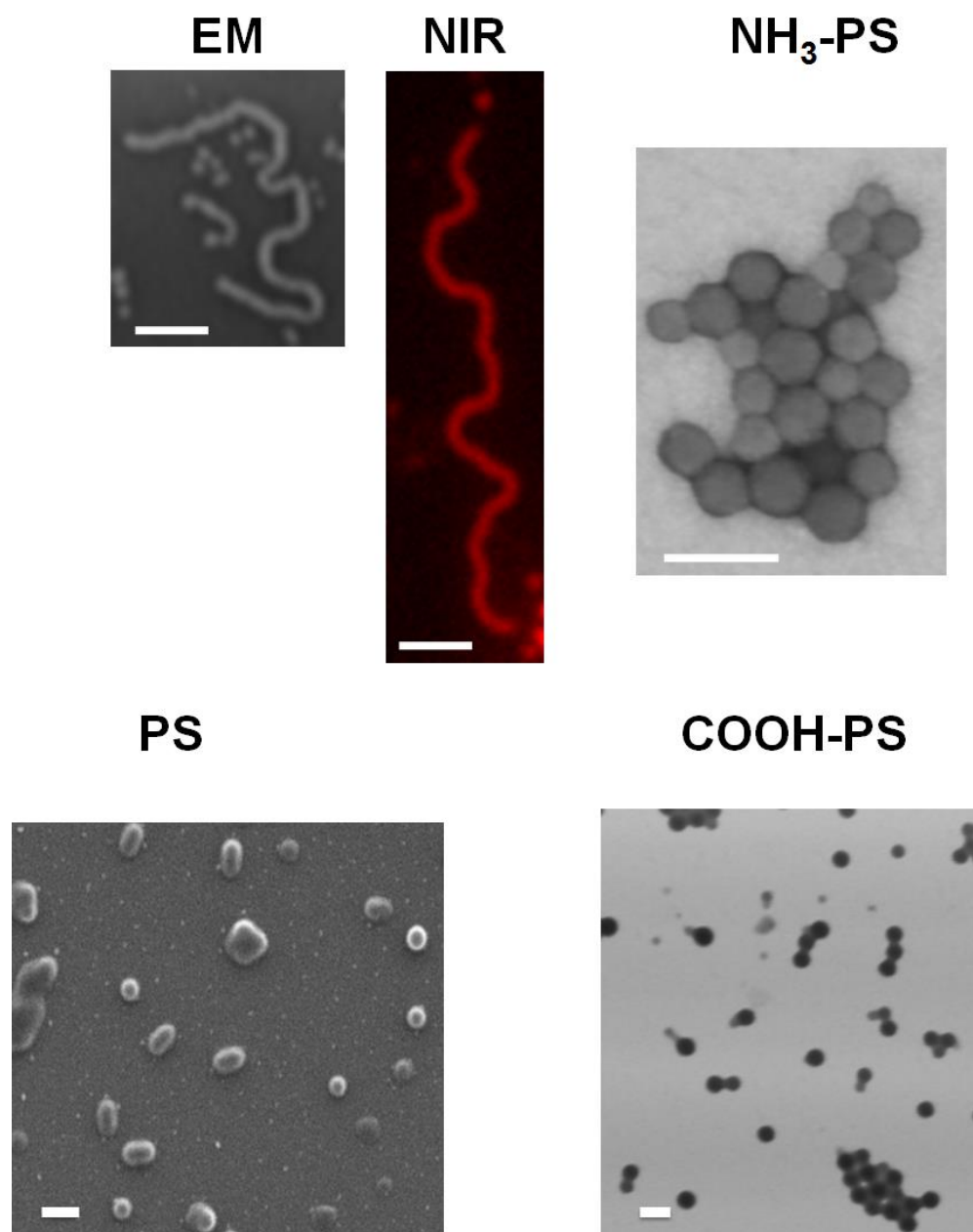


Figure 3-1 Filomicelle and Polystyrene Structures

Filomicelles imaged on a scanning electron microscope (EM) and fluorescent microscope loaded with DiR dye. Scale bar is 1 μm . Polystyrene particles imaged on an electron microscope. Scale bar is 100 nm. The particle chemistries shown are pristine polystyrene (PS), amine modified ($\text{NH}_3\text{-PS}$) and carboxylic acid modified (COOH-PS).

over pixel area. Amine modified spherical polystyrene nanoparticles having 50 nm diameters (Sigma-Aldrich, #L0780-1ML) were also used. The spheres should be removed from the circulation of mice in a matter of hours and thus the filomicelles are more suitable for long-term biodistribution studies. My goal was to use these in vitro experiments to elucidate the uptake of nanoparticles in varying fat environments.

3-4 Materials and Methods

Macrophage Cell Culture

To prepare latex beads, 10 μ L of 1 mg/mL DiR dye (Life Technologies; #D12731) solubilized in 20% EtOH was added to 100 μ m of 50 nm amine functionalized polystyrene spheres (Sigma Aldrich) [58]. The beads were centrifuged at 17000xg for five minutes to remove excess dye and resuspended in PBS. Macrophages were cultured at 37°C, 5% CO₂ in media consisting of 70 % Dulbecco's Modified Eagle Medium, DMEM (ATCC), 10 % fetal bovine serum (FBS) and 20% LADMAC conditioned media containing a growth factor, CGF-1. Arachidonic acid was added to media to emulate an in vivo high fat environment at concentrations of 10mM and 100mM and incubated overnight. 100 μ L of the resuspended beads were added to cell culture and incubated for two hours. Cells were pelleted at 400xg for two minutes and washed with PBS to remove beads that were not internalized. Cells were imaged using an AMG EVOS fluorescent microscope and fluorescence per cell was quantified in ImageJ [59]. Significance was determined using a Student's t-test. Flow cytometry was subsequently performed on these samples using the Accuri C6 for cell counting and fluorescence. Additional experiments were done with alternate polystyrene beads, both pristine and functionalized with carboxylate.

For macrophage cultures and induction of lipid droplets, oleic acid was solubilized in bovine serum albumin (BSA) and PBS. Cultures without oleic acid were given BSA at the same concentration that solubilized the oleic acid. The protocol for determining cell uptake was repeated as done previously with arachidonic acid cell cultures.

HepG2 cells were cultured in Eagle's Minimum Essential Medium, EMEM (ATCC), with 10% FBS and 10 mL/L of a stock Penicillin-Streptomycin solution (10,000 units penicillin and 10 mg/mL streptomycin, Sigma). HepG2 cells were incubated in oleic acid rich media, 100 mM and 300 mM. Oleic acid concentrations of 500 mM and greater caused a loss in cell viability. Polystyrene beads loaded with DiR dye were also added to HepG2 cultures to determine cell uptake. Analysis in ImageJ was done by cell area.

Mouse Tissue Harvesting and Imaging

Nanoparticles loaded with DiR dye were injected into mouse tail veins along with controls of dye without nanocarriers and PBS. The liver, heart, lung, spleen, kidneys, and subcutaneous WAT were harvested from mice sacrificed by cervical dislocation under isoflurane anesthesia in accordance with the IACUC approved protocol (#2231-0214). Blood was collected by retro-orbital bleeding and plasma was separated through centrifugation with a lithium heparin blood collector and plasma separator (BD Microtainer). Fluorescence of harvested tissue was measured using an IVIS Lumina imaging system with an ICG filter for near-IR fluorescence. Intensity of DiR dye was quantified using Living Image software (Xenogen) and tabulated as both total organ fluorescence and fluorescence by organ weight. Organ fluorescence was normalized by

the data for the control PBS injection to account for background fluorescence values. The intensity of an injection of the DiR dye without particles was also considered as a control of injection consistency between experiments. The control mice were C57BL/6J from the Jackson Labs diet induced obesity program. Leptin deficient *ob/ob* (B6.Cg-*Lep^{ob}*/J) mice were used for morbidly obese mice and *A^y/J* (B6.Cg-*A^y*/J) mice were used as a model for less extreme obesity. As suggested by the Jackson Labs diet induced obesity program, Research Diets feed with 10% fat and 60% fat were used to induce a range of metabolic states among the different strains. Kupffer cells from the livers were extracted from the tissue as previously outlined by Li *et al.* [34]. Extracted Kupffer cells were imaged using an AMG EVOS fluorescent microscope to determine cell fluorescence.

3T3 Pre-adipocyte Differentiation

Fibroblasts of the 3T3 cell line were cultured on 6-well plates in growth media consisting of DMEM (ATCC), 10% FBS (Sigma), 2mM freshly supplemented glutamine (Sigma), 6 µg/mL biotin (Fischer Scientific) dissolved in 1M NaOH, and 10 mL/L of a stock Penicillin-Streptomycin solution (10,000 units penicillin and 10mg/mL streptomycin, Sigma) for a differentiation protocol done previously by Brasaemle *et al.* [60] Fibroblasts are passed before reaching confluence with a 0.25% trypsin- 0.53 mM EDTA solution (ATCC). When differentiating, cells must reach complete confluence before adding differentiation media. Differentiation media consists of the previously detailed growth media, with 0.5 mM IBMX, 10 µg/ml insulin, and 10 µM dexamethasone. Differentiated cells do not need additional fatty acid to induce the formation of lipid droplets. Pristine 50 nm polystyrene particles loaded with DiR dye were incubated with

differentiated adipocytes for two hours. BODIPY lipophilic dye was added to determine co-localization of particles and lipid droplets. After incubation with dye and nanoparticles, wells were washed with 2 mL of PBS prior to fluorescent microscopy to remove particles not internalized or associated with the adipocytes.

3-5 Results and Discussion

Nanoparticle Biodistribution in Mice

I studied the biodistribution of nanoparticles as a function of mouse weight, particle shape, charge and surface chemistry using *ob/ob* mice and diet induced obesity. Particles used were filomicelles and polystyrene spheres (Figure 3-1). A comparison of biodistribution as a function of weight and normalized fluorescent intensity (Figure 3-2) shows the impact of obesity on the liver and spleen using filomicelles as the drug carrier model. Fluorescent intensity of each organ is normalized by the corresponding background fluorescence of organs from a PBS injection. *A^y/J* mice were used as a less extreme metabolic state compared to the *ob/ob* mice, however these mice still suffered from non-alcoholic fatty liver disease on a high fat diet. While decreased liver biodistribution by weight may be a result of decreased uptake caused by high lipid content, it is likely that the diseased livers cannot function at the basal levels seen in healthy mice. As seen in Figure 3-2 and 3-3, the livers of a diet induced obesity mouse show a higher uptake by mass than those with non-alcoholic fatty liver disease considering the livers of *ob/ob* mice are four fold more massive. Figure 3-3 shows that the liver uptake of nanoparticles in C57BL/6J mice on a high fat diet is higher than both low fat diet C57BL/6J mice and high fat diet *A^y/J* and *ob/ob* mice. The spleen does not significantly change in mass with mouse weight, and as the

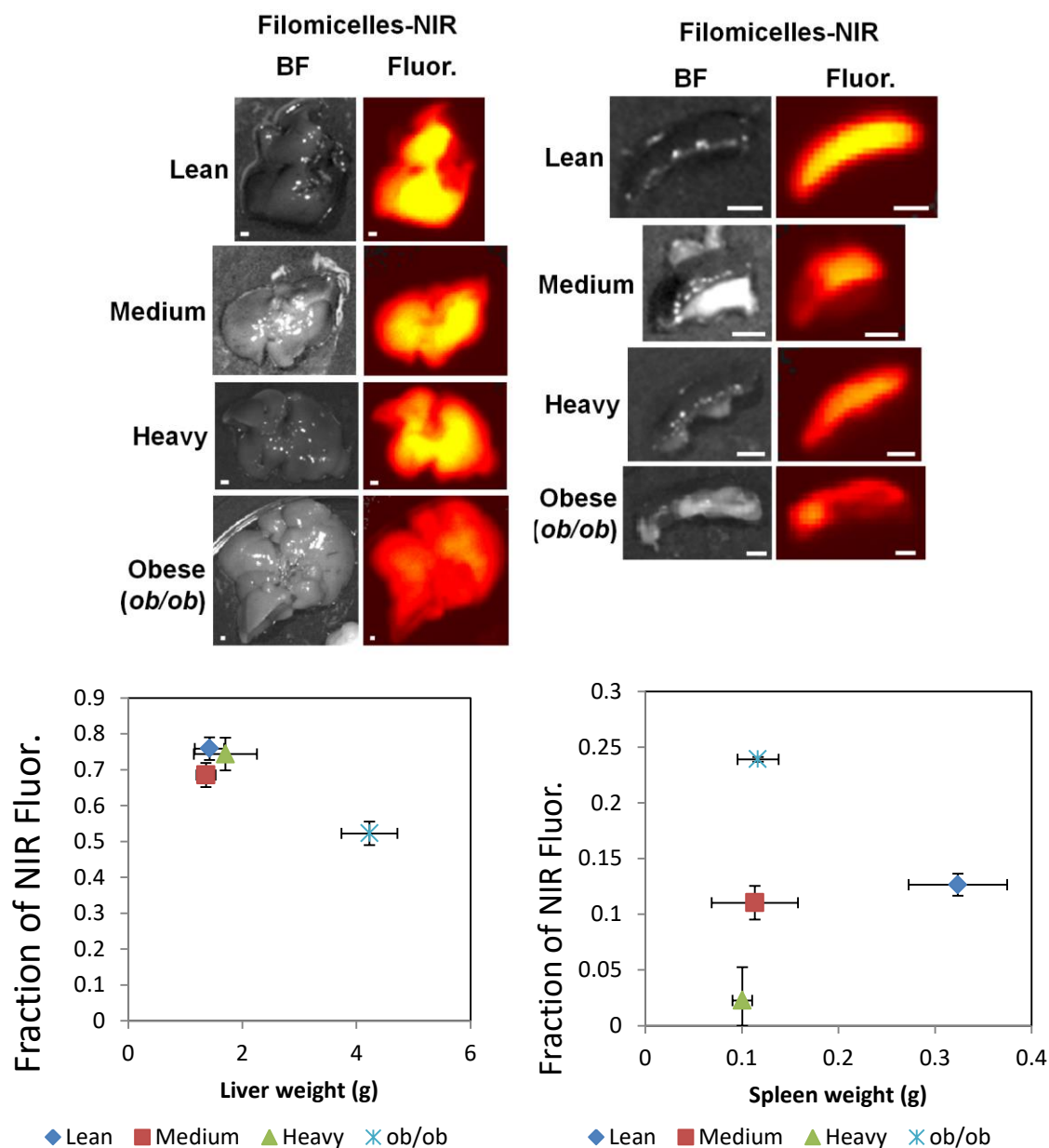


Figure 3-2 Liver and Spleen Fluorescence after Filomicelle injection

Filomicelles loaded with DiR dye were tail vein injected. Fluorescence was measured with IVIS fluorescent imaging two days after injection and tissue harvesting. Liver (Left) and spleen (Right). Scale bar is 1 mm.

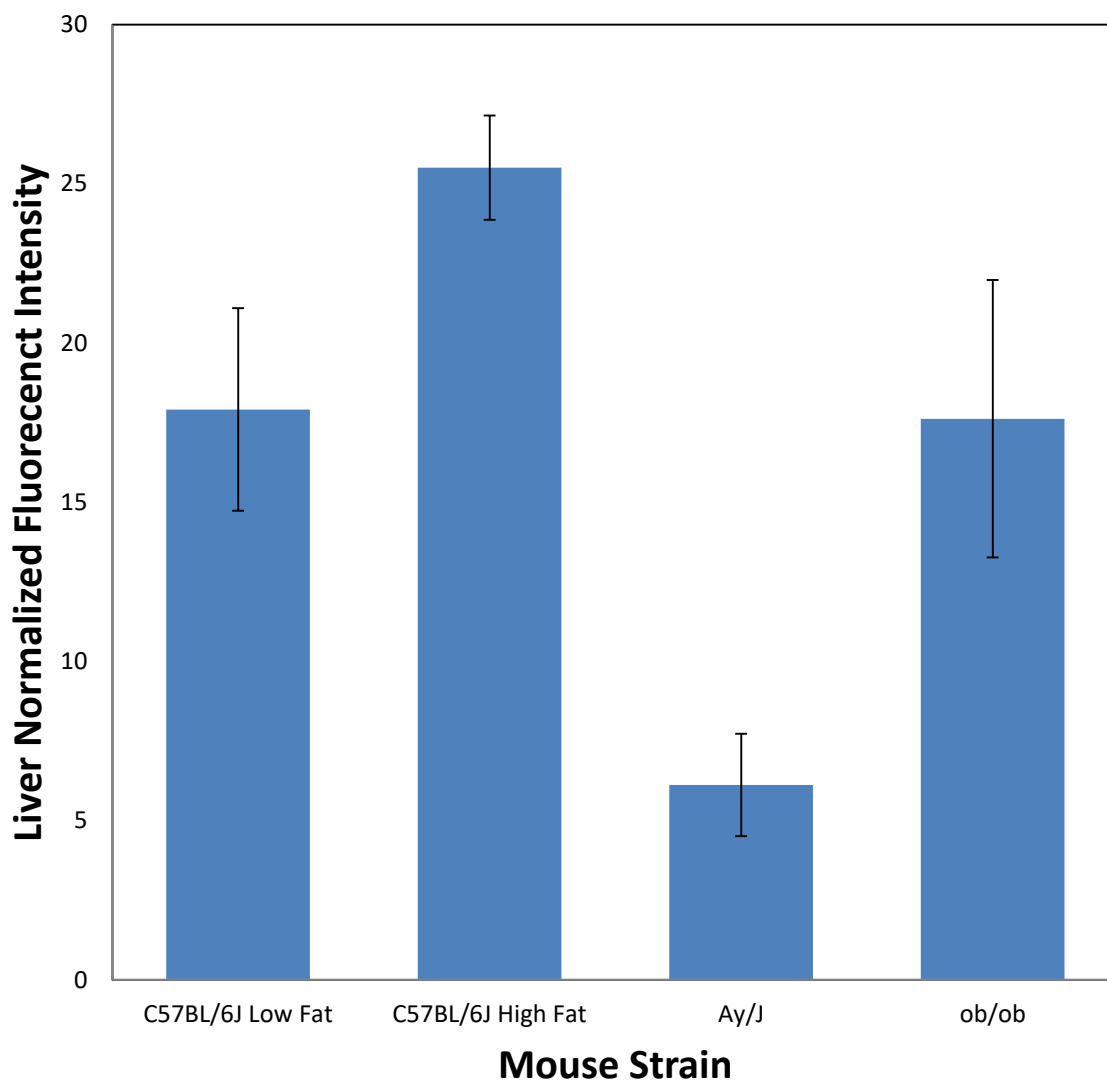


Figure 3-3 Liver Intensity with DiR filomicelles across mouse strains

Murine liver fluorescent intensity of DiR dye two days after injection imaged on IVIS quantified with Living Image software. Each mouse strain represents an average of the intensity of three mouse liver intensities relative to the background fluorescent value of the liver of a PBS injection. Average mouse weights for each strain were: C57BL/6J low fat, 26.6 g; C57BL/6J high fat, 38.1 g; *A^y/J*, 44.2 g; *ob/ob*, 55.3 g.

liver and WAT increase in size the fraction of filomicelles in the spleen decreases. Spleen uptake was reduced in C57BL/6J fed a 60% fat diet for 15 weeks, noted as “Heavy” in our experiments, compared to lean C57BL/6J mice and *ob/ob* obese mice. As the “Heavy” mice see increased liver uptake, this agrees with results in literature where uptake to the spleen and liver are inversely proportional. These decreases potentially represent the impact that removing drug carriers from circulation would have on a tumor as greater potential for drug sinks exist in non-ideal metabolic states. Organs other than the liver, spleen, and WAT show a smaller fraction of the biodistribution and show small decreases in fluorescence as mouse size increases (Figures 3-4, 3-5). While WAT does not increase in average intensity, indicating similar concentrations, the increased amount of WAT linearly increases fluorescence (Figure 3-5). Excluding the liver, all normalized filomicelles fluorescent organ data is shown on Figure 3-6.

The *in vivo* experiments in mice using filomicelles were repeated using 50 nm spherical polystyrene particles of three surface chemistries: amine, carboxyl, pristine polystyrene. A similar trend to filomicelle data was seen in the liver uptake (Figure 3-7) using all three particles, particularly amine and carboxyl particles seeing decreased liver uptake by mass. Spleen uptake did not vary significantly (Figure 3-8) as with previous experiments. The WAT particle fraction (Figure 3-9) increases with the tissue weight with all three particles. The normalized organ fluorescence is shown on Figure 3-10.

While organ fluorescent intensity after necropsy only shows a single time point of particle capture, this biodistribution is useful in comparison to the fate of nanoparticles used in cancer treatment. Both the particles removed from clearance by active sinks,

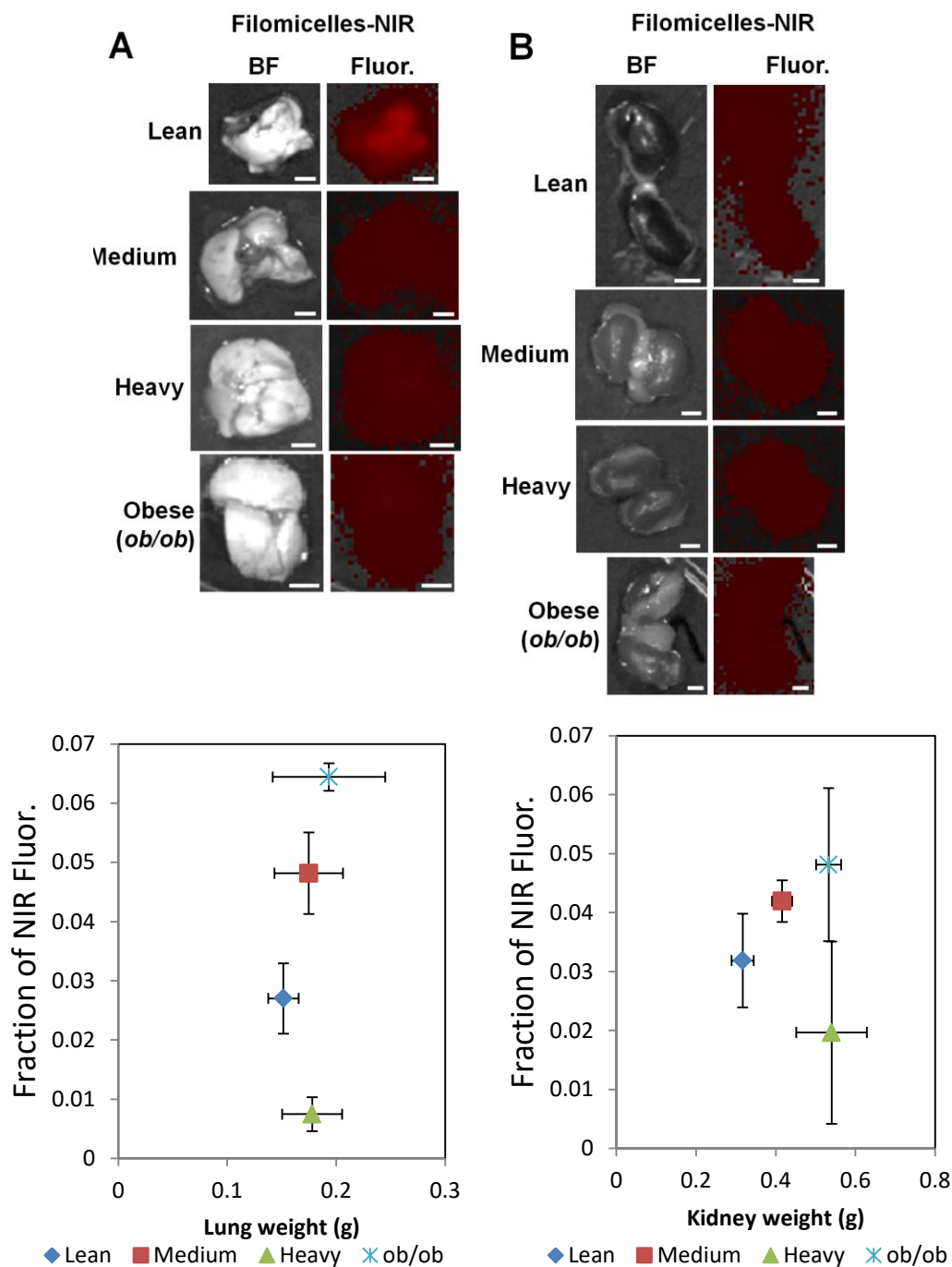


Figure 3-4 Lung and Kidney Fluorescence

Filomicelles loaded with DiR dye were tail vein injected. Fluorescence was measured with IVIS fluorescent imaging two days after injection. Organs shown are A, lungs and B, kidneys. Scale bar is 1 mm.

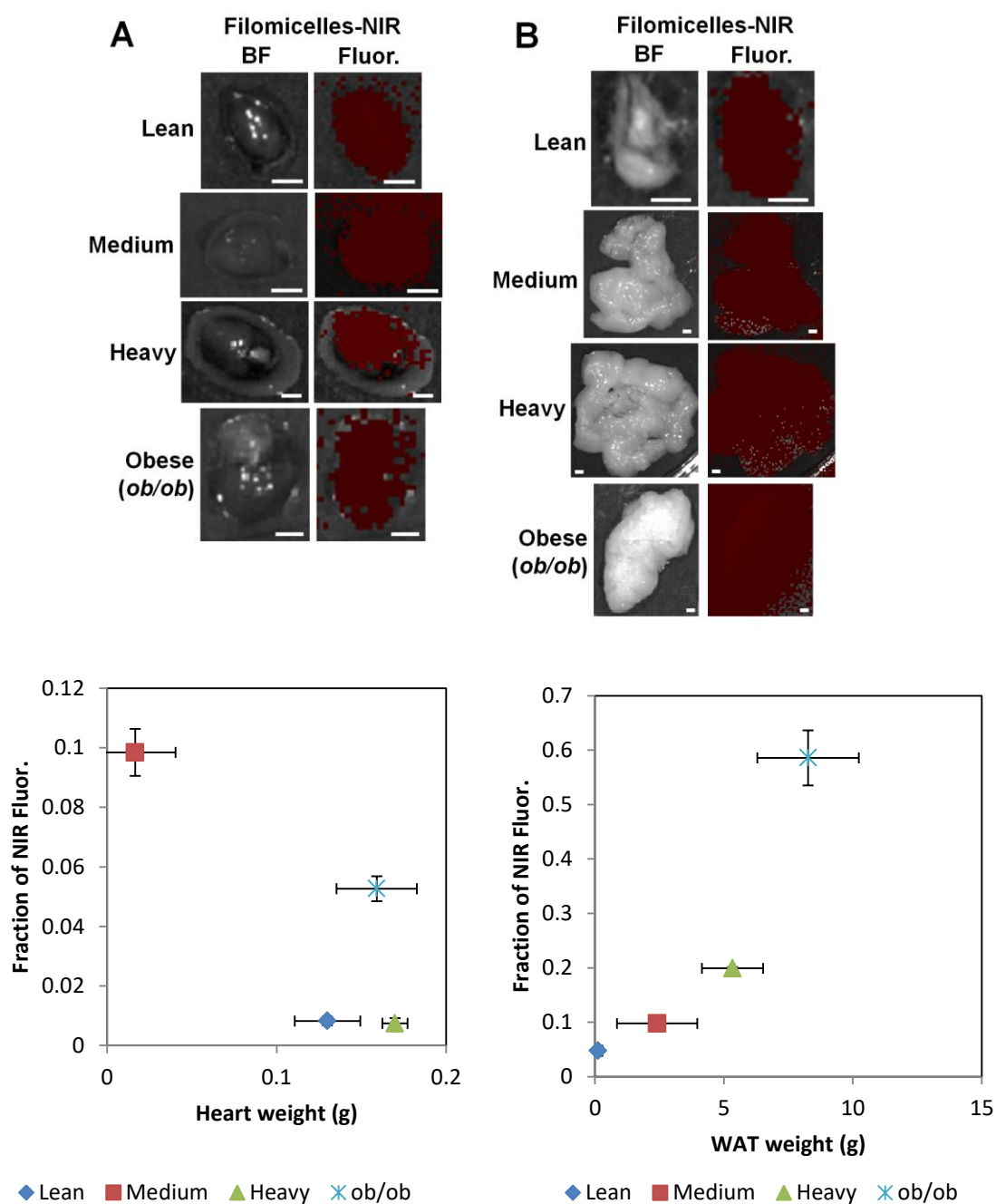


Figure 3-5 Heart and WAT Fluorescence

Filomicelles loaded with DiR dye were tail vein injected. Fluorescence was measured with IVIS fluorescent imaging two days after injection. Organs shown are A, heart and B, WAT. Scale bar is 1 mm.

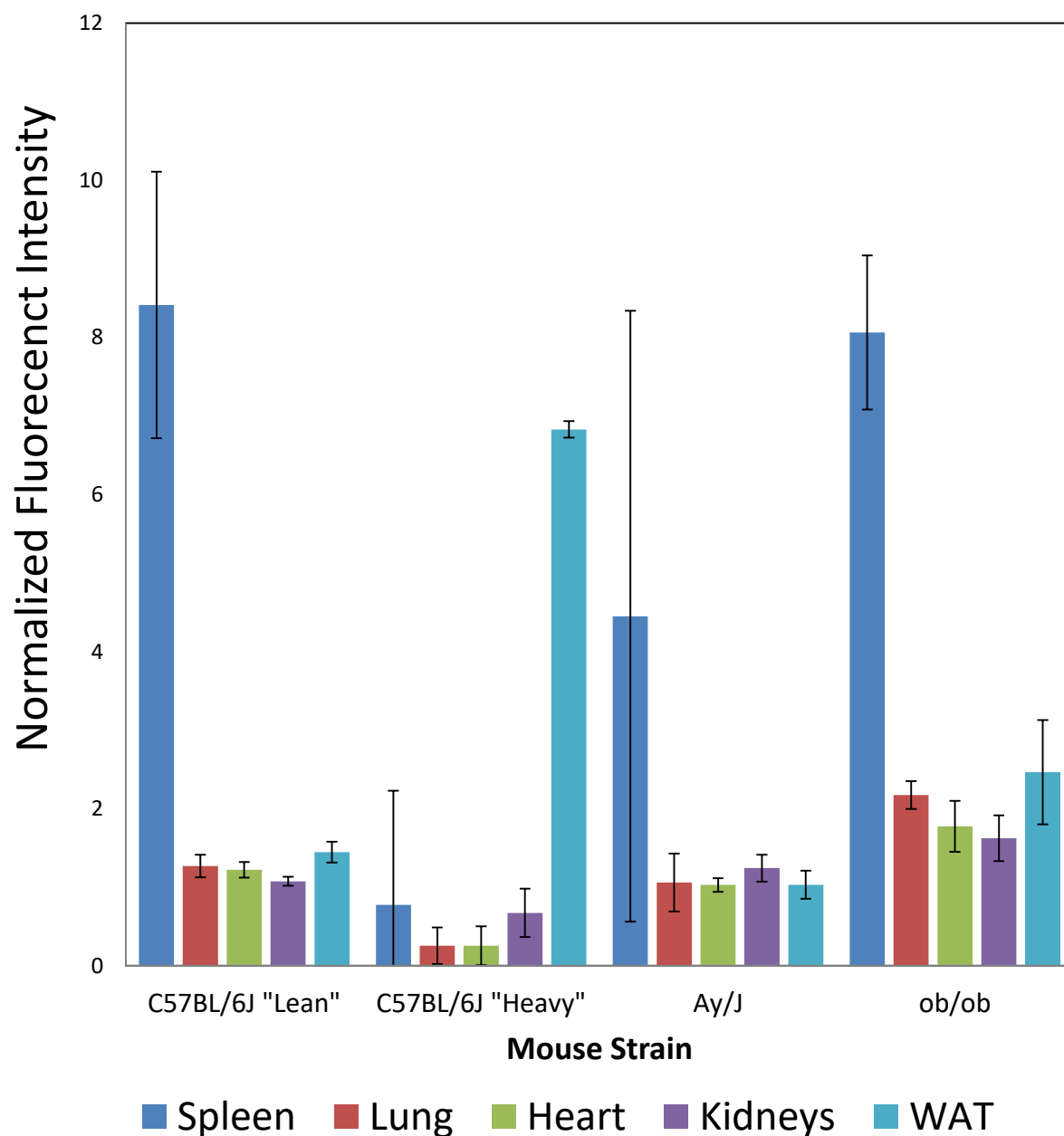


Figure 3-6 Mouse and Organ weight and fraction of fluorescent intensity

Mouse organs harvested two days after filomicelles injection, excluding liver normalized by background fluorescence of a PBS injection. A notable difference is the comparatively high amount of fluorescence in WAT and low intensity in the spleen of "Heavy" mice.

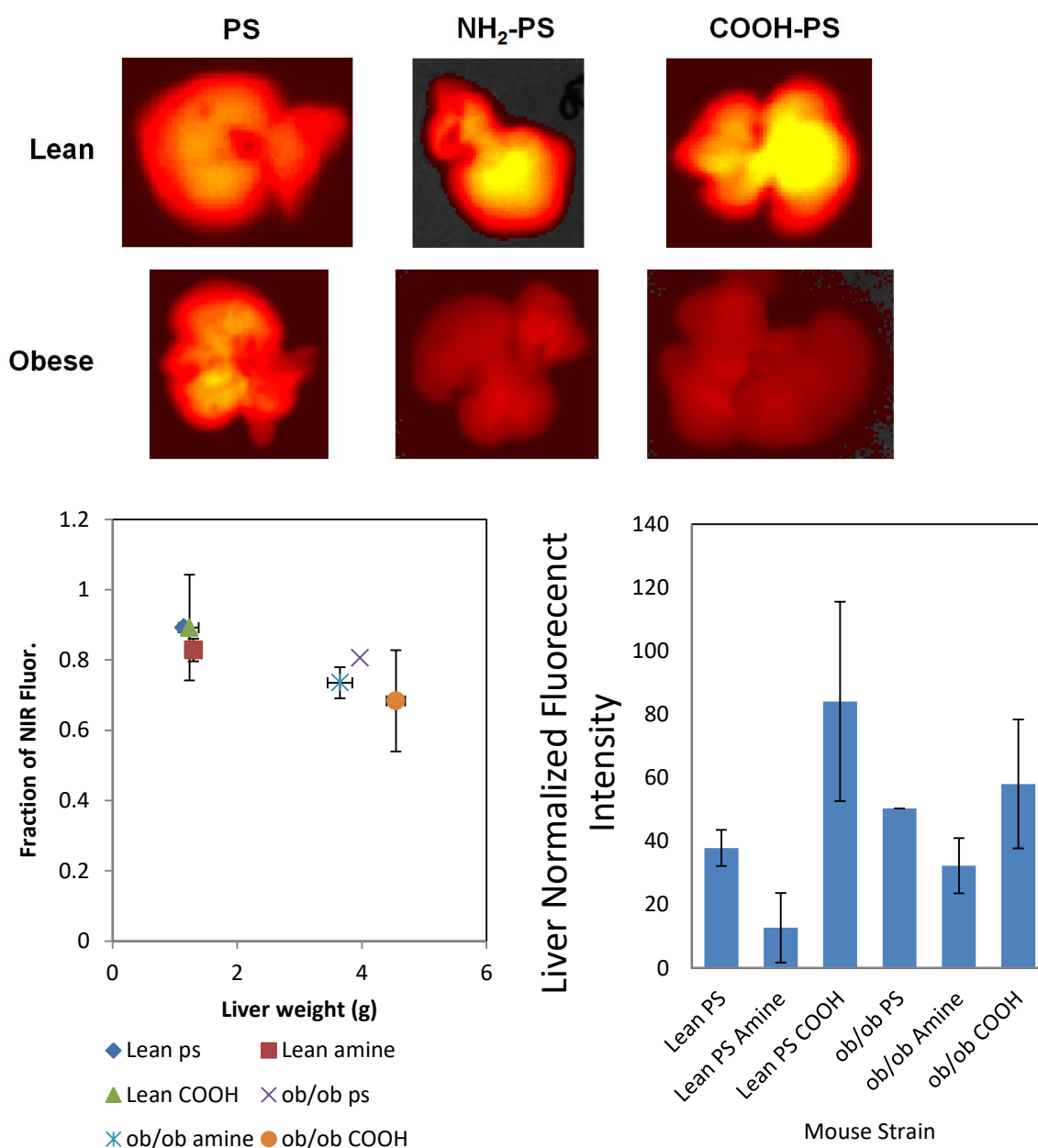


Figure 3-7 Liver intensity with NiR loaded PS spheres with modified surface chemistry

Murine liver fluorescent intensity of DiR dye two days after 50 nm polystyrene sphere injection taken with IVIS Lumina fluorescent imaging quantified with Living Image® software (Xenogen). A decrease in liver uptake is seen in obese mice.

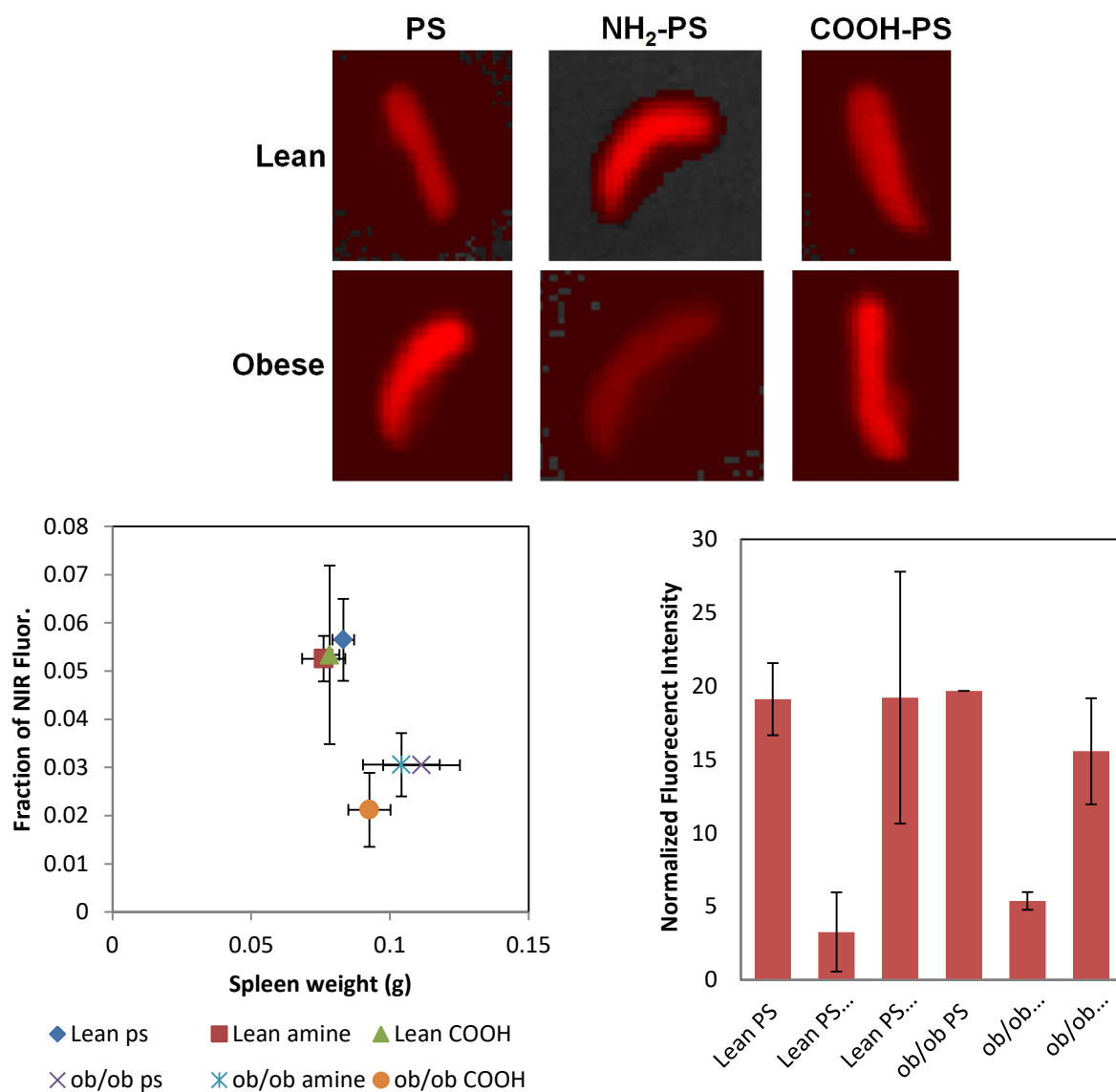


Figure 3-8 Murine spleen fluorescent intensity taken with IVIS with polystyrene sphere.

Spleen uptake does not vary significantly with mouse weight between comparable PS injections.

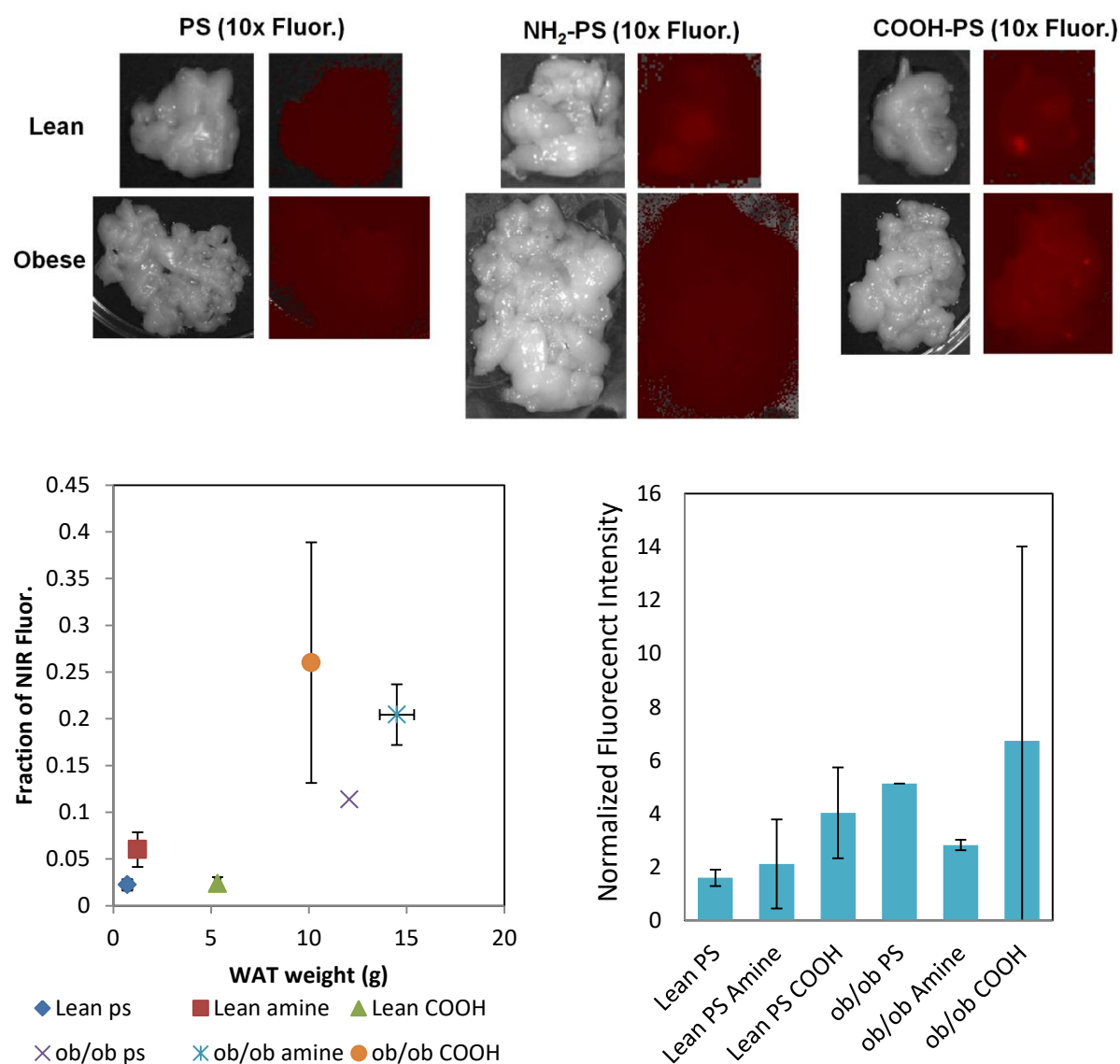


Figure 3-9 White adipose tissue polystyrene sphere uptake

Fluorescent intensity of white adipose tissue in lean and obese mice with multiple polystyrene sphere chemistries. Average intensity, from particle concentration, does not increase significantly, however the fraction of particles increases with WAT mass.

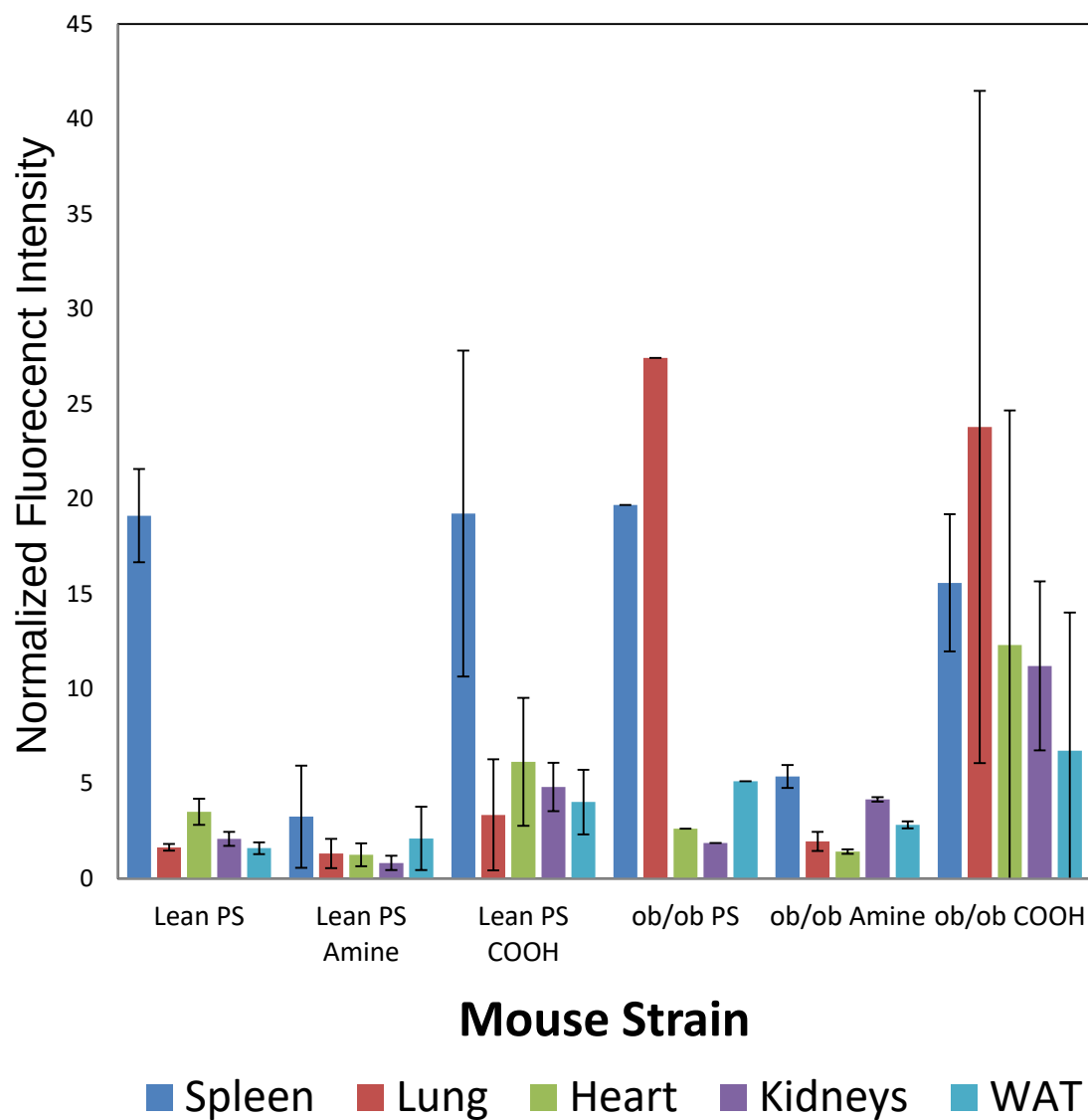


Figure 3-10 Mouse and Organ weight and fraction of fluorescent intensity

Mouse organs harvested two days after filomicelles injection, excluding liver normalized by background fluorescence of a PBS injection. Relatively high lung fluorescence in *ob/ob* mice with PS and PS-COOH injections is possibly due to particle aggregations. Two mice died after receiving the *ob/ob* PS injections; as such, this data set represents only one mouse.

such as the liver and spleen, and those that are passive such as white adipose tissue, limit the available drug for treatment of the intended target and increase the necessary effective dose. Mice with fur, particularly the black fur of C57BL/6J and *ob/ob* mice make it difficult to measure organ fluorescence *in vivo* using IVIS fluorescent imaging. For future experiments, nude or shaved mice could be used to image living mice with near infrared dye at multiple time points *in vivo*. This would allow for the IVIS to detect fluorescence in the live mice to get real time biodistribution changes. Blood collection and a Western blot with PEG as the epitope tag to determine circulating PEG levels could also be used to measure freely circulating drug carriers over time.

Nanoparticle Uptake in vitro

I studied the cellular uptake of nanoparticles in varying levels of arachidonic acid and oleic acid, which simulate a cell environment caused by a diet high in fat [47]. Arachidonic acid was initially chosen for the enhanced ability to induce lipid droplets in macrophages. However, while present in physiological settings, arachidonic acid, a 21:4 fatty acid, would not be a primary component of lipid droplets *in vivo*. Arachidonic acid may also act as a signaling molecule and change the activity of the macrophages; however, this does not appear to be the case as results using oleic acid and arachidonic acid showed similar trends [61-62]. The shorter chain, less saturated fatty acids, oleic acid and palmitic acid, 18:1 and 16:0 fatty acids respectively, are primary components of the high fat diet fed to my mice in obesity studies. I repeated these studies with oleic acid induced lipid droplets to reproduce the results in a more representative physiological setting. The formation of lipid droplets was induced using both oleic and arachidonic acid (Figure 3-11) in macrophages and HepG2 cells. For both positively

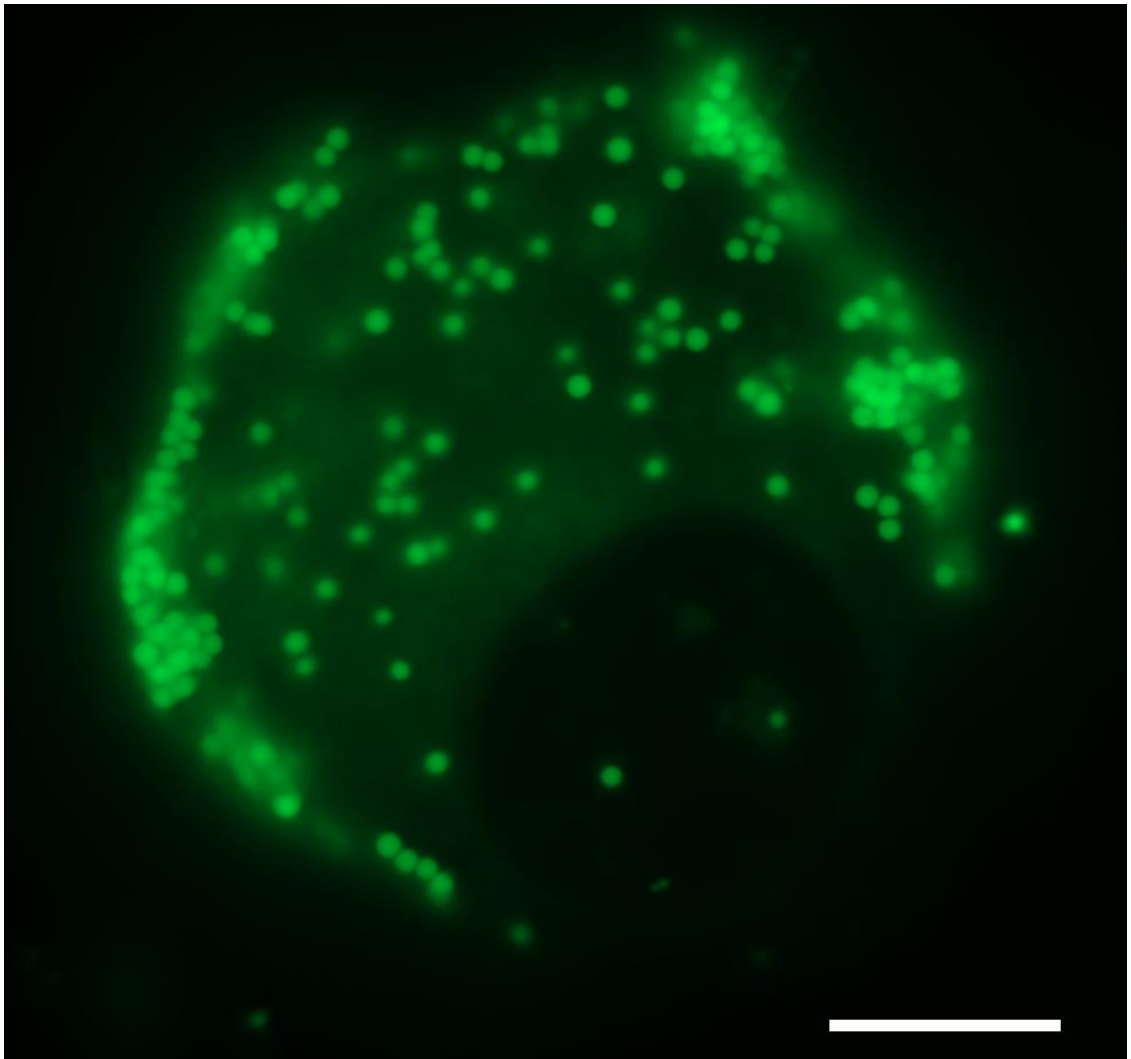


Figure 3-11 Lipid Droplets in Murine Macrophages

Lipid Droplets formed in 10 μ M arachadonic acid stained with BODIPY (green). Scale bar 10 μ m

charged amine coated polystyrene beads and pristine polystyrene beads, I saw an increase in uptake ($p < 0.05$) in macrophages with high amounts of both oleic and arachidonic acid compared to no additional fat. (Figure 3-12) Both arachidonic acid and oleic acid were able to produce a comparative increase in size and number of lipid droplets with increased fatty acid in media. The increase in lipid droplet formation can be seen in Figure 3-13 where lipids are stained with BODIPY. Increasing fatty acid content typically increases both the size and quantity of lipid droplets in these macrophage experiments. The DiR dye loaded into nanoparticles does not exclusively localize to lipid droplets in macrophages despite the relationship between fatty acid concentration and internalization although there may be some association between the particles and the lipid droplet surface.

Carboxylate modified polystyrene beads did not show any significant trend based on fatty acid concentration. These experiments produced a comparatively lower amount of particle uptake compared to the pristine and amine functionalized beads. While most cells showed little uptake, some regions of cells showed extremely high points of fluorescence, which led to high standard deviation and little statistical significance between oleic acid concentrations. As seen in Figures 3-14 and 3-15 the uptake of particles was minimal with carboxylate beads and did not vary with increased oleic acid as in pristine polystyrene beads. These results agree with previous immune studies, which found that nanoparticles with negative charge at physiological pH—specifically surfaces modified with carboxyl functional groups—have reduced cell uptake and immune detection. However size is often a stronger predictor of cell uptake than charge [18]. The carboxylate nanoparticles had a tendency to form large aggregates especially

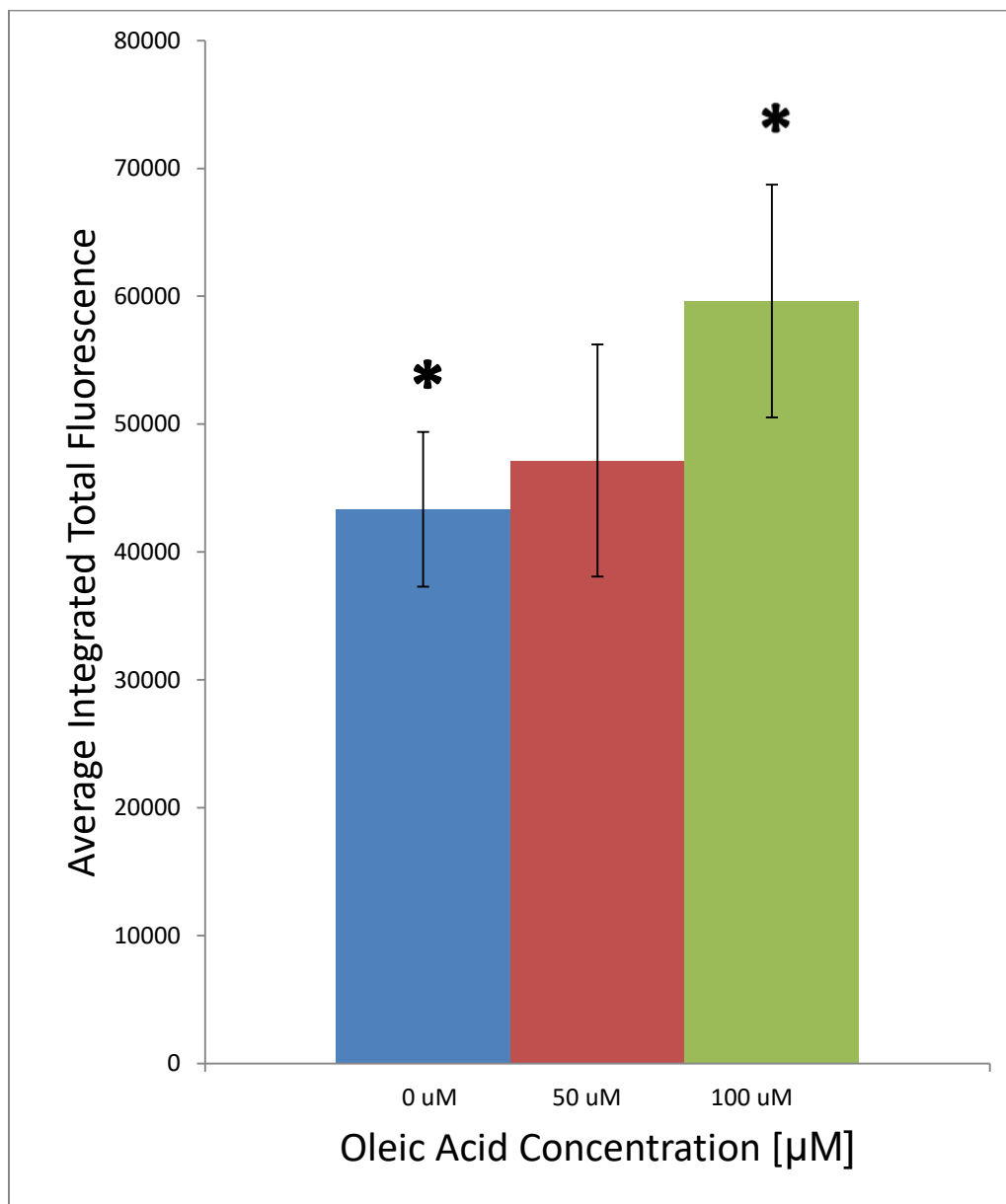


Figure 3-12 Average fluorescence per macrophage in elevated oleic acid oleic acid

Cells were incubated with 50 nm Amine modified polystyrene beads loaded with DiR near infrared dye. Cells were imaged on a fluorescent microscope and fluorescence was quantified using ImageJ. Representing the Student's t-test, “*” shows $p < 0.05$

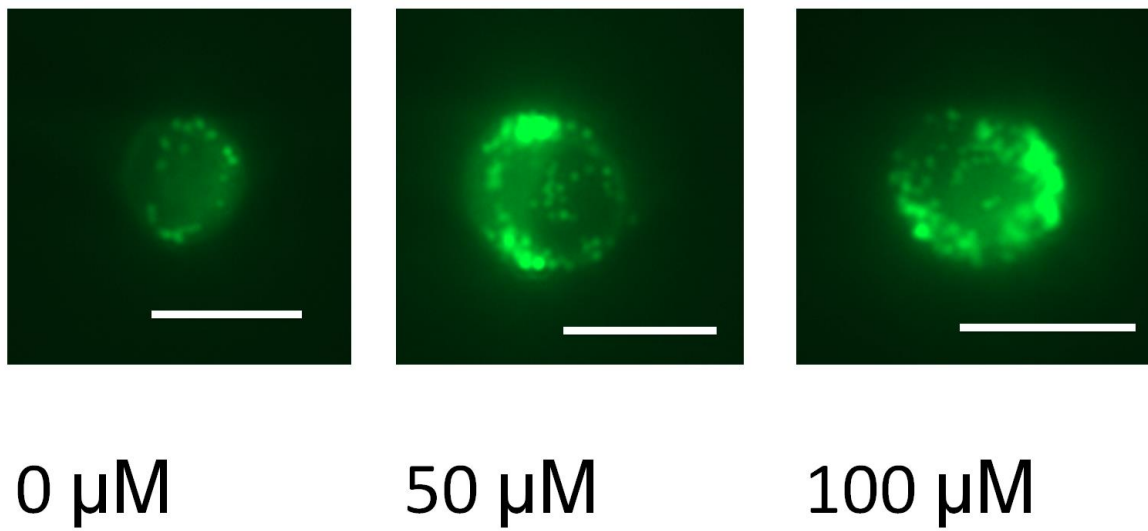


Figure 3-13 Lipid droplet formation in macrophages in oleic acid rich media

Lipid droplets are stained with BODIPY dye. Scale bar is 10 μm .

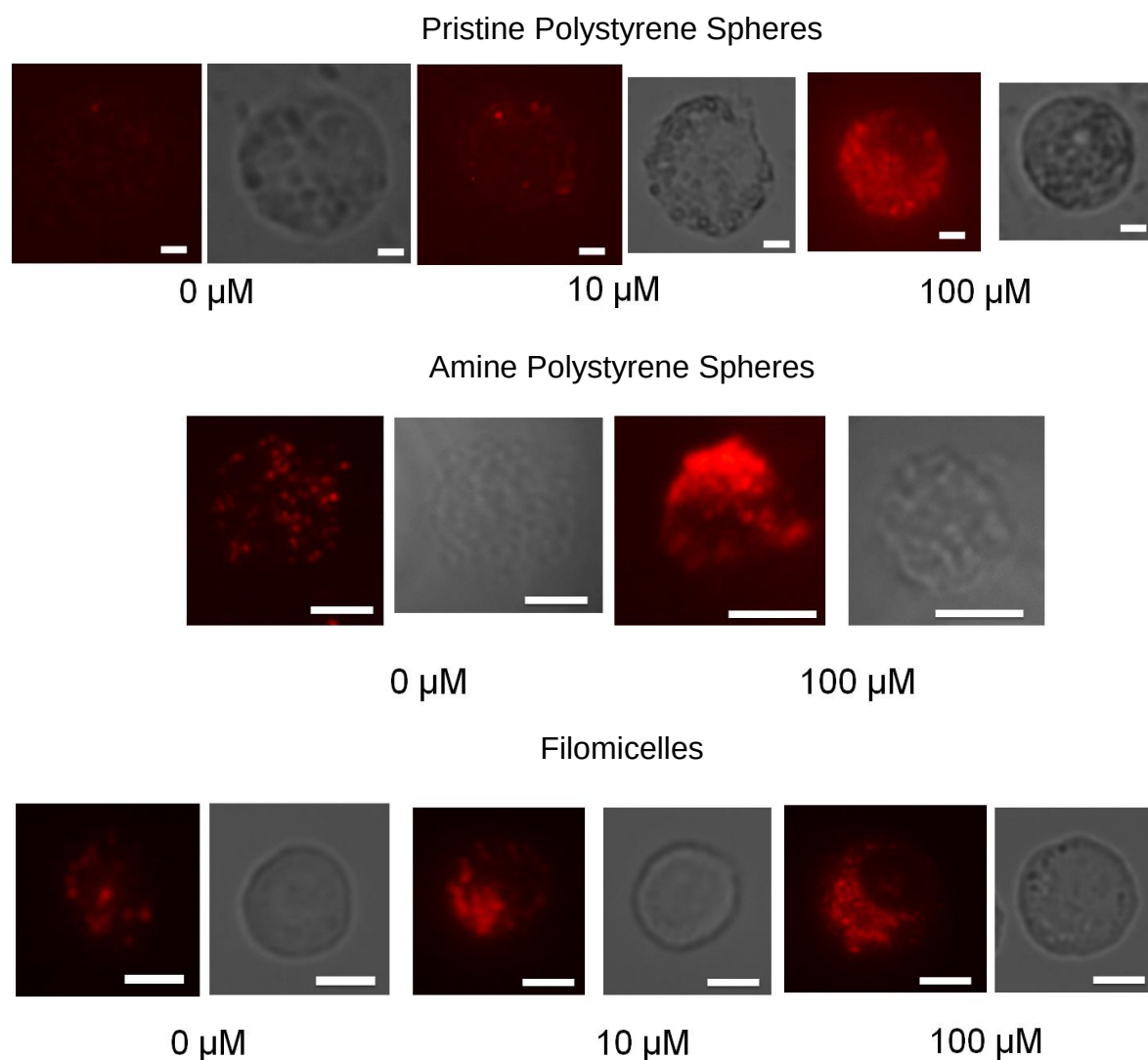


Figure 3-14 Nanoparticle uptake in macrophages incubated in increasing fatty acid

50 nm Polystyrene spheres and filomicelles incubated in macrophage culture for 2 hours. Results were similar in oleic and arachidonic acid; in this figure, pristine polystyrene spheres and filomicelles were incubated in oleic acid and amine spheres were incubated in arachidonic acid. DiR dye loaded into nanoparticles is shown in red. Scale bar is 10 μm .

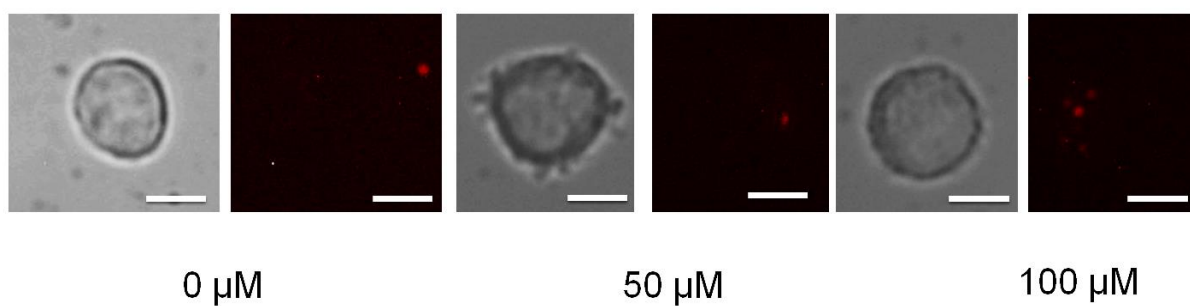


Figure 3-15 Carboxylated polystyrene bead uptake in murine macrophages.

50 nm Carboxylated polystyrene spheres incubated in macrophage culture for 2 hours. DiR dye loaded into nanoparticles is shown in white. Scale bar 10 μm.

in the presence of proteins and, while the beads were sonicated and centrifuged to remove large aggregates, it is possible the nanoparticles formed these complexes in the protein rich cell media. These carboxyl functionalized particles saw uptake *in vivo*, with similar localization in the spleen and liver as other nanoparticles, including isolated Kupffer cells. The fluorescent DiR dye does not fluoresce significantly outside of the nanoparticles. To show the measured macrophage fluorescence was due nanoparticle uptake and not due to the DiR dye alone, the dye was added to macrophages without particles. As seen in Figure 3-16, the macrophages with dye alone did not show significant fluorescence compared to the nanoparticle incubations.

HepG2 cells required higher concentrations of oleic acid for significant changes in nanoparticle uptake. My experiments found a drop in cell viability at concentrations of 500 μ M and higher. Similar to the macrophages, higher levels of lipid droplets resulted in a higher cell uptake with polystyrene beads (Figure 3-17) resulting in a significant difference in fluorescence ($p < 0.01$). To ensure measured fluorescence was a result of particle internalization, cells were washed with PBS, before imaging. The HepG2 experiments showed similar trends with fatty acid concentration compared to those with macrophages.

To explore extreme cases of metabolic states, adipocytes were differentiated from 3T3 fibroblasts. As with adipocytes in the body, these cells produce large lipid droplets without additional fatty acid in media. As done with macrophages and HepG2 cells, polystyrene beads loaded with DiR dye were incubated for two hours. To determine co-localization of nanoparticles with lipid droplets, the lipid marker BODIPY 493/503 was also added. While the polystyrene particles do not appear to be

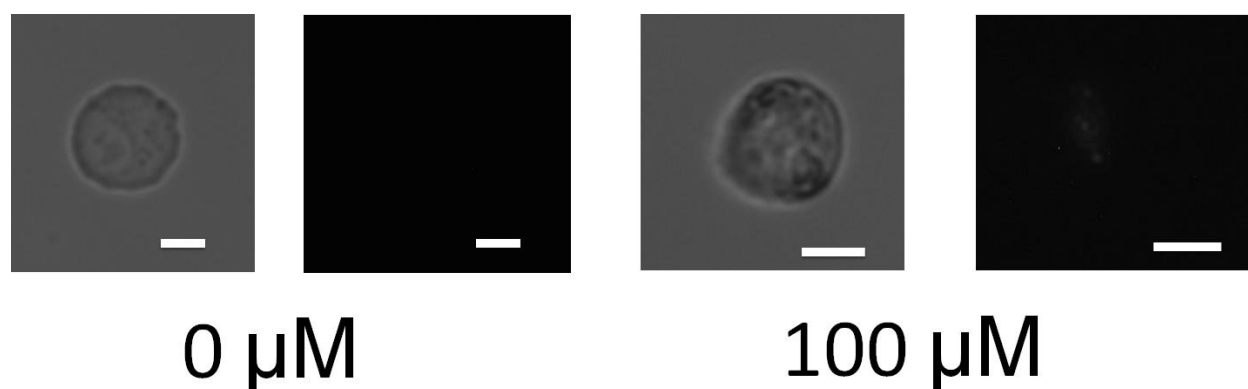


Figure 3-16 Murine Macrophages with DiR dye without nanoparticles

Macrophages incubated in with and without oleic acid with DiR dye added as done with nanoparticles. DiR dye does not show significant fluorescence without nanoparticles. While some cells showed some fluorescence, the difference between the concentrations was not significant. Scale bar is 10 μm .

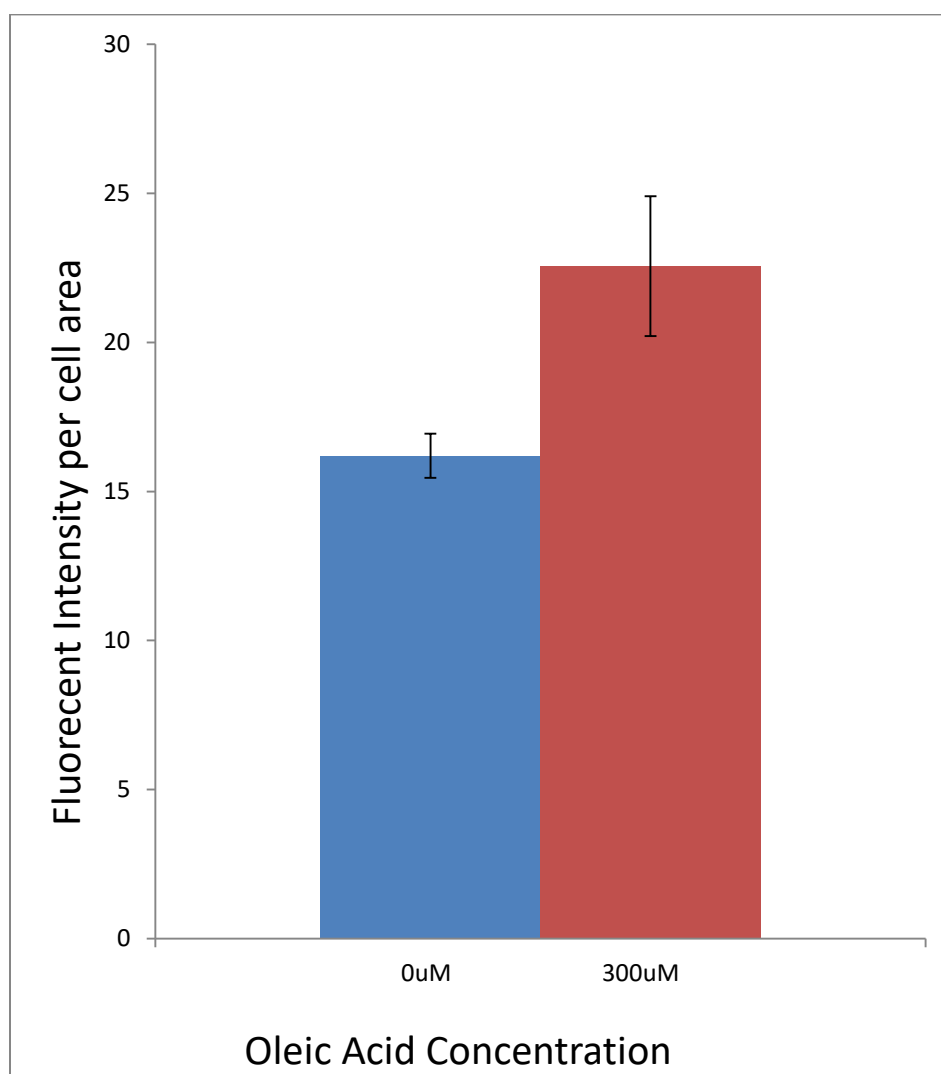


Figure 3-17 HepG2 Polystyrene Particle uptake in Oleic Acid

HepG2 hepatocytes were incubated in oleic acid rich media overnight. Polystyrene beads were then incubated for 2 hours. Following a wash with PBS, cells were visualized on a fluorescent microscope. Due to the confluence of the HepG2 cells, fluorescence was quantified per cell area rather than per cell. Student's t-test $p < 0.01$

internalized by lipid droplets in adipocytes, the overlay of DiR and BODIPY 493/503 dye at the periphery of lipid droplets, seen as a red circle around the green lipid droplets (Figure 3-18), shows an association between the particles and the surface of the lipid droplets. The surface association of the nanoparticles may suggest they are trafficked to the surface of the lipid droplets. This transport may contribute to the increase in particle internalization in high fatty acid concentrations seen in these experiments.

Previous experiments by Lundqvist *et al.* showed that lipoproteins bind to a variety of nanoparticle surface chemistries in both a charge and size dependent manner, with 50 nm amine modified beads showing affinity to the most lipoproteins [58, 63]. These lipoprotein levels are increased in obese mice, which may have an effect on particle fate. My results in *ob/ob* and diet induced obesity mice showed the impact of body composition as related to biodistribution. These results go beyond simple increased ratio of fat tissue of whole mouse mass that would be expected from a simple mass balance leaving less drug available to targets.

Larger livers in *ob/ob* mice do not result in a proportional increase in nanoparticles. Non-alcoholic fatty liver disease interferes with hepatocellular clearance, negatively affecting the activation and signaling of Kupffer cells [36]. Fluorescent Kupffer cells isolated from the livers of C57BL/6J mice after carboxylated polystyrene bead injection is shown in Figure 3-20. More data is needed to quantify the difference between Kupffer cell uptake as a result of nanoparticle injections. Comparisons from experiments previous to this macrophage isolation showed the average fluorescence per cell area in the *in vitro* experiments is typically ten-fold higher in the cultured macrophages than that of HepG2 cells. As the macrophages appear to internalize a

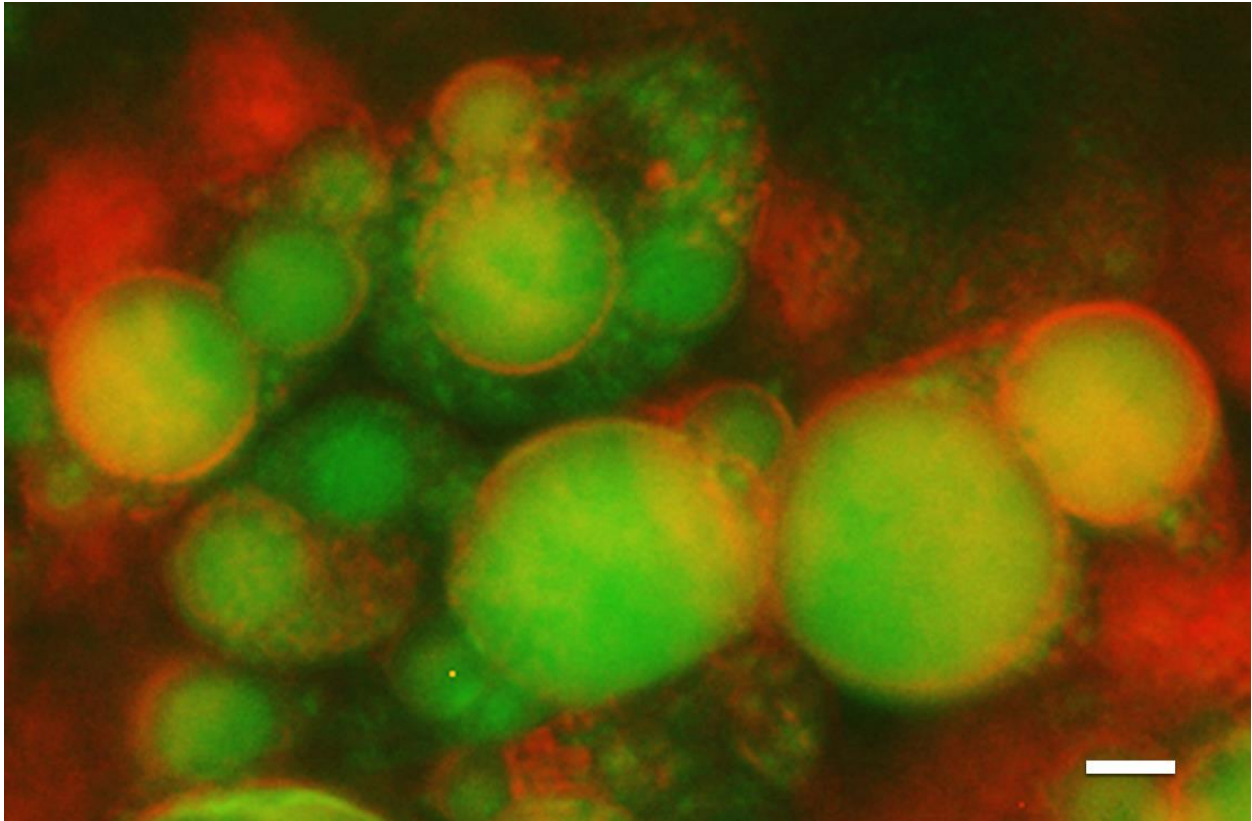


Figure 3-18 Merged Fluorescent microscopy of polystyrene particles and lipid droplets in Murine Adipocytes

Differentiated Adipocytes from the 3T3 cell line were incubated with 50 nm polystyrene spheres loaded with DiR dye, shown in red, for two hours. Lipid droplets stained with BODIPY, shown in green. Scale bar is 10 μm .

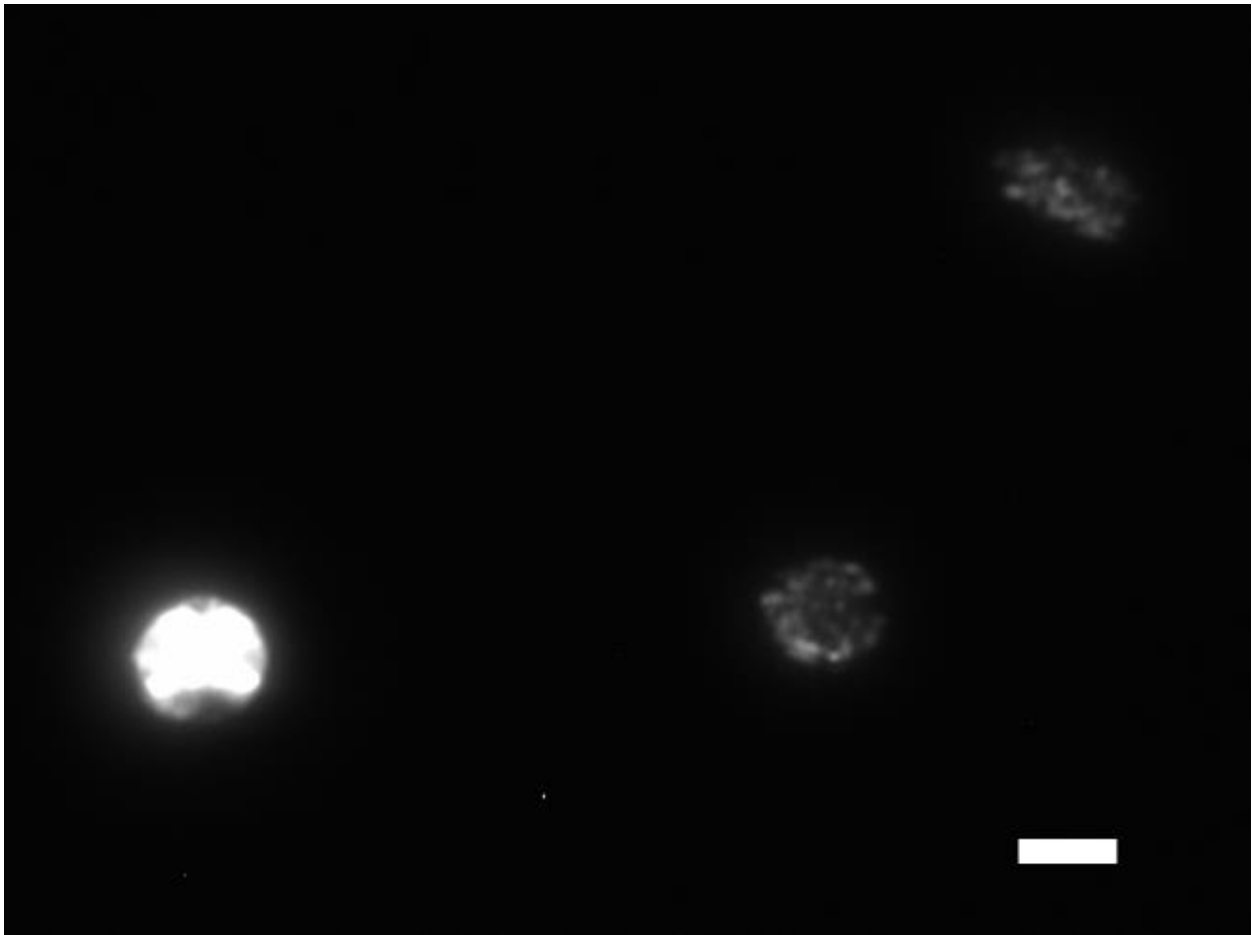


Figure 3-19 Isolated Kupffer cells after carboxylate polystyrene injection in C57BL/6J mice

Fluorescent microscopy of Kupffer cells extracted from the combined livers of three C57BL/6J mice on a low fat diet, two days after COOH modified polystyrene bead injection, loaded with DiR dye.

greater number of particles, this agrees with the idea that a disruption in Kupffer cell activity is a large contributing factor in the lower than expected liver uptake in *ob/ob* and *A^y/J* mice with fatty liver disease.

Chapter 4: Conclusions and Future Work

4-1 Conclusion

I have shown that obesity affects the biodistribution of model nanoparticles in mice. I used three mouse models of obesity: diet induced, A^y/J , and ob/ob . The localization of nanoparticles to the liver increases in diet induced obese mouse models. However, this trend does not continue in advanced cases of obesity where animals have developed NAFLD. I confirmed these findings *in vitro* using cultured macrophages, hepatocytes, and adipocytes. Oleic acid and arachidonic acid were used *in vitro* to emulate a diet high in fat, which influenced particle uptake. Macrophages and hepatocytes showed a significant increase in particle uptake with increased fatty acid media with both pristine polystyrene beads and amine modified beads *in vitro*. The results of nanoparticle injections in diet induced obesity mice with healthy livers compared to A^y/J and ob/ob with NAFLD show that this effect may only be present in normally functioning livers.

Due to the role of Kupffer cells in the signaling of non-alcoholic fatty liver disease, the change in liver uptake in ob/ob and A^y/J mice is not surprising. Kupffer cells act as one of the primary factors of particle clearance in the liver. A disruption in Kupffer cell function would result in diminished liver function. Preliminary isolations of Kupffer cells from mice showed it may be possible to compare the fluorescence of these isolated macrophages between mouse models and different nanoparticle injections. Exploring the changes in the body in non-ideal metabolic states is vital in understanding drug efficacy and fate.

4-2 Future Work

Future work should expand on the interaction of nanoparticles with the immune system in obese mouse models and explore the signaling and response to nanoparticles in high fat environments and in models for related diseases such as diabetes. Obese mouse models and protein interactions with nanotechnologies should be considered in all future drug development to ensure the highest chance of effectiveness at the clinical level. Design considerations such as surface chemistry and charge are already considered to minimize undesirable side effects and immune response. These parameters should also be tuned to minimize obesity related pitfalls of drug delivery, specifically minimizing the specific binding affinity of lipoproteins. To design successful drugs, the development must consider the constraints that will affect the real world treatment efficacy already seen by dosing complications in obese patients.

Research has been done where nanoparticles have been pre-incubated in whole or fractions of blood plasma before either determining particle clearance *in vivo* or particle internalization *in vitro*. Using both specific and non-specific protein-nanoparticle interactions, it could be determined which plasma proteins that increase with non-ideal metabolic states such as lipoproteins, can specifically accelerate or prolong blood clearance and alter the biodistribution. Preincubation of nanoparticles with HDL and LDL cholesterol and their protein components could be studied with macrophage particle internalization in media increasing amounts of fatty acids. Additionally these particle uptake studies should be done with *in vitro* cancer models in the presence of increasing fatty acid concentrations.

The *in vitro* experiments in macrophages and hepatocytes were done after an overnight incubation in fatty acid. At this point most free fatty acid has been incorporated into lipid droplets. It's possible the transport of drug carriers into cells could be affected in areas where excess fatty acid is present, which may be relevant as obesity progresses in the body. Nanoparticle internalization may be increased as fatty acid is incorporated into lipid droplets. The potential surface association with nanoparticles and lipid droplets could be explored through an isolation of lipid droplets. Lipid droplets can be isolated from lysed cells through ultracentrifugation and the associated nanoparticles could be quantified outside the cell. Although DiR dye without nanoparticles did not produce significant fluorescence in macrophages and hepatocytes, the trafficking of particles could be decoupled from dye by using drug carrier with bound fluorophore rather than hydrophobic association. Dye may diffuse out of the polystyrene spheres and associate with the surface of lipid droplets rather than the nanoparticles.

As the future direction of drug delivery involves actively targeted treatment, examining how the changes in a non-ideal metabolic state change targeting affinity and particle internalization should be the focus of additional research. Many functionalized nanoparticles employ targeting strategies that improve internalization, especially in drug resistant cancers. Since I have found internalization to be affected by fatty acid concentration, these targeting moieties could experience altered macrophage particle uptake in the presence of fatty acid. For the ultimate goal of improved drug efficacy and targeting, these internalization experiments can also be done *in vitro* with various cancer models such as those of B-cell lymphocytes.

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Appendix

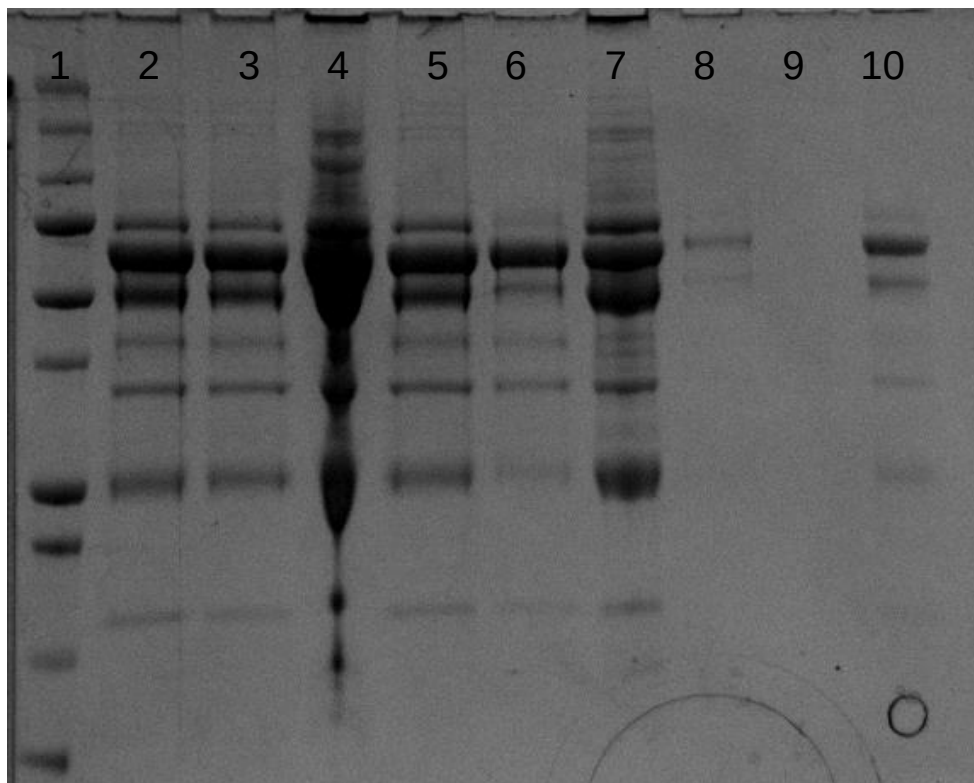


Figure A-1 Murine plasma depletion gradient

Example protein gel depicting the supernatants and pellet of protein-nanoparticle incubations. Lanes eight through ten are plasma without nanoparticles.

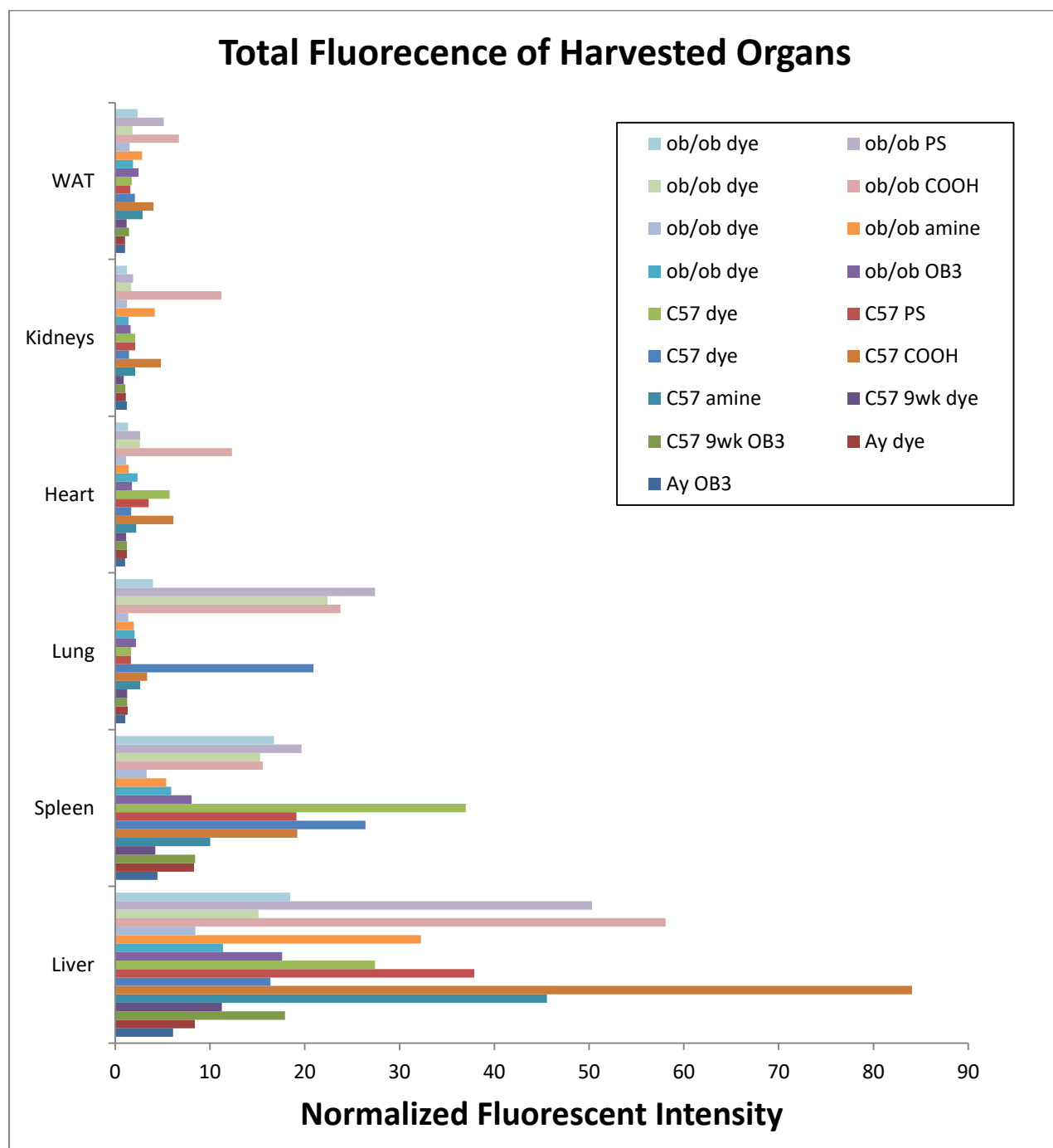


Figure A-2 Fluorescent Intensity per organ across mouse strains and diets

Total fluorescent intensity, measured as photon flux, was taken using IVIS fluorescence imaging. Data was normalized by the PBS control injection in each group, representing changes in background fluorescence. OB3 represents the PEG-PBD filomicelles.

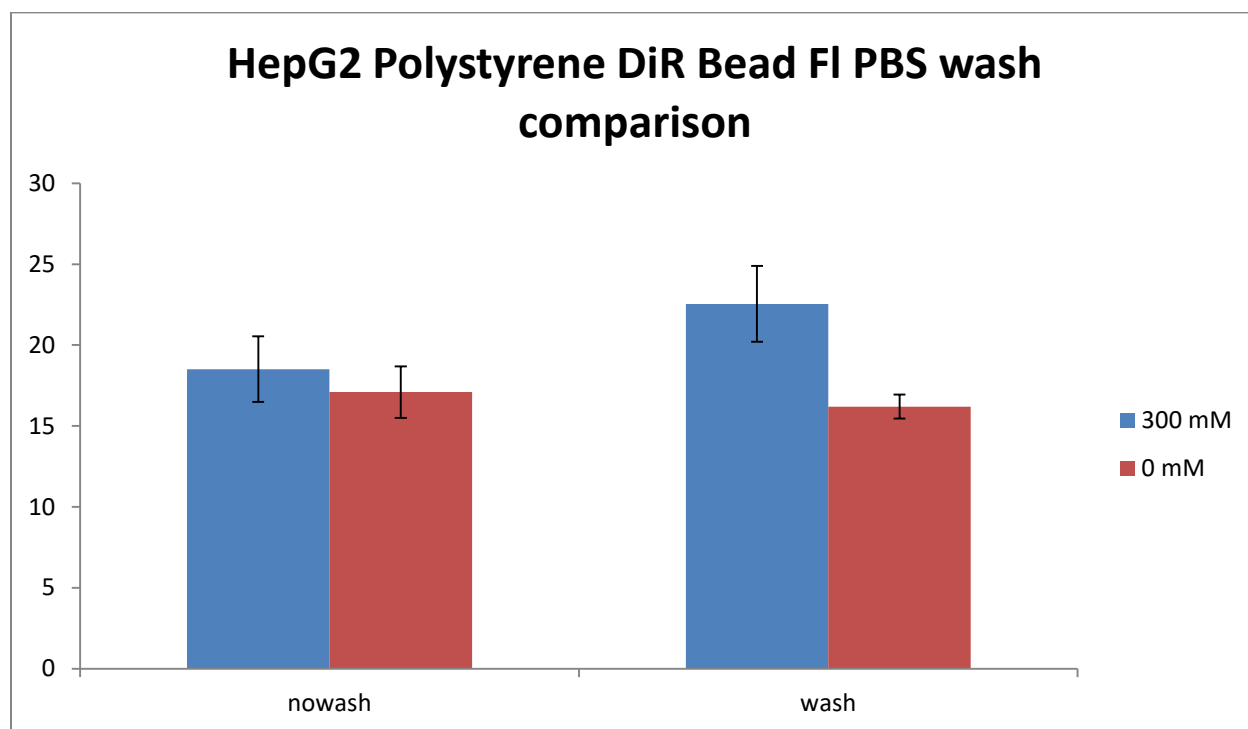


Figure A-3 Comparison of washed and unwashed HepG2 Fluorescence

Comparison of HepG2 fluorescence before and after a wash with PBS. Washing with PBS removes loosely associated and free particles while leaving adherent HepG2 cells and particles that are surface associated and phagocytosed.

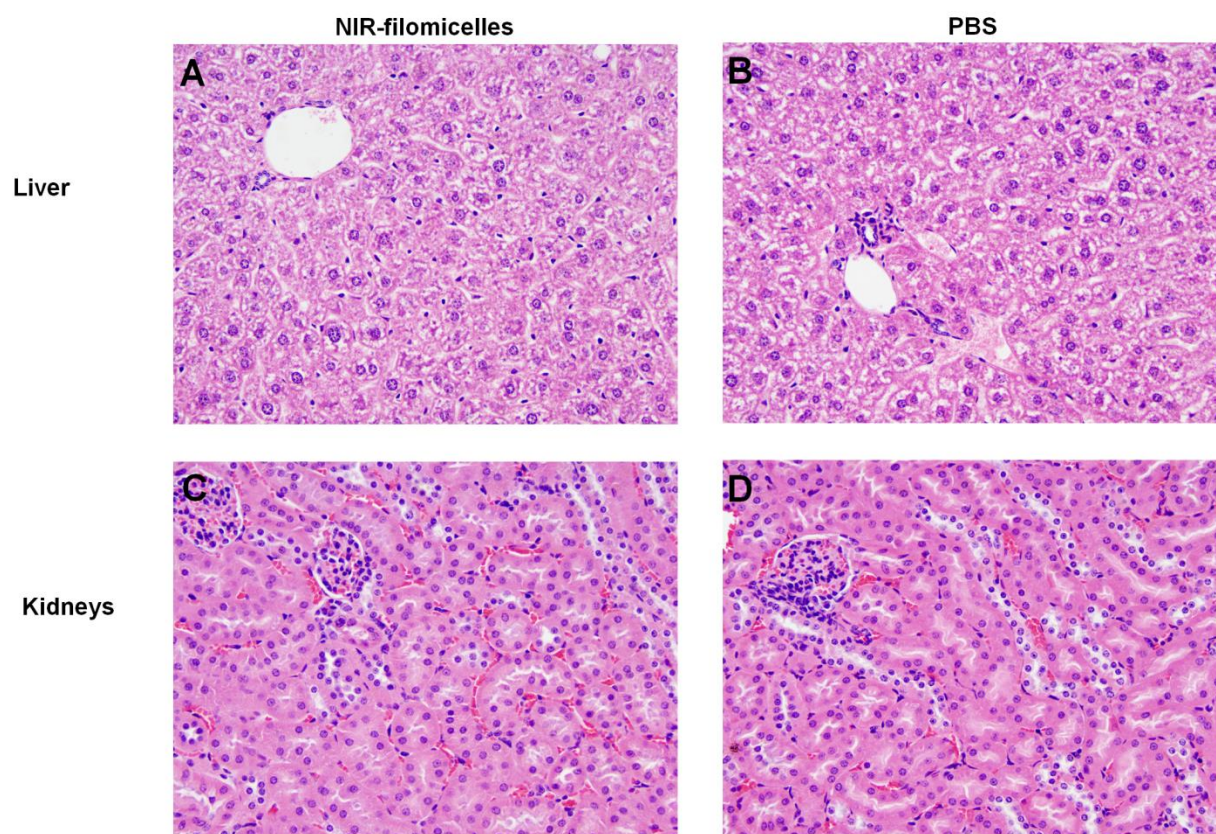


Figure A-4 Filomicelle and PBS control injection histology of liver and kidneys

Histology comparing filomicelle injections to PBS control. The filomicelles did not show significant changes from control injections.

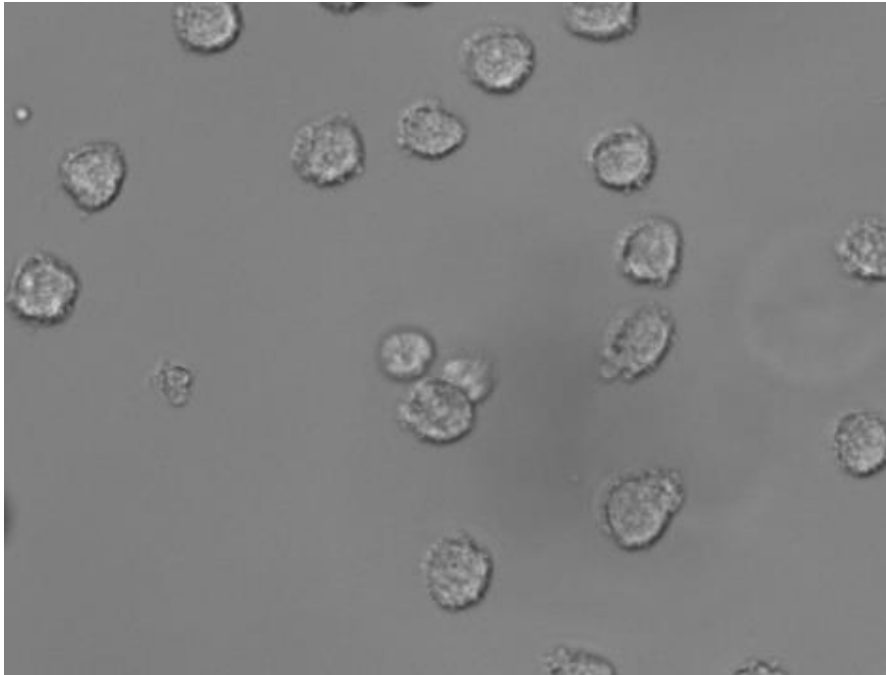


Figure A-5 LADMAC cell culture

LADMAC Bone marrow cells used to produce CSF-1 rich media, a colony stimulating factor used in the proliferation of macrophages.

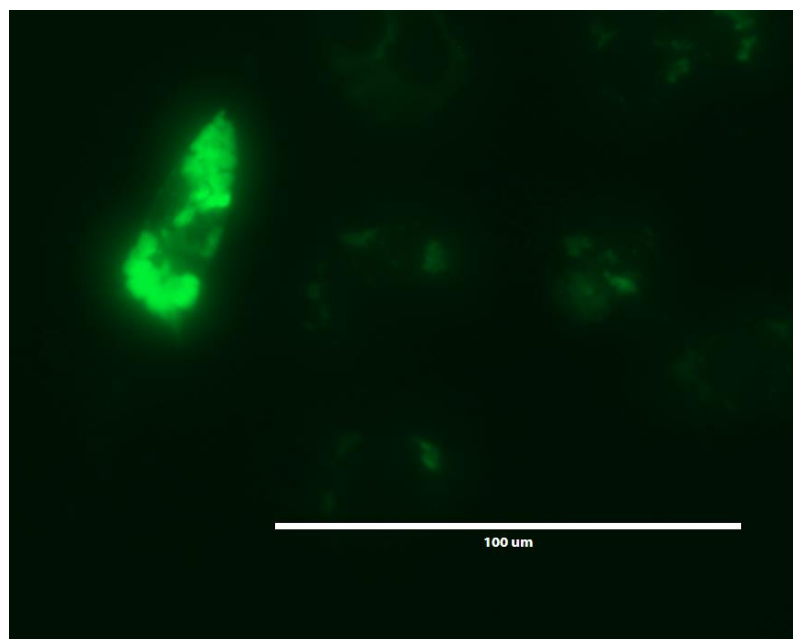


Figure A-6 HepG2 Lipid Droplets

HepG2 culture with lipids marked with green fluorophore.

Vita

Kevin Quigley graduated from the University of Notre Dame in the spring of 2011 with a Bachelor of Science in Chemical Engineering. His undergraduate research involved measuring the growth and ethanol production in strains of budding yeast on complex sugars. In the fall of 2011, he began work in Dr. Paul Dalhaimer's laboratory in the Department of Chemical and Biomolecular Engineering at the University of Tennessee Knoxville.