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# PHARMACOKINETICS AND TOXICODYNAMICS OF INTRAVENOUSLY ADMINISTERED RIGID MICROPARTICLES THAT PASSIVELY TARGET THE PULMONARY CIRCULATION OF RODENTS

by

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## **ABSTRACT OF THE DISSERTATION**

Pharmacokinetics and Toxicodynamics of Intravenously Administered Rigid Microparticles that Passively Target the Pulmonary Circulation of Rodents by HILLIARD L. KUTSCHER

> Dissertation Director: Professor Patrick J. Sinko

Systemic treatment of localized diseases, currently the most widely used method of drug delivery, often results in dose limiting toxicity. Reducing the amount of drug administered to minimize toxicity often reduces treatment effectiveness. Many drug delivery systems actively target their site based on an over-expression of cell surface receptors that may differ during disease or treatment progression.

The purpose of this thesis was to determine the optimal size/number of intravenously administered rigid polystyrene microparticles (MPs) that are passively filtered by the pulmonary circulation system prior to causing doselimiting toxicity and to develop an appropriately sized rigid yet biodegradable MP for future use.

Passive entrapment of MPs in the lung depended upon size. In a rat model, rigid, non-degradable 10  $\mu$ m polystyrene MPs were trapped in the lung

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capillary and remained for the duration of the 1-week study. Smaller MPs (6  $\mu$ m) were initially trapped in the lung but migrated to the liver and spleen over 48 h whereas 3  $\mu$ m MPs eluded the lung's filtering capability and became entrapped in the liver by 1 h.

To devise a non-invasive technique to detect early toxicity, a mathematical algorithm based on a clinically-relevant, non-invasive method developed in humans was adapted to study pulmonary gas exchange in young CD-1 mice. A threshold MP dosage that resulted in a rapid decrease in function was found for different MP sizes. The ventilation-perfusion ratio (V<sub>A</sub>/Q) was dramatically reduced from pre-treatment to Day 1 post-treatment when  $\geq$ 550,000 10 µm MPs/g,  $\geq$ 40,000 25 µm MPs/g or  $\geq$ 4,000 45 µm MPs/g were administered. Shunt increased slightly with MP burden but was not a consistent early marker for impaired gas exchange from microemboli. Of interest was that by Day 7, the resulting hypoxemia was resolved.

Finally, the manufacture of biodegradable, albumin-based MPs was optimized to create an appropriately-sized narrow distribution using an emulsion technique. Increased heating (150 °C vs. 120 °C) caused an increase in lysinoalanine formation and decreased the lung clearance rate, while not changing MP size.

In summary, understanding of the pharmacokinetics, toxicodynamics, and design of passively targeted intravenously administered MPs was significantly advanced.

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# DEDICATION

To my parents,

Harlan and Carole,

my brother,

Brett,

and

my love,

Katie

Thank you for your endless love, support and sacrifice.

.

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## 1. Introduction

Many researchers have used microparticles (MPs) as sustained release drug delivery devices for the administration of therapeutics by either intramuscular or subcutaneous injections or oral administration. In addition, radiolabeled particulates have been used to diagnose blood perfusion in multiple organs (e.g., liver, spleen, bone marrow, brain and lung). These particulates have been made of both organic and inorganic radiolabeled compounds (e.g., <sup>99</sup>Tc labeled albumin MPs and macroaggregated albumin for lung perfusion scanning and colloidal <sup>198</sup>Au for liver scanning).

Microparticles >10  $\mu$ m become entrapped in the first capillary bed encountered after injection, and in the case of an intravenous injection are retained by the lungs (Kutscher et al., 2010a). MPs between 5 and 10  $\mu$ m are retained in the lung for a period of time and are eventually cleared to the liver and spleen. MPs< 5  $\mu$ m become entrapped in the liver and spleen. Even smaller MPs (e.g., nanoparticles, colloids and aggregated proteins) have been shown to localize in the bone marrow, brain or to target the enhanced permeation and retention (EPR) of tumors. As a result of this passive, yet tissue specific targeting, the systemic dose of highly potent therapeutics may be lowered, which potentially reduces dose-limiting side effects; while at the same time significantly increases the localized tissue concentration of drugs leading to improved outcomes (Chao et al., 2010). Microparticle size is critical in determining not just the specific tissue to be targeted but also the site of entrapment within that tissue. Moreover, a vascularly entrapped MP functions as a microembolus, interfering with the normal blood flow and potentially inhibiting tissue function. The resulting toxicodynamic outcome is also directly related to the number of MPs administered with effects ranging from non-detectable to morbidity or even mortality. Therefore, developing an optimal drug delivery system based upon a microembolization strategy requires a precise understanding of how the organ will respond to both the size and number of MPs administered.

One of the major functions of the lung is to provide the exchange of oxygen and carbon dioxide to and from the body, respectively. Pulse oximetry is a simple, non-invasive method to measure the oxygen saturation  $(SpO_2)$  in the blood.  $SpO_2$  is a good biomarker for pharmacodynamic changes that occur due to lung impairment caused by embolization. Sapsford and Jones (Sapsford and Jones, 1995) and later Karbing et al. (Karbing et al., 2011) studied ventilation-perfusion mismatching and physiological shunting using a physiologically based computer model based solely upon varying the administered fraction of inspired oxygen ( $F_1O_2$ ) and measuring the  $SpO_2$ . Their modeling approaches, which were developed in humans, offer the opportunity to understand the degree to which MP embolization effects pulmonary gas exchange and have never been studied in another species.

The major purposes of this research are: 1) to determine the pharmacokinetics and biodistribution of intravenously administered MPs; 2) to

study and model the toxicodynamics of MP entrapment in the lung by measuring the impairment of pulmonary gas exchange; and 3) to create biodegradable MPs that can be passively trapped within the lung.

## 2. Background and Significance

### 2.1. Current routes of administration that target the lung

The current standard route of administration for lung-related illnesses is inhalation. However, pulmonary administration of therapeutics is an inefficient process with only 10 - 30% of administered drugs reaching the systemic circulation (Derendorf et al., 2001; Hirst et al., 2002; Janssens et al., 1999; Leach et al., 2002; Melchor et al., 1993; Newman et al., 1999; Pritchard, 2001; Washington et al., 2001). Decreased bioavailability can be attributed to an individual patient's breathing capacity (Leach et al., 2005) and / or inhaler technique (Cochrane et al., 2000; Goodman et al., 1994) as well as to characteristics of the drug formulation itself, such as its aerodynamic particle size (Pritchard, 2001). In addition, assuming that particles have been properly administered by inhalation, the particulate or drug contained within must still cross through the protective mucous layer that is continuously turned over, the epithelial cell layer, the interstitial space that contains macrophages, and finally the endothelial cell layer to achieve systemic administration.

Thus optimal design of an inhaled drug relies on a complex interplay of the pathophysiology of patient disease, patient technique and drug formulation.

# 2.2. Novel route of administration based upon the lung's capacity to filter particles

Particulates (e.g., MPs, nanocarriers, liposomes, etc.) have been used as drug delivery systems for a number of years to increase circulation of an active component, to create immune responses for immunizations or to elute a drug compound. MPs have also been shown to be able to passively target various organ systems over a wide range of species based only on the size of the material.

Our group has investigated the optimal threshold size of MPs in rats to become entrapped in the lung following IV injection (Kutscher et al., 2010a). It is important to note that the lung is the first capillary bed (i.e., filter) that venous blood encounters. Therefore any injected particulate matter is filtered in the lung prior to reaching systemic circulation. In this study, rigid 10  $\mu$ m PS MPs were lodged in the lungs of rats for the 1 week duration of the study (Figures 2.1 and 2.2). MPs that were 3  $\mu$ m were found to quickly locate to the liver and spleen. However, MPs that were 6  $\mu$ m were found to transiently locate in the lung with the majority being eliminated to the liver after 48 h and the remainder eluting to only 10% of the injected dose after 168 h.

These results were similar to findings in dogs that show larger MPs will become entrapped in the lung while smaller MPs will circulate to the liver and other capillary beds. The slight increase in pulmonary capillary size between dogs ( $6.23 \pm 1.5 \mu m$ ) and rats ( $5.15 \pm 1.3 \mu m$ ) could account for the difference in why 5  $\mu m$  MPs had different biodistribution and pharmacokinetic characteristics

in the dog compared to the slow removal of 6  $\mu$ m MPs in the rat (Short et al., 1996).

Furthermore, MPs >10  $\mu$ m have been shown to accumulate in the lung of humans and are currently used for pulmonary perfusion experiments. Pulmonary perfusion experiments using radiolabeled macroaggregated albumin (MAA) or human serum albumin MPs (HAMs) have been used since the 1960s with few reports of adverse events. These MPs are between 10 - 90  $\mu$ m and none can be larger than 150  $\mu$ m. Indicating that size plays a critical role in the safety of MPs.

In a series of studies, the effect of increasing the number and size of rigid MPs administered to rats found that very high MP burdens of large PS MPs (2.1E7, 15  $\pm$  5 µm MPs) administered by IV injection resulted in death in less than 15 h. However, lower MP burdens (1.6E7, 15  $\pm$  5 µm MPs) did not result in deaths (Cuenoud et al., 1978). In another study a 2-fold reduction in MP burden but with increased MP size (~8E6, 24  $\pm$  1 µm MPs) resulted in a 60% 16 h survival rate in rats whereas this dosage was non-lethal to dogs (Jones et al., 2003). Therefore, the optimal dose of MPs to be administered to an animal is both number and size dependent. In all of these studies, MPs were rigid and non-biodegradable.

In summary, injected MPs of specific sizes have the ability to selectively target the capillary beds of various tissues, but the toxicodynamics of these MPs is dependent in MP number and size and varies substantially in different species.

# 2.2.1. Pulmonary targeted drug delivery system for the treatment of lung cancer

Our group has shown previously that by administering 8.4E5 6  $\mu$ m camptothecin loaded MPs/g b.w., we were able to see improved tumor response with a reduced dose of camptothecin by 10-fold compared to free drug and there were no adverse events due to the MPs alone (Chao et al., 2010). In addition, increasing the dose to 2.1E6 6  $\mu$ m MPs/g b.w. resulted in only 1 of 3 rats showing signs of distress (Chao, 2006).

Interestingly, using 6  $\mu$ m MPs at much higher total MP burden (7.56E6 MPs/g b.w. (8.4E5 MPs/g b.w. every 3 days for 27 days)), we saw no noticeable signs of inflammation (Chao et al., 2010). However, since 6  $\mu$ m MPs were found to mostly leave the lung within 24 h (Figures 2.1 and 2.2), this could explain why there were no obvious signs of inflammation (Chao et al., 2010a).

These studies demonstrate the clinical potential of using a MP-based drug delivery system for the targeted treatment of disease.

### 2.3. Albumin Microparticles

Microparticles made of human serum albumin (e.g., HAMs or MAAs) were used to study regional blood perfusion since the 1960s and have been studied as a carrier system for a wide array of anti-cancer, antibiotic or anti-inflammation drugs. These MPs were made by several different techniques and are physically and chemically stable, amenable to large-scale production, metabolized and quickly cleared from the lung (Chilton and Ball, 1989) and have been used in humans with few adverse events reported (Dworkin et al., 1966).

As a naturally occurring protein, human serum albumin (HSA) has been used as the basis of a non-toxic and biodegradable polymer matrix for the formation of a parenterally administered, passive lung targeted drug delivery system. HSA is a globular unglycosylated serum protein consisting of 585 amino acids with a molecular weight of 66472.02. HSA contains 59 lysine, 24 arginine, 35 cystine and 16 histidine residues. HSA makes up approximately one half of the blood serum protein (Meloun et al., 1975).

In particular, the effect of temperature on amino acids and their subsequent cross-linking has been well studied. Lysinoalanine, ornithoalanine, histidinylalanine and lanthionine have all be found to exist after high temperature processing of natural products (Damodaran, 2008). These unusual amino acids form by the  $\beta$ -elimination of oxidized cystine and / or cysteine to dehydroalanine (DHA) followed by reaction with the amine group on the side chain of other amino acids via a Michael addition (Damodaran, 2008). These new amino acids (lysinoalanine and ornithoalanine) are more stable to trypsin digestion than lysine and arginine, respectively.

Thus albumin, a naturally occurring biodegradable substance, can be readily cross-linked or modified to become useful as drug delivery polymer.

### 2.4. The size and flexibility of circulating cells

The relative size of circulating cells among species appears to be conserved (Table 2.1 and 2.2). Because a rat and dog have similarly sized cells, and their pulmonary capillary size is similar, the use of red blood cell size as a method of capillary size comparison amongst species could be inferred. Furthermore, the LD<sub>50</sub> of PS MPs administered to rats and mice is similar across a large size range, again, indicating a strong correlation between size of red blood cell being a good allometric scaling factor. Therefore, using mice to understand the role of microembolism potential of an injectable lung drug delivery system in humans seems plausible.

However, size alone does not influence how quickly a cell is able to pass through the lung capillary bed. Stiffened erythrocytes due to either glutaraldehyde cross-linking or a particular disease state have been shown to take a much longer time to cross the lung capillary bed than healthy cells. In addition, neutrophils also take a longer time to cross the lung capillary bed. Although this could be for several reasons (e.g., chemoattraction) neutrophils are known to be stiffer than erythrocytes when measured by micropipette aspiration or AFM. This suggests that stiffness is much more important than size for lung retention.

### 2.5. A brief introduction to pulmonary physiology

The lung is a highly complex organ that has five main functions. First, its primary role is to perform gas exchange of  $CO_2$  out of the venous blood and  $O_2$ 

into the arterial blood. This exchange takes place in a fraction of a second and is driven solely by diffusion. Second, the lung acts as a filter to remove any natural or foreign debris such as a detached thrombosis clots or abused pharmaceutical products. Third, it provides a reservoir of blood to flow into the left ventricle. Fourth, it functions to remove fluid from the alveoli. Finally, the circulation within the lung provides nutrition to the alveoli and alveolar ducts (Comroe, 1966). In addition, the lung is a unique organ because it contains two separate blood compartments: the pulmonary circulation, whose primary task is to oxygenate the blood and to filter out debris; and the bronchial circulation, whose primary task is to supply oxygen and nutrients to the lung tissue. From now on, unless explicitly stated, any reference to blood flow and circulation will refer to the pulmonary circulation.

#### 2.5.1. The Lung – the Airway

The partial pressure of oxygen (PO<sub>2</sub>) flowing into the lungs and eventually supplying cells' mitochondria follows several very large step changes (Figure 2.3). The fractional amount of oxygen in air is 20.93% (approximated as 21% for these calculations). First, as air is inhaled, it is assumed to be instantly warmed to 37 °C and saturated with water. This increases the water vapor partial pressure (P<sub>H2O</sub>) to 47 mmHg and decreases the total PO<sub>2</sub> from 21% x 760 mmHg  $\approx$  160 mmHg to 21% x (760 - 47 mmHg)  $\approx$  150 mmHg. Upon entering the alveolar compartment, air is mixed with waste air that contains CO<sub>2</sub> (end tidal CO<sub>2</sub> is ~40 mmHg) and therefore the O<sub>2</sub> content is diluted further to yield ~100

mmHg of available  $O_2$  to be involved in gas exchange. The  $PO_2$  in the venous blood of healthy humans is ~40 mmHg and this  $PO_2$  gradient drives gaseous diffusion towards the blood. At the same time,  $CO_2$  is diffusing in the opposite direction, from  $PCO_2$  ~46 mmHg (blood) to ~0 mmHg (fresh air). This exchange takes place in <1 second in an at rest human and does so in less than one-third of the available time for a red blood cell to traverse the capillary (West, 1985b). However, there also exists a diffusional barrier across the alveolar membrane, spatially heterogeneous ventilation-perfusion mismatch (V<sub>A</sub>/Q inequality) and the mixing of oxygenated and venous blood (shunt).

### 2.5.2. The Lung – the Blood

The lung is a highly complex organ that has several unique features that are important in drug delivery. First, it accepts the entire venous blood output from the heart. Second, the transit time of a red blood cell through the lung is 4 -5 seconds and through the capillary is only 0.75 seconds (West, 1985a) whereas leukocytes have been reported to have significantly longer transit times (Hogg et al., 1994). Third, because the lung accepts the entire venous blood out from the heart, the lung is the first capillary filter in which anything may become trapped. Fourth, the lung is able to recruit underutilized capillaries during exercise or following embolization to maintain adequate blood pressure and flow. Finally, the lung facilitates the diffusion of oxygen and carbon dioxide from the air to the blood and vice versa.

West and others have shown that there are 3 zones of capillaries in the deep lung (i.e., alveolar sacs) (Fraser and Paré, 1977; West, 1985b). Capillaries at the base of the lung (zone 3, when alveolar pressure (P<sub>A</sub>) is less than arterial pressure (P<sub>a</sub>)) are the most dilated and have the most blood passing through them (Figure 2.4), as blood tends to "fall" during its transit through the lung vasculature due to gravity. However, capillaries in the most inferior position have slightly less blood flowing through them, which is thought to be a result of the weight of the lung tissue above compressing the capillary segments. Capillaries that are at the apex of the lung (zone 1,  $P_A > P_a$ ) are the least dilated and have the least amount of blood passing through them. Finally, capillaries that are in the middle (zone 2, when venous pressure  $(P_v)$  is less than  $P_A$ ) contain minimally utilized vessels and blood flow increases due to recruitment. When the orientation of the lung is changed (as in the case of rodents, or when lying down in humans), the blood flow through the lungs also changes, however the three regions still exist.

During exercise or in the case of a blockage, the pressure of blood flowing through the base of the lung increases thereby recruiting underused vessels found higher in the lung, all the while maintaining constant right ventricular systolic pressure (RVSP). At rest, only 30% of the human lung is utilized suggesting a large residual capacity to filter out material. In fact, it is estimated that 2.80E11 capillaries are present in the lung; therefore injection of 1E9 particles would result in only a 0.36% reduction of the lung capacity assuming every particle blocked an individual capillary.

In addition to the influence of gravity on capillary diameter, the lung is also able to constrict or dilate the vessels due to a feedback loop based on the partial pressure of oxygen and / or carbon dioxide in the capillaries. This results in shunting the blood to areas of the lung that have better ventilation, allowing for better oxygen exchange.

Finally, a true shunt in the lung passes blood from the pulmonary artery to the pulmonary vein without oxygenation or carbon dioxide offloading occurring. This mechanism is thought to be a method of protection for the right heart to avoid undue stress caused by increasing the pressure that it must pump against.

In summary, the lung is an organ with a vascular supply capable of adjusting to the body's oxygen demands by opening up capillary beds adjacent to alveoli to enable gas exchange as metabolically needed. These capillary beds also function as filters, and this filtration function can be exploited to trap MP drug delivery systems designed to take advantage of this property.

### 2.6. Understanding V<sub>A</sub>/Q and Shunt

The flow of air in the lungs is referred to as ventilation ( $V_A$ ) and the flow of blood through the circulation system is referred to as perfusion (Q). The lung acts as a bellows transporting oxygen-rich air from the atmosphere into close proximity of the blood flowing through the pulmonary circulation. However, oxygen and carbon dioxide exchange occurs in the capillaries solely based on diffusion. Oxygen is brought near the alveolar sacs where it then diffuses through the alveolar air to reach the capillaries and be exchanged with the carbon dioxide. Carbon dioxide meanwhile diffuses from the blood to the alveolar space, through the alveolar air and is removed through exhalation.

In a diseased lung, where changes to the pulmonary circulation result in perfusion changes such as right-to-left shunts, embolism, or bronchial anastomosis to the pulmonary circulation, and assuming true shunt, there will be an overall reduction in the oxygenation of the blood even at high  $F_1O_2$ . Conversely, in diseases where changes in the ability to ventilate the lung are more dominant such as asthma, emphysema and COPD then, assuming no shunt, the arterial oxygen saturation will reach 100% at high  $F_1O_2$ , but will have a dramatic decrease in SpO<sub>2</sub> at lower  $F_1O_2$ .

Because the lung is a dynamic organ, shunt and  $V_A/Q$  are inherently interconnected. Nevertheless, these two properties can be analyzed concurrently, thereby providing a method to clinically differentiate between various disease states. In particular, they can be used to follow the microembolism effect of a MP drug delivery system.

### 2.7. Modeling Pulmonary Gas Exchange Function

In general, ventilation-perfusion mismatch and shunt are determined for the entirety of the lung and are both reported as single numbers to estimate the overall function of the lung. However, in actuality there could be as many estimated compartment values as there are alveoli or capillaries in the lung. In the early 1970's West and Wagner developed a method to study ventilation perfusion and shunt by intravenously injecting 6 inert gasses in solution and monitoring the arterial blood for their disappearance called the multiple inert gas elimination technique (MIGET). MIGET used up to 50 compartments to describe ventilation and blood flow in the lung (Wagner et al., 1974). Although this method was invasive to the patient and computationally and labor intensive, it provided tremendous insight into the pulmonary gas exchange functioning of the lung.

Sapsford and Jones and Karbing et al. have since developed much simpler models to describe ventilation-perfusion mismatch and shunt based on changes in SpO<sub>2</sub> after varying  $F_1O_2$ . These models (Figures 2.5 and 2.6, respectively) only consist of one or two airway compartments, respectively (Karbing et al., 2011; Sapsford and Jones, 1995). The advantage of this model is that the data is obtained non-invasively and the does not require any further techniques for sample handling.

Jones et al. has shown in patients who suffer from pulmonary diseases that as the modeled amount of shunting increases, the patient's  $SpO_2$  decreases following a linear trend whereas an increase in  $V_A/Q$  results in a nonlinear increase of  $SpO_2$  (Jones et al., 2008). Nevertheless, increasing the amount of vascular or airway occlusion has deleterious effects on the patient's ability to exchange oxygen (as measured by  $SpO_2$ ) and it is difficult to truly separate these two phenomena.

This mathematical modeling approach to ventilation-perfusion mismatch is based solely upon two easily measured clinical parameters,  $F_1O_2$  and  $SpO_2$ . This approach, which has already been demonstrated to have clinical correlations in

humans, offers a way to make *in vivo* assessments of the effect of a MP drug delivery system passively targeted to the lung. Moreover, the non-invasive aspect of the overall technique should be applicable to various species including rodents where invasive testing is, at best, difficult to perform.

### 2.8. Role of Embolism on Pulmonary Gas Exchange Function

Pulmonary embolism affects more than 150,000 people in the US alone every year and is the third leading cause of death (Laack and Goyal, 2004). Most patients who die due to pulmonary embolism, die in the first few h. There are several possible reasons for this outcome. First, embolism increases the right ventricle systolic pressure (RVSP), which may lead to right heart failure. Second, embolism decreases the amount of oxygen present in the blood, essentially suffocating the patient from the inside out. Finally, embolism could elicit a massive inflammatory response.

While embolism normally occurs through natural processes, pulmonary perfusion scanning is based upon the temporary occlusion of pulmonary capillaries using radionucleotide labeled embolus. In addition, talcosis, as a result of IV drug use, has been found to occlude pulmonary capillaries (Marchiori et al., 2010).

To better understand the safety of the pulmonary perfusion scanning material, Davis and Taube studied the relationship of MP number and size necessary to cause death in rodents using rigid polystyrene (PS) MPs between 13 and 90  $\mu$ m (Davis and Taube, 1978). They found that the number of MPs that

can be safely administered is directly dependent upon their size and as MP size decreases, the number of MPs administered needed to elicit a toxic response in the animals increases in a non-linear fashion. This is most likely due to the number of capillaries or arterials that become blocked by the MPs and that there are fewer arterials than capillaries. Therefore, when comparing the same number of MPs, smaller MPs should have less of an effect on the function of gas exchange in the lung.

In addition, Davis and Taube indicate that the  $LD_{50}$  is taken at 24 h, but state that it is similar to an  $LD_{50}$  at 6 h. Moreover, the mice tended to expire in a few min post injection whereas the rats generally died within 2 h. This indicates that an acute response to the MP injury occurs and is most likely due to the animals having poor oxygen exchange or right heart failure. However, even at lower doses of PS MPs where animals have survived through 18 h, there is evidence of massive inflammatory response and pulmonary edema well below the  $LD_{50}$  of the MPs in rats (Zagorski et al., 2003); loss of pulmonary function (decrease in  $P_aO_2$ ); and an increase in right ventricle systolic pressure (Toba et al., 2010).

However, MP burdens well below the LD<sub>50</sub> (3.3E4 MPs/g b.w., 25  $\mu$ m (Davis and Taube, 1978)) of MPs caused inflammation. At 1.55E4 MPs/g b.w. SpO<sub>2</sub> significantly decreased by about 25 mmHg, the alveolar-arterial difference increased by about 30 mmHg and the RVSP increased by about 18 mmHg (Toba et al., 2010). Finally, a burden of 2E4 MPs/g b.w. caused lung leak (i.e., pulmonary edema) (Zagorski et al., 2003). Unfortunately, one aspect that both of

these groups did not study was the time dependence or possibility of resolving function or inflammation following embolism.

In summary, the clinical effects of pulmonary embolization are well known and potentially lethal. The toxicodynamics of pulmonary microembolization was initially studied in conjunction with the development of diagnostic pulmonary scanning techniques and, more recently, with an emphasis on the study of pulmonary pathophysiology. The data reported gave a starting point for the dosages to be used in the model of the proposed thesis.

### 2.9. Summary

In summary, the lung is an organ with a vascular supply capable of adjusting to the body's oxygen demands by recruiting underutilized pulmonary capillaries adjacent to well-ventilated alveoli to enable gas exchange as metabolically needed. These capillary beds also function as filters, and this filtration function can be exploited to trap MP drug delivery systems designed to take advantage of this property. Indeed, our group has demonstrated that the pulmonary filtration system can be used to trap MPs to treat pulmonary disease (Chao et al., 2010; Kutscher et al., 2010a; Kutscher et al., 2010b).

However, significant obstacles remain in developing this approach for the treatment of pulmonary diseases.

First of all, each injected MP represents a microembolism that can adversely affect the circulation in which it is entrapped. The data established from the use of MPs in studies related to the development of diagnostic pulmonary scanning and pulmonary pathophysiology can be used as a starting point to assess MP toxicodynamics in terms of particle size and number. However, the toxicodynamics of these particles have demonstrated both significant intra- and inter-species variation.

A potential approach to this variability is to build upon previous modeling work in humans based upon the non-invasive measurement of  $SpO_2$  while varying the  $F_1O_2$  of the subject. Although up until now this approach has been used as a diagnostic tool in the evaluation of pulmonary disease including shunts resulting from embolization, the technique itself can be adopted to permit realtime *in vivo* assessment of the toxicodynamics of an MP system. In addition, the technique permits the titration of the MP system in individual subjects whose pulmonary tolerances to embolization may differ substantially because of intraspecies variation as well as disease. Moreover, the non-invasive aspect of the overall technique should be applicable to various species including rodents where invasive testing is, at best, difficult to perform.

Another potential approach to the toxicodynamics of the MP system is to build the MPs from a biodegradable material. Since albumin is a naturally occurring biodegradable substance that can be readily cross-linked or modified to become useful as a drug delivery polymer, it can be used as the basis for such a targeted drug delivery vehicle. Because of its biodegradability, an albumin-based MP's toxicodynamic profile would be expected to differ significantly from that of the non-degradable MPs used by our group. These two approaches will form the basic avenues of research involved in this thesis.



**Figure 2.1.** Pharmacokinetics and biodistribution studies of MPs in the filter organs and heart of healthy rats was determined using a fluorescent plate reader (n = 3) at different time points after IV injection: (A) 2  $\mu$ m; (B) 3  $\mu$ m; (C) 6  $\mu$ m; and (D) 10  $\mu$ m (Kutscher et al., 2010a). Figure reprinted with permission (Kutscher et al., 2010a).



**Figure 2.2.** Pharmacokinetics and spatial distribution of MPs in healthy rat lung. A representative picture of the spatial distribution of 3, 6 and 10 µm fluorescently labeled carboxylate PS MPs in the lung of healthy rats, imaged using the IVIS® 100 at 1 h, 12 h, 24 h and 168 h post injection. The MPs were highly distributed throughout the lung tissue (Kutscher et al., 2010a). Figure reprinted with permission (Kutscher et al., 2010a).






**Figure 2.4.** The influence of gravity on blood flow in the lung. "Diagram Depicting the Influence of Gravity on Perfusion of Acinar Units in Upright Man. *In the upper unit*, alveolar pressure ( $P_A$ ) may in certain circumstances become higher than pulmonary artery pressure ( $P_a$ ) so that the capillaries are virtually empty. *In the midlung*, alveolar pressure ( $P_A$ ) is lower than arterial pressure ( $P_a$ ) but higher than venous pressure ( $P_v$ ), so that blood flow occurs through the arterial end of the capillaries but is impeded on the venous end. *The unit at the base of the lung* shows dilated capillaries throughout since both arterial ( $P_a$ ) and venous ( $P_v$ ) pressures are greater than alveolar pressure ( $P_A$ )" (Fraser and Paré, 1977). Figure reprinted with permission (Fraser and Paré, 1977).



**Figure 2.5.** Basic schematic diagram of the lung model of the lung. The airway space is considered as a single compartment, and the blood is either fully oxygenated or passes through the true shunt (blood that passes through the lung unoxygenated) (Karbing et al., 2007). Figure reprinted with permission (Karbing et al., 2007).



**Figure 2.6.** Improved schematic diagram of the lung. The airway space is considered as two compartments (high V<sub>A</sub>/Q and low V<sub>A</sub>/Q); and the blood can either pass through the highly ventilated area of the lung (normally the apex, V<sub>A</sub>/Q >0.9), the normally ventilated area of the lung (V<sub>A</sub>/Q  $\approx$  0.9) or the true shunt (Karbing et al., 2007). Figure Reprinted with permission (Karbing et al., 2007).

### 3. Specific Aims

The global objective of this research is to develop an intravenously (IV) administered lung-targeted microparticle (MP) delivery system for the localized treatment of lung related diseases (e.g., asthma, chronic obstructive pulmonary disease (COPD), interstitial pulmonary fibrosis (IPF) and primary or metastatic lung cancer). Based on extensive preliminary data in the Sinko Lab, this passively targeted drug delivery system was effective in the reduction of tumor burden in an orthotopic non-small cell lung cancer (NSCLC) rat model.

The toxicodynamics of rigid MPs to be used as a drug delivery system was studied. Using several well-characterized non-degradable rigid MPs, the pulmonary gas exchange function was non-invasively studied to determine a maximum safely administered dose. Second, biodegradable rigid MPs were created to reduce the potential toxicity of the delivery system itself.

Little is currently known about the toxicodynamics of microembolization of rigid MPs in mouse lung. This was of interest to study because physiologic changes such as ventilation-perfusion ( $V_A/Q$ ) matching or pulmonary circulation shunting (shunt) may be introduced through the entrapment of rigid MPs, which should result in a functional change to pulmonary gas exchange. These effects could potentially lead to poor gas exchange and subsequently death.

Thus, in order to better understand the specific effect of MP size and MP number on pulmonary gas exchange in healthy mice, rigid monodisperse nondegradable polystyrene (PS) MPs were used as a well defined model for passively targeted, IV administered, lung drug delivery systems. In particular, the use of these highly uniform and non-degradable MPs eliminated the need to take into account any issues related to variations in size or deformability, either initially or during biodegradation, that would occur with HAMs.

Finally, rigid biodegradable HAMs were engineered and *in vitro* degradation and pharmacokinetics evaluated as an IV injectable lung delivery system. HAMs of various sizes and degrees of biodegradability were created and tested *in vivo* for their potential utility.

Therefore, the specific aims of the present research are:

Specific Aim 1: <u>Determine the pharmacokinetic and biodistribution profile</u> of differently sized, rigid, monodisperse PS MPs to passively target the pulmonary capillaries of rats using an *ex vivo* imaging technique.

**Hypothesis 1.1:** Rigid PS MPs that are smaller in diameter to erythrocytes will pass through the pulmonary capillary bed and become predominantly entrapped in the liver or spleen.

**Hypothesis 1.2:** Rigid PS MPs that are larger in diameter to erythrocytes will become entrapped in pulmonary circulation.

**Hypothesis 1.3:** Rigid PS MPs that are similar in diameter to erythrocytes will become transiently entrapped in the pulmonary capillary bed and eventually become entrapped in the liver or spleen.

Specific Aim 2: Non-invasively determine the toxicodynamics of rigid MP microemboli on pulmonary gas exchange in spontaneously breathing mice. By measuring  $SpO_2$  while varying  $F_1O_2$ , early determination of impaired pulmonary gas exchange related to microembolization can be detected. The effect of intravenously administered microemboli on pulmonary gas exchange in healthy mice will be studied by using rigid PS MPs of known size and number. The effect of these microemboli on the mouse's  $SpO_2$  at a given  $F_1O_2$  will be directly and non-invasively measured.

**Hypothesis 2.1:** Increasing the number of microemboli will result in a decrease in SpO<sub>2</sub> at a given  $F_1O_2$ .

**Hypothesis 2.2:** Increasing the size of the microemboli will result in a decrease in SpO<sub>2</sub> at a given  $F_1O_2$ .

**Hypothesis 2.3:** The immediate reduction of SpO<sub>2</sub> following microembolization will revert to pre-embolization SpO<sub>2</sub> values over time.

Specific Aim 3: <u>Develop a non-invasive</u>, <u>physiologically based</u> <u>computational model that describes pulmonary gas exchange as it relates</u> to microembolization in spontaneously breathing mice.

From data obtained in Specific Aim 2, a virtual model of shunt and  $V_A/Q$  will be developed using based upon the Sapsford and Jones model that relates SpO<sub>2</sub> and F<sub>1</sub>O<sub>2</sub> (Sapsford and Jones, 1995). The modeled values of shunt and V<sub>A</sub>/Q will further explain/provide insight into (and increased sensitivity to) changes in pulmonary gas exchange compared to a single measurement SpO<sub>2</sub>. **Hypothesis 3.1:** This model will demonstrate that increasing the size or number of rigid MP microemboli will cause an increase in shunt and a decrease in  $V_A/Q$ .

**Hypothesis 3.2:** Based upon the data obtained in Hypothesis **2.3**, the model will demonstrate a decreasing shunt and an increasing  $V_A/Q$  over time.

Specific Aim 4: Engineer biodegradable and biocompatible micron-sized HAMs that can passively target the pulmonary circulation of mice and have controlled degradation.

HAMs of an appropriate size to principally block the pulmonary arterial circulation at the capillary level following IV administration will be made using a technique that is both suitable for mass production and available to the lab.

**Hypothesis 4.1:** HAMs of a sufficient size to primarily block the pulmonary capillaries (i.e., >10  $\mu$ m) and small enough to pass into or through the pulmonary arterioles (i.e., <75  $\mu$ m) can be created using a water-in-oil (w/o) emulsion technique using an overhead stirrer.

**Hypothesis 4.2:** HAMs can be made of sufficient size and narrow enough distribution (i.e., mean 20  $\mu$ m, polydispersity<0.5) to almost completely focus on solely blocking the distal pulmonary capillary circulation.

**Hypothesis 4.3:** Increasing the processing temperature of the w/o emulsion used to crosslink the HAMs will prolong their degradation time *in vitro*.

**Hypothesis 4.4:** Increasing the human serum albumin concentration in the water phase of the w/o emulsion will prolong the HAM's degradation time *in vitro*.

Specific Aim 5: <u>Determine the pharmacokinetic profile of biodegradable</u> and biocompatible micron-sized HAMs that passively target the pulmonary circulation of mice and have controlled degradation.

**Hypothesis 5.1:** HAMs loaded with a near-infrared (NIR) dye will be able to non-invasively monitor their biodistribution and pharmacokinetics.

**Hypothesis 5.2:** Appropriately sized HAMs will be entrapped in the pulmonary capillaries following IV administration.

**Hypothesis 5.3:** Increasing the processing temperature of the w/o emulsion used to crosslink HAMs will decrease their biodegradability resulting in a decreased clearance of the HAMs that are entrapped in the pulmonary capillary bed.

**Hypothesis 5.4:** The *in vitro* degradation of HAMs (Specific Aim 4) correlates to *in vivo* observations in mice.

4. Pharmacokinetic and biodistribution profile of differently sized, rigid, monodisperse PS MPs to passively target the pulmonary capillaries of rats using an *ex vivo* imaging technique

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#### 4.1. Introduction

The standard route of administration for treating lung diseases is inhalation whereby drugs and delivery vehicles are applied to the mucosal surface of the lung. Inhaled drugs must first penetrate the protective mucus layer and then the epithelial cell layer in order to enter the lung tissues. Once a drug traverses the air-blood interface it is rapidly cleared from the lung into the systemic circulation. Pulmonary delivery by means of inhalation is typically an inefficient process with only 10 - 30% of administered drugs reaching the systemic circulation (Derendorf et al., 2001; Hirst et al., 2002; Janssens et al., 1999; Leach et al., 2002; Melchor et al., 1993; Newman et al., 1999; Pritchard, 2001; Washington et al., 2001). Total drug absorption after inhalation occurs by two pathways: directly through the lung mucosa and indirectly by the intestine (i.e., after deposition in the mouth and pharynx or after it is moved by the mucociliary escalator in the upper respiratory tract and swallowed). Exposure of the lung mucosal surface to the applied drug is also a function of the breathing capacity of the patient (Leach et al., 2005), particle size (Pritchard, 2001) and inhaler technique (Cochrane et al., 2000; Goodman et al., 1994).

A patient with compromised lung function resulting from impaired deep lung inhalation will not have extensive mucosal surface coverage in the alveolar sacs and the resulting drug bioavailability will be lower than the already low theoretical maximum.

The capillary beds of "filter" organs (i.e., the lung, liver and spleen) act as mechanical filters that efficiently entrap MPs. In fact, over the years a number of reports on the gross biodistribution of rigid particulates such as silica dust (Gardner and Cummings, 1933; Simson, 1937), glass (Brewer and Dunning, 1947; Niden and Aviado, 1956; Prinzmetal et al., 1947), carbon (Biozzi et al., 1953; Stehbens and Florey, 1960) and polystyrene (Adlersberg et al., 1969; Dobson and Jones, 1952; Gesler et al., 1973; Kanke et al., 1980; Schoenberg et al., 1963; Schoenberg et al., 1961; Schroeder et al., 1978; Singer et al., 1969) have been published. Following intravenous (IV) administration, MPs larger than the diameter of capillaries become entrapped in the pulmonary circulation offering a unique opportunity for passive drug targeting. Although MP biodistribution and toxicity are generally thought to be dependent upon their size,

rigidity and dose, a systematic study of the relationship between MP size and lung retention, intra-lung distribution or blood vessel entrapment has not been performed. Most recent studies have focused on rigid MPs. Several studies have found that smaller rigid particulates (<4  $\mu$ m) pass through the lung and become entrapped in the reticuloendothelial system (RES) whereas the vast majority (80 - 90%) are found in the liver with the remainder in the spleen (5 - 8%) and bone marrow (1 - 2%) (Davis, 1975; Dobson and Jones, 1952; Scheffel et al., 1972; Yapel, 1985a). Larger particulates (>10  $\mu$ m) became entrapped in the lung.

There is also a body of work dating back to the 1960's that focuses on MPs that are somewhat flexible. Radiolabeled macroaggregated albumin (MAA) particles are used for perfusion scanning of various organs in mice, rats, dogs, monkeys, rabbits and humans (Bolles et al., 1973b; Sahin et al., 2002; Taplin et al., 1964b). MAA is a non-spherical, somewhat flexible biodegradable MP that is administered by IV injection and is currently approved for determining lung perfusion in humans (Pulmolite®, Pharmalucence, Inc. Bedford, MA and DraxImage®, Draxis Health, Inc. Kirkland, Québec, Canada). These products adhere to guidelines set forth in the USP that state ">90% of MPs must have a size between 10 - 90  $\mu$ m and no MPs may be larger than 150  $\mu$ m" (2008). Interestingly, humans have over 280x10<sup>9</sup> capillary segments with an average diameter of 7 - 10  $\mu$ m (Krahl, 1965; Weibel, 1963). The diameter of interior pulmonary capillaries in dogs and rats are 6.23 ± 1.5  $\mu$ m and 5.15 ± 1.3  $\mu$ m, respectively (Short et al., 1996). Given the wide array of factors that control

particle retention and persistence in the lung, it is not clear if a speciesindependent optimal particle size can be determined.

Following a standard dose of MAA ( $1x10^{6}$  MPs) and assuming that every MP is entrapped in an individual capillary, only 0.5 - 0.7% of a healthy lung becomes occluded (Davis, 1975). However, Davis found that clearance was not just dependent upon size but also on deformability since the clearance rates of similarly-sized MAA were significantly shorter than more rigid human albumin microspheres (HAM) (Davis, 1975). Human MAA dosages are much lower (<0.2 mg/kg or ~350,000 MPs) than the corresponding LD<sub>50</sub> for mice, rats, dogs and monkeys (72.2 mg/kg, 43.8 mg/kg, 68.1 mg/kg and 82.6 mg/kg, respectively) (Bolles et al., 1973b).

The IV injection of MPs and passive lung targeting affords the opportunity to treat lung diseases from the vascular side eliminating the need to traverse the mucus/surfactant and mucosal layers. This would be particularly useful in situations where lung capacity is compromised and inhalation is not a viable option. Higher deposition efficiency and prolonged retention may translate into reduced doses, less frequent administration and lower bioavailability variability. In addition, it is possible to treat deep lung injury regardless of the patient's ability to inhale the large volumes of air that are necessary for deep lung penetration. In the current report, the effect of size on the passive pulmonary targeting, lung distribution and persistence rigid MPs is reported in rats.

#### 4.2. Materials and methods

#### 4.2.1. Materials

Fluorescent, internally labeled, polystyrene MPs of various sizes (2, 3, 6 and 10 µm) were purchased from Polysciences Inc. (Warrington, PA). Polycarbonate filters with a 0.8 µm pore size were purchased from Osmonics Laboratory Products Inc. (Minnetonka, MN). 2-Ethoxyethyl acetate was purchased from Acros/Fisher Scientific (Fair Lawn, NJ). Male Sprague Dawley rats were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA). Rats were fed a standard rat diet, had free access to water and were housed in a room with a 12 h light-dark cycle for at least one week before the study. All rat studies were performed in AAALAC accredited animal facilities under approved protocols from the Rutgers University Animal Use and Care Committee.

#### 4.2.2. Methods

#### 4.2.2.1. Preparation of MPs for IV injection

Yellow-green fluorescent polystyrene MPs used in the fluorescent plate reader studies with various diameters (2, 3, 6 and 10  $\mu$ m) and blue fluorescent polystyrene MPs used in the IVIS® 100 studies of various diameters (3, 6 and 10  $\mu$ m) were washed several times using distilled water before being suspended in 0.9% NaCl containing 0.1% Tween 80 (20 mg MPs/mL). MPs were fully suspended in solution by sonicating and vortexing immediately prior to IV administration.

#### 4.2.2.2. Particle size analysis

The size distribution of the polystyrene MPs were determined using a Multisizer<sup>TM</sup> 3 Coulter Counter (Beckman Coulter, Inc. Miami, FL) with a 70  $\mu$ m aperture tube, which is able to detect particles from 1.4–42  $\mu$ m. MPs were added drop wise to 20 mL of ISOTON II dispersed phase until the concentration of particles was acceptable (<10%). 1 mL of dispersed phase was counted.

#### 4.2.2.3. Scanning electron microscopy (SEM)

MPs were washed twice with methanol and dried using a CentriVap concentrator (Labconco Corp., Kansas City, MO) for 30 min. The MPs were fixed on aluminum stubs with conductive tape, and coated with gold-palladium for 2 min at 30 mA and 5×10<sup>-2</sup> mbar in an argon atmosphere, using a Balzer SCD 004 Sputter Coater. The MP coated stubs were then examined using a SEM (AMRAY 1830 I) with an EDX 9800 X-ray system and a Robinson backscatter detector.

# 4.2.2.4. Biodistribution studies quantitated using a fluorescence plate reader

Groups of three male Sprague Dawley rats weighing  $300 \pm 50$  g were used for this study. Yellow-green fluorescence MPs (4 mg) of various diameters (2, 3, 6 and 10 µm) were suspended in 0.9% NaCl containing 0.1% Tween 80.

MPs were administered intravenously into the lateral tail vein of conscious rats. The animals were kept under close observation for any signs or symptoms of embolism for 6 h or until euthanized. At predetermined time points, animals were euthanized by CO<sub>2</sub> asphyxiation. The lung, right kidney, heart, spleen and a portion of the right lobe of the liver were collected for further processing. The rest of the liver was removed and placed in a separate tube for the calculation of the proportion of the MPs in the whole liver.

#### 4.2.2.4.1. MP recovery from organs

The analytical method for quantification of polystyrene MPs in organs has been described elsewhere and modified as follows (FMRC, 1999). Briefly, the organ samples were digested using freshly prepared 4N KOH in 2% Tween 80. An internal standard of 6  $\mu$ m bright blue fluorescent polystyrene MPs (0.5 mL, 0.025 w/v%) was added to each sample. The digested samples were then filtered through 0.8  $\mu$ m polycarbonate filters, washed with 2% Tween 80 (2 x 10 mL), and finally washed once with phosphate buffer (10 mL, 43.2mM KH<sub>2</sub>PO<sub>4</sub> and 131mM K<sub>2</sub>HPO<sub>4</sub>). After lightly vacuum drying the MPs, the filter with fluorescent MPs was carefully transferred to a polypropylene tube. 2-ethoxyethyl acetate (2 mL) was added to each sample and the samples were mixed thoroughly before being stored in the dark at room temperature for further analysis.

#### 4.2.2.4.2. Quantitation of MPs using a fluorescent plate reader

Samples containing fluorescent MPs were analyzed in triplicate using a GENios fluorescence plate reader (Tecan U.S. Inc., Research Triangle Park, NC). Sample aliquots were diluted to a total volume of 100  $\mu$ L in 96-well polypropylene microplates. The MPs (yellow-green fluorescence) were measured at  $\lambda_{ex} = 430 \pm 35$  nm and  $\lambda_{em} = 510 \pm 20$  nm. The internal standard MPs (bright blue fluorescence) used during tissue processing were measured at  $\lambda_{ex} = 360 \pm 42$  nm and  $\lambda_{em} = 465 \pm 35$  nm.

#### 4.2.2.5. Fluorescence imaging

Fluorescence imaging of rats was performed using an IVIS® 100 small animal imaging system (Caliper Life Sciences, Hopkinton, MA). CY5.5 excitation  $(\lambda_{ex} = 615 - 665 \text{ nm})$  and ICG emission  $(\lambda_{em} = 810 - 875 \text{ nm})$  filters were used. Identical illumination settings, including exposure time (5 s), binning factor (4), fstop (2), and fields of view (15 × 15 cm), were used for all image acquisition. Fluorescent and photographic images were acquired and overlaid. The pseudocolor image represents the spatial distribution of photon counts within the organs. Background fluorescence was subtracted prior to analysis. Images were acquired and analyzed using Living Image 2.5 software (Caliper Life Sciences, Hopkinton, MA).

Internally dyed, bright blue fluorescent polystyrene MPs of various diameters (3, 6 and 10  $\mu$ m) were washed twice in 0.1% Tween 80 in phosphate buffered saline (PBS, pH = 7.4). MPs were volumetrically dispensed and

weighed to yield a 4 mg dose of MPs and resuspended in sterile PBS (200 mL) containing 0.1% Tween 80.

Groups of three male Sprague Dawley rats weighing  $200 \pm 50$  g were used for *in vivo* imaging studies and fed a standard rat diet (AIN-93G). Rats were de-epilated immediately prior to reduce any auto-fluorescence effects due to diet or hair. Rats were anesthetized by isoflurane using an EZ-3500 Multi-Animal Anesthesia System (Euthanex Corp., Palmer, PA). A tail vein catheter was then inserted and used as the injection site. MPs (4 mg) in 0.1% Tween 80 in PBS (200 µL) were injected through the tail vein catheter. The tail vein catheter was flushed with up to 0.8 mL of 0.1% Tween 80 in PBS.

Three animals were euthanized at each time point and the heart, lung, liver, spleen, and kidney were removed and imaged intact. Imaging time points were as follows: No injection, 1 h, 12 h, 24 h, 168 h. Rats were euthanized by  $CO_2$  asphyxiation.

#### 4.2.2.6. Histology studies

Upon euthanasia after the administration of MPs, organs were collected for gross and histological inspection. The lung was inflated via tracheal cannulas with 10% buffered formalin solution (Fisher Scientific, Fair Lawn, NJ) till the pleura were smooth. The inflated lung along with the liver, kidney, heart and spleen were fixed by immersion in fixative for at least 24 h before being further processed. Sections (2x2x2 cm<sup>3</sup>) of the lung were dissected and used to prepare the tissue section slides. Tissue sections were then embedded in glycol methacrylate-based polymer (GMA) and tissue slices (6 µm thick) were cut and mounted onto glass slides. The tissue slides were stained using hematoxylin and eosin (H&E) dye, and observed histopathologically under a microscope to check for any possible tissue damage.

#### 4.2.2.7. Statistical analysis

Experimental values are expressed as mean  $\pm$  standard deviation. Differences between experimental groups were tested using a Student's t-test at  $\alpha = 0.05$  using GraphPad Prism v.4 (GraphPad Software, San Diego, CA). The regression analysis of the standard curves was performed using least squares linear regression (Microsoft Excel v.9.0). Figure 4.3 was generated by GraphPad Prism v.4.

#### 4.3. Results

#### 4.3.1. Polystyrene MP characterization

Polystyrene MPs were characterized for size and polydispersity. The manufacturer's reported values of the polystyrene MPs differ from the experimental results obtained by Coulter Counter measurements, and the results are shown in Figure 4.1. However, the measured values were statistically similar to their reported values by Student's t-test. There is no overlap between 3 and 6  $\mu$ m MPs; however, the amount of overlap between the 6 and 10  $\mu$ m MPs is 4.86%. All MPs were further characterized by SEM to determine their size,

shape and surface morphology. A representative scanning electron micrograph of 6 µm polystyrene MPs is shown in Figure 4.2. The polystyrene MPs appears spherical with a smooth surface, are discrete, and have low polydispersity.

## 4.3.2. Biodistribution of polystyrene MPs using the fluorescence plate reader

The biodistribution of fluorescently labeled carboxylate polystyrene MPs of four well defined size distributions was examined after IV injection in rats at 1, 6, 48 and 168 h post injection. The biodistribution of these MPs in the lung, liver, spleen and kidney was highly dependent on the size injected. Small MPs (2, 3)  $\mu$ m) were found to distribute quickly (within about 1 h) to the liver (43.12 ± 5.05) and 42.2 ± 6.74% of administered 2 and 3 µm MPs, respectively) and spleen  $(5.05 \pm 0.23 \text{ and } 13.64 \pm 4.18\%, \text{ respectively})$  (Figure 4.3A, B). Recovery of the 2 µm MPs slightly increased after 48 h in the liver and spleen (46.73 ± 1.86 and 5.37  $\pm$  1.3%, respectively). Interestingly, the majority of the 3  $\mu$ m MPs were detected in the liver and spleen (66.81  $\pm$  1.4 and 18.57  $\pm$  1.89%, respectively) at 48 h. Large MPs (10  $\mu$ m) distribute quickly (within 1 h) to the lung (97.77 ± 4.62%) and remain there for at least one week ( $107.75 \pm 3.51\%$ ) (Figure 4.3D). However, 6  $\mu$ m MPs were found to quickly distribute to the lung (64.86 ± 7.46%) and liver  $(7.84 \pm 1.85\%)$  after 1 h but then migrated from the lung  $(8.92 \pm 1.6\%)$ , 168 h) to the liver (83.2 ± 15.59%, 168 h) over time (Figure 4.3C).

## 4.3.3. Distribution of polystyrene MPs in the lung using the IVIS® 100 small animal imaging system

Since standard gross biodistribution studies only give an indication of the total number of MPs associated with an organ, a IVIS® 100 small animal imaging system was used to determine MP distribution in the lung of rats after IV injection. Rats were euthanized at 1, 12, 24 and 168 h post injection and the organs were removed and imaged. MP distribution pharmacokinetics were highly size dependent (Figure 4.4). Consistent with gross MP biodistribution (Figure 4.3), 3 µm MPs did not become entrapped in the lung (Figure 4.4, top row) and 10 µm MPs quickly distributed (1 h) to the lung, remaining there for at least 168 h (Figure 4.4, third row). However, 6 µm MPs were found to distribute quickly to the lung (Figure 4.4, second row) and then migrate from the lung over time. As can be seen in Figure 4.4, the perfusion distribution shows that the MPs are spaced in a fairly uniform manner when observed from an anterior-posterior While the IVIS® 100 was successfully used to demonstrate lung view. distribution of the MPs ex vivo, it was unable to detect the fluorescent signal from the internally labeled MPs in whole body animals and in organs such as the liver.

#### 4.3.4. Histology studies

It was observed that the injected 10  $\mu$ m MPs deposited throughout the lung (Figure 4.5C) and upon closer inspection in the pulmonary capillaries, especially at the septal microvessel area (junctions among alveoli), but not in small arterioles (Figure 4.5D). Few 10  $\mu$ m MPs were found in the liver and

spleen through microscopic observation. Emboli or tissue infarction were not observed macro- and / or microscopically in the tissue samples.

#### 4.4. Discussion

One of the lung's main functions is to filter out cellular debris and clots on a daily basis (Ganong, 2005a). In fact, the lung at rest only uses ~30% of its capacity and is therefore able to recruit other capillaries to avoid massive increases in the arterial pressure when blockages do occur (Ganong, 2005a). At rest, the bulk of blood passes through the base of the lung due to gravimetric forces (Ganong, 2005a). However, as blood pressure increases during obstruction or exercise, the level or height to which blood flows through the lung also increases thereby recruiting minimally used capillaries (Ganong, 2005a). The objective of this study was to evaluate the potential of achieving high lung targeting using a parenterally administered delivery system that exploits the body's natural filtering system.

The current study explores the threshold size necessary for optimal passive entrapment of MPs in the pulmonary capillaries of rats. Small MPs ( $\leq$ 3 µm) were quickly entrapped in the liver and spleen with a very low percentage in the lung. The relatively low recovery of  $\leq$  3 µm MPs is most likely due to their entrapment in other capillary beds, excretion through the feces or sequestration by macrophages in other organ systems. Larger MPs (10 µm) were nearly completely entrapped in the lung and remained there for a prolonged period of time. The current results are consistent with a series of studies reported in rats

and dogs by DeLuca and colleagues. They examined the biodistribution of  $^{141}$ Ce-labeled polystyrene MPs of various sizes (3, 5, 7 and 12 µm and 3, 8, 15 and 25 µm) in dogs (Kanke et al., 1980; Schroeder et al., 1978) and 3 µm in rats (Yokel et al., 1981). In dogs, MPs <7 µm were transiently retained in the lung eventually passing into the liver while MPs >8 µm became entrapped in the lung. In both dogs and rats, 3 µm particles were found to distribute to the liver and spleen. Although this suggests that the rat model could be used as an alternate to dogs as the lung delivery strategy is further optimized, additional studies would be required to determine the inter-species correlation.

There is no doubt that larger (>10 µm) MPs can be delivered to and filtered by the lung where the MPs can serve as a depot for drug release. Several groups have tried to exploit this mechanism for the sustained delivery of anticancer drugs such as carboplatin (Lu et al., 2003), doxorubicin (Jones et al., 1989) and adriamycin (Willmott et al., 1985b). Our group has shown that a camptothecin (CPT) prodrug immobilized on 6 µm MPs was more effective in reducing the amount of cancerous areas than a 10-fold higher dose of free CPT in an orthotopic lung cancer model in nude rats (Chao et al., 2009). However, since larger MPs occlude larger vessels, any potential toxic effect is multiplied since many smaller downstream vessels would also be blocked. As such, it would be advantageous to lodge MPs in as small a blood vessel as is practical since this would limit adverse downstream effects. Therefore, the goal was to determine the ideal MP size that transiently targets the rat lung with high efficiency and entrapment in the pulmonary capillaries. In the current studies, the

6  $\mu$ m MPs were predominately and very quickly entrapped in the rat lung (<1 h) after which they slowly eluted over time and accumulated in the liver. Our 6 µm MP results in rats are consistent with the biphasic elimination of 5 µm MP in dogs. The first elimination phase decreased 79% between 6 and 48 h with a more sustained second phase that only decreased 15% between 48 and 168 h. The slow terminal elimination of the 6 µm MPs is possibly due to the redistribution of the 6 µm particles after their first escape from the lung. However, the biphasic elimination pattern of the 6 µm MPs from the lung is compounded by the fact that these MPs are not as monodisperse as the 3 and 10 µm MPs (as characterized by Coulter Counter). These >6 µm MPs found in the 6 µm group may be retained longer in the lung as their size approaches 10 µm; similar results were found in dogs (Kanke et al., 1980; Schroeder et al., 1978). These results suggest that a mixture of 6 µm and larger MPs may be useful for delivering short-term loading doses followed by longer-term slow delivery. Such treatment strategies may be useful for diseases like cancer, asthma, emphysema or interstitial pulmonary disease.

Use of the IVIS® 100 provided for the first time evidence of the spatial distribution of various sized MPs in rat lung. Rigid MPs measuring 10 µm and smaller are widely distributed throughout the lung (Figure 4.4) and become entrapped in pulmonary capillaries but not arterioles (Figure 4.5). Targeting the septal microvessel area (i.e., junctions among alveoli) allows for a greater lung distribution pattern, more consistent drug gradients throughout lung tissues and a lower fraction of lung branch occlusions. Moreover, the 6 µm MPs appear to first

sequester in the lung and then after 48 h sequester in the liver, suggesting an intriguing pathway for potentially treating metastatic cancer.

Interestingly, the current results are also in good agreement with the transit times of stimulated neutrophils (mean diameter 8 µm) and leukocytes (6 -8  $\mu$ m), which are more rigid than erythrocytes (mean diameter 8  $\mu$ m) (Downey et al., 1990; Worthen et al., 1989). Neutrophils are larger than the pulmonary capillaries (mean diameter 5.5  $\mu$ m) and generally take a few seconds to >20 min to cross the pulmonary capillary bed (Lien et al., 1987; Lien et al., 1990). In comparison, erythrocytes are able to deform more easily than neutrophils and transit through the pulmonary capillary bed in less than one second (Levitzky, 2003). Nevertheless, the ability of neutrophils to deform allows them to transit through the pulmonary capillary bed, even if it occurs slowly. Rigid MPs larger than the capillary bed most likely rely on the ability of the capillaries to deform due to pressure changes in both the pulmonary and cardiovascular system. However, at some critical size and dose load, occlusion would occur due to the inadequate clearance rate. In an attempt to model erythrocytes and neutrophil transit through pulmonary capillaries, the passage of non-deformable MPs (>8) µm) was simulated (Huang et al., 2001) and they found that only 12% were predicted to pass through the lung capillary beds at increased pressures. This prediction was consistent with studies performed in dogs (Ring et al., 1961). These simulations also confirmed the findings of Wiggs et al. that only 15% of 5.85 µm polystyrene MPs were able to pass through the lung of rabbits whereas

86% of 3.08 μm MPs pass through the lung after less than 10 seconds (Huang et al., 2001; Wiggs et al., 1994).

Standard gross biodistribution studies involve destructive sampling of tissues in order to quantify compounds of interest. One of the purposes of the current study was to evaluate the IVIS® 100 for studying biodistribution, intraorgan distribution and to reduce the number of animals required for future Unfortunately, in vivo detection of fluorescently labeled MPs was studies. unsuccessful. There are two possible reasons for this. First, the dye in this commercially available MP was optimized for laser excitation wavelengths available in flow cytometry rather than for the available IVIS® 100 filter set. This means that the signal may not have been strong enough due to inadequate excitation. Second, the amount of dye encapsulated in the MP might not have been optimal for these imaging studies leading to poor signal strength. However, the IVIS® 100 was able to give an indication of the intra-lung distribution of MPs. This represents the first time that fluorescence has been used to demonstrate the widespread distribution of MPs in lung ex vivo after IV administration. In order to demonstrate the distribution patterns in vivo, dyes with stronger signals (e.g., near IR dyes) would have to be used.

In the current studies, neither emboli nor tissue infarction was observed either macro- and / or microscopically in any of the tissue samples. This is remarkable considering some of the MP dose loads that were administered (i.e., 3 µm MPs,  $2.6x10^8$  MPs; 6 µm MPs,  $3.3x10^7$  MPs; 10 µm  $7.3x10^6$  MPs). Previously, it was shown that dogs subjected to a very high dose of large

polystyrene MPs (8x10<sup>6</sup> MPs, 40 µm) died acutely, and upon further investigation all the dogs had MPs in the lung, half of the dogs had MPs in the heart and only 2 animals had MPs in the liver (Gesler et al., 1973). However, doses of fewer MPs (4x10<sup>5</sup> MPs) or smaller sizes (8x10<sup>6</sup> MPs, 10 µm) did not result in adverse events. In several studies, rats were subjected to high doses of polystyrene MPs resulting in pulmonary embolism. In one case, a very high dose of large polystyrene MPs ( $2.1 \times 10^7$  MPs,  $15 \pm 5 \mu$ m) was administered by saphenous vein injection in rats under anesthesia and the animals died in less than 15 h. However, lower doses  $(1.6 \times 10^7 \text{ MPs}, 15 \pm 5 \mu \text{m})$  did not result in deaths (Cuenoud et al., 1978). Another study involving the induction of pulmonary embolism in rats used a dose similar to the non-lethal dog dose of polystyrene MPs ( $\sim 8 \times 10^6$  MPs, 24 ± 1 µm) and the 16 h survival rate was 60% (n = 15) (Jones et al., 2003). These observations support the conclusion that larger rigid MPs (>10 µm) block larger upstream vessels effectively blocking larger areas of the pulmonary bed and strongly suggest that MP size should be minimized with the ultimate target being pulmonary capillaries. The lung has a variety of ways to compensate for natural MP exposure. Besides recruiting unused vessels, arteriovenous shunts can be created allowing pathways for MPs, which are larger than capillaries, to pass relatively unhindered into the venous circulation. In fact, MPs that are >25 µm have been found in the pulmonary venous blood after injection possibly due to arteriovenous shunts in the lung (Hopkins et al., 2008; Lovering et al., 2009; Lovering et al., 2007; Niden and Aviado, 1956; Prinzmetal et al., 1947; Rahn et al., 1952).

#### 4.5. Conclusion

In the current study, the effect of particle size on the lung distribution and retention of rigid MPs was assessed in rats. Taken as a whole, the current results suggest that the threshold size for optimal lung entrapment and transient retention of rigid polystyrene MPs is >6  $\mu$ m but <10  $\mu$ m in Sprague Dawley rats. Given the observed lung targeting and retention properties of injectable MPs, it is entirely likely that once per week administration is possible with highly potent therapeutics, a potentially significant alternative to multiple daily administrations using inhalation. Entrapping MPs with long residence times in the pulmonary vasculature may have advantages in primary lung diseases over inhaled medications that may be more readily removed through exhalation or absorption and clearance into the systemic circulation. Finally, close proximity of the MPs to the alveolar sacs and subsequent wide distribution throughout the bronchial tree itself suggests that MPs have the potential for treating asthma, emphysema, interstitial pulmonary disease and disseminated lung cancer. We are now in the systematically process of extending these studies by altering the deformability/rigidity of MPs and examining lung targeting, distribution and retention.

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**Figure 4.1.** Numeric (solid) and Volumetric (dashed) distribution of polystyrene MPs: 3  $\mu$ m (red); 6  $\mu$ m (green), 10  $\mu$ m (blue). The size of the 3  $\mu$ m, 6  $\mu$ m and 10  $\mu$ m polystyrene MPs as reported by the manufacturer were: 3.005 ± 0.112  $\mu$ m, 6.098 ± 0.573  $\mu$ m, 10.94 ± 0.396  $\mu$ m, respectively; the volumetrically measured size of polystyrene MPs were: 3.066 ± 0.236  $\mu$ m, 5.915 ± 0.205  $\mu$ m, and 11.06 ± 0.309  $\mu$ m, respectively.



**Figure 4.2.** SEM of 6  $\mu$ m polystyrene MPs. MPs appear to be discrete with a smooth surface and have low polydispersity.



**Figure 4.3.** Biodistribution studies of MPs in the filter organs and hearts of healthy rats was determined using a fluorescent plate reader (n = 3) at different time points after IV injection: (A) 2  $\mu$ m; (B) 3  $\mu$ m; (C) 6  $\mu$ m; and (D) 10  $\mu$ m.



**Figure 4.4**. A representative picture of the spatial distribution of 3, 6 and 10  $\mu$ m fluorescently labeled carboxylate polystyrene MPs in the lung of healthy rats, imaged using the IVIS® 100 at 1 h, 12 h, 24 h and 168 h post injection. The MPs were highly distributed throughout the lung tissue.



**Figure 4.5.** H & E stained healthy rat lung in GMA polymer matrix: control rat lung (Panel A & B); rat lung 48 h after administration of 10 µm MPs (Panel C & D). MPs are indicated by arrows; showing they were entrapped in the pulmonary septal microvessels. Magnification of Panels A & C: 20x; Panels B & D: 60x.

### 5. Toxicodynamic Effect of Rigid Non-Degradable Microparticles on Pulmonary Gas Exchange in Spontaneously Breathing Mice

#### 5.1. Introduction

An intravenously injected rigid polystyrene microparticle (MP) based drug delivery system was successful in treating primary lung cancer in an orthotopic rat model (Chao et al., 2010) by entrapment in the pulmonary capillary bed of these passively, size-targeted MPs (Kutscher et al., 2010a). However, the main concern with using an injectable MP approach is the potential for these microemboli to adversely effect pulmonary gas exchange. Therefore, we sought to understand the maximum tolerable single dose of MPs that could be safely administered prior to a significant change in pulmonary gas exchange using three differently sized MPs large enough to limit the MPs ability to leave the pulmonary capillary bed.

To measure the toxicodynamic outcomes of a microemboli-based drug delivery system, we chose to study changes to the estimated ventilationperfusion ratio (V<sub>A</sub>/Q) and shunt (i.e., pulmonary gas exchange) in order to find a maximum tolerable single dose prior to a statistically significant physiologic change. Currently there are a variety of commonly used techniques or indexes used by intensive care clinicians to assess pulmonary gas exchange including:  $PaO_2/F_1O_2$ , arterial-venous difference, nitrogen washout, multiple inert gas elimination (MIGET) and ventilation-perfusion scans (Rowe et al., 2010). Each method has limitations and strengths, but no method is without fault and require either advanced methods, costly equipment or are not feasible in a mouse. Moreover, while measuring the individual components of  $V_A/Q$ , namely alveolar ventilation and cardiac output is possible, they may be influenced by physiologic conditions and experimental protocols. In addition, the interpretation of the ratio of  $V_A/Q$  may be complicated by disease status. However, the physiologic importance of a  $V_A/Q$  shift in both magnitude and direction can not be understated.

We did this analysis by using a relatively new technique, originally developed and validated for use in humans that estimates 2 parameters (V<sub>A</sub>/Q and shunt) through a non-linear physiologically based mathematical modeling algorithm. This technique is based on the use of a pulse oximeter to measure SpO<sub>2</sub> while varying the percentage of oxygen inhaled ( $F_1O_2$ ) (Karbing et al., 2007; Kjaergaard et al., 2001; Sapsford and Jones, 1995). The technique was specifically established both to demonstrate that alterations consistent with clinical patho-physiologic changes could be determined by mathematical modeling P<sub>a</sub>O<sub>2</sub> from the non-invasive measurement of SpO<sub>2</sub> rather than from direct, but invasive, measurement of the P<sub>a</sub>O<sub>2</sub> by arterial sampling.

We present, for the first time, both this technique's usefulness in monitoring the effects of pulmonary capillary microembolism and its applicability to estimating  $V_A/Q$  and shunt in mice. The technique provides a method that is more sensitive to physiologic changes than simply measuring the SpO<sub>2</sub> while
breathing room air. Finally, we report how the technique allows for self controls to provide a method for studying disease progression, disease model development, and / or therapeutic intervention in the future.

# 5.2. Materials and Methods

#### 5.2.1. Animals

Male CD-1 mice (6-8 wks, ~30g) were purchased from Charles River Laboratories (Wilmington, MA). Mice were fed a standard mouse diet, had free access to water and were housed in a room with a 12-hour light–dark cycle for at least 1 week before the study. All animal studies were performed in AAALAC accredited animal facilities under approved protocols from the Rutgers University Animal Use and Care Committee.

#### 5.2.2. Methods

## 5.2.2.1. Preparation of MPs for IV injection

Polystyrene MPs of various sizes (diameters equal to 10, 25 and 45 µm) were purchased from Polysciences Inc. (Warrington, PA). MPs were washed 5 times using 0.1% Tween<sup>™</sup> 80 in PBS followed by centrifugation for 5 min at 600g. MPs were re-suspended in a final volume (5 mL/kg) of 0.1% Tween<sup>™</sup> 80 in PBS. MPs were fully suspended in solution by vortexing immediately prior to tail vein injection to conscious mice. MP suspensions were counted on a hemocytometer to confirm MP dose.

The 10  $\mu$ m MP doses were 150,000, 200,000, 250,000, 300,000, 350,000, 400,000, 450,000, 500,000, 550,000, 600,000, 650,000, 700,000, 750,000 and 800,000 MPs/g. The 25  $\mu$ m MP doses were 12,000, 15,000, 18,000, 21,000, 30,000, 40,000 and 50,000 MPs/g. The 45  $\mu$ m MP doses were 2,000, 4,000, 6,000 and 8,000 MPs/g. The vehicle control animals were administered 0.1% Tween<sup>TM</sup> 80 in PBS at 5 mL/kg.

### 5.2.2.2. Determination of pulmonary gas exchange

Free breathing mice were anesthetized by isoflurane (1.5%) in air using an EZ-3500 Multi-Animal Anesthesia System (Euthanex Corp., Palmer, PA). Upon anesthesia induction, animals were transferred to a single nose cone breather unit and placed in a supine position on a water heated surgical bed to maintain body temperature under anesthesia. Mixtures of gas were delivered by blending nitrogen and oxygen and the oxygen concentration was measured using a MaxO2 Oxygen Analyzer (Maxtec Inc., Salt Lake City, UT). The oxygen analyzer was calibrated using compressed breathing air at the beginning of each day. Arterial hemoglobin oxygen saturation (SpO<sub>2</sub>) in the blood was monitored on the mouse's thigh using a MouseOx® pulse oximeter (STARR Life Sciences, Oakmont, PA) at 1 and 3 day pre-MP exposure and 1, 3, 5 and 7 day post-MP exposure. Initially the lower portion of the  $SpO_2$  vs.  $F_1O_2$  curve was defined by decreasing  $F_1O_2$  sequentially from 21 to 18, 16, 14, 12 and 10%  $O_2$ . After a rest period of 3 minutes, breathing 21%  $O_2$ , we then increased  $F_1O_2$  to 24, 28, and 32% O<sub>2</sub>. Animals were maintained at each  $F_1O_2$  for 2 min or until the SpO<sub>2</sub> readings stabilized. The SpO<sub>2</sub> vs.  $F_1O_2$  data points were analyzed with a computer algorithm lung model previously defined in humans (Sapsford and Jones, 1995) as described below.

To determine the pulmonary gas exchange function of the lung, several parameters were measured or derived. Measurement of the PaO<sub>2</sub> can take place through arterial blood gas sampling, or can be related by the oxygen-hemoglobin dissociation curve, which in humans can be approximated by (Severinghaus, 1979):

$$S_x O_2 = \frac{1}{\frac{23400}{P_x O_2^3 + 150 \cdot P_x O_2} + 1}$$
(5.1)

where  $S_xO_2$  is the saturated oxygen hemoglobin value (%),  $P_xO_2$  is the partial pressure of  $O_2$  in the blood in a particular compartment and where x is the arterial (a), venous (v) or mixed pulmonary capillary (c) compartment.

Equation **5.1** incorporates a simplistic model of the oxygen dissociation curve and does not take into account the effects of base excess, concentrations of methemoglobin (MetHb), carboxyhemoglobin (COHb), 2,3-DPG, or CO<sub>2</sub> (i.e., the Haldane Effect), pH (i.e., the Bohr Effect), and temperature. Therefore  $P_aO_2$  may be inaccurately calculated from the measured SpO<sub>2</sub>. However, in mice, the measurement of multiple arterial blood gasses is significantly more difficult than in larger species because the volumes of blood necessary are large relative to a mouse and locations of accessible arteries are difficult to reach (Sahbaie et al., 2006).

The standard shunt equation is as follows (Aboab et al., 2006; Sapsford and Jones, 1995):

$$S = \frac{Q_s}{Q_t} = \frac{C_c O_2 - C_a O_2}{C_c O_2 - C_v O_2}$$
(5.2)

where S is shunt;  $Q_s$  is the amount of blood which is not oxygenated after passing through the pulmonary circulation;  $Q_t$  is the total amount of blood flowing through the lungs;  $C_xO_2$  is the concentration of  $O_2$  in the blood in a particular compartment. Concentration values used are per 100 mL.

The concentration of  $O_2$  in the blood stream is dependent upon the amount of  $O_2$  carrier present (hemoglobin) and the solubility of  $O_2$  in blood, which is based on the partial pressure of  $O_2$  present. The standard equation for determining the concentration of  $O_2$  in a particular blood compartment is (Aboab et al., 2006):

$$C_x O_2 = 1.34 \cdot Hb \cdot S_x O_2 + 0.003 \cdot P_x O_2$$
 (5.3)

where *Hb* is hemoglobin concentration.

In addition, the amount of change between the  $C_aO_2$  and  $C_vO_2$  is referred to as the arterial-venous difference (avDO<sub>2</sub>) and is generally accepted to be 5 (Chiang, 1968; Sapsford and Jones, 1995) for healthy resting humans:

$$C_a O_2 - C_v O_2 = av D O_2 = 5$$
(5.4)

Therefore substituting equation **5.3** into **5.1** and upon rearrangement:

$$C_c O_2 - C_a O_2 = \frac{S}{1 - S} \cdot av DO_2$$
 (5.5)

and substituting equation 5.3 into 5.5 results in:

$$(1.34 \cdot Hb \cdot S_c O_2 + 0.003 \cdot P_c O_2) - (1.34 \cdot Hb \cdot S_a O_2 + 0.003 \cdot P_a O_2) = \frac{S}{1 - S} \cdot av DO_2$$
(5.6)

 $P_cO_2$  can be approximated from the amount of  $O_2$  inspired and corrected for water vapor and carbon dioxide:

$$P_{c}O_{2} \approx P_{A}O_{2} = F_{I}O_{2} \cdot (P_{b} - P_{H_{2}O}) - \frac{P_{a}CO_{2}}{R} \cdot (1 - (F_{I}O_{2} \cdot (1 - R)))$$
(5.7)

where  $F_1O_2$  is the fraction of inspired  $O_2$  (20.9% in air);  $P_b$  is barometric pressure (assumed to be 760 mmHg);  $P_{H2O}$  is partial pressure of water vapor at 37°C (47 mmHg);  $P_aCO_2$  is the partial pressure of carbon dioxide that is remaining in the respiratory dead volume; and R is the respiratory quotient which is dependent upon metabolism in the body and diet (0.9 in mice (Zwemer et al., 2007)).

Finally, the alveolar ventilation-perfusion equation (Rahn and Fenn, 1955) is:

$$\frac{V_a}{Q} = \frac{8.63 \cdot R \cdot av DO_2}{P_a CO_2}$$
(5.8)

which upon rearrangement is:

$$\frac{P_a C O_2}{R} = 8.63 \cdot a v D O_2 \cdot \frac{Q}{V_a}$$
(5.9)

Substitution of equations **5.1**, **5.7** and **5.9** into **5.6**, solving for  $P_aO_2$  and back-substituting into equation **5.1** results in a model similar to the first one proposed by Sapsford and Jones (Sapsford and Jones, 1995), which can be viewed schematically in Figure 5.1.

### 5.2.2.3. Histology and Immunohistochemistry

The lung was inflated via the trachea with 3% paraformaldehyde 2% sucrose and immersed in fixative. After 24 h at 4 °C, the lung was transferred to 2% sucrose for an additional 24 h at 4 °C. The left lung was then embedded in OCT media and frozen until sectioning. Tissue sections (10 µm) were rehydrated in PBS, and following a citrate antigen retrieval step were blocked with 100% goat serum at room temperature for 2 h. Tissue sections were then incubated overnight at 4°C with primary rabbit affinity purified polyclonal antibodies against proliferating cell nuclear antigen (PCNA) (1:500, Abcam Cambridge, MA). Slides were then incubated for 30 min with biotinylated goat anti-rabbit secondary antibody (Vector Labs, Burlingame, CA). Antibody binding was visualized using a DAB Peroxidase Substrate Kit (Vector Labs).

### 5.2.2.4. Statistical analysis

The logit weighted [log(Y/(1-Y))] best fit curve parameter estimates of V<sub>A</sub>/Q and shunt and were calculated using Maple v.15 (Waterloo Maple Inc., Waterloo, ON) with the DirectSearch optimization package v.2 (Moiseev, 2011). Statistical analyses were performed using GraphPad Prism v.4.0c or GraphPad Instat v.3.1a (GraphPad Software Inc., La Jolla, CA). Experimental values are expressed as mean ± SD. The significance of a single factor in groups was tested by analysis of variance (ANOVA) at a=0.05 with a Dunnett's post-hoc test. All the graphics in this article are generated by GraphPad Prism v.4.0c.

#### 5.3. Results

Initially mice were analyzed for signs of distress following tail vein injection of MPs. Higher 10 µm MP loads were found to be lethal; thus after 750,000 and 800,000 MPs/g 2 of 4 mice died, while 1 of 4 died after injection of 650,000 and 700,000 MPs/g. In some animals lethal effects of the MP were evident immediately post administration while others died overnight.

To understand the theoretical changes in the SpO<sub>2</sub> vs.  $F_1O_2$  curve, values for shunt or V<sub>A</sub>/Q were substituted in the model; the relevant shift of the SpO<sub>2</sub> vs.  $F_1O_2$  curve is shown in Figure 5.2. A decrease in shunt causes a downward shift of the plateau while a decrease in V<sub>A</sub>/Q causes a rightward shift of the vertical portion of the curve. It is important to note that these two parameters are independent of one another and therefore measuring SpO<sub>2</sub> at a single fixed  $F_1O_2$ is inadequate to characterize pulmonary gas exchange (Sapsford and Jones, 1995). Moreover, simply measuring the change in SpO<sub>2</sub> of animals breathing 21% O<sub>2</sub> between pre- and post-treatment groups did not show a statistical difference between treatment and control groups using Dunnett's post-hoc analysis (Figure 5.3).

Representative SpO<sub>2</sub> vs.  $F_1O_2$  plots for animals treated with either a low or high dose of 10 µm MPs on Day -3 (i.e., pretreatment) and Day 1 are shown in Figure 5.4. For animals receiving low doses (e.g., 150,000 MPs /g) of MPs (Figure 5.4A and 4C) the raw data and best-fit curves overlap, indicating that there is little or no shift in V<sub>A</sub>/Q or shunt. In contrast, for animals receiving high doses (e.g., 550,000 MPs/g) of 10 µm MPs (Figure 5.4B and D), there is a

statistically significant rightward shift (i.e., reduced V<sub>A</sub>/Q) but relatively little downward shift (i.e., increased shunt). All SpO<sub>2</sub> vs. F<sub>1</sub>O<sub>2</sub> data were fit individually using the algorithm described above. The parameters ( $V_A/Q$  and shunt) were plotted vs. time as individual points for each animal with the means connected by a line (Figure 5.5). The temporal dependence of  $V_A/Q$  and shunt is apparent. Due to inherent animal variability, animals were normalized to the average of their pretreatment measurements (Day -3 and Day -1) for both  $V_A/Q$  and shunt (Figure 5.6). A statistically significant normalized reduction of  $V_A/Q$  was observed on Days 1 and 3 compared to pretreatment (Days -3 and -1) and the magnitude of the  $V_A/Q$  drop is relatively consistent across the three particle sizes (Figure 5.7). Interestingly, by Day 5, the animals had recovered pulmonary gas exchange relative to their pretreatment  $V_A/Q$  values and by Day 7 were fully recovered. The change in shunt between Day -3 and Day 1 is only statistically significant at 40,000 and 50,000 25 µm MPs/g (45.4 and 38.9%, respectively), and most likely due to recruitment and distention of the capillary bed. This lack of visible shunt may be a result of the size of MPs administered (i.e., blockade of capillaries and pre-capillary arterioles vs. arteries), method of administration (bolus vs. infusion) or age of mice (i.e., developing vs. adult) and never reached the higher clinical values reported by MIGET or  $V_A/Q$  scans observed in humans with pulmonary embolism.

In humans, the physiologic shunt is <7% (Chiang, 1968) and  $V_A/Q$  approximately 1 (Wagner, 2005) with a normal range of 0.8 – 1.2 (Itti et al., 2002). The average  $V_A/Q$  during pretreatment (Days -3 and -1) for all animals

had a mean and standard deviation of  $1.07 \pm 0.22$  and  $1.04 \pm 0.28$ , and a range of 0.69-1.58 and 0.63-1.82, respectively. Although some of these mice had low or high V<sub>A</sub>/Q values, the mean and standard deviation are similar to those reported in healthy humans, pigs and sheep (Harris et al., 2002; Itti et al., 2002; Rizi et al., 2004).

To check for signs of remodeling in the lung, actively dividing cells were stained for PCNA. On Day 7, animals receiving MPs, independent of size or number, exhibited markedly higher expression of PCNA throughout the lung compared to control animals (Figure 5.8). Significantly greater staining was noted in cells surrounding or adjacent to MPs. Increased PCNA expression in the septal walls was also detected in animals that received high numbers of MPs. This may be a result of a nearby MP that were not visible due to thickness of the section. In other words, if there was a MP lodged out of plane from the cut, but the resulting septal wall/capillary was present, then increased staining would be observed, even though no MP was present in the 2D slice.

#### 5.4. Discussion

The lung has several physiological functions including gas exchange, filtering particles from the mixed venous blood, a blood reservoir for the left heart and removal of excess fluid from the alveoli (Comroe, 1966; Ganong, 2005b; West, 2008b). The entire venous output from the heart passes through the pulmonary circulation and during rest red blood cells pass through the capillaries in <1 second. The lung is also a unique organ because it contains two separate

circulatory systems. The pulmonary circulation is fed by the right atrium of the heart and provides the lung with deoxygenated blood to reoxygenate. The bronchial circulation is fed by the aorta and drains back into the right atrium of the heart via the intercostal or azygous vein or anastomosis with the pulmonary vein draining to the left atrium. The bronchial circulation feeds the bronchial tissue and offers minimal oxygenation of the blood. However, in a mouse, there is no functional bronchial circulation beyond the mainstem bronchi (Mitzner and Wagner, 2004). Therefore any deoxygenated blood detected in this model would most likely be a direct result of microemboli and not a natural, anatomical shunt found in other species.

Blood flow (Q) distribution through the lung of an upright healthy human is dependent on localized vascular pressures and gravity (West, 1985a). In general, the distribution of blood flow is highest at the base of the lung and decreases from the base to the apex. The distribution of air flow in the lung caused by ventilation (V<sub>A</sub>) also decreases from the base to the apex, although less dramatically. Therefore, the V<sub>A</sub>/Q ratio is not constant throughout the lung. In the case of a microembolic event the average of the localized V<sub>A</sub>/Q ratios is reduced even though global V<sub>A</sub> and Q have not dramatically changed. This is most likely due to the fact that the distribution of blood flow has changed due to capillary occlusion in the base and subsequent recruitment in the apex. This difference is physiologically important; because areas with increased V<sub>A</sub>/Q where maximum oxygenation has already occurred cannot overcome the decreased

oxygenation of blood in areas of decreased  $V_A/Q$ . Therefore this reduction in  $V_A/Q$  will result in hypoxemia.

Ventilation-perfusion mismatch and shunting has been shown to occur in humans in various disease states (West, 2008a). A change in  $V_A/Q$  implies that there is a change to the amount of air or blood flowing through the lung by either a restrictive or obstructive process in the airways and/or vessels. In particular, the distribution of blood flow through the capillaries may be influenced by vascular obstruction from emboli as well as by ischemia, pulmonary hypertension or hypoxia. The lung can adjust the amount of airflow by increasing either the rate of respiration or increasing the overall inspiration and expiration volumes. The lung can also adjust the amount of blood flow by either recruiting capillaries that ordinarily are not necessary for oxygenation or by dilating the size of the capillaries to allow for greater flow. Therefore the lung is a highly dynamic organ controlled by multiple neurohumoral and / or mechanical feedback loops.

In a clinical report, Jones et al. reported that a young man suffering a long bone fracture following a motorcycle accident had reduced  $V_A/Q$  and increased shunt based on the measurement of SpO<sub>2</sub> while varying  $F_1O_2$  (Burnstein et al., 1998). These changes in pulmonary gas exchange resolved over a 10-day period. The cause of the change in pulmonary gas exchange was most likely fat embolization, which is known to occur following long bone fracture. To accurately assess the effects of microemboli on pulmonary gas exchange, techniques beyond measuring the SpO<sub>2</sub> to detect loss of function are required (Figure 5.3). This has also been shown to be true in humans suffering from various lung diseases (Jones et al., 2008). There is the potential for non-healthy patients to exhibit relatively normal SpO<sub>2</sub> values (SpO<sub>2</sub> >92%) breathing air at sea level. However, upon changing the  $F_1O_2$  to simulate typical cabin pressures in an airplane ( $F_1O_2 = ~15\% O_2$  at sea level, similar to 8000 ft elevation), their SpO<sub>2</sub> value dropped below 80% (Jones et al., 2008). Therefore, a single SpO<sub>2</sub> value when breathing air at sea level is inadequate to describe the extent of pulmonary gas exchange, or the health of the patient.

Techniques for investigating ventilation or perfusion abnormalities in mice, rats, dogs, monkeys, rabbits and humans have been available since the late These techniques use radioactive gases such as  ${}^{15}O_2$  or  ${}^{133}Xe$  or 1950's. radiolabeled MPs such as <sup>99</sup>Tc macroaggregated albumin (MAA) or human albumin microspheres (HAMs) to look for areas absent of ventilation or perfusion (Davis, 1975; Knipping et al., 1955; Taplin et al., 1964a, c). When overlaid together these two diagnostic tests provide a picture of  $V_A/Q$  mismatch. During the 1970's, Wagner and West developed the multiple inert gas elimination technique (MIGET) to characterize lung function (Wagner, 2008; Wagner et al., 1974). While more complex, this technique allows for quantification of blood shunting, ventilation dead space and areas of the lung not receiving proper ventilation or perfusion rates. Although this approach is considered the gold standard for pulmonary gas exchange experiments, it is not widely used in a clinical setting due to its complex technique (Wagner, 2007). However Rees et al. (2010) have found a good agreement between dead space volume, shunt and the heterogeneity of the lung and MIGET analysis (Rees et al., 2010) when

compared to a more complicated, 2 airway compartment model similar to ours, despite the fact that these two methods are not directly comparable (Karbing et al., 2011).

Since the 1960's, <sup>99</sup>Tc-MAA MPs have been clinically used for perfusion scanning of the lung in humans. This method of detection (i.e., injecting MPs) has been safely used with few adverse events reported. One reason for the safety of this visualization technique is that while the MPs are designed to occlude the smallest vessels possible, the injected MP dose (1 x 10<sup>6</sup> MPs) is significantly lower than the number of capillary (280 x  $10^9$ ) or precapillary arteriole segments (300 x 10<sup>6</sup>) in humans (Davis, 1975). At rest, a healthy human lung uses only 30% of its capacity (Ganong, 2005b), which results in a large reserve capacity to filter out debris from the blood. To date, the number of pulmonary capillary segments in a mouse is undetermined. However assuming allometric scaling based on lung weight of a 70 kg human (lung weight ~650 g) to a 30 g mouse (lung weight ~0.311 g) (Lindstedt and Schaeffer, 2002), a gross estimate of the number of capillary segments 8-16 µm in diameter should be  $\sim$ 1.34 x 10<sup>8</sup>. Injection of 500,000 10 µm MPs/g results in only an  $\sim$ 11% occlusion without any statistical shift in  $V_A/Q$  or shunt.

The lack of shunt detected by our experimental method throughout these experiments is justified due to the small size and relatively low numbers of MPs used. The technique did not detect shunt because there was little change of SpO<sub>2</sub> when breathing room air for all animals. While the standard method of detecting a shunt is to administer 100% oxygen and measure the difference

between SpO<sub>2</sub>, our SpO<sub>2</sub> values were >96% in all healthy animals and on average were >98%. After administration of MPs, and not including the noticeable outliers (see Figure 5.3B) the average was >97, indicating that there was a only a slight decrease in SpO<sub>2</sub> which could be attributable to either shunt or V<sub>A</sub>/Q change, but is less than normal physiological shunt of 7% in humans. Moreover, the advantage to using this mathematical model is that SpO<sub>2</sub> will asymptotically approach 100% when breathing 100% FIO<sub>2</sub>, however by generating several data points past the knee in the curve will all for the model to solve for shunt.

In addition, because of the large capillary reserve capacity of the lung, redistribution of blood flow through the underutilized capillary bed or distension of well-used capillary segments should occur well before a redirection of blood through a non-ventilated vessel would occur and be detectable by simply measuring SpO<sub>2</sub>. We speculate that the cause of this redirection would be the result of active neuro-hormonal control or passive vessel pressures, which are beyond the scope of this article to understand. This redirection theory could be tested *in situ* by increasing the number or size of MPs injected and measuring pleural and sub-pleural vessel diameters, however this would not be applicable for our purpose of targeting the capillary bed for site directed drug targeting and administering a maximal therapeutic but non-toxic dose.

In mice and rats the number and size of MPs administered is directly correlated with mortality. For example, in mice, the  $LD_{50}$  of albumin MPs has been reported to range from 72-200 mg/kg (Bolles et al., 1973a; Zolle et al.,

1970), while the LD<sub>50</sub> for 13.5  $\mu$ m MPs was 96,000 MPs/g; for 25.7  $\mu$ m MPs, 24,000 MPs/g; and for 45.4 µm, 6,000 MPs/g (Davis and Taube, 1978). In the current studies the animals showed significantly higher allowable MP burden with an LD<sub>50</sub> of 750,000 MPs/g for the 10  $\mu$ m MPs. The LD<sub>50</sub> was not determined for the other MP sizes studied since the purpose of the study was to understand MP burden in terms of changes to  $V_A/Q$  and shunt rather than mortality. Differences between previous studies and the current study include the age, weight and sex of the mice; the volume of vehicle injected; and the vehicle itself in which the MPs were administered. The most likely reason for the  $LD_{50}$  difference is the age of the animals in terms of their lung size relative to body weight and developmental stage. In rats, the relative lung to body weight was found to be significantly higher at a younger age (Brain and Frank, 1968). Thus is would be expected that, younger mice would require more MPs when dosed on a per gram body weight than an older mouse to effect a similar level of toxic occlusion. The lung of a Swiss Webster mouse, which is an outbred strain similar to CD-1, does not fully mature until 6 months of age (Mauderly, 2000). Therefore, the resolution of  $V_A/Q$  in our CD-1 mice following MP administration was likely a result of either lung growth due to natural development and/or remodeling due to MP administration.

Pulmonary arterial pressure has been reported to increase in mice, dogs and pigs following pulmonary obstruction by MPs (Allen et al., 1974; Hedenstierna et al., 2000; Tuchscherer et al., 2006). For example, administration of MAA to dogs resulted in increased pulmonary artery pressure and decreased femoral artery pressure, early signs of toxicity, which were directly related to the number and size of MAA (Taplin and MacDonald, 1971). In humans, an increase in pulmonary arterial pressure was found to correlate with increased vascular obstruction determined by angiogram and a reduction in  $S_aO_2$  (McIntyre and Sasahara, 1971). In Sprague Dawley rats administered 13,000 25 µm MPs/g, there was no effect on right ventricle systolic pressure (RVSP) or  $P_aO_2$  when compared to control. However, increasing the dose to 20,000 25 µm MPs/g resulted in hypoxemia, a near doubling of RVSP and a 62% survival rate at 18 hours (Zagorski et al., 2003). Administration of ~41,000 25 µm MPs/g to an *in situ* C57BL/6 mouse lung resulted in a doubling of pulmonary arterial pressure (Tuchscherer et al., 2006). In the current study, the survival rate for a similar dose of MPs was 100%.

For each size MP, there was a threshold number of MPs administered that greatly reduced the calculated  $V_A/Q$  value on Days 1 and 3 post-MP injection. However, by Day 5, the  $V_A/Q$  returned towards baseline levels. This finding was unexpected, since the large MPs remain in the lung throughout the duration of the study, indicating that there is some form of compensation or remodeling occurring. For instance, following left pulmonary artery ligation (LPAL) in 6-8 wk old C57BL/6 mice, angiogenesis of the intercostal arteries results in new vessels attaching to the pleura of the lung in 5 days (Mitzner et al., 2000). Although we did not observe new vessels adhering to the plural wall or enlarged vascular pooling in the pleural space by microscopy (Wagner et al., 2006), our method of causing ischemia (i.e., microembolization) was more diffuse than complete artery ligation.

Our technique to assess changes in ventilation-perfusion matching and shunting is non-invasive and suitable for clinical application in humans. In fact, this procedure was first developed in humans (Sapsford and Jones, 1995) and requires only a finger pulse oximeter to measure  $SpO_2$  and the ability to change  $F_1O_2$ . Although there have been some improvements in the original technique including incorporating  $P_aCO_2$ , or multiple compartments (Jones et al., 2008; Karbing et al., 2011), to date, this technique has not been performed in rodents. Recently, a pulse oximeter (MouseOx®, STARR Life Sciences) for rodents was developed and validated in rats (Strohl et al., 2007), allowing for the measurement of  $SpO_2$  in a non-invasive manner in a spontaneously breathing animal.

Our model is based on Sapsford and Jones' original model which was developed and used on 9 healthy, free breathing volunteers and 35 patients undergoing major surgery that were mechanically ventilated (Sapsford and Jones, 1995). Subsequently, it has been used to study pre-term infants and conscious adults with different underlying pulmonary diseases, and the original assumptions have not changed (Jones et al., 2008; Rowe et al., 2010). In the original model, the authors assumed a constant arterial-venous oxygen difference (avDO<sub>2</sub>); did not adjust for changes to the oxygen dissociation curve caused by changes to base excess, pH of the blood, which would affect SpO<sub>2</sub> readings; and did not require patients to be on a mechanical ventilator, to be able

to control end tidal  $CO_2$  ( $P_{et}CO_2$ ) by changing ventilation rate. Additionally, we made 2 additional assumptions that all animals had a constant hemoglobin concentration and respiratory quotient (R) and did not measure them.

A limitation of our methodology is assuming a constant avDO<sub>2</sub> because  $avDO_2$  is dependent upon cardiac output (Q) and oxygen consumption (VO<sub>2</sub>) and can vary substantially (Aboab et al., 2006). In fact, in a small sample of humans (n=7) following massive pulmonary embolism with resulting arterial hypoxemia, a near doubling of avDO<sub>2</sub> was calculated and a decrease in cardiac output was measured (Jardin et al., 1979). However, a change to cardiac output would have a similar effect on three terms,  $avDO_2$ ,  $V_A/Q$  and shunt because Q is found in the denominator of each term. In our model, when holding  $V_A/Q$  and shunt constant, doubling avDO<sub>2</sub> causes a noticeable rightward and slight downward shift of the  $F_1O_2$  vs.  $SpO_2$  curve. To "return" the shifted curve after doubling avDO<sub>2</sub>, our estimate term  $V_A/Q$  must also double to account for the left-right shift, and our estimate of shunt will also decrease by a factor of 1.6 to address the vertical movement of the plateau. Without measuring either oxygen consumption  $(VO_2)$ or alveolar ventilation  $(V_A)$  it is impossible to determine which term  $(avDO_2 \text{ or }$  $V_A/Q$ ) is actually changing. Nevertheless, by holding  $avDO_2$  constant, we are able to separate out the effects of V<sub>A</sub>/Q and shunt and can quantify these changes to be able to look for disease resolution or therapeutic intervention. Most importantly, multiple investigators have developed models to estimate changes to  $V_A/Q$  and shunt based on the shifting of the  $F_1O_2$  vs. SpO<sub>2</sub> curve in

humans and have found that these changes are consistent with clinically evident, pulmonary diseases.

In order to account for changes to the oxygen dissociation curve, an arterial blood gas measurement would be required, which would be an invasive and potentially lethal procedure in a mouse and could potentially interfere with our subsequent measurements until the animal's blood returned to normal (Sahbaie et al., 2006). Therefore, while there could be a change to our calculated  $PaO_2$  from our measured  $SpO_2$  values, we assumed that it would not have a marked effect on our estimates of  $V_A/Q$  and shunt. We did not place our animals on a mechanical ventilator nor did we measure  $P_{et}CO_2$ . This was not a requirement of the original model, and by allowing our animals to be free-breathing, they were able to adjust their  $P_{et}CO_2$  accordingly. An advancement of the original Sapsford and Jones model put forth by Karbing et al. makes use of blood and airway concentrations of  $CO_2$  to improve the fit of their model (Karbing et al., 2011) and the advancement of rodent sized equipment now makes this possible.

Subsequent models accounted for PaCO<sub>2</sub>, but the difference between models did not have a dramatic effect on the overall shape of the curve. In fact, addition of a second "airway" compartment to our model significantly increased the computational time. However, this only resulted in changing the model values, and not the overall left-right placement of the curve. Future work will focus on improving the number of values determined through the "knee" to justify an additional compartment. In addition, significant improvement to controlling the concentration of  $O_2$  used will be beneficial to statistical analysis for determining faster methods of looking for a change that has occurred. By simply administering  $O_2$  at a single, fixed percentage (e.g., 15%) any detectable change to  $V_A/Q$  could be quickly inferred. Infact, Jones has recommended using a number of pre-drawn curves for common  $V_A/Q$  and shunts to quickly and easily determine  $V_A/Q$  and shunt rather than the need for running the complete mathematical model (Rowe et al., 2010). Proving that an even further simplification of this model can have clinical importance. Regardless of future improvements or simplifications, the current model is more than adequate for clearly demarcating the significant degree of pulmonary toxicity or loss of function.

This demarcation immediate applicability has in terms of the toxicodynamics of an injectable MP pulmonary delivery system. The advantage of this type of delivery system is its ability to localize drugs to their site of action in order to minimize systemic concentrations and unwanted side effects. Drug delivery system targeting using specialized ligands and other modalities such as monoclonal antibodies is showing promise but these technologies are clearly in their infancy. On the other hand, treating lung diseases using drugs administered by inhalation has been used since 1500 B.C. and remains an important and well established route of administration today (Sanders, 2007). As a route of administration, inhalation relies on significant aerodynamic engineering of the drug delivery system to target the desired location within the lungs and as well as patient's breathing capacity and inhaler technique (Kutscher et al.,

2010a). Drug delivery via inhalation predominantly targets the large airways and bronchioles due to aerodynamic limitations. As a consequence downstream vessels such as pre-capillary arterioles and capillaries are largely out of reach.

Recently, our group has shown that rigid, non-degradable, polystyrene (PS) microparticles administered intravenously target the lung in a size and surface charge dependent manner (Kutscher et al., 2010a; Kutscher et al., 2010b). Using this pulmonary capillary entrapment design strategy, an amino acid ester prodrug of camptothecin (CPT) with similar activity to the parent drug (Deshmukh et al., 2010) was attached to the surface of PEGylated 6 µm PS MPs for the treatment of lung cancer in tumor bearing rats (Chao et al., 2010). This delivery system was designed to provide sustained therapeutic drua concentrations of CPT localized in the lung with low plasma concentrations. Rats administered the CPT-MP drug delivery system had a similar reduction in tumor burden to those receiving a 10-fold higher concentration of free CPT alone; however the systemic blood levels of CPT were found to be nearly undetectable in the CPT-MP treatment group (Chao et al., 2010). These results suggest that the passive targeting approach was successful in selectively delivering therapeutic drug concentrations to the lung, while minimizing systemic concentrations. However, there were three limitations of this particular system: 1) extremely low payload (0.22mg drug/100mg particle); 2) lack of biodegradability; and 3) enlargement of the spleen at higher MP burdens (150 mg/kg) because the 6 µm MPs escaped entrapment of the lung over 48 h and

thereby limited total drug administration. In addition, and most importantly, there is a rational fear of increased MP administration.

Previous work demonstrated that MPs  $\geq 10 \ \mu m$  become entrapped for 7 days or longer in the lung of rats, and 6  $\mu m$  MPs would become entrapped in the lung initially but over 48 h would migrate to the liver and spleen (Kutscher et al., 2010a; Watts et al., 2011). In addition, repetitive dosing of 6  $\mu m$  MPs every 3 days (150 mg MPs/kg) to rats resulted in enlargement of the spleen whereas lower doses (50 or 100 mg MPs/kg) did not; a single dose of 250 mg MPs/kg resulted in distress (Chao et al., 2010). Based on this knowledge, several sizes of MPs  $\geq$  10  $\mu m$  were selected for investigation in the present studies to only study pulmonary toxicity associated with MPs being entrapped in the lung. We hypothesized that large MPs would occlude larger vessels (i.e., upstream) and as a consequence smaller downstream vessels; and that this would change V<sub>A</sub>/Q and shunt which would lead to a decrease in oxygen exchange ultimately causing hypoxemia.

The results reported herein show that there is an optimal tradeoff between pulmonary toxicity and the combination of both MP number and size to be used for drug delivery that can be used effectively for passively targeted, IV administered, pulmonary drug delivery. To compare these results to our previously reported anti-cancer system that utilized surface attachment to 6  $\mu$ m MPs is difficult due to the transient nature of the 6  $\mu$ m MPs. However, when comparing the total surface area of 10, 25 and 45  $\mu$ m MPs (i.e., number of MPs times MP surface area) prior to a significant change in V<sub>A</sub>/Q or shunt, the 10  $\mu$ m

MPs have ~2.67 fold and ~12.35 fold more surface area than the 25 and 45  $\mu$ m MPs, respectively. Therefore assuming a similar attachment group density on the surface of the MPs, there is a significant advantage to using smaller particles when therapeutics are only surface bound. However, if physical entrapment, and thus a w/w loading of the drug within the MPs is a viable option, then the total volume (i.e., number of MPs times MP volume) of the MPs administered is a good indicator of total drug loading and the 10  $\mu$ m MPs (500,000 MPs/g = ~275 mg/kg) are ~1.07 and ~2.74 fold higher than the 25 and 45  $\mu$ m MPs, respectively. Therefore picking between 10  $\mu$ m and 25  $\mu$ m MPs moving forward may be a result of manufacturing technique rather than ideal drug delivery if therapeutics can be passively entrapped in MPs.

Other factors which may influence the decision of 10 or 25  $\mu$ m MPs include the distribution of MPs throughout the lung, the elimination rate of the MP from the lung, the release rate of the drug to be delivered, and the therapeutic half-life / efficacy. Dosing smaller MPs will enable a more diffuse drug delivery to the lung, the smaller MPs will remain suspended in solution longer offering the potential for a diffuse infusion in larger animals vs bolus dose, further improving the distribution to the lung. It is difficult to comment on how two differently sized MPs would degrade within the lung or release drug without actually creating uniform populations of MPs and doing the controlled experiments. The increased surface area of the 10  $\mu$ m MPs should drive an increased drug release rate. However, the biodegradation of the 10  $\mu$ m MPs may allow for an earlier elimination of MP from the lung capillary bed prior to all drug being released.

The resulting fragments would then most likely be found in the liver or the spleen. The biodegradation rate of similarly sized HAMs can be controlled by their manufacturing process, which may eliminate the need to worry about complete drug release prior to elimination from the lung (Yapel, 1985b). Finally, a therapeutic with a relatively long half-life may have significantly different drug delivery needs than one with a short half-life.

Our computer algorithm method allows for the non-invasive detection of change to  $V_A/Q$  and shunt and indicates that when dosing rigid, non-degradable MPs, there is a critical point at which a large change occurs when utilizing the each animal itself a healthy self-control. From a clinical stand point, providing a method which has been shown to detect physiologic changes prior to death, will enable a clinician to titrate the dose of drug containing MPs to significantly higher levels based on a patients condition all the while knowing that a return of function within one week is possible.

#### 5.5. Conclusion

In the current studies, the effect of particle number and size of permanently entrapped, rigid, non-degradable MPs was assessed in 6-8 wk CD-1 mice. The threshold MP dose limit resulting in a significant reduction in pulmonary gas exchange in mice is a size dependent and large. High doses of 10  $\mu$ m (500,000 MPs/g) and 25  $\mu$ m (30,000 MPs/g) rigid non-degradable MPs do not cause significant changes to pulmonary gas exchange and these MP doses are larger than what is needed for effective drug delivery. Larger 45  $\mu$ m MPs can still be

administered safely, but at a much lower dose (2,000 MPs/g). Given the observed immediate yet transient reduction in pulmonary gas exchange over a 1 week period, even at larger MP doses, it is entirely likely that the benefits of localized lung therapy may outweigh any potential dyspnea in the treatment of diseases such as disseminated lung cancer, interstitial pulmonary disease, or COPD.

The current studies also present a simple, clinically relevant method and mathematical model to detect changes of impaired pulmonary gas exchange that previously were unable to be determined non-invasively in mice. This method has the potential to detect toxicity of drug therapies, drug delivery systems or disease states that are ordinarily not detectable in a standard room air environment in species ranging from mice to humans. This technique also has the potential to allow for the titration of a drug with known pulmonary toxicity by measuring the subtle ventilation-perfusion changes that may occur prior to a clinically significant reduction in SpO<sub>2</sub>.



**Figure 5.1.** Model of a 1 compartment lung.  $P_xO2$  is partial pressure of  $O_2$  and  $C_xO2$  is the concentration of  $O_2$  in compartment x, where x is the arterial (a), venous (v) or mixed pulmonary capillary (c) compartment; Q[dot] is blood flow;  $F_1O_2$  is the fraction of inspired  $O_2$ ; Shunt is the percentage of unoxygenated blood passing through the lung. Blue indicates unoxygenated blood, red indicates oxygenated blood.



**Figure 5.2.** Theoretical SpO<sub>2</sub> vs.  $F_1O_2$  curves. Values of shunt increase (0, 5, 10, 20, 30%) resulting in a downward shift. Decreased V<sub>A</sub>/Q (1.1, 1.0, 0.9, 0.8, 0.7, 0.6) resulting in a rightward shift. There is little apparent change to the shape of the curves. Dotted line indicates  $F_1O_2$  of room air at sea level.











**Figure 5.3.** Change of SpO<sub>2</sub> breathing room air ( $F_1O_2 = 21\%$ ) between Day -3 (pretreatment) and Day 1 (post-treatment). ANOVA analysis indicates p<0.05 however Bonferonni post-hoc analysis does not indicate that any groups are statistically different.



**Figure 5.4.** Representative SpO<sub>2</sub> vs.  $F_1O_2$  curves of animals receiving low (150,000 10 µm MPs/g) or high (550,000 10 µm MPs/g) shown on Day -3 (pretreatment, black) and Day 1 (red). There are 4 different shades for each day indicating different animals. The relative shade of the line in each color indicates the same animal on different days. The connecting lines are for clarity (A, B). The best-fit line (solid) and 95% confidence interval (dashed) are shown below (C, D). There is a noticeable rightward shift for the high dose treatment group, indicating a decrease in V<sub>A</sub>/Q.

















0.3-

0.2



























**Figure 5.5.** Time course of V<sub>A</sub>/Q and Shunt for MPs of 10, 25 and 45  $\mu$ m MPs from pre-treatment (Day -3 and Day -1) to post-treatment (Day 1 through Day 7). Black is shunt and red is V<sub>A</sub>/Q. The connected line is through the mean of the value. Error bars indicate SEM. Individual V<sub>A</sub>/Q is plotted as open symbols and shunt is closed symbols. Similar shapes indicate the same animal (i.e., a red open box is animal 1's V<sub>A</sub>/Q and a closed black box is animal 1's shunt).



**Figure 5.6.** Data grouped by MP size to look at changes in  $V_A/Q$  over time. Left, raw data; Right, normalized data to the individual animal using their average  $V_A/Q$  value on Day -3 and Day 1 (pretreatment).





C.

Α.

D.



**Figure 5.7.** Changes in  $V_A/Q$  (A, B) and Shunt (C, D) from pretreatment (Day - 3) and Day 1 compared to total cross-sectional volume (left) and area (right). Total area or volume on the x-axis is defined as the Number of MPs times the area or volume of an individual MP.



**Figure 5.8.** Immunohistochemistry staining for PCNA on Day 7. Control animals have little to no staining in the septal walls of the lung (A). Injection of different sized MPs does not change PCNA localization (B, D). Injection of increased number of MPs does not change PCNA relative expression (C, D). Original magnification, 4x. IgG controls (not shown) were negative.
# 6. Preparation, *In Vitro* and *In Vivo* Characterization of Human Serum Albumin Microspheres (HAMs)

## 6.1. Introduction

Drug delivery systems that range from the nano to macro scale offer effective methods in the controlled and sustained release of a therapeutic agent by many different routes of administration. The usefulness of these systems includes: the ability to tailor the degradation of the carrier matrix, to adjust the release rate of the drug, to improve the circulatory half-life, and to target the drug to the site of action. However, there are a few limitations put on the carrier matrix. The matrix must be non-toxic, be non-immunogenic (unless that would enhance the properties of the drug delivery system), and be able to incorporate which may be hydrophobic or hydrophilic, through either the drug. physicochemical entrapment or chemical attachment. Targeting the drug to the site of action may be accomplished by active targeting moities, such as cell penetrating peptides (e.g., DV3 or TAT), tumor targeting peptides (e.g., RGD, BH3 or LHRH) or specific cell targeting moities (e.g., mannose and N-Formyl-Methionyl-Leucyl-Phenylalanine[fMLF] target macrophages), as well as by antibodies or through passive entrapment due to size. Previously, our group has studied the effective use of polystyrene microparticle (MP) entrapment in the capillary bed of the lung for the treatment of cancer and has systematically studied the effect of uniformly sized MPs on entrapment efficiency to the lung and the toxicodynamic changes to pulmonary gas exchange that occur at higher

MP burdens. However, in order to make a commercially viable drug delivery system, a biodegradable carrier matrix would definitely be preferable, particularly at higher MP burdens.

To form this biodegradable carrier, human serum albumin (HSA) was chosen as the polymer matrix basis for the formation of a parenterally administered, passive lung targeted drug delivery system. HSA makes up approximately one half of the blood serum protein (Meloun et al., 1975). HSA was used as a non-toxic and biodegradable polymer matrix throughout these studies. HSA is a globular unglycosylated serum protein consisting of 585 amino acids with a molecular weight of 66472.02. HSA contains 59 lysine, 24 arginine, 35 cystine and 16 histidine residues.

In general, proteins consist of a highly complex interplay of primary, secondary and tertiary structures. However, after undergoing the physical processing such as that necessary to create the drug delivery system described in this chapter, the protein will no long retain its tertiary structure and possibly lose much of its secondary structure. In fact, thermal processing has been found to also change the primary structure of amino acids. The effect of temperature on amino acids and their subsequent cross-linking has been well studied. Lysinoalanine, ornithoalanine, histidinylalanine and lanthionine have all be found to exist after high temperature processing of natural products. As can be seen in Figure 6.1, dehydroalanine (DHA) forms by the  $\beta$ -elimination of oxidized cystine and / or cysteine. Lysinoalanine is formed when the  $\epsilon$ -lysino sidechain reacts with the dehydroalanine via a Michael addition (Damodaran, 2008). The effect of

manufacture processing temperature on the formation of lysinoalanine was studied using amino acid analysis, differential scanning calorimetry and degradation of material using a 0.25% trypsin solution.

The use of a particles (e.g., human serum albumin microspheres (HAMs) or macroaggregated albumin (MAAs)) made from albumin to study regional blood perfusion has been used since the early 1960s by several different techniques including water/oil emulsion, spray-drying, solvent coaccervation, or pH coaccervation. Both HAMs and MAAs are physically and chemically stable, amenable to large-scale production, are metabolized and guickly cleared from the lung (Chilton and Ball, 1989) and have been used in humans with few adverse events reported (Dworkin et al., 1966). HAMs have been studied as carrier systems for a number of drugs (e.g., adriamycin, (Gupta et al., 1989; Willmott et al., 1985a; Willmott et al., 1985b) mitoxantrone, (Bozdag et al., 2004) mitomycin C, (Fujimoto et al., 1983; Fujimoto et al., 1985; Morimoto and Fujimoto, 1985) 5-fluorouacil, (Sugibayashi et al., 1979) doxorubicin, (Chen et al., 1987; Cummings et al., 1991; Jones et al., 1989) vancomycin, (Nettey et al., 2007; Nettey et al., 2006) ciprofloxacin, (Li et al., 2001; Pavanetto et al., 1994) clarithromycin, (Ozkan et al., 2000a; Ozkan et al., 2000b), prednisolone, dexamethasone, (Pavanetto (Burgess et al., 1987) et al., 1994), progesterone, (Lee et al., 1981) tartrazine, (Rubino et al., 1993) amiloride hydrochloride, (Rubino et al., 1993) chlorothiazide, (Katti and Krishnamurti, 1999) bupivacaine, (Bernardo et al., 2000; Blanco et al., 1999) gentamicin sulphate, (Haswani et al., 2006) terbutaline sulfate, (Sahin et al., 2002) and a potent peptidic inhibitor of human leukocyte elastase (Martodam et al., 1979)). These formulations have been used to treat multiple diseases (e.g., cancer, bacterial infections, asthma, tuberculosis, emphysema).

Despite the widespread study of HAMs as drug carriers, there was a clear need to develop HAMs with a specific size and narrow size distribution that can primarily target the pulmonary capillary circulation. In addition, it was important to understand how the manufacturing processes that could produce HAMs with varying degradation and drug release properties.

This chapter provides a detailed description of the formation of HAMs and in-depth characterization into the process by which HAMs are formed and degrade in both the *in vitro* and the *in vivo* setting. Fractional factorial designs were used to determine which manufacturing parameters were the most important in controlling size for optimal pulmonary capillary entrapment and *in vitro* and *in vivo* degradation characteristics.

## 6.2. Materials and Methods

#### 6.2.1. Materials

Human serum albumin (HSA) was purchased through three separate vendors LifeBlood Medical, Inc. (Adelphia, NJ), Gemini Bioproducts (West Sacramento, CA), and Sigma Chemicals (St. Louis, MO). Indocyanine green (ICG), a near-IR dye was purchased from Sigma Chemicals (St. Louis, MO). Dexamethasone Sodium Phosphate (DSP) was purchased from Spectrum Chemicals and Manufacturing Corporation (New Brunswick, NJ). Several grades of mineral oil were generously donated by Calumet Specialty Products Partners (Karns City, PA). Cottonseed oil was purchased from MP Biomedicals (Solon, OH). Nylon net filters (11 μm) and glass filtration setup were purchased from Millipore Corporation (Billerica, MA). A Barnant Company (Barrington, IL) microprocessor controlled high speed-high sheer overhead stirrer and stirrer paddles were purchased from Fisher Scientific (Waltham, MA). Glassware was purchased through Ace Glass (Vineland, NJ). Digital temperature controller was purchased through J-KEM Scientific, Inc. (St. Louis, MO). All remaining material was purchased through Fisher Scientific or VWR.

#### 6.2.2. Animals

Male C57BL mice were purchased from Harlan Animal Lab (Indianapolis, IN) and were housed in standard mouse cages. Female SKH1 hairless mice were purchased from Charles River Laboratories (Wilmington, MA) and were housed in sterile microisolation cages for *in vivo* imaging studies. Mice were fed a standard mouse diet, had free access to water and were housed in a room with a 12 h light-dark cycle for at least one week before the study. All animal studies were performed under approved protocols from the Use and Care of Animal Committee at Rutgers University in AAALAC accredited facilities.

#### 6.2.3. Methods

## 6.2.3.1. Experimental Design of HAM Formation

HAMs were formed using a water in oil (w/o) emulsion followed by high temperature cross-linking. Due to the complexity of this process, a number of variables were determined to play an important role in the formation and size distribution of HAMs. Variables studied included: water phase composition (water:oil ratio, protein concentration in water phase); reactor setup conditions (location of needle, oil temperature upon initial droplet formation, impeller size); emulsion formation conditions (impeller speed, rate of water phase addition); and cross-linking conditions (temperature of final oil bath, length of processing time). In order to quickly determine which variables were the most important in understanding the size dependency of MP formation and cross-linking, two fractional factorial designs were performed. Both a  $2^{7-4}$  and  $2^{4-1}$  fractional factorial design were created to optimize the formation of HAMs (Box et al., 2005). The factors that were studied to understand their effect on HAM size were as follows: impeller diameter, impeller speed, PBS volume, protein concentration, location of needle during addition, rate of addition, and starting temperature (Table 6.1). The factors that were studied to understand their effect on HAM cross-linking were: processing time, processing temperature and protein concentration (Table 6.2). Both of these fractional factorial designs are resolution III indicating that the main effects are not confounded with one another but are potentially confounded with two-factor interactions.

# 6.2.3.2 HAM Formation Methodology

## 6.2.3.2.1. HAM Formation Methodology for *in vitro* analysis

HAMs were made by a water-oil emulsion technique. HSA was solubilized in phosphate buffered saline (PBS) and transferred to a 5 mL syringe. Cottonseed oil containing 0.25% v/v Span 85 was heated and maintained at 30 °C prior to the start of water phase addition. Using a syringe pump (Harvard Apparatus, Holliston, MA), mini-bore extension tubing and 12" 20G needle, the HSA solution was added drop wise to the stirred oil phase. The emulsion underwent constant stirring (750 RPM) for 15 min and was subsequently heated to processing temperatures (75 - 180 °C) for a fixed duration (0 - 80) min. The oil phase was continuously stirred throughout the process. The emulsion was allowed to cool by air to 75 °C and was filtered through an 11  $\mu$ m nylon net filter using vacuum filtration followed by washing in hexane. Dried HAMs were then weighed and placed in a -20 °C freezer until size analysis was performed. HAMs were analyzed for particle size distribution by a Beckman Coulter Multisizer<sup>TM</sup> 3 Coulter Counter, SEM and / or light microscopy.

## 6.2.3.2.2. HAM Formation Methodology for *in vivo* analysis

HAMs were made by a water-oil emulsion technique as described above. DSP (10% or 20% w/w HSA) or ICG (0.1% w/w HSA) was added to 375  $\mu$ L reagent ethanol, 125  $\mu$ L methanol and 2 mL double distilled (DDI) water and solubilized by vortexing and probe sonication. HSA (10% w/v) was solubilized in the above solution and transferred to a 5 mL syringe. Cottonseed oil containing 0.25% v/v Span 85 was heated and maintained at 30 °C prior to the start of water phase addition.

# 6.2.3.3. Particle Sizing

## 6.2.3.3.1. Use of Coulter Counter

HAMs were resuspended in ISOTON II solution and were briefly sonicated using a probe sonicator. After sonication, a small amount of this suspension was added to 20 mL of ISOTON II solution and HAMs were analyzed for particle size distribution by a Beckman Coulter Multisizer<sup>™</sup> 3 Coulter Counter.

# 6.2.3.3.2. Use of LS13320 Laser Diffraction Particle Size Analyzer

HAMs were resuspended in 0.1% Tween 80 in PBS solution and were briefly sonicated using a probe sonicator. After sonication, a small amount of this suspension was added to sample chamber of the Beckman Coulter LS13320 Particle Size Analyzer until the obscuration reached an acceptable level.

# 6.2.3.4. Scanning Electron Microscopy (SEM)

MPs were washed twice with hexane and dried using a CentriVap concentrator (Labconco Corp., Kansas City, MO) for 30 min. The MPs were fixed on aluminum stubs with conductive tape, and coated with gold-palladium for

2 min at 30 mA and 5x10<sup>-2</sup> mbar in an argon atmosphere, using a Balzer SCD 004 Sputter Coater. The MP coated stubs were then examined using a SEM (AMRAY 1830 I) with an EDX 9800 X-ray system and a Robinson backscatter detector.

# 6.2.3.5. Thermogravimetric Analysis (TGA)

Thermogravimetric analysis was performed on a Perkin-Elmer (Waltham, MA) system consisting of a TGA 7 analyzer with TAC 7/DX instrument controller. Perkin-Elmer Pyris software was used for data collection on a Dell OptiPlex GX110 computer. Samples (10 mg) were heated under dry nitrogen gas at a heating rate of 10 °C/min. Decomposition temperature was defined as the onset of decomposition.

# 6.2.3.6. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry measurements were performed on a Q200 DSC (TA Instruments, New Castle, DE). HAMs were weighed to 3 - 5 mg and hermetically sealed in aluminum pans. During each run, the cell temperatures were increased from -10 °C to 260 °C at a rate of 5 °C/min. Data was analyzed using TA Instruments Universal Analysis 2000 software v4.3A. Raw data was smoothed using the "Smooth function 3 points" and analyzed for local minimums using the "Signal Maximum" function.

## 6.2.3.7. *In vitro* stability in 0.25% trypsin using Turbidity

HAMs were resuspended in 0.1% Tween 80 in PBS (pH = 7.4) and were briefly sonicated using a probe sonicator. Freshly sonicated HAMs were then added to 0.25% trypsin at 37 °C using a water bath circulator and constantly stirred. The turbidity of the solution was monitored every 5 min at  $\lambda_{abs}$  = 650nm for 24 h using a Hitachi U-3010 Spectrophotometer (Hitachi High Technologies America, Inc., Pleasanton, CA). Absorbance was normalized using the first measurement after HAM addition to account for any difference in turbidity due to more HAMs being initially present. For the duration of the study, the spectrophotometer was zeroed before each measurement using a cuvette filled with the same material found in the blank (PBS containing 0.1% Tween 80 and 0.25% trypsin). A cap was applied to the cuvette and sealed with parafilm to limit evaporation. The initial concentration of MPs was 0.5 mg/mL in 3 mL quartz cuvettes.

## 6.2.3.8. Amino Acid Analysis

Amino acid analysis was performed by Ansynth Service B.V. using a Protein Hydrolysate System (Biochrom Ltd., Cambridge, UK). Ansynth Service has a well developed method for detecting lysinoalanine using sodium citrate buffer. Ansynth also attempted to detect lanthionine with a standard supplied by Sigma Aldrich.

## 6.2.3.9. Dexamethasone Sodium Phosphate HPLC Assay

The high-performance liquid chromatography (HPLC) system consisted of a Waters model 510 pump, a Waters 717 Autosampler, and a Waters 486 fixed wavelength UV detector (Waters Associates, Milford, MA). Separations were achieved using a Waters Symmetry® C18 column (150 × 4.6 mm, 5  $\mu$ m). The isocratic mobile phase consists of 25% (v/v) acetonitrile in water containing 50 mM ion-pairing agent (ammonium acetate) with the aqueous phase adjusted to a pH of 4.5 using glacial acetic acid. The mobile phase was degassed by 20 mL/min helium sparge. The flow rate of the mobile phase was 1.4 mL/min. The retention time of DSP is 4.77 min. The detection wavelength was  $\lambda_{abs} = 254$  nm.

Three separate DSP standards were made to create the standard curve (Figure 6.14). The linearity ( $R^2$ ) of the individual standards was 0.9999 - 1.0 and the overall linearity of the three standards was 0.9996. The lower limit of quantitation was found to be 100 ng/mL. The upper limit of quantitation was found to be 250 µg/mL.

# 6.2.3.10. DSP Release from HAMs

Microspheres (10 mg) were weighed into microfuge tubes and 1 mL of 37 °C PBS was added. The microfuge tubes were agitated in an incubator maintained at 37 °C. At sampling time points (5 min, 30 min, 1 h, 3 h, 6 h, 9 h, 24 h, 48 h, 96 h, 192 h, 240 h, 336 h) microfuge tubes were centrifuged and 50  $\mu$ L aliquots were taken of the supernatant without replacement. Aliquots were then frozen at -20 °C until being analyzed using HPLC.

# 6.2.3.11. *In vivo* imaging

Optical *in vivo* fluorescence imaging was performed with either an IVIS® Imaging System 100 Series small animal imaging system (Caliper Life Sciences, Hopkinton, MA) or In-Vivo Multispectral (MS) FX Pro (Carestream Molecular Imaging/Carestream Health, Woodbride, CT). Animals were anesthetized for imaging by 1.5% isoflurane in 100% O<sub>2</sub> using an EZ-3500 Multi-Animal Anesthesia System (Euthanex Corp., Palmer, PA).

# 6.3.11.1. Caliper Life Sciences IVIS

An ICG excitation filter (710 - 760 nm) and an ICG emission filter (810 - 875 nm) were used for acquiring the fluorescence images. Identical illumination settings, such as exposure time (5 s), binning factor (4), f-stop (2), and fields of view (25 x 25 cm (whole body)), were used for acquiring all images. Fluorescent and photographic images were acquired and overlaid. The pseudocolor image represents the spatial distribution of photon counts within the organs. Background fluorescence was measured and subtracted by setting up a background measurement. Images were acquired and analyzed using Living Image 2.5 software (Caliper Life Sciences, Hopkinton, MA).

## 6.2.3.11.2. Carestream MS FX Pro

The camera settings used for fluorescence and reflectance imaging were: (fluorescence) 60 secs, 2x2 bin, 0.95 f-stop, 135 mm fov, Ex/Em: 760 nm/830 nm; and (reflectance) 0.175 secs, no bin, 0.95 f-stop, 135 mm fov, Ex/Em: 600/0, 540/0, 440/0 (three images combined to yield full color image).

# 6.2.3.12. Biodistribution of HAMs using an IVIS

HAMs containing ICG (0.1% w/w HSA) produced by methods stated above were weighed to yield a 0.4 mg dose of MPs and resuspended by sonication in 100  $\mu$ L sterile PBS containing 0.1% Tween 80.

Female SKH1 hairless mice weighing  $20 \pm 5$  g were used for *in vivo* imaging studies and fed a standard mouse diet. HAMs were administered by tail vein injection. Animals were euthanized at 18 h and the lung was removed for histology. Imaging time points were as follows: No injection, every 10 min for 90 min. Mice were euthanized by pentobarbitol overdose followed by exsanguination.

## 6.2.3.13. Lung Distribution of HAMs by Histology

HAMs (10% w/w HSA) produced by methods stated above were weighed to yield a 0.4 mg dose of MPs and resuspended by sonication in 100  $\mu$ L sterile

PBS containing 0.1% Tween 80. An internal standard of 15  $\mu$ m black NEN-TRAC MPs were added to the HAMs prior to injection.

Groups of three male C57BL mice weighing  $21 \pm 3$  g were used for *in vivo* degradation studies and fed a standard diet. Three animals were euthanized at each time point and the lung was removed for histological observation. Time points were as follows: 1 h, 6 h, 12 h, 24 h, 36 h, 48 h, 96 h and 120 h. Mice were euthanized by pentobarbitol overdose followed by exsanguination.

Lungs were fixed in ice cold 10% neutral buffered formalin, embedded in parrafin and sectioned into 6  $\mu$ m slices. Slides were stained using hematoxylin and safranin. HAMs were counted for lung retention.

# 6.2.3.14. Fluorescent Microscopy of Lung Sections Containing HAMs

The presence of ICG loaded HAMs in the lung was further confirmed by fluorescent microscopy using a Leica TCS SP2 spectral confocal microscope. Unstained sections were imaged using the 488 or 633 nm excitation laser and the emission spectrum window was 504.2 - 556.4nm or 721.3 - 797.1. The images were co-localized and can be seen in Figures 6.21 - 6.23.

# 6.3. Results and Discussion

## 6.3.1. HAMs preparation

#### 6.3.1.1. Experimental design

MPs have been manufactured using various materials including natural proteins and synthetic polymers by several techniques including spray drying or emulsions. One method readily available to our lab was an overhead stirrer emulsion technique. There are a number of factors that influence the initial droplet formation and steady state size distribution of an emulsion. Arshady proposed the following equation (**6.1**) as a method for understanding and controlling particle size due to changes in processing conditions (Arshady, 1990).

$$\overline{d} \propto K \frac{D_{v} R \nu_{d} \gamma}{D_{s} N \nu_{m} C_{s}}$$
(6.1)

where  $\overline{d}$  is the average particle diameter; K is a factor dependent on apparatus setup; D<sub>v</sub> is the diameter of the vessel; R is the volume ratio between the dispersed phase and the continuous phase; v<sub>d</sub> is the kinematic viscosity of the dispersed phase;  $\gamma$  is the surface tension between the two immiscible phases; D<sub>s</sub> is the diameter of the stirrer; N is the stirring speed; v<sub>m</sub> is the kinematic viscosity of the continuous phase; and C<sub>s</sub> is the stabilizer concentration.

Arshady's equation suggests that increasing viscosity of the dispersed medium (water phase) will result in increased particle size. However, Morris et al. created nanoparticles of magnetic albumin whose size increased with decreasing albumin concentration (Arshady, 1990; Morris et al., 1984). In addition, Figures 6.2 and 6.6 give conflicting results showing that increasing protein concentration results in a decrease in particle size during the fractional factorial design performed in cottonseed oil but an increase in particle size with protein concentration when using mineral oil. Moreover, Arshady's equation includes the use of a factor (K) dependent upon glassware used and other conditions inherent in the individual system, suggesting that this equation may not be reproducible between labs. Nevertheless, understanding which factors are important in controlling particle size was an important goal of this project, and was studied through the use of both fractional factorial design, and variation of conditions based on Arshady's findings, namely oil viscosity and surfactant concentration.

The use of a fractional factorial experimental design was able to quickly determine which variables were the most important in effecting the mean particle size of the HAMs (Table 6.2, Figure 6.2). As can be seen in Figure 6.2, impeller speed, PBS volume and needle position played an important role in determining particle size. Each of these 3 factors created a >15  $\mu$ m change in the mean particle size. Surprisingly, the diameter of the stirrer, which is intrinsically related to the impeller tip speed velocity and thus similar to changing RPM, played a negligible role in determining microsphere size. In addition, starting temperature appears to play an inverse role in particle size. This was most likely due to the fact that during original conditions, the heating was started immediately upon the dispersion of the entire HSA solution into the oil phase, thereby not allowing

enough time for the impeller to shear particles to their final size. Denaturation of HSA in solution occurs at ~58.5 °C (Hayashi et al., 1989). Above that temperature, the size of the HAMs has been fixed and further heating results in evaporation of water and cross-linking of HSA. Therefore the increased time to reach 58.5 °C from 30 °C instead of 50 °C enables the HSA solution droplets to be reduced to smaller sizes. However, the rate of addition of the HSA solution to the oil phase played a minimal role considering that the "+" rate (1000  $\mu$ L/min) is 4 fold faster than the "-" rate (250  $\mu$ L/min) and therefore took anywhere from 6 to 12 min longer to add completely to the oil phase.

After understanding the variables that appeared to play the greatest role in particle formation, the second fractional factorial design was utilized to determine the effect of protein concentration and processing time and processing temperature on the stability of the HAMs as determined by differential scanning calorimetry (DSC). Processing time was investigated to determine if small changes in processing time (40  $\pm$  5 min) would change the stability of the particles as had been mentioned by Yapel (Yapel, 1979, 1985b). In addition, because protein concentration may also play a role in the cross-linking process and be detectable by changes in the endotherm temperature.

As can be seen in Table 6.2, protein concentration was the most influential in changing endotherm temperature followed by processing temperature and lastly processing time. This suggests that the largest impact on degradation of material will be due to protein concentration and processing temperature. Because the endotherm temperature varied only slightly while changing processing time, small variations (± 5 min) in processing time were considered to have little effect on cross-linking.

Upon the conclusion of these studies, additional work was performed to determine if there were any correlations between protein concentration, processing temperature and the output of endotherm temperature (Figures 6.10 and 6.11). The increase of protein concentration from 10 - 40%, at either 120 °C or 150 °C, increases the endotherm temperature in a linear fashion (Figure 6.11). In addition, increasing the processing temperature from 120 °C to 150 °C increases the endotherm temperature. These two conclusions suggest that degradation of material can be controlled by processing temperature and / or protein concentration.

However, although processing temperature plays little role in effecting mean particle size, protein concentration does play a significant, non-linear role in controlling particle size when using mineral oil (Figure 6.6). This is most likely due to the increase in material per droplet as this material was stirred for 15 min prior to heating, suggesting that all particle droplet formation had already occurred.

While viscosity of the oil phase plays a critical role in particle size, the choice of oil phase is predominately dependent upon the solubility of the drug to be incorporated in the microsphere. As the drug to be studied could change based upon future results, two different types of oil phases were studied: cottonseed oil and mineral oil. Mineral oil is produced in a wide array of viscosities and densities and the effect of these parameters on particle size was

studied and compared to cottonseed oil. The viscosity of cottonseed oil, Drakeol 19 and Drakeol 35 at 40 °C is 35, 36 and 68 cST, respectively. The density of cottonseed oil, Drakeol 19 and Drakeol 35 at 25 °C is ~0.92, 0.86, and 0.87 g/mL, respectively. The In addition, multiple surfactants and surfactant concentrations were studied to develop the best water/oil (w/o) emulsion. A standard method of determining the best surfactant for use in a stable w/o emulsion is based on the hydrophilic-lipophilic balance (HLB) scale. The smaller the number on the HLB scale, the better the surfactant is able to mix with a lipophilic base. The overall HLB scale number of a solution is able to changed by varying the ratio of two or more surfactants. The recommended HLB scale number to make stable w/o emulsions for cottonseed oil and mineral oil are readily available.

The use of surfactants to create w/o emulsions in cottonseed oil show that varying both the number on the HLB scale number (i.e., type of surfactant) and surfactant concentration have little or no effect on particle size. However, for mineral oil, surfactant selection and concentration do play an important role in initial w/o emulsions and subsequent particle size. Increasing either surfactant concentration or the HLB value is able to decrease particle size.

Finally, increasing the oil viscosity resulted in decreasing the particle size. In this case, Arshady's equation was able to correctly predict the direction of particle size change, but not necessarily the magnitude of the change.

### 6.3.1.2. Particle size and morphology

HAMs prepared were spherical in shape and appear by scanning electron microscopy (SEM) to have a smooth surface without any visible pores when in a dry state. In addition, HAMs that were fractured appeared to have a monolithic matrix as opposed to a core and shell fabrication.

#### 6.3.2. HAMs matrix modification

As previously mentioned, HAMs were characterized in terms of endotherm temperature to determine the degree of cross-linking occurring due to various processing conditions. Further studies increased the understanding of endotherm temperature by processing HAMs from 75 – 180 °C. These studies showed endotherm temperatures increased in a non-linear fashion with respect to processing temperature. The next step was to understand how processing temperature changed endotherm temperature. Thermal processing of proteins result in the cross-linking of amino acid side chains to create non-natural amino acids (Carr and Lewis, 1993; Damodaran, 2008; Friedman, 1999; Kuboki et al., 1984; Taylor and Wang, 2007). The most commonly formed amino acid is called lysinoalanine (LAL) and is a result of the  $\varepsilon$ -amino side chain of lysine reacting with a reactive species (dehydroalanine, DHA) formed from cystine, serine, and other amino acids. LAL has been shown to be toxic to the rat, however not to the mouse (Sternberg et al., 1975).

During the creation of HAMs, the side chains of both lysine and arginine are subjected to reaction with DHA forming lysinoalanine and ornithoalanine, respectively. Ornithine is formed upon the heating of arginine. Poly-L-ornithine and di- and tri-ornithine have been found to be resistant to pepsin, carboxypeptidase, and papain as well as trypsin, an enzyme that cleaves the carboxyl side of peptides following lysine and arginine when not followed by proline (Erlanger, 1957). Therefore, the use of a trypsin solution to digest HAMs would at first glance be problematic since, assuming complete conversion of lysine and arginine to lysinoalanine and ornithoalanine, digestion should not occur. However,  $\alpha$ -chymotrypsin, a common contaminant found in tryspin solutions, will digest peptides at the carboxyl side of tryptophan, tyrosine, phenylalanine, leucine, and methionine residues which are unaffected by thermal processing.

In the current study, lysinoalanine was produced during the manufacturing process of HAMs. The amount of LAL led to a decrease in the effectiveness of trypsin to digest the HAMs. In addition, the use of pure trypsin (2500:50, trypsin:chymotrypsin) versus cell-culture grade trypsin (250:75,trypsin:chymotrypsin) was found to result in a decrease in the effectiveness of trypsin to digest the HAMs, further supporting the formation of lysinoalanine and / or ornithoalanine after thermal processing. As can be seen in Figure 6.12, the formation of LAL is linear with respect to processing temperature suggesting that increases in endotherm temperature have a direct correlation with increases in LAL formation. These results correlate well with several studies showing that LAL formation is merely due to the heat (and time of processing) but not to environmental conditions surrounding the reaction (Sternberg et al., 1975). The

resulting formation of LAL and other non-natural amino acids also has an additional consequence in terms of the ability for natural enzymes to degrade microspheres containing these non-natural formations and the abundance of side chains for future chemical attachment or the potentially unwanted reaction between drug and matrix during cross-linking.

#### 6.3.3. Degradation of HAMs in vitro

There is a clear effect of thermal processing on the degradation of HAMs in a 0.25% trypsin solution (Figure 6.7). This correlates well with the endotherm temperature showing that HAMs processed at <135 °C degrade quickly in 0.25% trypsin solution and have similar endotherm temperatures. Increasing the processing temperature clearly slows the degradation of HAMs in 0.25% trypsin, correlating to increased lysinoalanine content (Figure 6.12).

In addition, increasing the protein concentration from 10 to 40% w/w had little effect on trypsin degradation of HAMs compared to the effect of processing temperature (Figure 6.8). Because the degradation rate of HAMs by trypsin was more affected by processing temperature, the clearance of HAMs from the lung based on changes in processing temperature was studied *in vivo* (Section 6.3.5.).

# 6.3.4. Release of dexamethasone sodium phosphate in vitro

Dexamethasone sodium phosphate (DSP) is the phosphate ester prodrug of dexamethasone, a potent corticosteroid. DSP is freely soluble in water and was chosen as a model compound for release by HAMs for its potential usefulness in the treatment of chronic asthma. The molecular weight of DSP is 516.41. As can be seen in Figure 6.15, there was an initial burst of DSP followed by a slight increase in DSP by 336 h, although not statistically significant. As well, the increase at 10 and 24 h is not statistically different from the initial burst for all 3 groups. The most likely reason for this burst and non-sustained release was that albumin matrix swells rapidly and does not limit diffusion of water into and subsequent release of DSP out of the HAMs. In addition, DSP may not be well incorporated within the HAMs or only surface bound and due to its high water solubility was easily dissolved into the release media. Therefore, the incorporation of a less water-soluble form of DSP, such as dexamethasone, may limit the immediate release. However, more hydrophobic molecules may either partition into the non-polar, hydrophobic oil phase, become difficult to incorporate into the polar, hydrophilic phase of the emulsion, and / or not be able to redissolve due to crystallization following the thermal cycling necessary to make the HAMs.

The amount of DSP released was dependent upon the processing conditions. Incorporation of increased DSP resulted in increased total release, but did but the release rate did not change, indicating that release was most likely not diffusion limited. HAMs produced at a higher temperature (150 °C vs. 120 °C) released a lower amount of DSP. Sugibayashi et al. demonstrated that thermal cross-linking decreases the total amount of 5-fluorouracil (5-FU, molecular weight 130.08) released from albumin nanocapsules (Sugibayashi et al.

al., 1979). In addition, these nanocapsules showed a burst release that occurred over 1 day, followed by a sustained but slow release until day 7.

The burst release followed by slow release has been shown in ovalbumin MPs that encapsulated muramyl dipeptide. These MPs were made by chemical cross-linking and showed a slight burst release followed by slow release until 48 days, followed by a change in release rate dependent upon processing conditions (e.g., pH and glutaraldehyde concentration) (Puri et al., 2000). However, based on previous literature reports of relatively fast degradation of HAMs from lungs, this secondary release would not be achieved (Yapel, 1985b). Therefore, due to the burst release of DSP from the HAMs, without a secondary increase in release over the given time period, DSP was not considered a viable option for study in a mouse model of asthma.

## 6.3.5. Biodistribution and degradation of HAMs in vivo

## 6.3.5.1. In vivo imaging

In this section, the effect of HAM processing temperature on *in vivo* biodegradation and biodistribution in mice is presented. Initially, ICG was chosen as a suitable dye to facilitate the *in vivo* studies using an *in vivo* imaging system. However, this dye also proved to be a good method of detection *in vitro*. ICG has an excitation and emission in the near-infrared (NIR) wavelength range. The advantage of using NIR dyes is the reduction in autofluorescence caused by biological samples. The disadvantage of the NIR dyes is reduced quantum efficiency. As can be seen in Figure 6.21, HAMs without ICG fluoresce when

excited with 488 nm light, but not when excited with 633 nm light, indicating that the protein itself is fluorescent. Addition of ICG to similar HAMs (Figure 6.22) results in emission at the NIR wavelength filter (721.3 - 797.1 nm). Excitation at the lower (488 nm) wavelength also resulted in NIR emission. Finally, these ICG loaded HAMs were injected into SKH1 mice, and their presence in the lung was confirmed (Figure 6.23).

The *in vivo* biodistribution of fluorescently labeled HAMs administered by tail vein injection to SKH1 mice, was measured using an IVIS® 100 system. Independent of processing temperature, the biodistribution pattern was similar. Initially, HAMs appeared to be entrapped in the lung; however within 90 min the signal appeared to reach the abdomen. ICG was only passively entrapped within the HAMs and therefore is able to release from the HAMs when put in media such as serum. The immediate release of DSP (Figure 6.15) would suggest that ICG, which is less water soluble than DSP, would have an initial burst of dye followed by negligible release. Once released, ICG is profoundly bound to protein in serum, confounding subsequent dye detection and localization *in vivo*. ICG is eliminated in the liver and subsequently excreted through the bile duct into the intestine.

In a single animal injected with ICG loaded HAMs followed by euthanization at 30 min, organs were examined for fluorescence using the CareStream MS FX Pro *in vivo* imager (Figures 6.17-18). The X-ray co-localized images suggest that fluorescence is not in the lung. However, upon *ex vivo* examination, the lung, liver and intestine fluoresce. There were distinct zones of the intestine that were fluorescent, indicating the location of bile duct emptying and gastric motility over the course of 30 min. This is the likely explanation for the movement of fluorescent signal seen in Figure 6.16. However, because no obvious fluorescent signal was detected in the lungs of the intact mice, a more invasive method was used to determine the clearance of HAMs in the lung over time.

## 6.3.5.2. Lung clearance of HAMs

One of the global aims of this thesis was to develop HAMs that could remain in the lung for a controlled period of up to one week. C57BL/6 mice were used to study the lung clearance of HAMs because many disease models are based upon this strain. Mice were administered HAMs by tail vein injection and euthanized at specific time points. The time points chosen were based on previous reports that HAMs produced at 135 °C last only for 30 - 36 h in mouse lung (Yapel, 1979, 1985b). Only the lung was studied because: 1) HAMs were a sufficient size to be entrapped in the capillary bed; 2) HAMs were not radiolabeled making gross tissue detection difficult and / or non-sensitive; 3) the lung architecture allowed for easy detection of spherical protein MPs through common histological techniques.

The lungs were sent for histology and the total number of HAMs found in a single lobe of the lung was counted in duplicate (i.e., successive sections) for 3 animals. A representative picture of a control animal and one receiving HAMs can be seen in Figure 6.20. The number of HAMs found was plotted on a log-

linear plot (Figure 6.19) and the pharmacokinetic profile of these HAMs was determined using a one-compartment model with a  $1/SD^2$  weighting to determine the half-life of the HAMs. Increased processing temperature (120 °C vs. 150 °C) resulted in an increased half-life ( $t_{\frac{1}{2} [120 \circ C]} = 7.116$  h vs.  $t_{\frac{1}{2} [150 \circ C]} = 38.67$  h), thus prolonging the localized residence and potential for drug delivery.

There were several difficulties encountered in counting HAMs within the lung tissue. First, because HAMs are spherical, a slice through the center would result in the ability to measure the diameter, however a slice through the end may result in only a very small circular piece of HAM remaining. Further adding to this problem is that the size distribution of the HAMs is not uniform. Finally, because the HAMs are made of protein, detection by staining for protein may result in non-specific staining. Therefore, the MPs were counted by eye and every effort was made to differentiate between MP and non-specific staining. In addition, to confirm the location and staining of the MPs for histology, a fluorescent confocal microscope was used to confirm the presence of MPs.

Of particular note, as can be seen in Figure 6.1719, HAMs manufactured at 150 °C for 40 min were still present in the mouse lung after 168 h clearly demonstrating that HAMs with proper engineering can be made to degrade within one week in a controlled fashion in the mouse lung. These results achieve the aim of this portion of the project.

# 6.4. Conclusions

HAMs of a sufficient size (>10  $\mu$ m) to be entrapped in the pulmonary capillary bed were made using a w/o emulsion technique with thermal crosslinking. HAMs were found to be spherical and have a smooth surface by SEM. Cross-linking was found to increase in a non-linear fashion using both differential scanning calorimetry (DSC) and a linear fashion using amino acid analysis for lysinoalanine (LAL). In addition, degradation of HAMs using 0.25% trypsin was used to predict the degradation of HAMs *in vivo*.

Furthermore, HAMs have been shown to be biodegradable and biocompatible as can be seen by histological sections. Increasing the degree of cross-linking by temperature increased the residence time of the HAMs in the lungs. Therefore, the residence time of the drug delivery system (HAMs) in the lung can be tailored to deliver an optimal amount of drug over a specific amount of time.

Output											
Starting Temperature	I	+	+	ı	+	ı	ı	+		50 °C	30 °C
Rate of Addition	+	+	ı	ı	ı	ı	+	+		1000 µL/min	250 μL/min
Needle Location	+	I	+	I	I	+	I	+		In	Out
Protein Concentration	+	I	I	+	+	I	I	+		25% w/w	10% w/w
PBS Volume	I	I	I	I	+	+	+	+		2 mL	1 mL
Impeller Speed	I	ı	+	+	ı	ı	+	+	meters Used	1250	750
Impeller Diameter	I	+	I	+	I	+	I	+	imental Paraı	2"	1.5"
Run #	~	2	3	4	5	9	7	8	Exper	+	I

Table 6.1. 2<sup>7-4</sup> III Fractional Factorial Design

#u	Processing Time	Processing Temperature	Protein Concentration	Endotherm Temperature ( °C)	Mean Particle Size (μm)
		+		187.49	10.5
	+	+	+	197.76	13.2
	ı	Ι	+	191.52	12.3
	+	I	-	185.29	13.6
otherm berature	4.04	8.66	16.5		
icle	4	2.2	1.4		
rimental F	<sup>o</sup> arameters Use	þé			
	80 min	150 °C	25% w/w		
	0 min	120 °C	10% w/w		

Table 6.2. 2<sup>3-1</sup> III Fractional Factorial Design



**Figure 6.1.** Formation of amino acid side chain cross-linking formed by thermal processing.



Factors

**Figure 6.2.** 2<sup>7-1</sup> Fractional Factorial Design . '+' and '-' values are shown in Table 6.2. Impeller speed, PBS volume and needle position were the most important variables in controlling the size of the MPs.



Figure 6.3. Scanning Electron Micrograph of HAMs.



**Figure 6.4.** Representative Particle Size Distribution of HAMs from Multisizer 3 Coulter Counter using a 70  $\mu$ m aperature tube. MPs were produced using mineral oil. The volumetric mean, standard deviation, coefficient of variation and polydispersity ((D<sub>90</sub>-D<sub>10</sub>)/D<sub>50</sub>) are 21.05  $\mu$ m, 6.073  $\mu$ m, 28.8%, 0.71, respectively. The numeric mean, standard deviation, coefficient of variation and polydispersity ((D<sub>90</sub>-D<sub>10</sub>)/D<sub>50</sub>) are 7.363  $\mu$ m, 7.019  $\mu$ m, 95.3%, 4.55, respectively. Based on light microscopy and SEM, the volumetric statistics appear more plausible.



Name	Oil	Surfactant Concentration	HLB	Mean Size (μm)	Poly- dispersity		
HSA#52	Cottonseed	Span 80 0.25% (1.25 mL)	4.3	19.39	1.07		
HSA#53	Cottonseed	Span 80 1% (5 mL)	4.3	21.74	0.97		
HSA#56	Cottonseed	Span 85 0.25% (1.25 mL)	1.8	18.89	0.83		
HSA#64	Drakeol 19	Span 85 0.25% (1.25 mL)	1.8	418.2	1.24		
HSA#66	Drakeol 35	Span 85 0.25% (1.25 mL)	1.8	268.2	1.26		
HSA#71	Drakeol 35	Span 80 1% (5 mL)	4.3	3.853	1.54		
HSA#72	Drakeol 19	Span 80 1% (5 mL)	4.3	6.068	1.95		
Figure 6.5. Effect of Surfactant and Oil Phase on HAM sized using Beckman							

Coulter LS13320. Increased oil viscosity resulted in a decrease in HAM size. Surfactant had little effect on HAM size when using cottonseed oil, but dramatically influenced HAM size using mineral oil.



**Figure 6.6.** Effect of Protein Concentration on the HAM size. Protein concentration increases the mean particle diameter of HAMs in a non-linear fashion.


**Figure 6.7.** Degradation of HAMs (10% HSA) in 0.25% Trypsin due to changes in processing temperature. The rate of degradation is decreases as processing temperature is increased.



**Figure 6.8.** Degradation of HAMs in 0.25% Trpsin due to changes in protein concentration and processing temperature. There is no clear trend to increased protein concentration resulting in changes to degradation time, however, the importance of temperature remains.



**Figure 6.9.** Thermogravimetric Analysis of HSA. The decomposition temperature is 276 °C, limiting the maximal temperature used in the DSC experiments to 260 °C.



**Figure 6.10.** Effect of Protein Concentration and Temperature on DSC Endotherm Temperature. Increased protein concentration (10 – 40 % w/w) and / or increased processing temperature (150 °C vs. 120 °C) increases the second endotherm temperature.



**Figure 6.11.** Effect of Protein Concentration on DSC Endotherm Temperature. The second endotherm peak is increased when protein concentration is increase, suggesting better *in vivo* stability.



**Figure 6.12.** Effect of Processing Temperature on the Formation of Lysinoalanine. The formation of lysinoalanine increases in a predominantly linear fashion as manufacturing temperature is increased.



**Figure 6.13.** Effect of Processing Temperature on DSC Endotherm Temperature. The secondary endotherm temperature increases (seen in Figure 6.8) under higher HAM processing temperatures.



**Figure 6.14.** Dexamethasone sodium phosphate standard curve. DSP was weighed into three separate vials to create stock solutions. Standard curve was created from dilution of stock. The linearity ( $R^2$ ) of the individual standards was 0.9999 - 1.0 and the overall linearity of the three standards was 0.9999. The lower limit of quantitation was found to be 100 ng/mL. The upper limit of quantitation was found to be 250 µg/mL.



**Figure 6.15.** Dexamethasone sodium phosphate release from HAMs. HAMs were loaded with 10% (1 mg) or 20% (2 mg) DSP in a 10% albumin solution. DSP exhibited an initial burst release with negligible release thereafter. This release profile was independent of DSP concentration or processing temperature. Increased processing temperature did result in a reduction of total DSP released.



**Figure 6.16.** Representative *in vivo* imaging of SKH1 mice. Mouse on left injected with 120 °C HAMs and mouse on right injected with 150 °C HAMs. Both mice initially appear to have HAMs trapped in the lung, however the signal is found to quickly move to the abdomen. This is most likely due to the release of ICG, which was passively entrapped in the HAMs, rather than the HAMs quickly avoiding lung entrapment.



Β.

Α.



**Figure 6.17.** *In vivo* imaging of SKH-1 mouse. (a) Untreated (b) 200 µg ICG loaded MPs. (A) immediately following injection. (B) 30 minutes post injection. Tail fluorescence is due to missed injection.



FluorescenceReflectanceFluorescence and ReflectanceFigure 6.18. Ex vivo imaging of SKH-1 mouse.ICG fluorescence is detected inthe lung, liver and intestine.This pattern indicates HAMs are entrapped in thelung, and free ICG is being eliminated in the liver to the intestine.Organs: (a)heart, (b) lung, (c) kidneys, (d) spleen, (e) pancreas, (f) liver, (g) reproductivetract, and (h) intestinal tract (stomach to colon).



**Figure 6.19.** Entire safranin and eosin stained mouse lung sections were scanned and the number of HAMs found over time is shown. A one compartment model of degradation with a  $1/SD^2$  weighting. Degradation of HAMs follows a first order elimination ( $120 \ ^{\circ}C \ R^2 = 0.8198$ ,  $150 \ ^{\circ}C \ R^2 = 0.6666$ ). Higher thermal processing results in longer retention of HAMs in mouse lung ( $120 \ ^{\circ}C \ t_{1/2} = 7.116 \ h, 150 \ ^{\circ}C \ t_{1/2} = 38.67 \ h$ ).





**Figure 6.20** Representative safranin and eosin stained slides of HAMs found in mouse lung tissue. A, C Control animal; B, D HAM injected mouse. HAMs appear uniformly stained and are a different shade compared to red blood cells. HAMs are predominately found in the alveolar septal walls. (A&C, 10x; B&D, 40x)



Excitation with 633nm laser



Upper Left – 504.2-556.4nm emission wavelength Upper Right – 721.3-797.1 emission wavelength Bottom Left – bright field image Bottom Right – co-localized image

Excitation with 633nm laser



Figure 6.22. Confocal Microscopy of ICG encapsulated MPs in vitro (HSA#116)

Excitation with 633nm laser



Figure 6.23. Confocal Microscopy of ICG encapsulated MPs in vivo (HSA#116)

#### 7. Summary and Conclusion

This research is a continuation of work done by our group to advance a localized, passively targeted therapy for the treatment of lung cancer. Systemic administration of therapeutics has often led to dose limiting side effects. Previous work has shown that a localized sustained release therapy is able to reduce cancer burden similar to a 10-fold lower free drug dose. However, further refinement of this system was necessary to make it biodegradable and to determine a safe loading dose of MPs in the lung.

Biodegradable human serum albumin MPs that were a sufficient size to be entrapped in the capillary bed of the lung were developed. These MPs were found to have different *in vitro* degradation characteristics based upon the temperature of manufacture; where increasing the temperature resulted in more cross-linking to occur through lysinoalanine formation and slower material degradation by trypsin over a 24 h period. Subsequently, these large (mean ~20 µm) biodegradable MPs were administered by tail vein injection to young healthy mice and upon histological evaluation, were well distributed throughout the lung. In addition, MPs manufactured at a higher temperature (150 °C vs. 120 °C) were found to remain present in the lung longer with a ~3-fold increase in half-life (34.3 h vs. 10.7 h, respectively). This indicates that the residence time of the MPs in the lung is tailorable based upon the *in vitro* degradation with trypsin, increase in lysinoalanine content and the *in vivo* findings.

After creating these biodegradable MPs, the issue of dose-limiting MP load became paramount. Therefore, rigid, non-degradable polystyrene MPs of unique

sizes (10, 25 and 45  $\mu$ m) that were large enough to become entrapped in the capillary and pre-capillary arteriole following tail vein administration were studied in young healthy mice. These MPs caused blockage of the capillaries resulting in hypoxemia. The degree of hypoxemia was evaluated by using a simple, inexpensive, non-invasive, clinically relevant technique previously developed in humans that calculates ventilation-perfusion ratio (V<sub>A</sub>/Q) and shunt from a sophisticated computer algorithm. The number of MPs required to cause ventilation perfusion mismatch, and the resulting hypoxemia, was dependent upon their size. For each MP size, there is a number of MPs after which there was a marked and rapid drop of V<sub>A</sub>/Q allowing for a clear demarcation of toxicity prior to any clinically significant change to SpO<sub>2</sub> while breathing room air.

In summary, this research shows for the first time in mice, a method of noninvasively determining  $V_A/Q$  and shunt to assess pulmonary gas exchange that would not ordinarily be detectable in a standard room air environment. Previous methods to measure  $V_A/Q$  and shunt have not been feasible in rodents, where the bulk of disease models are tested. In addition, this non-invasive method offers the ability to follow disease progression and evaluate treatments over time in the same animal, and watch for pulmonary toxicity endpoints prior to death or morbidity. Although the computer algorithm was tested using pulmonary embolism, there is no reason that patterns of  $V_A/Q$  and shunt cannot be compared in other diseases such as disseminated lung cancer, infections or inflammatory diseases.

## 8. Future Directions

The current studies demonstrated that: (1) biodegradable HAMs are able to passively target the lungs and remain present depending upon their processing conditions and (2) the toxicodynamic marker of pulmonary gas exchange (namely  $V_A/Q$  and shunt) are able to predict adverse events prior to death. Therefore, the following directions are suggested for further studies:

- To incorporate a non-water soluble drug into the HAMs. This may be accomplished by either: 1) changing the oil phase media to avoid extraction at high temperature; 2) incorporating non-water soluble moities into NPs which are physically entrapped in the HAM matrix; or 3) covalently binding a prodrug to the HAMs.
- 2. Improve the size uniformity of the HAMs to specifically target the lungs with minimal toxicity. This may be accomplished by: 1) changing the oil phase or other processing parameters to improve polydispersity and reduce MP size; or 2) using another method such as spray-drying or microfluidics to create uniform MPs.
- 3. Understand the role of animal age or disease state on the changes in V<sub>A</sub>/Q or shunt after microembolization. For example, as age increases, the ability to grow new lung tissue rapidly may be reduced, thereby changing the relatively quick (<5 day) recovery to baseline V<sub>A</sub>/Q and shunt.

- 4. Improve the mathematical model by incorporating additional compartments to account for diseased lungs.
- Further refine the experimental conditions: have a more sensitive method to step F<sub>1</sub>O<sub>2</sub>; improve the MP suspension and reduce flocculation of MPs; develop an infusion method to improve non-aggregated MP delivery to the lung.
- Study the time course effects of non-rigid or non-spherical MPs on pulmonary gas exchange.
- Determine the effective functional lung unit in a mouse by using MPs larger than 45 µm and better understand the effect of microemboli between 10 and 45 µm MPs.

# 9. Appendix

#### 9.1. Maple Code

> restart;

> with(Statistics): with(plots): with(DirectSearch): with(ArrayTools):

with(LinearAlgebra): with(ExcelTools):

## >#1 compartment model

> eq1 := 134\*Hb\*Sc[1]\*(1/100)+3\*Pc[1]\*(1/1000)-134\*Hb\*Sa\*(1/100)-

3\*Pa\*(1/1000) = S\*avD/(1-S);

> eq1a := subs(134\*Hb\*Sc[1]\*(1/100) = Q, 3\*Pc[1]\*(1/1000) = R, Q+R = QQ,

S\*avD/(1-S) = T, eq1);

> eq20 := Sa = 1/(a1/(a2\*Pa^3+a3\*Pa)+1);

> eq30a := Sc[1] = 1/(a1/(a2\*Pc[1]^3+a3\*Pc[1])+1);

> eq40a := Pc[1] = FIO2\*(Pb-47)-PaCO2[1]\*(1-FIO2\*(1-R))/R;

> eq50a := Va[1]/Q[1] = L\*RQ\*avD/PaCO2[1]; eq50a := PaCO2[1] = solve(%,

PaCO2[1]);

> subs(eq20, eq1a); eq12 := Pa = solve(%, Pa);

> eq12a := subs(eq12, rhs(eq20));

> eq13 := subs(Q = 134\*Hb\*Sc[1]\*(1/100), R = 3\*Pc[1]\*(1/1000), T = S\*avD/(1-

S), eq12a);

> eq14h := subs(eq30a, eq40a, eq50a, RQ = R, Va[1] = HK[1]\*Q[1], Pb = 760,

avD = 5, Hb = 15, eq13);

## >#Call Data from spreadsheet

> C1 := Import("/Users/lab225/Dropbox/T115.xlsx", "Sheet1", "a115:gj128"):

> C := convert(C1, Matrix):

> m := RowDimension(C);

for k to ColumnDimension(C) do

A[k] := (1/1000)\*Vector([seq(C[j, k], j = 1 .. m)]):

I[k] := rtable\_scanblock(A[k], [rtable\_dims(C)], NonZeros):

```
B[k] := Vector([seq(A[k][j], j = 1 .. l[k])]):
```

end do:

> for i to (1/2)\*ColumnDimension(C) do

X[i] := B[2\*i-1]:

Y[i] := B[2\*i]:

end do:

#### >#Weight Vector

> for i to (1/2)\*ColumnDimension(C) do

W[i] := Vector([seq(log[10](Y[i][j]/(1-Y[i][j])), j = 1 .. l[2\*i])]):

end do:

> eq1compartmentfinal := subs(a1 = 23400, a2 = 1, a3 = 150, avD = 5, R =

8\*(1/10), L = 863/100, eq14h):

#### >#Data Fit – will output to excel file named Employees.xls on tab 26-30

```
>constr111a := [10 >= HK[1] and HK[1] >= 0, 1 >= S and S >= 1/1000];
```

```
startpoint111a := initialpoint = [S = 1/100, HK[1] = 1];
```

for i to (1/2)\*ColumnDimension(C) do

sol[i] := DataFit(eq1compartmentfinal, constr111a, X[i], Y[i], FIO2, W[i],

startpoint111a);

curve[i] := plot(subs(sol[i][2, 1], sol[i][2, 2], eq1compartmentfinal), FIO2 = 0.9e-1 ...

.35, .5 .. 1, title = [i]):

end do;

SHUNT := Vector([seq(rhs(sol[i][2, 1]), i = 1 .. (1/2)\*ColumnDimension(C))]);

Export(SHUNT, "Employees.xls", "26-30", "b1");

VAQ := Vector([seq(rhs(sol[i][2, 2]), i = 1 .. (1/2)\*ColumnDimension(C))]);

Export(VAQ, "Employees.xls", "26-30", "a1");

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# 11. Curriculum Vita

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## Education:

Bachelor of Science <i>With honors</i>	Clarkson University Potsdam, New York, 2001 <u>Major</u> : Chemical Engineering, <u>Minor</u> : Mathematics
Doctor of Philosophy	Rutgers, The State University of New Jersey New Brunswick, New Jersey, 2012 <u>Major</u> : Pharmaceutics, <u>Thesis Advisor</u> : Patrick J. Sinko

### Awards and Fellowships:

**2005-2006, 2006-2007, 2007-2008** AFPE Predoctoral Fellowship **2006-2007,** Ernest Mario Endowed AFPE Predoctoral Fellowship in the Pharmaceutical Sciences

**2005-2007** NanoPharmaceutical Science and Engineering IGERT (Integrative Graduate Education and Research Traineeship) Fellow, National Science Foundation; Principal Investigator: Fernando J. Muzzio, Co-Principal Investigators: Rajesh Dave, Carlos Velazquez, Patrick Sinko and Silvina Tomassone.

**2004** Novartis Student Internship, Novartis Pharmaceuticals, East Hannover, New Jersey 05/04 –9/04

1996 Eagle Scout, Troop 333, Neversink, Pennsylvania

## **Publications:**

Deshmukh, M., **Kutscher, H.L.**, Gao D, Sunil, V.R., Malaviya, R., Vayas, K., Stein, S., Laskin, J.D., Laskin, D.L., Sinko, P.J., 2012 Biodistribution and Renal Clearance of Biocompatible Lung Targeted Poly(ethylene glycol) (PEG) Nanogel Aggregates J Control Release, In Press, Accepted Manuscript, Available online 3 October 2012

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#### **Poster Presentations:**

**H. Kutscher**, K.J. Patel, J. Shen, C.B. Massa, L.B. Joseph, M. Deshmukh, S. Li, D. Gao, V.R. Sunil, D.C. Reimer, A.J. Gow, D.L. Laskin and P.J. Sinko, The Effect of Nitrogen Mustard Exposure on Pulmonary Gas Exchange in Rats, *The AAPS Journal*, 13 (S2) W5427, 2011.

**H. Kutscher**, M. Deshmukh, D. Gao and P.J. Sinko, Effect of Processing Temperature on the Formation, Biodistribution and Biodegradation of Human Serum Albumin Microspheres In Mice, *The AAPS Journal*, 11 (S2) R6374, 2009.

M. Deshmukh, **H. Kutscher**, K. Patel, J. Shen, S. Sundara Rajan, R. Malaviya, V. Sunil, S. Stein, D. Laskin, P.J. Sinko, Polyethylene Glycol (PEG) Nanogel Aggregate Drug Delivery System for Targeted Lung Delivery, *The AAPS Journal*, 11 (S2) R6406, 2009.

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**H. Kutscher**, M. Deshmukh, A. Menjoge, P.J. Sinko, Effect of Processing Temperature on Biodegradable Human Serum Albumin Microspheres, *The AAPS Journal*, 10(S2), W5107, 2008.

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**H. Kutscher**, Y. Singh, S. Stein, P.J. Sinko, Biodistribution of Silica Particles in Rats Using Non- Invasive In vivo Imaging as a Detection Technique, *The AAPS Journal*, 8(S2), T2333, 2006.

**H. Kutscher**, Y. Su, P.J. Sinko, Human Organic Anion-Transporting Polypeptide OATP-C (SLC21A6) is an Uptake Transporter of Saquinavir in Microinjected Xenopus laevis Oocytes, *The AAPS Journal*, *7*(S2), R6281, 2005.

#### **Patent Applications:**

**Hilliard Kutscher** with co-inventors: P.J. Sinko, M. Deshmukh, S. Gunaseelan, A. Menjoge, S. Stein, Y. Singh, "PEG Nanocarrier and Nanogel Compositions", Rutgers Docket number 08-063, 12/936,392, PCT/US2009/123768, (filed April 23, 2008), WO2009123768 published Oct. 8, 2009.