SUBCUTANEOUS DELIVERY OF PROTEIN THERAPEUTICS

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ABSTRACT OF THE THESIS

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Since their discovery monoclonal antibodies have been a worldwide medical phenomenon that has documented numerous novel therapeutic uses. However, delivery of such large therapeutic proteins remains a challenge in maintaining efficacious systemic concentrations as well as minimal costs. Furthermore, pharmacokinetic profiles of monoclonal antibodies begin to vary from the norm when considering obese populations and require additional analysis to avoid over-dosing. The goals of this thesis work were: 1) to develop a generic bioanalytical method for quantifying concentrations of multiple monoclonal antibodies without requiring the use of specific coating antibodies for each separate monoclonal antibody evaluated; 2) to evaluate the pharmacokinetics of rituximab in obese rats and normal-weight control animals; 3) to develop an in-vitro cell culture permeability assay for evaluating permeability properties of monoclonal antibodies.

<u>Acknowledgments</u>

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INTRODUCTION

Importance and impact of monoclonal antibodies in modern medicine

Several of today's most prominent medicines, particularly within the fight against cancer as well as a wide range of other diseases, involve the utilization of monoclonal antibodies (mAbs). As of 2013, the FDA has approved 47 monoclonal antibodies for the treatment of cancer, cardiovascular diseases, infectious diseases, transplantations, and chronic inflammatory diseases [4, 5]. Due to its versatility, the worldwide market value of antibody therapeutics is roughly $20 billion U.S. dollars per year [4].

Structure and function of antibodies

The structure of monoclonal antibodies closely resembles the structure of endogenous immunoglobulin G (IgG). They are complex proteins that are composed of four peptide chains consisting of two identical heavy chains and two identical light chains. Their spatial arrangement is that of a typical Y-shape as other antibody monomers (**Figure 1**). Each IgG has two antigen binding sites, which allows them to function as binding sites to identify and capture foreign antigens to be recognized and eliminated by phagocytes of the immune system [10]. Immunoglobulins (Igs) can attribute their name to and belong to the immunoglobulin super-family. Each peptide chain comprises one NH_2-terminal "variable" (V) domain and one or more COOH-terminal "constant" (C) domain. There are roughly 110-130 amino acids in each of the V or C domains; and the average molecular weight is 12,000-13,000 Daltons (Da). The Ig light chains always contain only one C domain, while Ig heavy chains typically contain three or four domains. When the heavy chain does contain three constant domains, there usually includes a spacer hinge region amid the first (CH1) and second (CH2) domains. The mass of a normal light chain will typically sum to 25 kDa, while a heavy chain containing its hinge will accumulate to a

mass of 55 kDa [10]. The total molecular weight of an IgG molecule is approximately 150-160 kDa.

Initial studies of Ig structure were augmented with the use of enzymes that could divide the IgG molecules into fragments. The enzyme papain could digest IgG into two Fab fragments, each of which can bind to their corresponding antigens, and one Fc fragment. Another enzyme, pepsin, can divide IgG into one Fc fragment and a single dimeric F(ab)2 that can bind to antigens as well as cross-link [10]. The Fab segment is encoded with information that allows highly specific binding to occur at this site. It is the variable domain of the Fab that contains the paratope, or antigen-binding site. Each arm of the Y-shaped antibody monomer therefore binds an epitope, the part recognized by antibodies on antigens. The Fc fragment is constant throughout all different types of immunoglobulins and is responsible for binding to different complement proteins and cell receptors. The single-arm Fc fragment in this way helps to arbitrate the distinctive physiological effects of antibodies [11].

The type of monoclonal antibody can be additionally classified based on their composition. Initially, mAbs were derived from mouse models; however, such murine mAbs were highly immunogenic in humans. Therefore, chimeric mAbs were engineered by combining murine variable domain regions and human constant domain regions. The majority of currently developed mAbs are humanized or fully human. They are derived using complex *in-vitro* methods and contain less than 5% of murine sequence[12]. These mAbs are much less likely to lead to immune reaction in patients.

There are five different classes of Igs that are categorized based on their isotypes, which are the determinants found in the C domain that define each class. IgM is the first

immunoglobulin expressed during early B cell development followed by IgD by means of alternative splicing. Later during development, other isotypes, IgG, IgA, and IgE, are generated though cytokine regulation and antigenic stimulation in a controlled process. Therefore, isotype switching does not happen through chance alone [10].

Due to their early generation during B cell development, IgM have low affinity to antigen because of their immaturity and may sometimes be referred to as natural antibodies. Their main functions are associated with a primary immune response and are thereby frequently utilized to diagnose acute exposure to a pathogen or an immunogen. These characteristics can be attributed to the variable domains within their heavy chains that have yet to undergo somatic mutation in response to antigen and can therefore be more poly-reactive than other isotypes. Alternatively, IgD is has a much higher affinity and sensitivity as it is found at very low levels in serum and has a short half-life in serum. The hinge portion of IgD has also been prone to proteolytic degradation. The function of IgD still remains unclear, however, their membrane-bound form have been reported to direct some level of signaling, regulate B cell development, and react with specific bacterial proteins [10].

The most extensively studied and predominant isotype found in the body is IgG with the longest half-life (up to 3 weeks) exhibited in serum compared to all Igs. IgG has also been categorized into four subclasses IgG1, IgG2, IgG3, and IgG4 where concentrations of each in serum are less than the former. The differences between subclasses have been accredited to the constant region of the heavy chain, particularly CH1 and CH3, which have been found to affect antibody functional affinity and antibody flexibility. Generally, their responsibility as a group is to initiate the complement cascade in the event of an invasion from a foreign pathogen and effectively clear them from the body.

The functional distinctions amongst the subclasses can be found in their affinity to the three classes of FcɣR (I, II, and III). Their functions however, have recognizable similarities when concerning participation in the secondary immune response and transplacental transport. Here, IgG1 and IgG3 are commonly stimulated in response to protein antigens while IgG2 and IgG4 are prompted with polysaccharide antigens. However, it has also been stated that specific subclasses have a greater presence in individual disease states than others [10].

Although more predominant in serum than IgM, IgA levels are still considerably lower than IgG. Contrariwise, IgA levels are much greater than IgG in secretions such as breast milk and saliva and at mucosal surfaces. In a child's early development, 50% of the protein the neonate receives from breast milk can be attributed to IgA. The main function of IgA is to protect mucosal surfaces from bacteria, virus, and toxins by direct termination or by inhibition of binding to the mucosal surface. Their protective capabilities are not limited to just mucosal surfaces but expand to intracellular activities as well, as they are important in preventing pathogenesis or viral infection. Lastly, IgE is known to be a very potent Ig but is found at the lowest concentrations in serum with the shortest half-life among the other Igs. IgE has mainly been correlated with allergic reactions and hypersensitivity, as well as defending against parasitic worm infections. Recent research progress has enabled the generation of anti-IgE antibodies for allergy and asthma therapy [10].
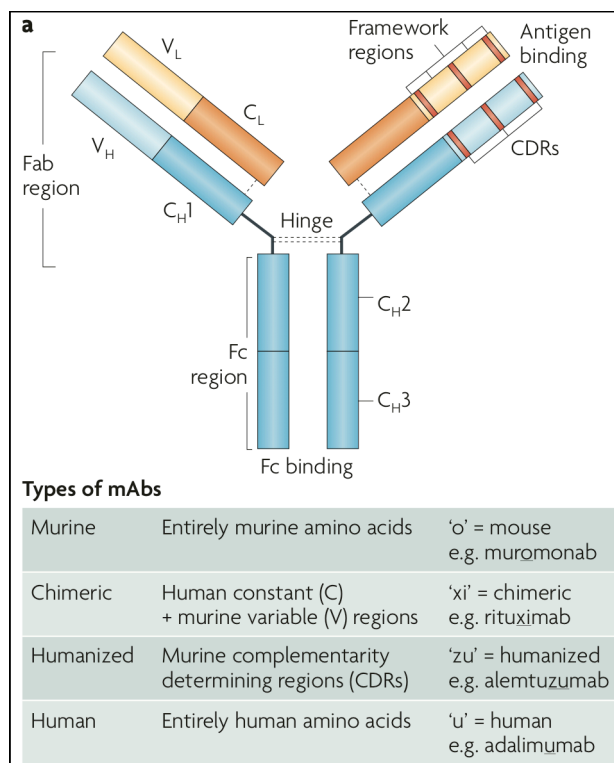
Figure 1 – Schematic of the basic structure of a mAb and description of different types of mAbs and their nomenclature [9]. VL: the variable domain of the light chain. VH: the variable domain of the heavy chain. CL: the constant domain of the light chain. CHX: the X-numbered constant domain of the heavy chain.

Protein drugs vs. small molecule drugs

Modern therapeutics encompasses two very different types of drugs: protein drugs and small molecule drugs. There are many distinctions that are drawn between the two types however; the most apparent difference can be illustrated by the size of biologics, which give rise to protein based drugs compared to classical small molecule drugs and other peptide drugs. Genentech has illustrated this difference between aspirin (21 atoms) and an antibody (~25,000 atoms) with the contrast in weight between a bicycle (~20 pounds) and a business jet (~30,000 pounds). This demonstration isn't far off from the reality when considering monoclonal antibodies, as they usually range around 20,000 atoms [13]. Therefore, another evident implication from the size contrast is the disparity of manufacturing price between the two types. This inference is supported by evidence in

the scientific industries' complex and strict process that describe the generation of biologics and other protein drug therapeutics when compared to the relatively simple, well defined, and independent manufacturing process of small drug molecules [14]. Typically, these processes for biologics tend to be produced in a living cell culture system, whereas small molecule drugs are synthesized chemically and have a predictable chemical process where identical copies can be made in bulk without the apprehension of heterogeneity. The production of biologics can be difficult to manage from starting to material to the final active pharmaceutical ingredient and have ongoing issues ensuring homogeneity. Depending on the type of biologic, some cannot be entirely characterized by heterogeneity and molecular composition and require strict conditions to maintain stability. On the other hand, small molecule drugs offer a complete contrast from the former complications, as they are comparatively stable and easy to characterize. Another major obstacle confronting clinicians and researchers alike is the immunological response stimulated by the presence of biologics when compared to small molecule drugs that are mostly non-immunogenic. The complexity with biologics only continues to exacerbate as they are generally used to treat chronic conditions, such as rheumatoid arthritis. With that said, in tandem with the difficulty in generating biologics, they offer some explanation into the discrepancy between small molecule drugs averaging a cost of a dollar a day, even though costing just cents to make, compared to an average cost of twenty-two dollars a day for protein therapeutics [14].

However, mAbs specifically do exhibit much longer half-lives than small molecule agents [15]. Pharmacokinetic studies provide evidence that plasma concentrations of small molecule drugs can fluctuate at a set dose between patients. This may be attributed, to some degree, to the route of administration of small molecule agents, which are generally given orally against intravenous administration of mAbs. There is also

continued speculation that the degradation kinetics of small molecule drugs can vary more between subjects when compared to mAbs or proteins. However, because of their size, mAbs only specialize in treating conditions that are expressed on the cell surface as they are embargoed by the blood brain barrier and other impediments alike [15].

Production of monoclonal antibodies

Monoclonal antibodies are monovalent antibodies, which allow them to bind to the same epitope and are produced through a special fusion process, referred to as the hybridoma technique. B-lymphocyte cells are extracted from the spleen of a selected animal of study, where the subject has already been induced with a disease and has developed an antigenic response to the foreign contagion. The cells are then merged with a myeloma cell line lacking the hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) and allowed to fuse together. After the fusion is complete, the hybridoma cells are placed in a special media containing hypoxanthine-aminopterin-thymidine [16]. This process is able to selectively allow only the B-lymphocytes that have successfully fused and attained the myeloma cell line's trait of "immortality", the hybridoma cells, to survive. The resulting media would contain a mixture of many different hybridoma cells each with their own unique specific antibody and epitope derived from the original B-lymphocyte [16]. The mixture can then be screened and separated into different batches of matching antibody types and further replicated and tested for usage [4].

Popularity of mAbs

The FDA approved the first mAb in 1986. Therapeutic use of mAbs has been steadily increasing since the late 1990s after the approval of several more mAbs (**Figure 2**). The increase has been so dramatic since the late 90s that the total revenue in 2013 was roughly 75 billion U.S. dollars. The popularity of mAbs can be attributed to their unique

characteristics, especially high specificity and high tolerability. The level of risk in human clinical trials when concerning unexpected safety issues is lower than for other kinds of therapeutic agents. For these reasons, mAbs can move rapidly towards mass production and thereby having a "first-to-market" advantage. Furthermore, the pharmaceutical market has seen steady tremendous growth the past decades and has transformed into a global business with efforts attempting to make more medicines accessible to more people. Because of the aforementioned and the increase in the standard of living from technological advances, mAbs have yet an additional cause to be popularized amongst clinicians. Also, the aging worldwide population has fueled the growth of mAbs use and sale across the globe. In addition, more mAbs are being approved in recent years as many clinical trials are coming to a close. As of November 10, 2014, six mAbs were recently approved, making it an average of about four mAbs approved per year (**Figure 3**). At this rate, by 2020, there will be roughly 70 mAbs on the market [2].
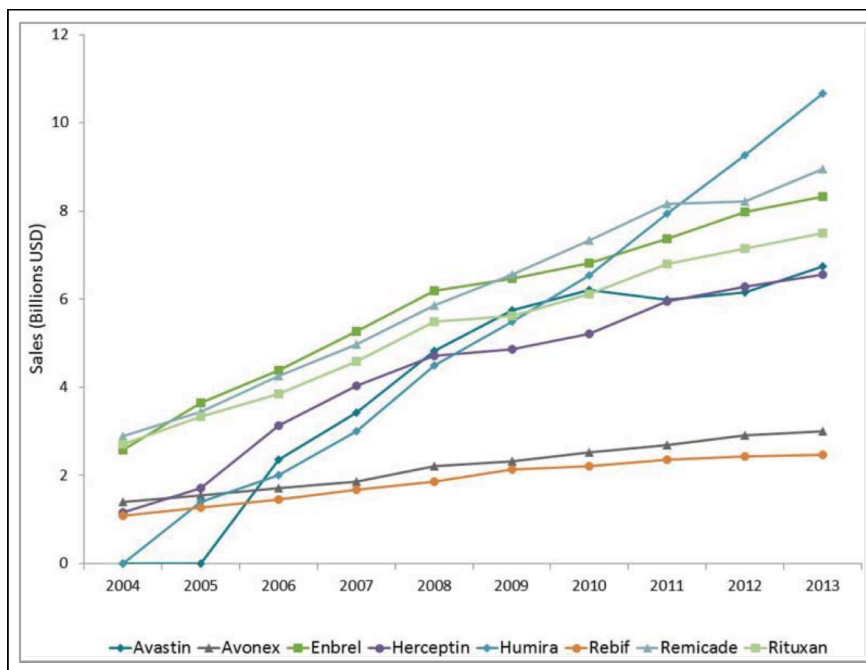
Figure 2 – Annual sales of the top six selling mAbs vs. non-antibody recombinant proteins. [2]
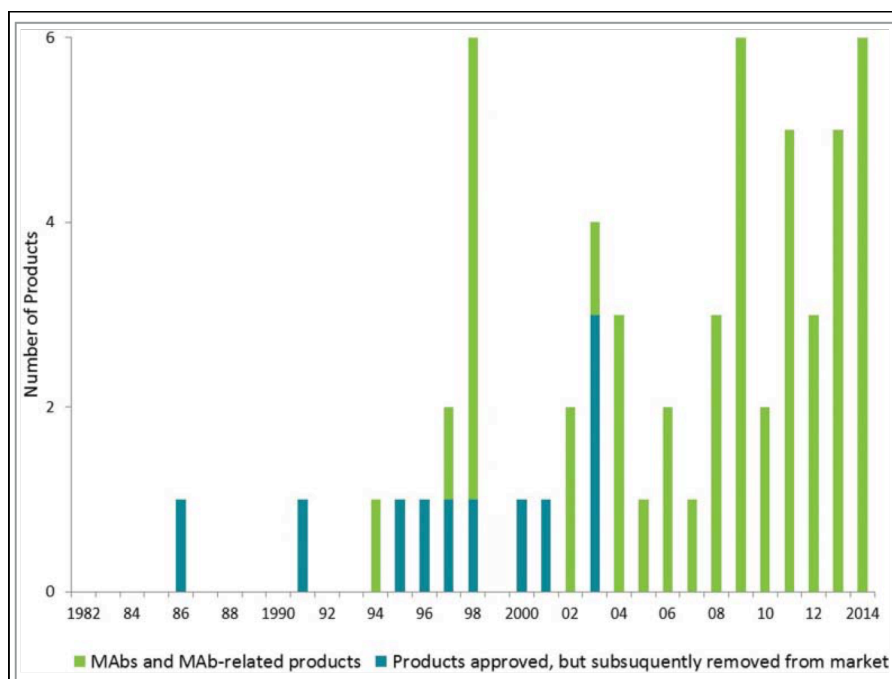

Figure 3 – Annual approval rate for mAb products. [2]

Therapeutic uses of mAbs

Monoclonal antibodies as a class of drug exhibit a significant versatility and therapeutic relevance in modern medicine. For example, rituximab has been utilized to treat both B-cell lymphoma and chronic lymphocytic leukemia by targeting the antigen CD20 (B-lymphocyte antigen) [17]. Another widely used antibody is trastuzumab, or Herceptin, which targets the HER-2 (human epidermal growth factor receptor 2) antigen to treat metastatic breast cancer as well as metastatic stomach cancer [17]. A myriad of conditions can be accounted for with the use of monoclonal antibodies including acute and chronic myeloid leukemia, metastatic colorectal and renal cancer, head and neck cancer, Crohn's disease, prophylaxis in premature infants, and prophylaxis in allogeneic renal transplantation patients in risk of rejecting the graft [17, 18]. Because of their (monoclonal antibodies) unique and enhanced ability to target antigens with high specificity they can effectively treat different types of cancer.

Drawbacks of intravenous injections of monoclonal antibodies

Whether the matter is activating the body's effector functions or conjugating with other compounds to treat an ailment, researchers are seeking to improve the delivery, absorption, and distribution of monoclonal antibodies in the body to minimize side effects and optimize the clinical response. The high cost of mAbs also prevents them from being a more common therapeutic class for treating diseases. These costs can be attributed to the multiple large doses that must be administered intravenously at regular intervals in order to maintain adequate systemic concentrations [6]. Additional costs can be accounted for by the requirement of needing a professional clinician to administer the appropriate dosages on a regular basis. Therefore, it is beneficial to explore an alternative method of delivery that can sustain mAbs within the required therapeutic window for long periods of time. Intravenous (IV) injections are not without risk and

drawbacks. For example, the risk of infection arises from the invasive procedure of implanting a catheter into the vein [19]. Failure to maintain complete sterility could result in inadvertently allowing bacteria to be introduced at the injection site. Other adverse reactions, infections, or cases of hemolysis are possible with IV injections despite the extreme rapid deliveries of high concentrations of drug [19].

Advantages of subcutaneous injections of monoclonal antibodies

Alternatively, subcutaneous (SC) injections have proven to be effective in delivering therapeutic macromolecules such as cytokines and hormones. Furthermore, SC injections can provide the advantages of simpler administration as well as the capability to extend the duration of release of antibody into the systemic circulation over longer periods and diminish the need for repeated dosing routines. Subcutaneous administration has also proven to cost considerably less when compared to its intravenous counterpart. Part of this reduced cost comes from the capability for patients to self-administer their medication rather than having a trained clinician on-site [19]. A study following the cost of administering the antibody alemtuzumab discovered the average cost of nursing labor to deliver the antibody was around $30 compared to $113 for the same antibody delivered intravenously. SC injections are generally easier to administer, which reduces risk of erroneous delivery or bacterial infection, and offer a multitude of additional injection sites as opposed to vein penetration. Even if an infection were to occur, the advantage of a subcutaneous injection limits the infection spread to a localized area rather than a widespread systemic contamination. Not only is the difficulty of application attributed to the discrepancy in cost, but also the time intensive difference between the two routes of administration. A little more than half of the participants in the study for SC injections responded that less than half an hour of labor was involved, while IV infusion time for labor would range between 4 and 6 hours. Additional costs

concerning consumable supplies, preparation and observation times were not captured in the study and signify a hidden cost [19]. However, subcutaneous injections of mAbs are not without complication as they may elicit a greater immune response and result in an incomplete/low bioavailability, which can be attributed to proteolytic degradation at the site of injection [20]. However, mAbs characteristically have longer circulating half-lives, a higher molecular weight, and other antibody related qualities that shield them from profound degradation. The most influential factors affecting subcutaneous absorption include the formulation excipients, the size of the macromolecule, and application of heat or pressure [20]. It can be argued the most prominent factor influencing absorption kinetics in the subcutaneous space the size of the protein. It has been previously reported and acknowledged that the blood capillaries absorb molecules smaller than 10 nanometers, while molecules between 10 and 100 nanometers are absorbed through the lymphatics [21]. However, there is no austere size limit when considering lymphatics absorption, although, molecules exceeding 100 nanometers have exhibited the tendency to become trapped at the site of injection for longer periods of time and thereby establishing reduced concentrations in systemic circulation [21]. Although initially disconcerting when considering antibody or large protein therapeutics, other studies have demonstrated reliable uptake of proteins of increasing molecular weight. A study observing lymphatic absorption in sheep utilizing human growth hormone reported 62% uptake by the lymphatics [22]. Another report demonstrated more than 75% absorption of recombinant human erythropoietin through the lymphatics in sheep and also generated a positive linear relationship between molecular weight and the absorptive capability of the lymphatics [21]. Human growth hormone is measured around 22 kDa and recombinant human erythropoietin around 37 kDa, which is comparable to monoclonal antibody dimensions. Furthermore, the most prevalent pathway for absorption of interleukin-2 in pigs following SC injection is also through the

lymphatics [23]. A study observing the absorption of ^{131}I albumin in dogs reported that almost the entire dosage appeared in systemic circulation through the thoracic duct and less than 3% utilized non-lymphatic routes [24]. In general, literature reports indicate that the efficacy and degree of lymphatic absorption could differ amongst varying species.

Although molecular size is the most predominant factor, there are other factors that impact lymphatic uptake. It has been previously reported that application of pressure or massaging of the site of SC injection improves the rate and uptake of liposomes into systemic circulation [25]. An additional experiment concentrating on the flow of lymph described an 83% increase when observing a normal human leg during a cycling exercise over a two-hour period [26]. The same study also indicated a 117% increase in lymph flow for a human during a warm bath over a two-hour period [26]. These findings advocate the possibility of exercise or the application of muscle exertion and heat as significant factors influencing lymphatic flow and thereby a realistic determinant of lymphatic transport of proteins.

The role of lymphatic system in macromolecule absorption in SC delivery

Once injected underneath the skin, absorption from the interstitial matrix (SC space) can happen either through the blood or lymphatic vessels (**Figure 4**). The rate of uptake of antibody is governed by a complex web of interactions likely between pre-absorption elimination, transport in the extracellular space, and diffusion into the proper vessels. Although not understood entirely, the physiology of the external cellular matrix and interstitial space have significant roles in how proteins and other drugs are absorbed. This space is heavily populated by collagen fibers to provide the main mechanistic structure support and elastin, which is a fibrous protein providing elasticity [21]. The concluding central component of this space would be the glycosaminoglycans that give

the area a consistency of a gel-like phase and is responsible for controlling hydraulic conductivity and interstitial fluid content [21]. Therefore, the charge of the utilized protein can influence the rate in which it is absorbed through the interstitial space. The collagen fibers do inherently have a slight positive charge about them at a physiological pH, but are greatly overcome by the substantial negative charge density generated by the glycosaminoglycans in the interstitial space [27, 28]. A study was able to test the difference between negatively and positively charged proteins with molecular weights within the range of 20-78 kDa [29]. It was observed that negatively charged proteins appeared at earlier times in lymph than their positively charged counterparts of similar molecular weight [29]. Lymph is formed due to a hydrostatic pressure gradient that allows interstitial fluid to enter into the initial lymphatic capillaries. Here, mAbs encounter diminutive obstruction since lymphatic capillaries lack tight junctions and allow the passage of many molecules from the interstitium [20]. Additionally, lymphatics have 'cleft-like' intercellular junctions across their surface that can be open or close depending on changes in interstitial pressure and volume [27]. In contrast, proteins, particulates larger than 100 nanometers in diameter, and other macromolecules have difficulty penetrating into blood capillaries due to their well-organized structure and inability to permeate across the vascular endothelium [30], thereby, making the lymphatics a preferred route of transport. The lymphatic system also functions as a one-way route for excess fluid, discharged plasma proteins, and cellular debris from the outlying surrounding tissues to the systemic circulation [8]. This unidirectional transport is initially comprised of discharged plasma materials as well as interstitial fluid that flow into tiny lymphatic capillaries that have a widespread intricate system throughout the body, which generally remain physically adjacent to blood capillaries. Lymph then travels through the lymph nodes to the thoracic duct, the biggest of the lymphatic vessels, and subsequently flows through the thoracic cavity into the systemic circulation [8].
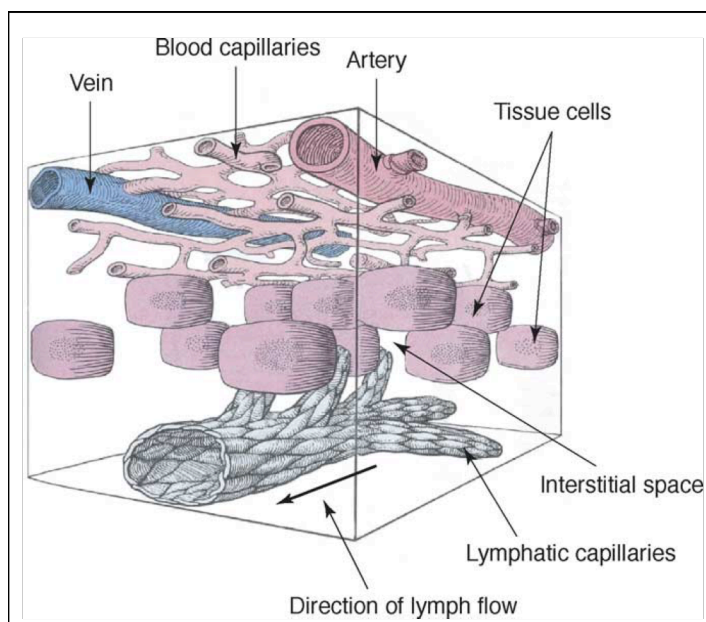
Figure 4 – Schematic of the SC interstitial space with lymphatic system interweaved with systemic circulation. [8]

Correlation between lymphatic absorption and molecular weight

Previously, researchers studying protein therapeutics and SC absorption have found that there exists a positive relationship between the proportion of dosage absorbed by the lymphatics and the molecular weight of a protein/macromolecule (**Figure 5**). Nearly complete uptake by the lymphatics system was observed in sheep injected with proteins approximately 30-40 kDa in molecular size. This demonstrates that the role of the lymphatics system is increasingly promoted as molecules with increasing size are injected subcutaneously [8].

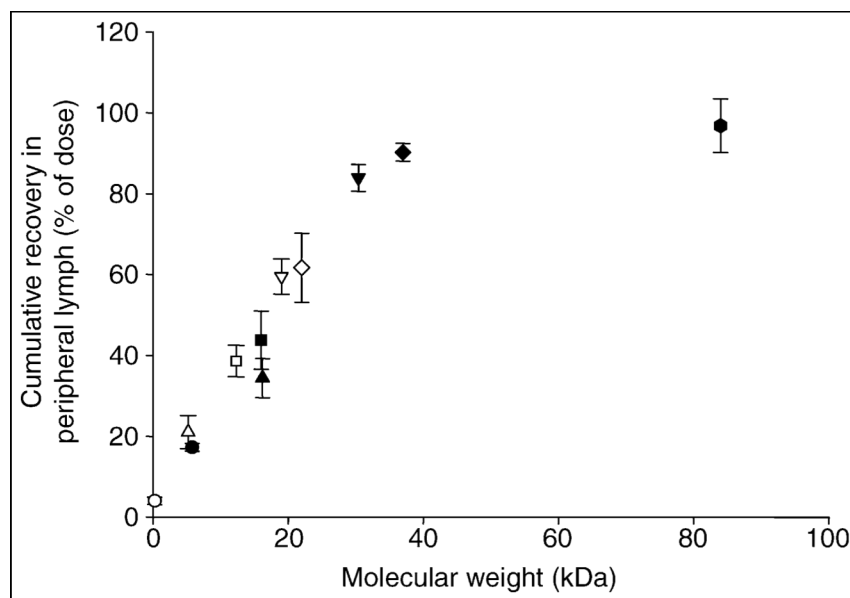**Figure 5** – Relationship between molecular weight (kDa) of selected compounds and proportion of dose absorbed into the peripheral lymph (% of dose). Data for interferon-**α** (▽), cytochrome c (□), inulin (△), fluorodeoxyuridine (○), human growth hormone (◇), darbepoetin alfa (♦), r-metHu-Leptin (▲), an analogue of Leptin (■), epoietin alfa (▼), soluble insulin (●), and a high molecular weight protein (●) [8].

Role of FcRn in pharmacokinetics of IgG

Fc receptor of neonates (FcRn) is a receptor that is expressed on a variety of cell that can bind Fc domain of IgG and mAbs. One function of FcRn is to help transport IgG from the gut to the bloodstream in neonates. It is also known that FcRn protects IgG from degradation by binding to it inside the endothelial cells [31]. FcRn is expressed in high concentrations in the endothelium of the vascular beds of the skin and skeletal muscle. There is evidence that supports the notion that FcRn intercepts IgG in these regions to internalize these proteins and return them to the systemic circulation (**Figure 6**) [31]. This process is pH-mediated, where a pH of 6.5 will facilitate the binding mechanism [6]. It is also known that FcRn is responsible for transcytosis of IgG into tissues and this uptake was illustrated *in-vitro* with human endothelial cell culture lines [31].
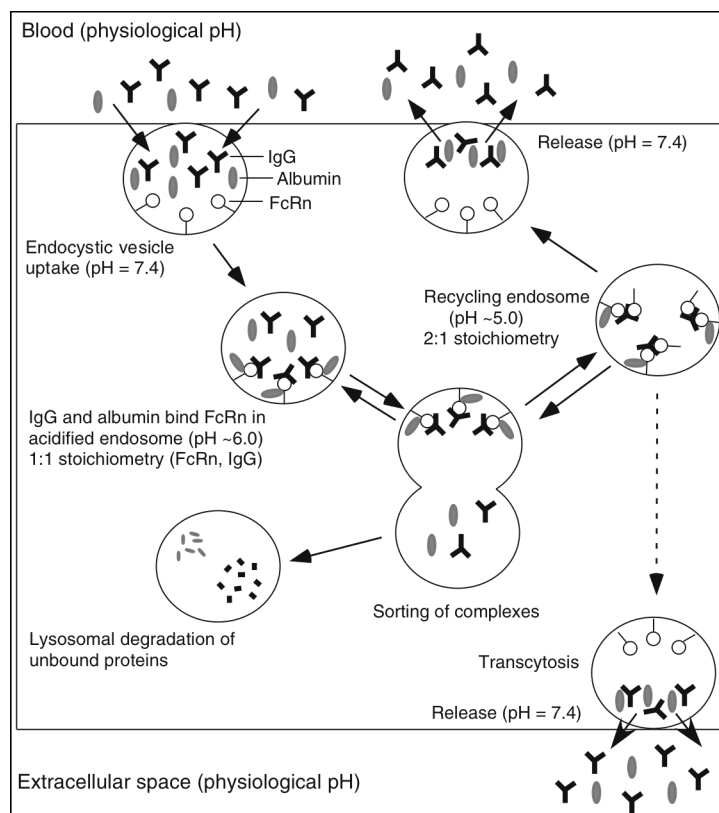
Figure 6 – Endosomal recycling of IgG [7]. Binding of IgG to FcRn is pH-dependent. IgG can be internalized into initial endosome, then the pH is decreased and IgG can bind at high affinity. Bound IgG are sorted to be returned in the blood, while free protein are sorted for proteolytic degradation.

Mechanism of SC absorption of mAbs

The rate-limiting step in the absorption of mAbs from SC injection is the transport of macromolecules across the intersititum. The diffusion across the interstitium is dictated by molecular size and the physical and electrostatic interactions between mAbs and the intersititum. However, in a study by Kagan et al., it was observed that there was a saturation of SC absorption of mAbs at high doses which suggested that the lymphatic uptake could not be the only means of transport [20]. There are three competing processes at the SC absorption site: systemic absorption, proteolytic degradation, and most likely some kind of binding phenomenon. The initial hypothesis claimed that binding protected the mAbs, specifically ritiximab in this study, from degradation.

Mathematical modeling was used to better understand the kinetics of the absorption processes. The first model proposed to describe this phenomenon with a depot created by the FcRn by protecting rituximab through binding. The second model builds upon assumption of dual role of FcRn, which not only protects rituximab from degradation, but also participates in drug absorption [6].

However, this is still not yet sufficient to succinctly prove the role of FcRn in the saturation of SC uptake of rituximab delivered subcutaneously. There are two approaches for testing binding. One is to create a situation where rituximab does not have anything to bind. This can be achieved by knocking out the FcRn gene in mice, making them FcRn-deficient and then observe to see if the saturation phenomenon persists. However, knockout animals can be a very expensive and time-consuming option. Alternatively, the system can be challenged with an agent that will compete with the binding. In order to test the hypothesis that FcRn is responsible for protecting rituximab from degrading and is responsible for absorption, rituximab was co-administered with a high dose (500 mg/kg) of non-specific IgG subcutaneously. From this, they were able to observe a predicted decrease in rituximab concentration in the systemic circulation when comparing this group with the control (rituximab injected alone). The area under the serum concentration-time curve (AUC) for rituximab was found to be 6.5- and 2.6- fold lower, depending on the rituximab dose that was tested (1 mg/kg and 10 mg/kg, respectively) when administered with IgG. The effect of IgG on the rituximab concentration was greater when dosages were smaller, as predicted by the pharmacokinetic model, see below [6].

Pharmacokinetic modeling of absorption

The two models previously described were then simulated *in silico* and compared to the *in-vivo* data that was gathered (**Figure 7**). It was observed that the second model

provided a better fit to describe the system and supports the notion that FcRn is responsible for both protection and absorption. The model begins with the co-administered dosage at the injection site in the subcutaneous layer of the skin. The dose is then transferred to an absorption site by a first order process (**Figure 8**). At the absorption site, free antibody can bind to a receptor to form a complex, which is assumed to occur rapidly, characterized by an equilibrium dissociation constant. Both the un-bound antibody and bound antibody can be absorbed into the systemic circulation, but have two different first order kinetic values for each type. The un-bound antibody also has a first order kinetic process that accounts for proteolytic degradation.
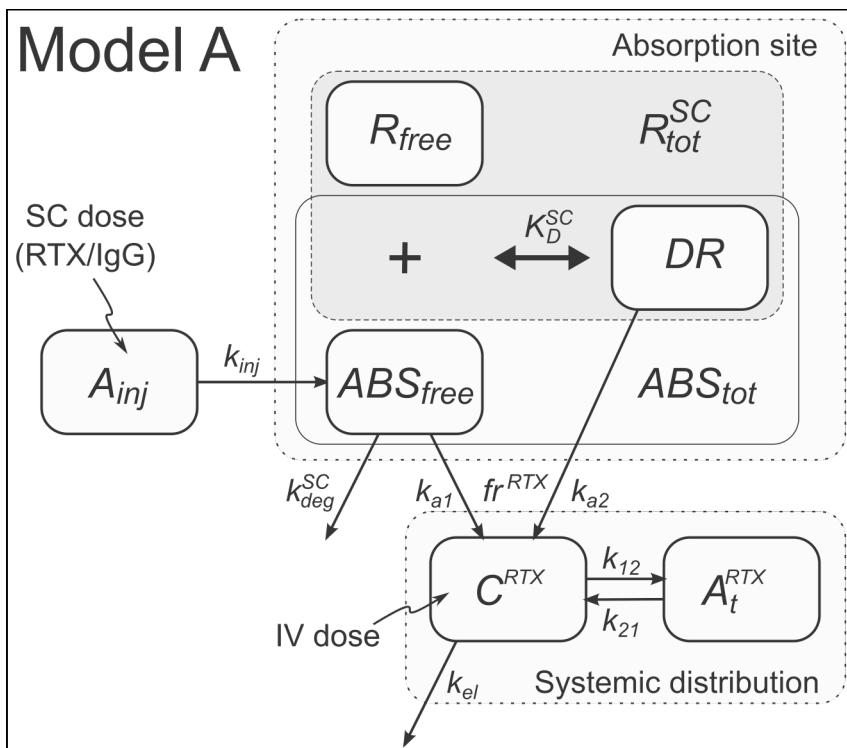
Figure 7 – *In silico* model of SC absorption of Rituximab and IgG. SC injection site (Ainj), kinetic first-order process (kinj), free antibody at the absorption site(ABSfree), receptor (Rfree), rituximab (RTX), drug-receptor complex (DR), equilibrium dissociation constant between DR and ABSfree and Rfree (KD), total antibody at the absorption site (ABStot), dose of RTX/total antibody dose (frRTX), free antibody kinetic degradation first-order process (k_{SC_deg}), free antibody kinetic absorption first-order process (ka1), antibody-receptor complex absorption first order process (ka2), systemic disposition of RTX (At), first-order elimination (kel), RTX in central compartment (CRTX), volume in central compartment (Vc), rituximab in peripheral distribution compartment (A RTX t), and first-order transfer rate constants between peripheral and central compartments (k12, k21) [6].

$$\frac{dA_{inj}}{dt} = -k_{inj} \cdot A_{inj} \tag{1}$$

$$\frac{dABS_{tot}}{dt} = k_{inj} \cdot A_{inj} - \left(k_{deg}^{SC} + k_{a1}\right) \cdot ABS_{free} - k_{a2} \cdot \left(ABS_{tot} - ABS_{free}\right) \tag{2}$$

$$ABS_{free} = 0.5 \cdot \left[\left(ABS_{tot} - R_{tot}^{SC} - K_D^{SC}\right) \right.$$
$$\left. + \sqrt{\left(ABS_{tot} - R_{tot}^{SC} - K_D^{SC}\right)^2 + 4 \cdot K_D^{SC} \cdot ABS_{tot}} \right] \tag{3}$$

$$\frac{dC^{RTX}}{dt} = k_{a1} \cdot fr^{RTX} \cdot \frac{ABS_{free}}{V_c} + k_{a2} \cdot fr^{RTX} \cdot \frac{\left(ABS_{tot} - ABS_{free}\right)}{V_c}$$
$$- \left(k_{el} + k_{12}\right) \cdot C^{RTX} + k_{21} \cdot \frac{A_t^{RTX}}{V_c} \tag{4}$$

$$\frac{dA_t^{RTX}}{dt} = k_{12} \cdot C^{RTX} \cdot V_c - k_{21} \cdot A_t^{RTX} \tag{5}$$

Figure 8 – Equations describing the *In silico* model. [6]

The need for controlled-release formulation of mAbs

Successful modification of SC injections into a controlled-release formulation would prove beneficial for the medical field of antibody delivery. The high cost of monoclonal antibodies renders them unattainable by a significant portion of patients. It was noted in 2012 that a year's worth of Campath, a drug to help treat chronic lymphocytic leukemia, is averaged at $60,000 U.S. dollars [32]. The average cost for a patient using a mAb considered in the top-nine biologics is priced at $200,000 U.S. dollars [32]. These high costs can be attributed to the complexity involved in manufacturing the medicines, the necessity to deliver them intravenously, and the need for multiple infusions. To date, no other FDA (Food and Drug Administration) approved clinical method of administration can successfully deliver the mAbs at therapeutic dosages safely and effectively. The discovery of an alternative method of administration involving slow-release mechanics has potential to prove clinically effective and cost-efficient for patients worldwide.

Advantages and disadvantages to controlled-release

To curb the extreme costs for intravenous delivery, a controlled-release formulation could eliminate the need for both intravenous delivery and multiple infusions. The capability to slowly-release the medicine provides greater durations of action between dosing intervals. Furthermore, such formulations may provide direct local delivery of mAbs to diseased locations and enhance therapeutic effects. There is also the possibility of selecting a local site of injection that could have additive or synergistic effects with clinically approved small molecule drugs that could lower dosing regiments for patients [33]. In addition to slow release profiles, researchers and clinicians could improve their control over release kinetics giving way to possibilities including slow basal kinetics, pulsatile, off-on, and longer-term. The current disadvantages to considering such formulations include their inability to provide a high antibody bioavailability when considering certain devices that derive from their poor loading capabilities. Some formulations have antibody instability issues when they are maintained inside the release device [33]. When manipulating controlled-release systems, one must understand site-specific antibody release, correlated target-site concentrations, systemic concentrations, and clearance or metabolism. Once fully realized and relinquished of obstacles, the controlled-release formulations have the potential to change the mAbs market.

Epidemic of Obesity

Although most predominant in the United States, obesity has developed into an epidemic in numerous countries. According to the CDC, a little over one-third of the American population is obese [34]. Obesity can be defined by a person's body mass index (BMI), which can be calculated by a person's body weight in kilograms divided by the square of the same person's height in meters. If this value falls between 18.5 and 25,

then the subject is considered normal. Though a figure above 30 is then considered obese [35, 36]. Obesity can be then subdivided into three categories: class 1, class 2, and class 3 with a BMI of 30-35, 35-40, and 40 or higher, respectively. Related conditions to obesity include type-2 diabetes, heart disease, certain forms of cancer, and all are leading causes of avertable mortality. The average medical cost is roughly $1,429 higher for persons with obesity than their non-obese counterparts [34]. On a national scale, obesity costs in the United States are estimated to surmount to $147 billion annually [34]. Recent research has shown that the socioeconomic status of an individual may affect a person's likelihood of becoming obese. Those of a lower income are more prone to obesity than those of a higher income. The level of education does not seem to have any significant influence on the incidence of obesity, though women with higher income are less prone to become obese than their lower income counterparts [34]. Obesity seems to more common in women than compared to men [37].

Childhood obesity

Childhood obesity has also become a serious issue in the United States in recent years and has seen a steady increase in the past 30 years [38]. In 2012, nearly 17% of children and adolescents aged 2-19 were obese and has steadily remained at that figure [39]. Similar to obesity in adults, there is a higher correlation of child obesity with parents of lower income and education levels. Obese children as young as 2 years old have been shown to have higher risks of remaining obese into adulthood and thereby are exposed to life-threatening conditions such as heart disease, osteoarthritis, etc. [38].

Causes of obesity

Some cases of obesity have been linked to a medical condition such as Cushing's syndrome, Prader-Willi syndrome, etc. [40]. However, other analysts indicate obesity can be attributed to two of the most commonly associated reasons for its increase, which are

diet and exercise. Components of these reasons may include, but not limited to, the artificial environment, the widespread utilization of high fructose corn syrup in products, larger portion sizes, convenient availability of food and other high-energy dense products in vending machines, and less physical education [41]. Other researches though are not as convinced that these factors are as significant as previously thought. A comparative study between diners frequenting fast food developments and those at restaurants illustrated that the net intake of energy/calories was relatively small (~200-300 kcal/day) [42]. A separate critical review also established the negligible difference between sucrose and high-fructose corn syrup as their metabolism and absorption kinetics should be relatively the same [43]. Further evidence also dismissed the correlation between a building environment such as sidewalks and physical activity [44] as well as the impact of physical education on adolescents [45]. There is also little support of the claim that obese individuals who eat less and gain weight is not substantiated [46]. Furthermore, the relative energy expenditure of obese individuals is generally higher than the average person because of the increased energy to sustain a heavier body weight [46, 47].

Current solutions to obesity

There are various methods to address obesity which include prescription weight-loss medication, exercise weight-loss programs, weight-loss surgery, as well as device implants [40]. Some common medications include lorcaserin (Belviq), liraglutide (Saxenda), orlistat (Xenical), buproprion and naltrexone (Contrave), and phentermine and topiramate (Qysmia). These medications do require close monitoring by a medical professional [40]. Implants present another solution by utilizing electrical impulses sent to the vagus nerve indicating the brain that the stomach is full and thereby preventing the patient from over-eating. Several different types of weight-loss surgeries are available including gastric bypass surgery, laparoscopic adjustable gastric banding,

biliopancreatic diversion with a duodenal switch, and implementing a gastric sleeve. All of these solutions however are questionable in their ability to provide long-term recovery from obesity without implementing changes to an individual's lifestyle and diet. Finding a long-term solution is more complex than just exercise and cutting back on calories. There are numerous studies suggesting that the type of diet or type of foods eaten can heavily influence the risk of becoming obese. Following this logic further supports the evidence that lower income families have more trouble managing obesity within their households as frozen foods and foods with additives and preservatives tend to be cheaper and more convenient than their produce equivalents.

Additional adverse effects of obesity

At the core of it, obesity primarily occurs when an individual intake too much energy and does not spend enough of it during the day. Being obese can have many additional adverse effects upon the body leading to higher levels of blood cholesterol and triglyceride levels, increased blood pressure, and can induce diabetes [48]. Much of the western world has stigmatized obesity in a negative light often associating those afflicted with lack of self-control, often instigating further feelings of shame and guilt. Though there are still some parts of the world where obesity is viewed as a sign of affluence and fertility [49]. Obese people may also have higher risks of depression, disabilities, sexual difficulties, and societal isolation. These increased risks coupled with modern society's technological advances can create a sedentary lifestyle leaving more individuals prone to obesity [40]. Childhood obesity also becoming more prevalent with societal culture shifting from playing outdoors to playing electronic games indoors. Additional studies demonstrate that certain foods such as candy can be addictive. These foods that are high in fat and sugar can change the chemical composition in the brain and can lead to a dependence on the high that they provide. The high can be characterized by dopamine

receptors that function somewhat as a reward system every instance the individual partakes of the unhealthful substance [50]. Due to the high statistics that indicate preventable deaths because of obesity and prevalence, the American Heart Association has indicated that doctors should treat obesity as a disease as of 2013 [51]. They also urge that healthcare providers more actively aid obese patients to finding solutions to weight loss before leading to heart disease and stroke, the United States number 1 and 5 killers, respectively.

Dosing difficulty of obese patients

Apart from the negative effect obesity has on an individual's health, it is becoming more problematic for clinicians to treat obese patients, as their physiology and body chemistry deviates from the norm. These changes have been documented in a certain number of cases where accommodation must be made for the obese population. One study concluded the need for safe and effective treatment of morbidly in obese patients undergoing anesthesia. The total body weight of obese patients can be a deceiving figure and should not be used when preparing the right dosage forms, even though the standard is to use this total. In general, this is due to the similarity between an average person's total body weight with their lean body weight and ideal body weight. An obese individual, however, has a large portion of their fat included in their total body weight and lean body weight and fat mass do not increase proportionally. And because a significant percentage of blood flow is still concentrated at lean tissue groups, a total body weight calculation could result in an overdose for a morbidly obese patient. Ideal body weight is also disregarded as it typically describes ideal weight for a measured height. Therefore, all patients are standardized for a particular height and the calculated ideal body weight for a morbidly obese patient will always be less than their total or lean body weight and will result in under dosing. The ideal standard for the morbidly of obese patient would be

the lean body weight, which is the total body weight minus the fat mass. This metric is useful since it can accurately describe cardiac output, which is a dominant factor in primary distribution kinetics of drugs. Also, there is a positive correlation between drug clearance and lean body weight, extending the impact of pharmacokinetic parameters to adjust to a morbidly obese patient's physiology. Depending on the type of a drug, ideal or total body weight may prove more useful as duration of action may be prolonged [51].

Goals and objectives

Obesity is associated with multiple comorbidities, which require administration of multiple drugs. Medications are commonly administered based on patients' weight. However, dosing modification requirements for obese patients are unclear as body composition of these patients changes. These changes may affect the biodistribution of drugs. Monoclonal antibodies are an important and rapidly growing class of drugs that can be frequently delivered subcutaneously. Subcutaneous delivery and controlled delivery of mAbs offers multiple advantages over other injection types. Effect of obesity on pharmacokinetic of monoclonal antibodies is not known.

The main goal of this study was to develop approaches for investigating subcutaneous absorption of monoclonal antibodies *in-vitro* and *in-vivo*.

Specific aims of this work are:

1) Develop a generic bioanalytical method for determining concentration of multiple monoclonal antibodies in buffer and animal serum.

2) Investigate the pharmacokinetics of rituximab in obese and control animals after intravenous and subcutaneous dosing.

3) Develop a cell culture model for screening the permeability of monoclonal antibodies *in-vitro*.

Methods

Materials

Enzyme-Linked Immunosorbent Assay (ELISA)

- Aluminum Foil Roll, Fischer Scientific

- Tween 20, Fischer Scientific, Fairlawn, NJ, LOT 153087

- Bovine Serum Albumin (BSA), Sigma-Aldrich Co., St. Louis, MO, LOT SLBN7989V

- Microcentrifuge Tubes 1.5mL Graduated Tube with Flat Cap, Fischer Scientific, LOT 14130846

- Multichannel Pipette 300, eppendorf

- Multichannel Pipette 1200, eppendorf

- P20 Pipette, eppendorf

- P100 Pipette, eppendorf

- P200 Pipette, eppendorf

- P1000 Pipette, eppendorf

- SIGMAFAST OPD, Sigma-Aldrich Co., St. Louis, MO, LOT SLBP2797V

- Anti-Human IgG (H+L), Sigma-Aldrich Co., St. Louis, MO, LOT RI21577

- Rat Anti-Rituximab

- Rat Serum, Sprague Dawley, BioreclamationIVT

- 96 Well Plate, Thermo Scientific, Rochester, NY

- Vortex Mixer, Fischer Scientific

Antibodies

- Avastin (bevacizumab), Genetech, Inc., South San Francisco, CA

- Erbitux (cetuximab), Bristol-Myers Squibb Company, Princeton, NJ

- Herceptin (trastuzumab), Genetech Inc., South San Francisco, CA

- Opdivo (nivolumab), Bristol-Myers Squibb Company, Princeton, NJ

- Perjeta (pertuzumab), Genetech Inc., South San Francisco, CA

- Rituxan (Rituximab), Genetech Inc., South San Francisco, CA

- Yervoy (ipilimumab), Bristol-Myers Squibb Company, Princeton, NJ

Blood Sampling

- BD PrecisionGlide Needle 23G (0.6mm x 25mm)

Cell culture

- Centrifuge 5810 R, Eppendorf

- Cell culture hood 1300 Series A2, Thermo Scientific

- Disposable Serological Pipette 5mL, Fischer Brand

- Disposable Serological Pipette 10mL, Fischer Brand

- Disposable Serological Pipette 25mL, Fischer Brand

- Fetal Bovine Serum (USDA approved source), Corning, Manassas, VA

- Hemacytometer, Hausser Scientific, Horsham, PA

- HERAcell 150i CO2 Incubator, Thermo Scientific

- Liquid Nitrogen Tank, Thermo Scientific, Marietta, OH

- MaxQ2000 Shaker, Thermo Scientific

- MEM Nonessential Amino Acids, Corning Cellgro, Manassas, VA

- Penicillin Streptomycin Solution, 100X, Corning, Manassas, VA

- Primo Vert Microscope, Carl Zeiss Microscopy GmbH

- Trypan Blue Solution 0.4% (w/v) in PBS, Corning, Manassas, VA

- Water Bath Isotemp, Fischer Scientific

- 0.05% Trypsin, 0.53mM EDTA, 1X, Corning, Manassas, VA

- 75cm2 Cell Culture Flask Canted Neck Nonpyrogenic Polystyrene, Corning, Manassas, VA

ELISA overview

ELISA is a plate-based assay that can quantify specific antigens or antibodies. Antigens include, but are not limited to, proteins, hormones, and peptides [52]. There are multiple types of assays to quantify different materials, however, a general antibody detection assay begins with test serum incubated in an antigen-coated polystyrene well or tube. Enzyme-labeled anti-immunoglobulin is added and allowed to adhere to the well and washed out after an allotted period of time, thereby yielding a measure of how much antibody was in the serum (**Figure 9**). Three similar approaches can be used to detect and quantify antigens in body fluids and include a competitive assay, a double-antibody ELISA, and an inhibition assay. The competitive assay employs tubes or wells coated with enzyme-labeled antigen and the antigens are mixed with the sample. There is an inverse relationship between the reaction product and the antigen present in the sample solution. A double-antibody ELISA or sandwich ELISA, utilizes an antibody coating on the well or tube walls. Test serum is then added and followed by a conjugate detection antibody labeled with the enzyme. There is a direct relationship between the reaction product and the amount of antigen present in the test solution. Finally, the inhabitation assay utilizes a test fluid comprising antigen and is incubated with antiserum. The concentrations of the leftover antibody are then quantified by a standard ELISA test in antigen-coated wells or tubes [53].
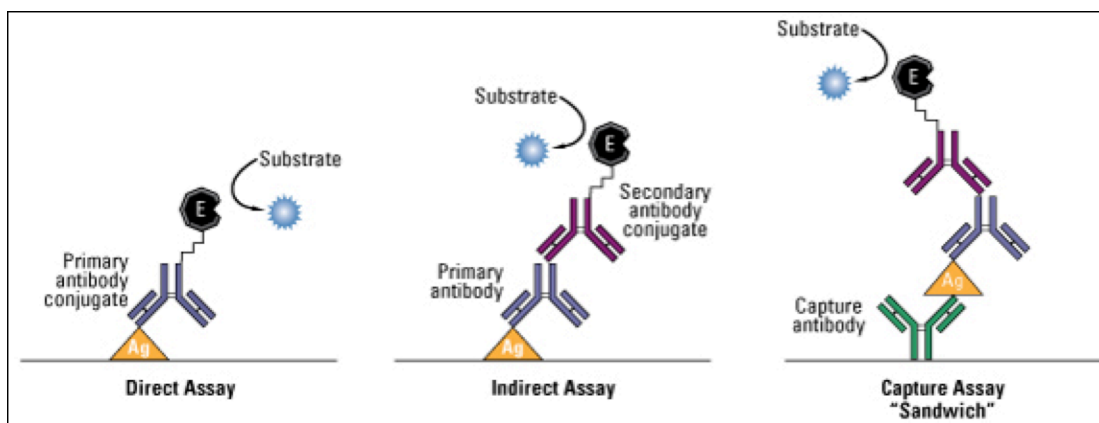
Figure 9 – Diagram of common ELISA tests. [1]

Rituximab ELISA protocol

For this studies purposes, ELISA was performed in order to quantify the concentration of rituximab in serum samples obtained from animal studies. The ELISA protocol is described below: 96-well plates were coated the previous evening with rat anti-rituximab antibody at 100 µl/well (1 µg/mL in carbonate buffer, pH 9.4, 0.2M) and allowed to incubate overnight at 4°C. Blocking solution (1X phosphate buffered saline (PBS), 1% bovine serum albumin (BSA)) was introduced at 150µl/well to prevent non-specific binding for 1 hour. Calibration standards (0.0122µg/mL – 0.5µg/mL) and samples were added at 100µl/well in duplicates for 1 hour. Goat anti-human IgG (Fc-specific)-peroxidase conjugate (1:50,000 in 1X PBS) was added for detection at 100µl/well for 1 hour. Revelation of the plate was achieved using OPD (*o*-Phenylenediamine dihydrochloride) that was newly generated and applied to the plate for 25 minutes in the dark. Immediately after, 50µl/well sulfuric acid (H_2SO_4, 1M) was added to stop further reactions. Plate was read at 492 nanometers. In between each step, the plates were rinsed three times with a wash solution (1X PBS, 0.05% Tween-20) to eliminate contamination and any preceding solutions. All incubation steps were performed at room

temperature and on an orbital shaker (300 rpm), with the exception of the overnight incubation.

Generic anti human mAb ELISA

In addition to the rituximab ELISA, a more versatile generic ELISA was developed in order to allow for testing multiple types of human mAbs with a single protocol. The procedure was based on the rituximab ELISA protocol with an alteration in the coating step where anti-human IgG Fc-specific antibody was used to coat the plate rather than anti-rituximab. This coating was tested at three different concentrations, 1µg/mL, 2µg/mL, and 4µg/mL in order to evaluate the efficacy of quantification at each level. After determining the optimal coating concentration, the working range of the assay was determined for multiple therapeutic mAbs.

Animal study

Male Long Evans rats (HsdBlu:LE, Envigo) were used for this study in accordance with an IACUC approved protocol. Animals were divided into two groups that were maintained on different diets. Obese group were obtained by feeding regular LE rats with a high-fat diet (60% Kcal from fat, Harlan Teklad Diet TD.06414) for approximately 2.5 months to obtain animals with an average body weight of 500 g. Regular rats weighing ~250 g (maintained on a standard Harlan Teklad Global Diet 2014S, 14% protein, 4% fat) were used as controls. The rats were caged and given free access to food and water and were maintained on a 12/12-hour light/dark cycle. Among the different groups, one set of rats were intravenously administered 10 mg/kg of Rituximab (Rituxan, 10mg/ml; Genentech, Inc., San Francisco, CA) through the tail and another set was subcutaneously administered the same dosage in the abdomen. Overall, the rats were

divided into four groups (n=2-3) according to the diet treatment and route of administration. Serial blood samples were collected from all animals using saphenous vein puncture. Isoflurane gas was utilized in isolated containers to anesthetize the animals. Following sedation, the animals were shaved down to minimize contamination as well as enhancing the ease of blood collection. Petrolatum ointment was applied to the site of puncture as to provide a hydrophobic slippery surface to ease the transport of blood from the wound into the collection tubes. The blood was then allowed to clot for at least a half hour before being centrifuged for 6 minutes at 1000g to separate the blood cells from the serum. The serum was then aliquoted at 3μl and frozen at -80°C. The aliquoted serum samples were thawed at the day of their ELISA analysis and rituximab content was quantified.

Cell monolayer permeability assay development

The biological half-life of mAbs is approximately 2 weeks in rats. Therefore, evaluation of absorption of multiple mAbs *in-vivo* would take a very long time. To rapidly evaluate absorption characteristic of multiple mAbs developing an *in-vitro* screening method can be very useful. Two types of cell cultures were evaluated for development of an *in-vitro* permeability screening for mAbs: Caco-2 (derived from colon adenocarcinoma) and HUVEC (human umbilical vein endothelial cells).

Caco-2 cells are commonly used model for assessment of permeability of small molecule drugs. The cells were obtained from American Type Culture Collection (ATCC) were thawed on the day of their arrival and seeded onto T-75 flasks obtained from Corning. EMEM provided by ATCC was used with the addition of 10% fetal calf serum as well as 1% nonessential amino acids. The cells were passaged every 3-4 days before confluency was reached in order to avoid mass apoptosis, which Caco-2 cells have been known to do at confluency. A trypsin/EDTA solution was used to help detach the cells

from the flask. The assay is made up of two major components: a top and bottom plate. The top plate is where the filter was located and where cells could be seeded. The bottom plate provided wells filled with media where transport and exchange of molecules could occur. Before seeding the cells, the Caco-2 cells were passaged until the 30th generation in order to achieve a state of maturation that allowed them to be safely seeded and maintained on filter supports. At maturation, the cells were seeded onto the filter supports at a density of 300,000 per well. Media was changed every other day in both top and bottom plates, which consisted of EMEM, 10% fetal calf serum, 1% nonessential amino acids, and 1% penicillin-streptomycin. During the media replacement, a transepithelial electrical resistance measurement was performed in order to evaluate and monitor the cellular density. The cells were allowed to grow for roughly 21 days before the cellular density began to plateau and reach maximum capacity and ready for the introduction of test samples. Test samples can then be added at varying concentrations at either the top or bottom plates to test both directions of transport, apical to basolateral and basolateral to apical sides [3].

Another cell culture model utilized HUVEC cells [54]. The cells were maintained on gelatin coated (0.2%) T-75 flasks using endothelial cell growth medium 2 + supplement mix (Promocell C-22011) that was changed every 24h. Trypsin solution was used to help detach the cells from the flask. For permeability assay, various growing conditions on Transwell plates were tested, including 30000, 50000, and 100000 cell/well seeding density, with and without coating with gelatin (0.2%) and collagen (0.2%). Transepithelial electrical resistance measurement was performed every few days for up to 21 days. On the day of experiment, the upper part of the well (donor) is replaced with a drug-containing solution and samples are taken over time from the lower part (acceptor). The apparent permeability coefficient is determined using the following equation:

$$Papp = (\frac{dQ}{dt}) \cdot (\frac{1}{A \cdot C0})$$

where dQ/dt is the slope of the curve describing change in the concentration in the acceptor over time, A is the permeability area, and C0 is the initial concentration. Schematic of the transwell assay is shown below (**Figure 10**).
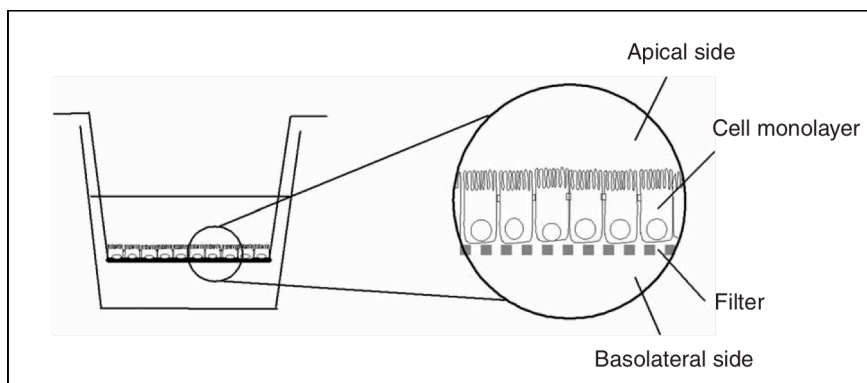

Apical side

Cell monolayer

Filter

Basolateral side

Figure 10 – Illustration of transwell filter membrane and its associated contents. [3]

Results and Discussion

Development of the generic ELISA method

The generic ELISA assay was developed in order to create a more robust assay to assess multiple monoclonal antibodies without the use of highly specific reagents. Particularly, this assay generalizes the antibody used to coat the ELISA plates rather than using specific anti-drug antibody coating, which can be expensive or unavailable for newly developed mAbs. Instead of using drug specific reagents such as anti-Rituximab antibody, the generic ELISA assay utilizes standard goat anti-human IgG Fc-specific antibody to coat the plate for attachment and detection of monoclonal antibodies in samples. This approach is based on the fact that all therapeutic mAbs contain very similar human Fc part.

To obtain a useful ELISA method, multiple protocol details should be optimized, such as reagents concentrations, volumes, incubation times, temperatures, washing steps, etc. Anti-Rituximab ELISA protocol was developed and optimized before by Dr. Kagan [6, 20]. In this work, it was assumed that optimization of only the first part of the protocol – coating step would be required, as the following steps – detection and plate development were not rituximab specific and could be potentially transferred without modification to this generic ELISA method. The coating concentration may impact the sensitivity and the range of the ELISA.

To optimize the generic ELISA assay, plates were coated at 3 different concentrations of goat anti-human IgG Fc-specific antibody: 1 µg/mL, 2µg/mL, and 4 µg/mL. The coating test was performed for rituximab, human IgG (IVIG), trastuzumab, ipilimumab, and bevacizumab. Each coating concentration was then tested with a range of standard dilutions of mAbs in rat serum of interest. As can be seen from these Tables 1 and 2 and Figures 11-13 increasing concentrations of the tested mAbs produced increasing signal;

therefore, it can be concluded that utilization of a single coating agent is a viable approach for detecting multiple mAbs. Furthermore, as can be seen from the graphed data, the points are almost indistinguishable, as they show no difference between the 3 varying concentrations. As various coating concentration provided a very similar signal in the ELISA it was concluded that all these coating concentrations can be used in the assay. However, to minimize the use of reagents, the lowest concentration of 1 µg/mL was selected for future analysis.

RTX [c]	RTX [c] ug/mL	coating [c]			signal to noise ratio		
		4 ug/mL	2 ug/mL	1 ug/mL	4 ug/mL	2 ug/mL	1 ug/mL
CC2	0.25	2.4627	2.3561	2.325	49.5	51.7	50.9
CC5	0.03125	1.0098	0.9964	1.0306	20.3	21.9	22.6
CC7	0.0078	0.3443	0.3361	0.3601	6.9	7.4	7.9
CC10	0.00098	0.08535	0.08745	0.0918	1.7	1.9	2.0
blank	0	0.0498	0.0456	0.0457			

Table 1 – Optical densities and signal to noise ratios for Rituximab under generic ELISA method at varying coating concentrations

IgG [c] (ng/mL)	coating [c]			signal to noise ratio		
	4 ug/mL	2 ug/mL	1 ug/mL	4 ug/mL	2 ug/mL	1 ug/mL
40	1.1482	1.12825	1.19115	24.4	22.8	25.8
20	0.66045	0.6582	0.7057	14.0	13.3	15.3
10	0.3648	0.38835	0.4027	7.7	7.9	8.7
2.5	0.131	0.137	0.1433	2.8	2.8	3.1
0.312	0.06545	0.0642	0.0724	1.4	1.3	1.6
blank	0.04715	0.04945	0.04625			

Table 2 – Optical densities and signal to noise ratios for IVIG under generic ELISA method at varying coating concentrations
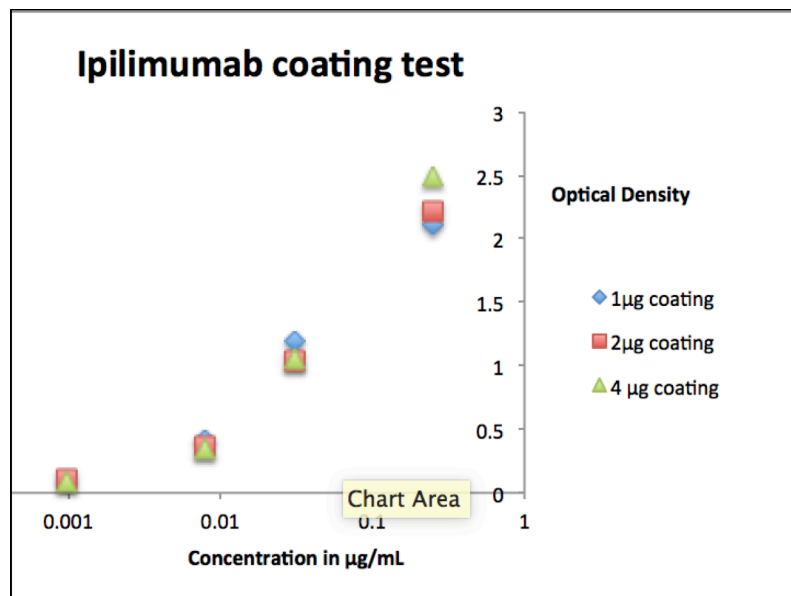
Figure 11 – Generic ELISA for Ipilimumab at 1, 2, and 4 μg coating concentrations.
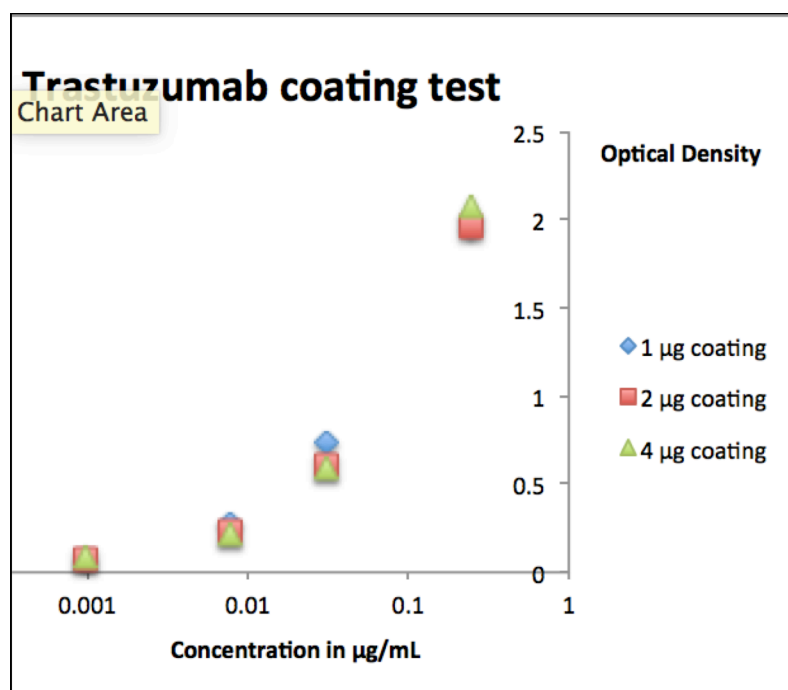
Figure 12 – Generic ELISA of trastuzumab at 1, 2, and 4 µg coating concentrations.
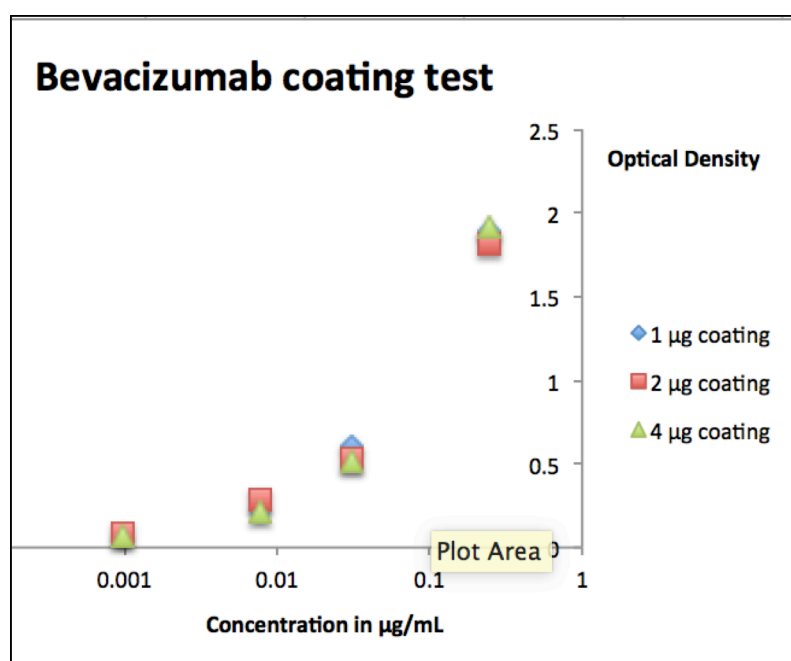

Figure 13 – Generic ELISA of bevacizumab at 1, 2, and 4 µg coating concentrations.

Following the optimization of the coating concentration, efforts were concerted towards building full calibration curves for multiple mAbs in rat serum to determine the dynamic range for multiple mAbs of interest.

Optical densities were obtained through generic ELISA method, as discussed in previous section, and then paired with their corresponding mAbs concentration in a table. The aforementioned data was then graphed on a scatter plot through Sigma Plot and yielded typical calibration curves observed through a regular ELISA assay (**Figure 14**).
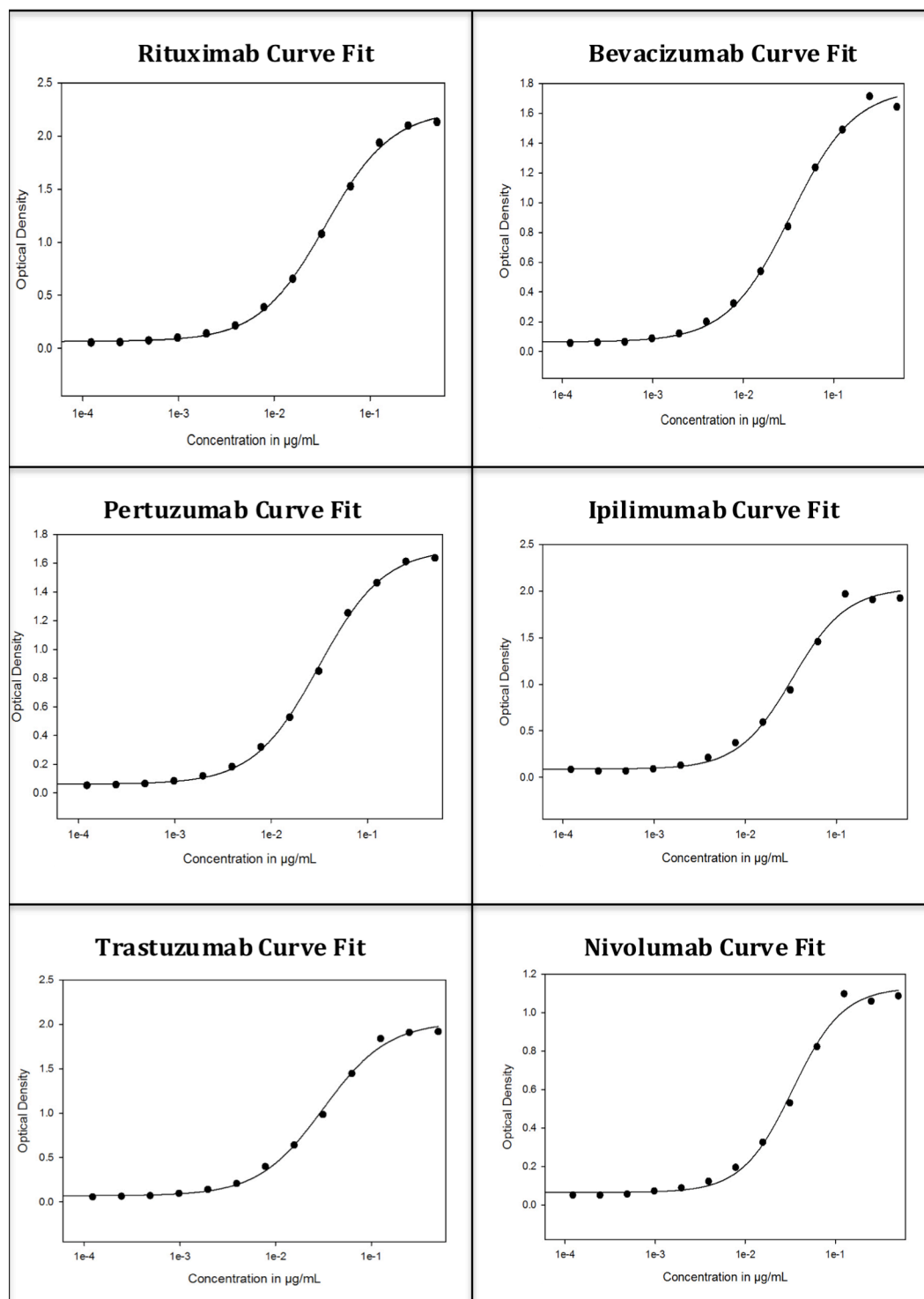
Figure 14 – Sigma Plot generated scatter plots of Rituximab, Pertuzumab, Bevacizumab, Ipilimumab, Trastuzumab, and Nivolumab

Each graph was then fitted using a four-parameter logistic equation as follows:

$$y = min + (\frac{max - min}{1 + \left(\frac{X}{EC50}\right)^{-H}})$$

The calibration curve parameters were extracted from Sigma Plot reports from the extrapolated graphs (**Table 3**). The parameters were used to recalculate the concentrations of the standards used to produce the curve. And the relative deviation of less than 20% from a nominal value was considered acceptable.

	min	max	EC50	H	Concentration range µg/mL
Rituximab	0.065	2.2448	0.0341	1.2494	0.001-0.25
Pertuzumab	0.0606	1.703	0.0313	1.2797	0.0009-0.25
Bevacizumab	0.0649	1.7688	0.0337	1.2391	0.0009-0.125
Ipilimumab	0.0873	2.035	0.0328	1.4638	0.007-0.0625
Trastuzumab	0.0692	2.0297	0.0314	1.2868	0.003-0.0625
Nivolumab	0.0646	1.1317	0.0335	1.5562	0.007-0.0625
Cetuximab	0.0786	1.8335	0.0349	1.6522	0.007-0.0625

Table 3 – Extrapolated values for four parametric logistic equation variables

The generic ELISA developed in this worked will be utilized in the future for *in-vitro* and *in-vivo* pharmacokinetic assessment of various mAbs.

Pharmacokinetics of rituximab in obese and control animals

Serum concentrations of rituximab were measured following administration of the 10 mg/kg of total body weight by intravenous and subcutaneous injection to obese and normal-weight control rats (Figures 15 and 16). Pharmacokinetic parameters for mean concentration-time profiles all groups are shown below (**Table 4**).

As can be seen, the initial concentrations after IV administration were higher in obese rats, which indicate a smaller initial volume of distribution in obese rats as compared to normal-weight control rats. While further investigation with a large number of animals is required this finding can be attributed to a less efficient disposition of mAbs into adipose tissue, which is disproportionally increased in obesity. Furthermore, the concentrations of rituximab were also higher in obese rats following subcutaneous injection, which may indicate that absorption of protein following subcutaneous injection may be dependent on the subcutaneous adipose tissue layer. Similar findings have been reported before for pegylated erythropoietin. Our data from this work and published data indicate that additional studies of effects of obesity on pharmacokinetics of protein drugs are required in order to provide optimal therapy for this patient population [55].
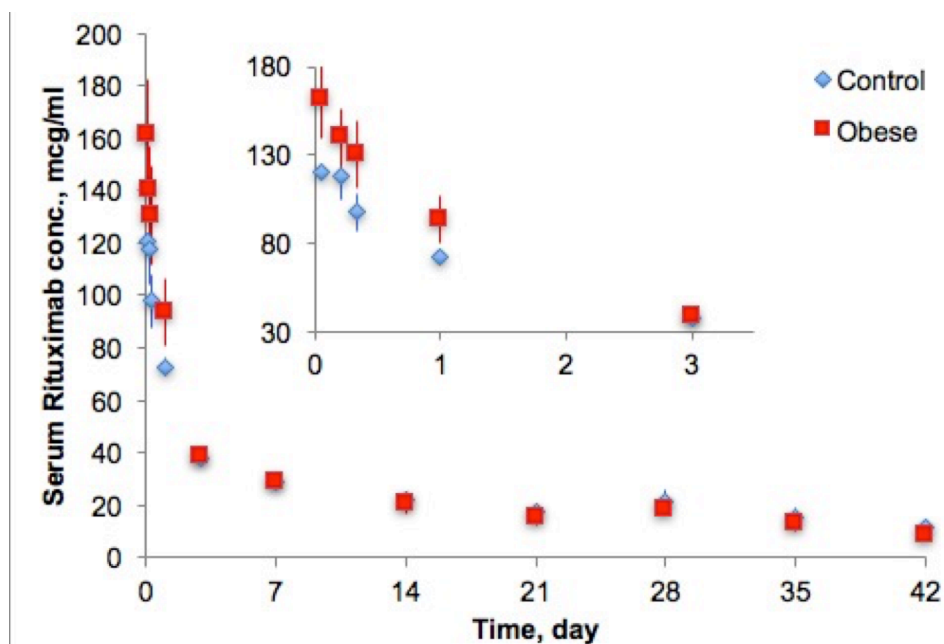
Figure 15 – Plasma concentration-time profiles of rituximab following intravenous administration (10 mg/kg) to obese and control rats.
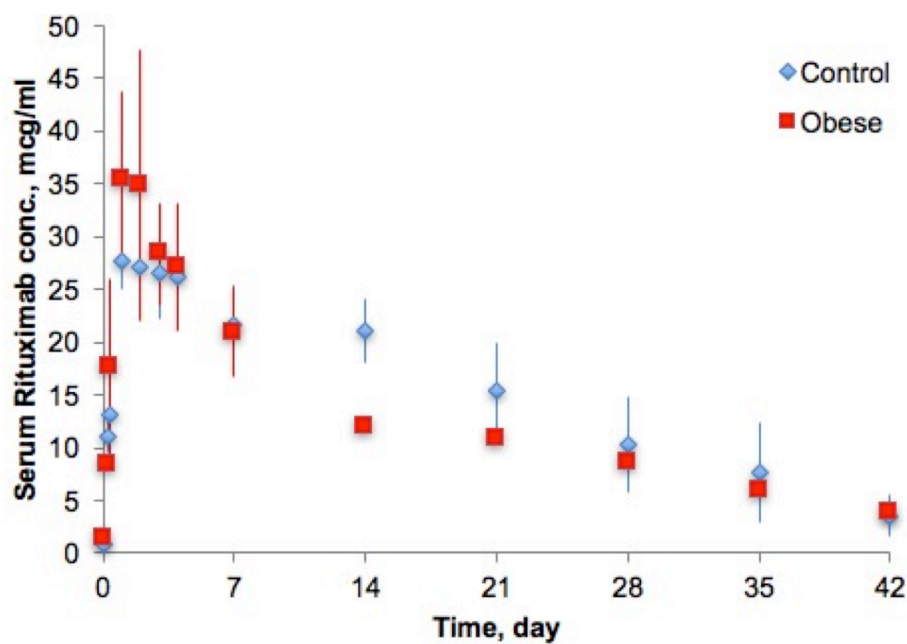

Figure 16 – Plasma concentration-time profiles of rituximab following subcutaneous administration (10 mg/kg) to obese and control rats.

Parameter	Units	Group			
		IV Obese	IV Control	SC Obese	SC Control
AUC	µg/mL•day	982	1000	530	632
CL or CL/F	mL/day/kg	8.93	7.96	16.76	14.83
VD or VD/F	mL/kg	149.2	179.4	287.3	176.6
Half-life	day	11.6	15.6	11.9	8.2

Table 4 – Pharmacokinetic parameters of rituximab in obese and control rats.

Development of the in-vitro permeability assay

Two different cell culture models were tested for developing an *in-vitro* screening method for assessment of permeability and subcutaneous absorption of mAbs.

The Caco-2 model is an established model for assessment permeability and prediction of oral absorption of small molecule drugs. The Caco-2 model was first validated using several small molecule compounds that are usually used as standards. The resulting permeability coefficient, Papp (mean ±SD, $x10^{-6}$), values for antipyrine, metoprolol, and propranolol were 11.1±0.5, 7.3±1.1, and 12.8±0.9 cm/s, respectively. While between lab variability in Caco-2 permeability is a known phenomenon these values are within the range of reported values [56]. However, when a permeability of mAbs was tested using this model no drug could be detected in the acceptor compartment. The lack of permeability can be potentially attributed to a high metabolic capacity of these cells or to the lack of transporting mechanism for antibodies.

The second tested model utilized HUVEC cells that has been utilized before as a cell model for endothelial cells and could be potentially more suitable for assessment of permeability of mAbs through blood capillaries. However, after multiple trials (with varying conditions) in establishing a barrier monolayer using these cells on transwell plates we could not observe a significant increase in transepithelial resistance or a

significant barrier for permeation of Lucifer yellow dye. The growing pattern in regular flasks was as expected. It appears that additional experimentation with growing conditions and coating maybe required for establishing this permeability model.

Conclusions

Optimization of dosing is important for achieving efficacious and safe medications. Published data and our study show that drug pharmacokinetics can be changed in obesity, and therefore, evaluation of such changes in models of obesity is important for drug development. In this work, we have developed bioanalytical methods that will allow for a rapid *in-vivo* assessment of changes in pharmacokinetics of various monoclonal antibodies in an animal model of obesity. We have also shown that serum concentration-time profiles were different between obese and control rats after intravenous and subcutaneous injection of rituximab. Developing *in-vitro* screening methods for prediction of subcutaneous absorption can facilitate pharmaceutical development of these drugs; however, additional research is needed to accomplish this goal.

Bibliography

1.   *Overview of ELISA*. 2016   [cited 2016 Nov. 14]; Available from: https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html.

2.   Ecker, D.M., S.D. Jones, and H.L. Levine, *The therapeutic monoclonal antibody market.* MAbs, 2015. **7**(1): p. 9-14.

3.   Hubatsch, I., E.G. Ragnarsson, and P. Artursson, *Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers.* Nat Protoc, 2007. **2**(9): p. 2111-9.

4.   Liu, J.K., *The history of monoclonal antibody development - Progress, remaining challenges and future innovations.* Ann Med Surg (Lond), 2014. **3**(4): p. 113-6.

5.   *Monoclonal Antibodies Approved by the EMA and FDA for Therapeutic Use (status 2013)*. 2013 23.12.2013 [cited 2016 Nov. 3]; Available from: http://www.actip.org/products/monoclonal-antibodies-approved-by-the-ema-and-fda-for-therapeutic-use/.

6.   Kagan, L. and D.E. Mager, *Mechanisms of subcutaneous absorption of rituximab in rats.* Drug Metab Dispos, 2013. **41**(1): p. 248-55.

7.   Dostalek, M., et al., *Pharmacokinetics, pharmacodynamics and physiologically-based pharmacokinetic modelling of monoclonal antibodies.* Clin Pharmacokinet, 2013. **52**(2): p. 83-124.

8.   McLennan, D.N., C.J. Porter, and S.A. Charman, *Subcutaneous drug delivery and the role of the lymphatics.* Drug Discov Today Technol, 2005. **2**(1): p. 89-96.

9.   Hansel, T.T., et al., *The safety and side effects of monoclonal antibodies.* Nat Rev Drug Discov, 2010. **9**(4): p. 325-38.

10.   Schroeder, H.W., Jr. and L. Cavacini, *Structure and function of immunoglobulins.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S41-52.

11.   Janeway CA Jr, T.P., Walport M, et al., *The structure of a typical antibody molecule.* Immunobiology: The Immune System in Health and Disease., 2001.

12.   *NCI Dictionary of Cancer Terms*. [cited 2017 Jan. 04]; Available from: https://www.cancer.gov/publications/dictionaries/cancer-terms?cdrid=470256.

13.   J., S. *Small Molecule, Peptide and Protein-Based Drugs - The Differences and Similarities*.   [cited 2017 Jan. 06]; Available from: http://www.portfoliomanagementsolutions.com/the-organization-of-pharmaceutical-rd/small-molecule-drugs-versus-biomolecular-drugs-biologics/.

14.   Declerck PJ, S.H., et al. *Small molecule versus biological drugs*. 2012 June 29, 2012   [cited 2017 Jan. 07]; Available from: http://www.gabionline.net/Biosimilars/Research/Small-molecule-versus-biological-drugs.

15.   Imai, K. and A. Takaoka, *Comparing antibody and small-molecule therapies for cancer.* Nat Rev Cancer, 2006. **6**(9): p. 714-27.

16.   Little, M., et al., *Of mice and men: hybridoma and recombinant antibodies.* Immunol Today, 2000. **21**(8): p. 364-70.

17. Modjtahedi H., A.S., Essapen S., *Therapeutic application of monoclonal antibodies in cancer: advances and challenges.* British Medical Bulletin, 2012. **104**(1): p. 41-59.

18. Drewe, E. and R.J. Powell, *Clinically useful monoclonal antibodies in treatment.* J Clin Pathol, 2002. **55**(2): p. 81-5.

19. Dychter, S.S., D.A. Gold, and M.F. Haller, *Subcutaneous drug delivery: a route to increased safety, patient satisfaction, and reduced costs.* J Infus Nurs, 2012. **35**(3): p. 154-60.

20. Kagan, L., et al., *Subcutaneous absorption of monoclonal antibodies: role of dose, site of injection, and injection volume on rituximab pharmacokinetics in rats.* Pharm Res, 2012. **29**(2): p. 490-9.

21. Richter, W.F., S.G. Bhansali, and M.E. Morris, *Mechanistic determinants of biotherapeutics absorption following SC administration.* AAPS J, 2012. **14**(3): p. 559-70.

22. Charman, S.A., et al., *Systemic availability and lymphatic transport of human growth hormone administered by subcutaneous injection.* J Pharm Sci, 2000. **89**(2): p. 168-77.

23. Chen, S.A., et al., *Plasma and lymph pharmacokinetics of recombinant human interleukin-2 and polyethylene glycol-modified interleukin-2 in pigs.* J Pharmacol Exp Ther, 2000. **293**(1): p. 248-59.

24. Porter, C.J., G.A. Edwards, and S.A. Charman, *Lymphatic transport of proteins after s.c. injection: implications of animal model selection.* Adv Drug Deliv Rev, 2001. **50**(1-2): p. 157-71.

25. Trubetskoy, V.S., et al., *Massage-induced release of subcutaneously injected liposome-encapsulated drugs to the blood.* J Control Release, 1998. **50**(1-3): p. 13-9.

26. Olszewski, W., et al., *Flow and composition of leg lymph in normal men during venous stasis, muscular activity and local hyperthermia.* Acta Physiol Scand, 1977. **99**(2): p. 149-55.

27. Swartz, M.A., *The physiology of the lymphatic system.* Adv Drug Deliv Rev, 2001. **50**(1-2): p. 3-20.

28. Aukland, K. and R.K. Reed, *Interstitial-lymphatic mechanisms in the control of extracellular fluid volume.* Physiol Rev, 1993. **73**(1): p. 1-78.

29. Reddy, S.T., et al., *A sensitive in vivo model for quantifying interstitial convective transport of injected macromolecules and nanoparticles.* J Appl Physiol (1985), 2006. **101**(4): p. 1162-9.

30. Strand, S.E. and L. Bergqvist, *Radiolabeled colloids and macromolecules in the lymphatic system.* Crit Rev Ther Drug Carrier Syst, 1989. **6**(3): p. 211-38.

31. Roopenian, D.C. and S. Akilesh, *FcRn: the neonatal Fc receptor comes of age.* Nat Rev Immunol, 2007. **7**(9): p. 715-25.

32. Shaughnessy, A.F., *Monoclonal antibodies: magic bullets with a hefty price tag.* BMJ, 2012. **345**: p. e8346.

33. Grainger, D.W., *Controlled-release and local delivery of therapeutic antibodies.* Expert Opin Biol Ther, 2004. **4**(7): p. 1029-44.

34. *Adult Obesity Facts.* 2016 [cited 2016 Oct. 3]; Available from: https://www.cdc.gov/obesity/data/adult.html.

35. *Obesity.* 2016 [cited 2016 Oct. 3]; Available from: https://medlineplus.gov/obesity.html.

36. *Defining Adult Overweight and Obesity.* 2016 [cited 2016 Oct. 3]; Available from: http://www.cdc.gov/obesity/adult/defining.html.

37.　*Obesity and overweight Fact sheet N311*, W.H. Organization, Editor. 2015.

38.　*Childhood Obesity Facts*. 2015　[cited 2016 Oct. 3]; Available from: https://www.cdc.gov/healthyschools/obesity/facts.htm.

39.　*Childhood Obesity Facts: Prevalence*. 2016　[cited 2016 Oct. 3]; Available from: https://www.cdc.gov/obesity/data/childhood.html.

40.　Staff, M.C. *Disease and Conditions: Obesity*. 2015　[cited 2016 Oct. 3]; Available from: http://www.mayoclinic.org/diseases-conditions/obesity/basics/definition/con-20014834?p=1.

41.　Crossley, N., *Global anti-corporate struggle: a preliminary analysis.* Br J Sociol, 2002. **53**(4): p. 667-91.

42.　Anderson, M., Matsa, DA *Are restaurants really super-sizing America*. 2007.

43.　Forshee, R.A., et al., *A critical examination of the evidence relating high fructose corn syrup and weight gain.* Crit Rev Food Sci Nutr, 2007. **47**(6): p. 561-82.

44.　Miles, R., et al., *Residential context, walking and obesity: two African-American neighborhoods compared.* Health Place, 2008. **14**(2): p. 275-86.

45.　Cawley, J., C. Meyerhoefer, and D. Newhouse, *The impact of state physical education requirements on youth physical activity and overweight.* Health Econ, 2007. **16**(12): p. 1287-301.

46.　in *Oxford Handbook of Medical Sciences (2nd edition)*. 2011: Oxford.

47.　Kushner, R., in *Treatment of the Obese Patient (Contemporary Endocrinology)*. 2007, Humana Press: Totowa, NJ.

48.　*Obesity Information*. 2014　[cited 2016 Oct. 3]; Available from: http://www.heart.org/HEARTORG/HealthyLiving/WeightManagement/Obesity/Obesity-Information_UCM_307908_Article.jsp - .V_PPy1dOJSU.

49.　*Obesity in art: A brief overview*. Frontiers of Hormone Research.

50.　Neuroscience, S.f. *Food for Thought: Obesity and Addiction*. 2012　[cited 2016 Oct. 3]; Available from: http://www.brainfacts.org/Across-the-Lifespan/Diet-and-Exercise/Articles/2012/Food-for-Thought-Obesity-and-Addiction.

51.　Ingrande, J. and H.J. Lemmens, *Dose adjustment of anaesthetics in the morbidly obese.* Br J Anaesth, 2010. **105 Suppl 1**: p. i16-23.

52.　Yolken, R.H., *Enzyme-linked immunosorbent assay (ELISA): a practical tool for rapid diagnosis of viruses and other infectious agents.* Yale J Biol Med, 1980. **53**(1): p. 85-92.

53.　*The enzyme-linked immunosorbent assay (ELISA).* Bull World Health Organ, 1976. **54**(2): p. 129-39.

54.　Chan, L.J., et al., *PEGylation does not significantly change the initial intravenous or subcutaneous pharmacokinetics or lymphatic exposure of trastuzumab in rats but increases plasma clearance after subcutaneous administration.* Mol Pharm, 2015. **12**(3): p. 794-809.

55.　Wang, W., et al., *Lymphatic transport and catabolism of therapeutic proteins after subcutaneous administration to rats and dogs.* Drug Metab Dispos, 2012. **40**(5): p. 952-62.

56.　Lee, J.B., et al., *Quantitative analysis of lab-to-lab variability in Caco-2 permeability assays.* Eur J Pharm Biopharm, 2017.