Image-Guided Predictions of Liposome Transport in Solid Tumours

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Medical Biophysics
University of Toronto

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Doctor of Philosophy
Medical Biophysics
University of Toronto
2014

Abstract
Due to the ability to preferentially accumulate and deliver drug payloads to solid tumours, liposomes have emerged as an exciting therapeutic strategy for cancer therapy. Unfortunately, the initial excitement was dampened by limited clinical results, where only negligible increases in patient survival following liposome therapy have been observed. What are the reasons for the limited clinical efficacy? Is the nanoparticle formulation optimal? Is the enhanced permeability and retention effect overstated? What are the barriers limiting the delivery of drugs to cancer cells? What is the optimal dosing and treatment schedule? Addressing these questions requires developing quantitative tools to understand the behaviour of liposomes in vivo, such as pharmacokinetics, biodistribution, intra-tumoural accumulation, and drug release. Central to each of these questions is the concept of transport – the collection of biophysical processes responsible for the delivery of molecules to tissues. Understanding transport means understanding the crucial links between the spatio-temporal accumulation of liposomes, the physicochemical properties of liposomes, and properties of the tumour microenvironment. In this thesis, a biophysical mathematical transport model is developed that when used in combination with non-invasive imaging methods can predict liposome transport in solid tumours. The mathematical transport framework is validated in its ability to predict the bulk and intra-tumoural accumulation of liposomes based on biophysical transport properties of solid tumours. Furthermore, novel imaging methods are developed and used to elucidate the crucial links between transport barriers and spatial heterogeneity in liposome accumulation. Finally, methods are presented to integrate quantitative imaging and mathematical modelling such that an accurate prediction of liposome transport in solid tumours is possible. In summary, this thesis presents and validates an image-guided mathematical framework that can be used to guide the rational application of liposomes for the treatment of solid tumours.
Acknowledgments

Roads? Where we’re going we don’t need roads. I’m lucky enough to have worked with a group of stellar researchers that have taught me the art of science. Particularly my supervisory committee: David Jaffray, Christine Allen, Mike Milosevic and Ian Tannock, without whom my success would have been precarious at best. To my supervisors David Jaffray and Christine Allen, I’m indebted to you. Your support, enthusiasm, and tireless effort in helping me move not only my project but also my career forward has been remarkable and greatly appreciated. David, your ability to create a supportive and creative environment is inspirational and I will do my best to copy it during my tenure as a scientist. Christine, our discussions on creating a successful scientific career has really encouraged me to brave the uncertain world of being an academic. I consider you both more than supervisors, but also friends. To Ian Tannock and Mike Milosevic, your insight and guidance has been matchless and your ability to always make time for me remarkable. As well, I’ve had the pleasure of working within the Hypoxia program at the Ontario Cancer Institute. It was extraordinary to work with such a highly productive and collaborative group of scientists. Thank you to all the people at STTARR who have helped me navigate the black hole of paper work and policies. I owe a great deal to the EIRR21 and NSERC scholarship programs, which have helped open doors during my academic career. A special thanks to all the summer students that I’ve had the pleasure of working with over the past five years. Most importantly thank you to Merle Casci, Chau Dang, Daphne Sears and Lothar Lilge who have provided an amazing level of support within the Department of Medical Biophysics.

Being a successful researcher is more than just doing great science, it also involves having people to laugh and commiserate with. I have made an amazing group of friends and network of colleagues. It has been a pleasure to serve on the Medical Biophysics Graduate Student Association in many different capacities. I got to work with a great group of inspired students who worked hard to make graduate student life in Medical Biophysics as fun as possible. The most important people have been my friends and family who have helped keep me sane over my graduate degree. Tracy Lui, simply put you’re the best. Every adventure is infinitely more exciting when you’re there. Greg Anderson, my brother in arms. Jocelyn Stewarts, whose intelligence knows no bounds. Jen Wolter, your smile brings a room to life. TD, a crucial member of the Fine Spirits Club. Jarvin (i.e. Janet and Arvin), Ross Williams, Keira Pereira, the Sunnybrook crew, the softball and volleyball teams, and the list goes on and on. Finally to my Mom, sisters, brothers, aunts, uncles and niece, thank you for the support. It’s been a long road!
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<td>AUC</td>
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<td>Area under the curve</td>
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<tr>
<td>CA</td>
<td>$C_a$</td>
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<td>CFC</td>
<td>$L_pS/V$</td>
<td>Capillary filtration coefficient</td>
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<td>CT</td>
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<td>Dynamic contrast enhanced computed tomography</td>
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<tr>
<td>ECM</td>
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<td>Extracellular matrix</td>
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<td>EPR</td>
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</tr>
<tr>
<td>Gd-DTPA</td>
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<td>Gadolinium diethylene-triamine penta-acetic acid</td>
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<td>H520</td>
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<td>HU</td>
<td>$P_i$</td>
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<td>VFI</td>
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<th>Title</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>$J_v$ ($cm^3 \cdot s^{-1}$)</td>
<td>Rate of transvascular fluid flow</td>
<td>The volumetric rate of fluid flow across the endothelium</td>
</tr>
<tr>
<td>$V$ ($cm^3$)</td>
<td>Volume of tissue</td>
<td>The sum of cellular, interstitial, and vascular volumes</td>
</tr>
<tr>
<td>$L_p$ ($cm \cdot mmHg^{-1} \cdot s^{-1}$)</td>
<td>Vascular hydraulic conductivity</td>
<td>Resistance to fluid flow across the endothelium</td>
</tr>
<tr>
<td>$S/V$ ($cm^{-1}$)</td>
<td>Surface area of blood vessels per unit volume of tissue</td>
<td>The total surface area of blood vessels within a volume of tissue</td>
</tr>
<tr>
<td>$P_i$ (mmHg)</td>
<td>Interstitial fluid pressure</td>
<td>The pressure felt by solid tissue components (e.g. cells) due to the presence of interstitial fluid</td>
</tr>
<tr>
<td>$P_v$ (mmHg)</td>
<td>Microvascular fluid pressure</td>
<td>The blood pressure in vessels that comprise microcirculation (e.g. capillaries)</td>
</tr>
<tr>
<td>$P_e$ (mmHg)</td>
<td>Effective pressure</td>
<td>The interstitial pressure that yields zero net volume of fluid flux out of the vasculature</td>
</tr>
<tr>
<td>$P_{ss}$ (mmHg)</td>
<td>Steady-state pressure</td>
<td>The interstitial pressure at which the efflux from the vasculature equals the influx into the lymphatics</td>
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<tr>
<td>$\sigma$</td>
<td>Reflection Coefficient</td>
<td>The fraction of molecules unable to transport across the endothelium due to size and charge constraints</td>
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<tr>
<td>$v_i$ ($cm \cdot s^{-1}$)</td>
<td>Interstitial fluid velocity</td>
<td>Velocity of fluid through the porous interstitial space of tissue</td>
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<tr>
<td>$K$ ($cm^2 \cdot mmHg^{-1} \cdot s^{-1}$)</td>
<td>Interstitial hydraulic conductivity</td>
<td>Resistance to fluid flow through the interstitium</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Conductivity Ratio</td>
<td>Measure of the ratio of interstitial to vascular resistance to fluid flow</td>
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<tr>
<td>$f$</td>
<td>Retardation coefficient</td>
<td>The fraction of molecules unable to transport through the interstitium due to size and charge constraints</td>
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<tr>
<td>$P$ ($cm \cdot s^{-1}$)</td>
<td>Microvascular diffusion/permeability coefficient</td>
<td>The rate that molecules diffuse across the endothelium</td>
</tr>
<tr>
<td>$D$ ($cm^2 \cdot s^{-1}$)</td>
<td>Interstitial diffusion coefficient</td>
<td>The rate that molecules diffuse across the interstitium</td>
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<tr>
<td>$C_i$ ($mg \cdot kg^{-1}$)</td>
<td>Interstitial concentration</td>
<td>Number of nanoparticles in the interstitial space per unit volume of tissue</td>
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<td>$C_{i,M}^m$ ($mg \cdot kg^{-1}$)</td>
<td>Interstitial concentration</td>
<td>Interstitial concentration of small molecules ($m$) and nanoparticles ($M$)</td>
</tr>
<tr>
<td>$T_{ss}^{m,M}$ (min)</td>
<td>Steady state time</td>
<td>The time to reach steady state plasma and interstitial concentration for small molecules ($m$) and nanoparticles</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
<td>Units</td>
</tr>
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<td>--------</td>
<td>------------</td>
<td>-------</td>
</tr>
<tr>
<td>$C_a$</td>
<td>Arterial blood concentration</td>
<td>$mg \cdot kg^{-1}$</td>
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<td>$Hct_{aorta}$</td>
<td>Arterial Hematocrit</td>
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<tr>
<td>$C_p$</td>
<td>Plasma concentration</td>
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<tr>
<td>$C_p^N M$</td>
<td>Plasma concentration of small molecules (m) and nanoparticles (M)</td>
<td>$mg \cdot kg^{-1}$</td>
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<tr>
<td>$\varepsilon_i$</td>
<td>Interstitial volume fraction</td>
<td>$ml \cdot ml^{-1}$</td>
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<tr>
<td>$\varepsilon_p$</td>
<td>Plasma volume fraction</td>
<td>$ml \cdot ml^{-1}$</td>
</tr>
<tr>
<td>$\varepsilon_p^{lipo}$</td>
<td>Plasma volume fraction using liposomes</td>
<td>$ml \cdot ml^{-1}$</td>
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<td>$F_p/V$</td>
<td>Perfusion</td>
<td>$ml \cdot min^{-1} \cdot ml^{-1}$</td>
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<tr>
<td>$PS/V$</td>
<td>Permeability surface area product per unit volume</td>
<td>$ml \cdot min^{-1} \cdot ml^{-1}$</td>
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<td>$K_{trans}$</td>
<td>Permeability/blood flow product</td>
<td>$ml \cdot min^{-1} \cdot ml^{-1}$</td>
</tr>
<tr>
<td>$E$</td>
<td>Extraction fraction</td>
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<td>$AUC_{iox}$</td>
<td>Area under the time intensity curve for free iohexol</td>
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</tr>
<tr>
<td>$AUC_{lipo}$</td>
<td>Area under the time intensity curve for liposomes</td>
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<tr>
<td>$C_{peak}$</td>
<td>Peak interstitial concentration of liposomes</td>
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</tr>
<tr>
<td>$r$</td>
<td>Radial position</td>
<td></td>
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<td>$R$</td>
<td>Tumour Radius</td>
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Chapter 1

Introduction
In order to reach target cells, nanoparticle drug delivery systems, such as liposomes, must transport through the systemic circulation, extravasate from blood vessels, and penetrate the interstitial tissue. The transport of nanoparticles in solid tumours is often described by the enhanced permeability and retention (EPR) effect (Matsumura & Maeda 1986). The EPR effect is an empirical description of the preferential accumulation of nanoparticles at tumour sites due to leaky vasculature (i.e. enhanced permeability) and impaired lymphatic drainage (i.e. enhanced retention), in comparison to normal tissue (Figure 1.1). The EPR effect is the rationale behind the development of numerous types of high molecular weight (MW) drug delivery vehicles, including polymeric nanoparticles, micelles and liposomes (Peer et al. 2007). Liposomes are one of only two clinically approved nanoparticle drug delivery systems used in oncology and have been shown to result in significant increases in tumour accumulation of drugs in comparison to that achieved following administration of free drug (Gabizon 1992; Drummond et al. 1999; Harrington et al. 2001). Yet, despite demonstrating substantial accumulation of drug in many pre-clinical and human tumours, clinically approved liposome formulations, such as Doxil®/Caelyx® (pegylated liposomal doxorubicin) and Myocet® (unpegylated liposomal doxorubicin), have only resulted in a modest increase in anti-tumour efficacy in many human cancers relative to the standard of care (Table 1.1). There are several major limitations of liposome-based drug delivery, including: (1) variability in the EPR effect and therefore, total tumour accumulation (Lammers et al. 2012); (2) limited tumour penetration (Yuan et al. 1994); and (3) slow or limited release of hydrophilic/amphiphilic drugs (Laginha et al. 2005; White et al. 2006).

Effective response to chemotherapy depends on the delivery of drugs to cancer cells at cytotoxic concentrations and therefore heterogeneity in both total tumour accumulation and intra-tumoural distribution remain major challenges for liposome-based therapeutics (Garcia et al. 1998; Gordon et al. 2000; O’Brien et al. 2004). Indeed, several pre-clinical studies have shown that achieving both an adequate concentration and consistent intra-tumoural distribution of cytotoxic drugs is crucial to the successful outcome of liposome-based chemotherapy (Huang et al. 1994; Kong & Dewhirst 1999; Kong et al. 2000; Ponce et al. 2007; Seynhaeve et al. 2007). The clinical challenge for nanoparticle drug delivery is the inability to assess if all cancer cells within a solid tumour are exposed to a sufficient concentration of cytotoxic agent.
Figure 1.1 The EPR Effect. Blood vessels in tumour tissue are leaky causing increased extravasation of molecules into the interstitial space. Small molecules extravasate on the order of seconds to minutes, while macro-molecules and nanoparticles take minutes to days. Tumour lymphatic vessels are non-functioning resulting in the retention of macro-molecules; meanwhile small molecules diffuse back into blood vessels and are cleared systemically. Conversely, blood vessels in most normal tissue are less leaky, which limits the extravasation of macro-molecules and, to a lesser extent, small molecules. Furthermore, functional lymphatics are present in healthy tissue and serve to remove molecules.

Recent advances in liposome design have resulted in the development of “image-able” systems allowing for non-invasive and quantitative in vivo detection of the drug delivery vehicle using imaging modalities such as gamma camera imaging or single photon emission computed tomography (SPECT) (Gabizon et al. 1991; Harrington et al. 2001), positron emission tomography (PET) (Seo et al. 2008), optical (Huang et al. 2013), magnetic resonance imaging (MRI) (Zheng et al. 2006), and computed tomography (CT) (Zheng et al. 2009). The application of imaging can be used for pharmacokinetic and biodistribution analysis, and to reveal the extent of inter-subject and intra-tumoural heterogeneity in liposome accumulation (Figure 1.2).
Table 1.1 Nanoparticle formulations clinically approved for the treatment of solid tumours. Reproduced from Jain (2012).

The ability to measure the intra-tumoural accumulation of liposomes using imaging provides an important tool to determine if an adequate intra-tumoural concentration of cytotoxic drugs has been achieved; however, it is difficult to determine from imaging alone what biological barriers have contributed to poor accumulation and distribution. This knowledge is paramount for the rational development of novel and more efficacious formulations, for the development of strategies to improve the intra-tumoural accumulation of liposomes, for treatment planning, and potentially for treatment response assessment (Lammers et al. 2010). It may be possible to identify the biological transport barriers using quantitative imaging by: (1) developing image-based methods to quantify properties of the tumour microenvironment that correlate with liposome accumulation, and provide a predictive capacity for response assessment; or (2) developing a mathematical model that describes the causal relationship between biophysical transport properties of tumour and liposome accumulation. The latter
is similar to CT and MRI based methods of quantifying vascular properties of tumours by detecting the kinetics of low MW contrast agents (CA) (O’Connor et al. 2011). In fact the combination of both approaches is ideal, whereby correlations between image derived metrics of transport and liposomes accumulation can be used to inform, refine, and ultimately increase the robustness of a mathematical model of liposome transport in tissue.

**Figure 1.2** Many imaging modalities provide the ability to track the pharmacokinetics, biodistribution, and inter/intra-tumoural accumulation of liposomes including: (1) computed tomography (Stapleton et al. 2013b); (2) optical imaging (unpublished data of optically labeled liposomes administered to mice bearing DsRed transfected cervix cancer cells implanted in a window chamber and imaged with two photon-microscopy); (3) magnetic resonance imaging (unpublished data of an MRI-Liposome contrast inject injected into mice bearing a patient derived primary cervix tumour); and (4) Scintigraphy/Nuclear Imaging (Harrington et al. 2001). Arrows denote the tumour mass.
The goal of this thesis is to develop and validate a biophysical mathematical model of transport that describes the delivery, extravasation, interstitial penetration, and retention of liposomes in solid tumours. Developing an accurate mathematical model of liposome transport in solid tumours requires understanding of the biophysical transport processes in tissue and the biological barriers that hinder accumulation. Furthermore, the accuracy of model-based predictions requires developing methods to independently inform model parameters, for example using quantitative imaging to derive metrics that reflect the tissue transport dynamics. The concept of using a mathematical model of liposome transport in combination with imaging provides an important tool for understanding fluid dynamics, and drug delivery barriers in solid tumours. It provides a theoretical basis to understand variability in the EPR effect and a framework for liposomal based drug delivery treatment planning.

In this chapter the biophysical processes that drive liposome transport in the systemic circulation, across blood vessel walls (endothelium), and through tissue are reviewed. Emphasis is placed on specific barriers of the tumour microenvironment that contribute to heterogeneous accumulation of liposomes. Mathematical approaches to model transport in tissue are reviewed and a framework for applying mathematical transport models to imaging data is presented.
Table 1.2 Properties related to fluid velocity in different biological tissues. N denotes normal tissue and T denotes tumour tissue. Mean fluid velocity across vessel walls and through the interstitial space consists of both direct and extrapolated measurements. Data compiled from various sources (Guyton et al. 1971; Hargens et al. 1981; Jain 1987a; b; 1988; Heldin et al. 2004; Truskey et al. 2004; Swartz & Fleury 2007; Hompland et al. 2012; Wiig & Swartz 2012).

1.1 Transport of Liposomes in Biological Systems: Convection and Diffusion

The transport of molecules through the systemic circulation, across blood vessel walls, and into the interstitial tissue occurs by convection and diffusion. Convection is the bulk flow of fluid as a result of a spatial pressure gradient, whereby the fluid acts as a carrier medium for molecules. Diffusion is driven by the random thermal motion of fluid molecules, resulting in rapid molecular collisions and transport of molecules from a region of higher to lower concentration. The relative importance of these two transport processes depends on the biological length scale, physicochemical properties (e.g. molecular weight, size, shape, charge, etc.) of the molecule being transported, and tissue properties (e.g. fluid viscosity, fluid pressure, porosity and permeability). In humans the biological length scales range over eight orders of magnitude (Figure 1.3). In mice, which are typically used to study cancer-drug interactions, the length scales range over seven orders of magnitude. In a healthy subject, convection typically dominates for transport at longer biological length scales (i.e. transport through the systemic circulation), while diffusion typically dominates for transport at smaller biological length scales (i.e. transport between cells). The Péclet number simple method to determine the relative importance of convection and diffusion based on experimental measurements. The Péclet number is defined as the ratio of the rate of mass transport by convection to diffusion (Truskey et al. 2004). When the Péclet number is less than one, diffusion is the dominant transport process, while a Péclet number greater than one signifies that convection dominates.
Liposomes typically have Péclet numbers greater than 1 indicating that convection is the predominant transport method in (a) moving blood, (b) across the capillary endothelium, and (c) through the interstitial space of tissue (see top most region of each graph). The x-axis represents the range of fluid velocity for a given tissue determined experimentally. The bars on the top indicate the range of fluid velocity in different normal and tumour tissues. The left y-axis shows the hydrodynamic radius for different molecules (indicated on the right y-axis). The y-axis is also representative of the rate of diffusion in a given tissue, whereby larger molecules tend to diffuse at a slow rate compared to smaller molecules. The dashed line corresponds to a Péclet number of 1.

Using data reported in the literature, it is possible to estimate the Péclet number for different normal and tumour tissues, different transport compartments (systemic circulation, microvasculature, interstitial space), and different molecules. Figure 1.4 shows the Péclet number of different tissues and different molecules, based on the transport properties given in Table 1.2 and Table 1.3. Transport through the systemic circulation of normal and tumour tissue occurs predominantly by convection for all molecules (Figure 1.4a). This is particularly true for nanoparticles that are similar in size to liposomes (MW > 10 MDa, HR = 45 nm), where the Péclet number is consistently greater than $10^5$ for all blood velocities. However, the spatio-temporal heterogeneity of blood flow in tumour capillaries results in flow stasis, suggesting that conditions exist where diffusion may contribute to liposome transport in plasma.

Transvascular transport (extravasation) of molecules in healthy and tumour tissue occurs by a combination of diffusion and convection (Figure 1.4b). Convection dominates for large molecules or for high transvascular fluid velocities, while diffusion dominates for small molecules or low transvascular fluid velocities. The extravasation of nanoparticles, including liposomes, occurs predominantly by
convection in normal and tumour tissues. Transvascular convective transport requires a spatial gradient between the microvascular pressure (MVP) and interstitial fluid pressure (IFP). It is generally accepted that a transvascular pressure gradient does not exist in the central region of tumours and therefore diffusion is the only mechanism for the extravasation (Jain 1987a; Baxter & Jain 1989; Boucher & Jain 1992). In Section 1.2.3, some data is presented that suggests intermittent transvascular pressure gradients may exist within the tumour volume. Regardless, the rate of diffusion of liposomes across the vessel wall is generally exceedingly slow. Based on the estimated microvascular permeability coefficient (Table 1.3), it may take liposomes up to 13 hrs to diffuse across the endothelium. Comparatively, simulations have indicated that in the presence of modest transvascular pressure gradients, convection driven liposome extravasation is estimated to be on the order of tens of minutes (Rippe & Haraldsson 1987). Therefore, transvascular transport of liposomes is typically dominated by convection; however, under certain circumstances the slow process of diffusion may be the only available mechanism of transvascular transport.

Interstitial transport (penetration) of molecules occurs by a combination of diffusion and convection (Figure 1.4c). Similar to transvascular transport, convection dominates for large molecules or for high transvascular fluid velocities, while diffusion dominates for small molecules or low transvascular fluid velocities. However, IFP is elevated in central tumour regions, which in turn abolishes interstitial fluid flow and results in diffusion as the dominant mechanism of interstitial transport (Jain 1987; Boucher and Jain 1992; Chauhan, Stylianopoulos et al. 2011). The reported rate of interstitial diffusion for liposomes varies considerably, but is in general exceedingly slow (Table 1.3). For example, using a random walk model of 3D diffusion and the range of reported diffusion coefficients for liposomes, it may take anywhere between 4 hours to 10 days for liposomes (MW > 10 MDa, HR = 45nm) to diffuse 100 μm in tumour tissue, and 4 to 240 days to diffuse 500 μm. At the tumour periphery, IFP drops precipitously, resulting in substantial interstitial fluid velocities and predominantly convection driven interstitial transport of liposomes.

In general, analysis of the Péclet number indicates that convection is the predominant transport mechanism for liposomes through the system circulation, across the capillary endothelium, and through the interstitial space of tumour tissue. Therefore, mathematical modelling of liposome transport in
tumour tissue should incorporate the mechanisms and barriers of convection driven transport. This requires a firm understanding of the nature of fluid dynamics within the tumour volume, and is the subject of the next section. It is important to note that the reported Péclet numbers are approximations that are highly dependent on the accuracy of experimental data used to inform the calculations. In some instances, the availability of measurements for both convective and diffusive based transport of molecules in different tissues is limited. For example, only one measurement of transvascular fluid velocity in tumours has been reported (Sevick & Jain 1991). There is a paucity of data on steric hindrance and electrostatic interactions between molecules and the endothelium or interstitial components. Furthermore, in vivo measurements of transvascular and interstitial diffusive transport are inherently biased by the presence of convection, and thus diffusion coefficients represent an effective rate of transport that includes both mechanisms (Jain 1987a). Simulations have suggested that convection accounts for at least 90% of the measured effective microvascular diffusion permeability coefficient, or put another way only, 10% of the effective microvascular permeability coefficient is due to diffusion (Curry 1984; Rippe & Haraldsson 1987). It is expected that as experimental techniques are refined and more measurements are made, a more complete picture of the relationship between convection, diffusion, and nanoparticle transport will emerge.

1.2 Pathophysiology of Fluid Dynamics in Solid Tumours

Fluid pressure gradients play an active role in maintaining tissue homeostasis by promoting interstitial fluid flow, which transports nutrients and waste products between blood vessels and cells, and macromolecules, such as antigens and cytokines, to local draining lymph nodes (Wiig & Swartz 2012). Fluid pressure gradients may also play an important role in regulating blood flow (Milosevic et al. 1999) and modulating important biological processes that contribute to morphogenesis, remodelling of the extracellular matrix (ECM), cell migration, and cell-cell signaling (Rutkowski & Swartz 2007; Swartz & Fleury 2007; Wiig & Swartz 2012). In healthy tissue, the relationship between fluid pressure and structural, physiological, and molecular factors allows cells to thrive, tissues to grow, and organs to function. In cancer, fluid pressure gradients become dysregulated, which leads to abnormal fluid flow and consequently reduced nutrient delivery and waste removal, as well as an altered immune microenvironment. The abnormal pressure gradients are also associated with more aggressive tumour behaviour such as increased metastatic potential and altered cellular function (Rutkowski & Swartz 2007; Lunt et al. 2008; Swartz & Lund 2012). The culmination of direct and indirect effects of abnormal
pressure gradients in solid tumours contributes to inter- and intra-tumoural heterogeneity of the EPR effect, manifests as a barrier to drug delivery (Baxter & Jain 1989; Minchinton & Tannock 2006), and contributes to impaired response to radiation therapy (Milosevic et al. 1999; Ueki et al. 2002; Yeo et al. 2009). In this section, the pathophysiology of abnormal fluid pressure gradients in solid tumours is reviewed with focus placed on the relationship between fluid flow and liposome transport.

1.2.1 Pressure Gradients in Normal tissue

The total pressure experienced by tissue is the combination of solid tissue pressure and interstitial fluid pressure (Guyton et al. 1971). Solid tissue pressure (STP) is the result of direct contact between solid elements such as cells (epithelial, fibroblasts, and immune cells), ECM proteins (collagen, elastin and fibronectin), and interstitial gel-like material composed primarily of hyaluronic acid. STP arises from tissue growth (cell division and vascular remodelling), tissue deformation (breathing), and cellular contraction (heart beating). IFP is the hydrostatic force exerted on the interstitium due to the accumulation of fluid. IFP arises from convection driven fluid flow out of capillaries, through the interstitium, and into lymphatic vessels or back into post-capillary venules via osmosis. Due to the poro-elastic nature of the interstitium, STP and IFP are intrinsically coupled.

In normal tissue, pressure gradients are actively regulated. STP gradients are regulated by: (1) cell contraction, which in turn applies mechanical stress on the ECM; (2) re-alignment of fibroblast cells to locally shield from stress; and (3) ECM remodelling to modulate the tension between cells and other solid components (Discher et al. 2005; Wiig & Swartz 2012). IFP is regulated by the relationships among key determinants of fluid flow into, through and out of the interstitium: trans-capillary fluid flow, interstitial fluid flow, and trans-lymphatic fluid flow. The steady-state (i.e. time independent) trans-capillary fluid flow into the interstitium is typically described using Starling’s law (Equation 1.1), that relates the trans-membrane plasma flux to hydrostatic and colloidal osmotic pressure differences between the capillary and interstitium (Figure 1.5a). Mathematically Starling’s law is given by,

\[ J_v = L_p S \left[ (P_v - P_i) - \sigma (\Pi_v - \Pi_i) \right] \] 1.1
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Tissue</th>
<th>MW (Da)</th>
<th>Hydrodynamic Radius (nm)</th>
<th>Plasma Diffusion Coefficient (cm² s⁻¹)</th>
<th>Microvascular Permeability Coefficient (cm s⁻¹)</th>
<th>Interstitial Diffusion Coefficient (cm² s⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>N</td>
<td>32</td>
<td>0.12</td>
<td>2.7x10⁻⁵*</td>
<td>3.6x10⁻³</td>
<td>3.6x10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.8x10⁻⁵</td>
</tr>
<tr>
<td>Urea</td>
<td>N</td>
<td>60</td>
<td>0.18</td>
<td>1.5x10⁻⁵</td>
<td>3.6x10⁻³</td>
<td>8.2x10⁻⁶‡</td>
</tr>
<tr>
<td>Creatinine</td>
<td>N</td>
<td>113</td>
<td>0.3</td>
<td>8.7x10⁻⁶</td>
<td></td>
<td>5.1x10⁻⁶‡</td>
</tr>
<tr>
<td>Evans Blue</td>
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<td>1.31</td>
<td>5.6x10⁻⁷†</td>
<td>1.2x10⁻⁵</td>
<td>1.0x10⁻⁶‡</td>
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<tr>
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<td>N</td>
<td>16,000</td>
<td>2.8</td>
<td>2.4x10⁻⁶</td>
<td></td>
<td>1.2x10⁻⁷‡</td>
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<tr>
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<td>67,000</td>
<td>3.5</td>
<td>1.9x10⁻⁷†</td>
<td>1.4x10⁻⁷</td>
<td>4.3x10⁻⁸‡</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.9x10⁻⁸‡</td>
</tr>
<tr>
<td>IgG</td>
<td>N</td>
<td>15x10⁴</td>
<td>7.5</td>
<td>8.7x10⁻⁸†</td>
<td>2.8x10⁻⁷</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2.0x10⁻⁸</td>
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<tr>
<td>Dextran</td>
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<td>1.7x10⁻⁸</td>
<td>3.3x10⁻⁹‡</td>
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<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>45</td>
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<td>10⁻⁹ - 10⁻¹¹</td>
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<td>T</td>
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</tbody>
</table>

Table 1.3 Diffusion coefficients in different mediums (water, plasma, endothelial, interstitial tissue) at physiological temperature of 37°C. *measurements made in water at 37°C. †Estimated plasma diffusion coefficient using the Stokes-Einstein equation. ‡Estimated interstitial diffusion coefficient using the empirical relationship developed by Swabb et al. (1974). Data compiled from various sources (Swabb et al. 1974; Gerlowski & Jain 1986; Jain 1987a; b; Wu et al. 1993; Yuan et al. 1994; El-Kareh & Secomb 1997; Dreher et al. 2006). Note that the microvascular permeability coefficients for liposomes were scaled to 10% of the literature reported values to remove the contributions of convection (Curry 1984; Rippe & Haraldsson 1987).

where \( J_p \) is plasma flux (cm³/s), \( L_p \) is the hydraulic conductivity coefficient (permeability to fluid) of the capillary wall (cm mmHg⁻¹ s⁻¹), \( S \) is the surface area of the vessel (cm²), \( P_v \) and \( P_i \) are the microvascular and interstitial hydrostatic pressures respectively (mmHg), \( \sigma \) is the osmotic reflection coefficient (a dimensionless number between 0 and 1), and \( \Pi_v \) and \( \Pi_i \) are the capillary and interstitial colloid osmotic pressures respectively (mmHg). Interstitial fluid flow is driven by IFP gradients and influenced by the hydraulic conductivity of the tissue (Figure 1.5b). Steady-state fluid velocity through a porous medium is described by Darcy’s law,
where $v_i$ is the interstitial fluid velocity ($\text{cm s}^{-1}$), $K$ is the hydraulic conductivity coefficient (resistance to fluid flow) of the interstitium ($\text{cm}^2 \text{ mmHg}^{-1} \text{ s}^{-1}$), and $\nabla P_i$ is the spatial IFP gradient. The parameter $K$ is an effective property that represents the combination of the pore size, ECM fibre arrangement, interstitial fluid viscosity, and volume of the interstitial space (Swartz & Fleury 2007).

The mechanisms driving trans-lymphatic fluid flow are still an active area of research, but it is generally accepted that IFP and interstitial fluid volume are primarily responsible. As interstitial fluid volume increases due to capillary filtration, strain is placed on the ECM that in turn places strain on lymphatic endothelial cells through anchoring filaments (Aukland & Reed 1993; Swartz 2001). The strain opens gaps between lymphatic endothelial cells, allowing pressure driven trans-lymphatic flow of interstitial fluid. Mathematical modelling of trans-lymphatic transport is still in its infancy, and attempts to describe lymph transport using Starling’s law have proven insufficient (Swartz 2001; Margaris & Black 2012). While several mathematical theories of trans-lymphatic transport have been proposed (Margaris & Black 2012), it is sufficient to understand the genesis of IFP gradients by assuming a trans-lymphatic fluid flow rate. Due to conservation of mass, the interstitial fluid velocity is equal the trans-capillary fluid flux (Equation 1.1) minus the trans-lymphatic fluid flux. That is,

$$v_i = J_v - J_L \tag{1.3}$$

where $J_L$ is the lymphatic fluid flux ($\text{cm}^3/\text{s}$). Combining Equations 1.2 and 1.3 reveals that steady-state IFP gradients are driven by the capillary and lymphatic hydrostatic pressures, capillary osmotic pressure, and are regulated by the hydraulic conductivities of the capillary wall, the interstitium, and lymphatic wall (Equation 1.4). All of the properties work in unison to regulate IFP in normal tissue, maintaining it close to or slightly less than atmospheric pressure.

$$\nabla^2 p_i = \left( \frac{\alpha}{V^{1/3}} \right)^2 (P_i - P_{ss}) \tag{1.4}$$
Figure 1.5 (a) The rate of trans-capillary fluid flow is related to the hydrostatic and osmotic pressure gradients between the vascular and interstitial spaces, as well as the permeability / hydraulic conductivity of the capillary endothelium (Equation 1.1). (b) The interstitial fluid velocity is driven by a spatial gradient in interstitial fluid pressure and depends on the hydraulic conductivity of the interstitial tissue (Equation 1.2). The interstitial hydraulic conductivity depends in part on the composition of the ECM.

where $\alpha = V^{1/3} \sqrt{(L_p S - L_{pL} S_L)/KV}$ is a measure of the ratio of interstitial to vascular resistance to fluid flow (unitless), $L_{pL} S_L$ is the lymphatic filtration coefficient (cm mmHg$^{-1}$ s$^{-1}$), and $P_{ss} = [L_p S (P_v - \sigma (P_i - P_i)) - L_{pL} S_L P_L]/[L_p S - L_{pL} S_L]$ is the steady-state pressure where the efflux of fluid from the vasculature equals the influx into the lymphatics (mmHg). The parameter $\alpha$ relates the effects of tissue growth, lymphatic drainage, and vascular and interstitial hydraulic conductivity to IFP. In general, as $\alpha$ increases so does IFP. In normal tissues $\alpha$ values are low (less than 1) corresponding to the ease of fluid transport from the vascular, through the interstitium and into the lymphatic vessels. In tumour tissue $\alpha$ increases, representing an abnormal and dysfunctional fluid transport environment, whereby fluid accumulates in the interstitial space.

1.2.2 Pressure Gradients in Solid Tumours
In solid tumours, tissue pressure gradients become dysregulated and are typically characterised by elevated STP and IFP. Structurally, tumours are less compliant than comparable normal tissues due to a higher density of malignant and non-malignant (stromal) cells, the higher elastic modulus of tumour
cells, as well as higher concentrations of ECM proteins such as collagen and fibronectin (PaszeK et al. 2005; Ng & Brugge 2009). Rapid tumour cell proliferation and growth in a confined space combine to cause elevated STP. Although difficult to measure, STP is believed to be elevated in the range of 28 to 120 mmHg in solid tumours (Helmlinger et al. 1997; Cheng et al. 2009). In comparison, IFP is elevated in the range of 10 to 100 mmHg in virtually all solid malignant tumours in both animal models and man (Boucher et al. 1990; Less et al. 1992; Milosevic et al. 2001a; Lunt et al. 2008; Vaupel et al. 2009), and is a consequence of abnormal vasculature and lack of functional lymphatic vessels within the tumour tissue (Baxter & Jain 1989).

Elevated STP in solid tumours is a consequence of growth-induced solid stress and compressive solid stress caused by confined growth (Skalak et al. 1996). Growth-induced solid stress, or residual stress, is caused by proliferating cancer cells that exert force on surrounding cells and ECM structures causing them to deform. The force is transmitted through direct contact or through compliant interstitial components including fibroblasts, other stromal cells, collagen, fibronectin and hyaluronan (Stylianopoulos et al. 2012). Additionally, the ECM is extensively cross-linked and bound to cellular structures such that contraction of ECM components contributes to increased solid stress (PaszeK et al. 2005). STP and IFP are intrinsically coupled and changes in STP, for example due to tissue deformation or cell contraction, will lead to changes in IFP. Alternatively, changes in IFP, for example caused by increased transvascular fluid flow, cause changes in STP as a result of tensile forces on the ECM, and shear stress on cellular and ECM components.

Elevated IFP in solid tumours is a consequence of morphologically and functionally abnormal blood vessels and a lack of functional lymphatic vessel (Baxter & Jain 1989; Vaupel et al. 2009). Unregulated angiogenesis results in tumour blood vessels that are spatially disorganized, tortuous, lack structural hierarchy, are often dilated, and contain excessive branching and shunts (Jain 1987a; Carmeliet 2005).
Introduction

Figure 1.6 Mathematical modelling demonstrates that steady-state IFP is elevated and uniform in the central tumour volume, and drops precipitously at the tumour periphery (a). Based on the state of IFP, the predicted rate of transvascular convection ($J_v$) is zero within the tumour volume, and fluid flow occurs predominantly along the peri-tumoural regions (b). The corresponding interstitial velocity is also zero in the central tumour region, and directed outwards at the periphery of the tumour (c). This simulation demonstrates that convection is negligible in the central tumour regions, and convective transport is limited to the peri-tumoural regions. The mesh denotes the boundary between healthy (outside) and tumour (inside) tissue and was obtained by a CT scan of an orthotopic cervix tumour xenograft mouse model. The simulation does not consider lymphatic transport.

These properties contribute to elevated viscous and geometric resistance to tumour blood flow, resulting in elevated MVP (Jain 1988; Boucher & Jain 1992; Rofstad et al. 2009). Unregulated angiogenesis also results in hyper-permeable tumour blood vessels, which have wide inter-endothelial junctions, large fenestrae, and large trans-endothelial channels (Hobbs et al. 1998). The increased permeability of tumour vessels results in a decreased trans-capillary osmotic pressure gradient (Stohrer et al. 2000) and increased vascular hydraulic conductivity - $L_p S$. Some studies reported that $L_p S$ is 10 to 1000 times higher in tumours compared to normal tissue (Sevick & Jain 1991; Bates & Curry 1996) and can result in as much as a 15 fold increase in trans-capillary fluid flux in solid tumours compared to normal tissue (Vaupel et al. 2009). Lymphatic vessels are present within the tumour but are generally non-functional (Leu et al. 2000; Padera et al. 2002). Instead, functional lymphatics are located along the tumour margins in the peri-tumoural tissue. The excess fluid leakage in combination with high interstitial resistance to fluid flow and the lack of functional lymphatic vessels causes the central IFP to rise until it nearly equals the mean capillary pressure (Boucher & Jain 1992).
Biophysical modelling using Equations 1.1 to 1.4 has suggested that tumour growth, the loss of lymphatic tissue and an increase in vascular hydraulic conductivity results in elevated IFP, with $\alpha$ values increasing to the hundreds (Baxter & Jain 1989). Figure 1.6a demonstrates that when $\alpha = 50$ the steady-state IFP becomes uniformly elevated in the central tumour volume, peaking at the MVP, and falling precipitously to zero near the interface between tumour and surrounding normal tissue. The transvascular and interstitial fluid pressure gradients become largely abolished in the center of tumour, and both transvascular and interstitial fluid flow only occurs at the periphery (Figure 1.6b and c). The predicted radial IFP profiles and MVP have been confirmed using an invasive point-based mapping method in animal tumours, and thus establish the general validity of mathematical model (Boucher et al. 1990; Boucher & Jain 1992).

In summary, in vivo measurements have demonstrated that IFP is elevated in solid tumours and can be described using a biophysical mathematical model of fluid transport. Several mechanisms drive abnormal fluid dynamics in tumour tissue, including tumour volume, lack of functional lymphatic vessels, and abnormal vascular and interstitial hydraulic conductivities. The culmination of elevated IFP is that fluid transport occurs predominately in the tumour periphery. Given that liposomes transport predominantly by convection, it is expected that liposome accumulation should be limited to the tumour periphery. However, spatio-temporal heterogeneity in IFP may exist as a result of variations in many microenvironment properties, including vascular density, tumour blood flow (TBF), and both vascular and interstitial hydraulic conductivities. Therefore, there may still be opportunities for convection driven transport of liposomes in the central tumour region. In the next section the relationship between tumour IFP, heterogeneity in transport properties, and liposome transport is further explored.

1.2.3 Interstitial Fluid Pressure, Tumour Heterogeneity, and Liposome Transport

As previously discussed, steady state IFP is elevated and relatively uniform through the central tumour volume, and then drops precipitously along the tumour periphery. Because liposomes transport occurs predominantly by convection it is expected that liposome accumulation occurs primarily along the tumour periphery where a substantial transvascular pressure is present. Indeed as shown in Figure 1.2, large tumours tend to have a predominately peripheral accumulation of liposomes. However,
considerable intra-tumoural heterogeneity in liposome accumulation has been observed, implying the presence of spatio-temporal heterogeneity in transport properties. Measurements have demonstrated that in practice considerable spatial-variations in IFP exist in pre-clinical studies of human malignant melanomas, breast carcinomas, and colorectal carcinomas (Baxter 1990; Boucher et al. 1991; Less et al. 1992). For example, in 77 human cervix cancers patients it was demonstrated that IFP can vary by up to 15 fold (median 2.4 fold) in an individual tumour (Milosevic et al. 1998). The chaotic vascular morphology and physiology of the tumour microcirculation and remodelling of blood vessels may also contribute to spatio-temporal heterogeneity in both IFP and liposome accumulation. In fact, a dynamic relationship likely exists between elevated IFP, abnormal vascular morphology/physiology, and abnormal interstitial morphology (Milosevic et al. 2001b; Vaupel et al. 2009). Therefore, the spatio-temporal relationship between these properties is believed to be the source of heterogeneous delivery, extravasation, and penetration of liposomes in solid tumours (Figure 1.7).

**Figure 1.7** Tumour angiogenesis results in abnormal capillary morphology and physiology, spatio-temporally heterogeneous blood flow, and increased fluid filtration into the tumour interstitium. Solid tissue pressure compresses blood vessels, further contributing to abnormal blood flow. A lack of functional lymphatics results in interstitial fluid accumulation and elevated IFP. The biophysical connections between angiogenesis, interstitial fluid pressure, and blood flow results in a cyclically deteriorating transport microenvironment. Ultimately, the abnormal transport environment results in spatio-temporal heterogeneity in drug delivery. Adapted from Milosevic et al. (2001b) and Vaupel et al. (2009).
The delivery of liposomes to tumour tissue is dependent on IFP, TBF and vascular density. Many tumours have a high density of vessels along the periphery compared to the tumour centre (Jain 1988; Jirtle 1988). Vascular constriction and collapse, caused by STP, may lead to increased flow resistance and spatio-temporal heterogeneity of blood flow (Milosevic et al. 1999; Sarntinoranont et al. 2003; Araujo & McElwain 2006; Stylianopoulos et al. 2012). STP and IFP may also influence the chaotic morphology and heterogeneous TBF through pressure induced modifications of the ECM and tumour blood vessels. STP and interstitial fluid flow actively participate in the remodelling of the ECM, which leads to the release of potent pro-angiogenic molecules and stimulates tumour angiogenesis (Rutkowski & Swartz 2007; Wiig & Swartz 2012). Clinical and experimental evidence has shown that vascular morphogenesis and remodelling occurs on a continuous basis (Patan et al. 1996; Patan et al. 2001). Experimentally, significant changes in blood flow have been observed on a time scale of seconds to hours (Intaglietta et al. 1977; Endrich et al. 1979; Chaplin & Hill 1995; Hill et al. 1996; Pigott et al. 1996; Cárdenas-Navia et al. 2008; Dewhirst et al. 2008). A high vascular hydraulic conductivity ($L_p$) suggests greater communication between blood flow, transvascular fluid exchange, and interstitial fluid flow in solid tumours compared to normal tissue. Therefore, temporal fluctuations in TBF may result in similar fluctuations in IFP (Netti et al. 1995). Mathematical modelling that takes account of the coupling between IFP and TBF has demonstrated that elevated IFP results in efferent capillary constriction, which in turn leads to flow stasis and re-distribution of perfusion from the center to periphery of the tumour (Netti et al. 1996; Baish et al. 1997; Milosevic et al. 1999). Ultimately, there exists a complex interplay between fluid pressure and TBF and results in spatio-temporal heterogeneity in liposome delivery to tumour tissue.

The accumulation of liposomes is dependent on the ability of the agent to extravasate from capillaries, and is governed by vascular permeability to both fluid (hydraulic conductivity) and liposomes and the transvascular fluid pressure gradient. Vascular permeability is driven by the expression of several factors including: bradykinin, nitric oxide, prostaglandins, and vascular endothelial growth factor (Maeda 2010). The pore size of tumour vessels can be up to 2 µm in diameter, and considerable heterogeneity has been observed within and between tumours (Yuan et al. 1994; Hobbs et al. 1998). Vascular permeability is also spatio-temporally heterogeneous as a result of fluctuations in stimulators of angiogenesis, which as previously mentioned are in part driven by abnormal pressure gradients (Yuan et al. 1994; Yuan et al. 1995; Monsky et al. 1999). Furthermore, increased permeability results in the abolishment of osmosis, increased vascular hydrostatic conductivity, decreased transvascular pressure gradients in the central
tumour region, and hindered transvascular convective transport of nanoparticles (Baxter & Jain 1989; Boucher & Jain 1992; Stohrer et al. 2000). As the Pécllet analysis demonstrated, diffusion is the dominant transport mechanism for liposomes in central regions where there are no transvascular pressure gradients. However, observations of intermittent blood flow suggest that transient spatio-temporal fluctuations in MVP and IFP may exist in the central tumoural region and temporarily restore the transvascular pressure gradient. Therefore, transvascular convection may also contribute to liposome extravasation in the central tumour regions, but this has yet to be experimentally confirmed. The largest effect of abnormal tissue pressure gradients appears to be limiting the extravasation of liposome to the tumour periphery, where substantial transvascular pressure gradients are present. While diffusive transport contributes to extravasation, theoretical modelling predicts that the rate of transvascular diffusion of macromolecules is substantially slower than convection in the presence of modest (~2 mmHg) transvascular pressure gradients (Rippe & Haraldsson 1987). IFP and STP may also play an indirect role in the heterogeneous extravasation of liposome by modulating vascular permeability through ECM remodelling and hypoxia driven angiogenesis. Ultimately, liposome extravasation depends on the spatio-temporally heterogeneous nature of vascular permeability and transvascular fluid pressure gradients.

The penetration of liposomes into the interstitial space is dependent on the ability for fluid and liposomes to navigate through the porous tissue. Experiments have shown that the interstitial penetration of 100 nm liposomes can be severely limited (Figure 1.8) and may only be a few cell layers deep (Yuan et al. 1994). The penetration of liposomes into the tumour interstitium is hindered by the lack of interstitial fluid pressure gradients, fluid viscosity, and the dense interstitial structure. Abnormal pressure gradients directly limit the penetration of nanoparticles by reducing interstitial fluid flow, particularly in the central tumour regions. Along the tumour periphery, steep interstitial pressure gradients serve to transport nanoparticles out of the tumour and into the surrounding normal tissue. Interstitial fluid pressure gradients are influenced by the abnormal stromal characteristic of solid tumours leading to hindered interstitial fluid flow and steric resistance to nanoparticle transport (Chauhan et al. 2012; Wiig & Swartz 2012). Molecules in the interstitial space such as hyaluronan increase the interstitial fluid viscosity and may impede the convective interstitial transport of high MW agents (Swabb et al. 1974). In addition, abnormal pressure gradients lead to altered blood flow
distribution, resulting in an increased inter-capillary distances that must be traversed by nanoparticles in order to reach target cells far away from blood vessels.

In summary, the complex relationship between elevated IFP, TBF, vascular density, vascular and interstitial hydraulic conductivities represent transport barriers that contribute to the heterogeneous accumulation of liposomes in solid tumours. These barriers are likely the driving force behind the observed variations in the EPR effect. In the next section, mathematical models are presented and evaluated in terms of their ability to accurately describe the biophysical process of liposome transport in solid tumours.

**Figure 1.8** Immunohistochemical images demonstrating extravasation and penetration of liposomes into a non-small cell lung carcinoma tumour implanted subcutaneously at 24 hrs post-injection. (a) Penetration of liposomes within the tumour volume. Note the preferential direction of extravasation, and that not all perfused vessels are a source of liposome extravasation. (b) Penetration of liposomes along the tumour periphery. Note that little extravasation and penetration occurs in normal tissue. In general, extravasation was heterogeneous and penetration was limited to between 10 to 30 μm from blood vessels in tumour tissue.

### 1.3 Mathematical Modelling of Nanoparticle transport

Several approaches have been proposed to model the transport of molecules in tumour tissue, including physiological based pharmacokinetic (PBPK) models and spatially distributed diffusion-convection
models (El-Kareh & Secomb 1997; Harashima et al. 1999; Gentile et al. 2008; Qin et al. 2009; Li et al. 2010; Liu et al. 2011; Hendriks et al. 2012). PBPK models are perhaps the most widespread as they provide a simple framework to describe the accumulation of molecules in tissue. They are based on compartment volumes, compartment concentrations, and rate constants reflecting exchange of molecules between compartments (Figure 1.9). In general, PBPK models provide a bulk description of transport in the entire tissue, or in independent tissue sub-volumes. Currently, there has been limited work on modelling liposome transport in solid tumours. Some examples include the following: Harashima et al. (1999) developed a pharmacokinetic/pharmacokinetic model of liposomal doxorubicin transport and drug release in order to predict the optimal rate of drug release. Cheng (2008) developed a PBPK model to compare the relative performance of bolus injection, continuous infusion and liposomal delivery of doxorubicin to tumour cells. Gasselhuber et al. (2010) developed a PBPK model coupled with a heat transfer to model to examine the spatio-temporal delivery of doxorubicin released from temperature sensitive liposomes using radiofrequency ablation. They further extended this model to compare the accumulation of free doxorubicin, liposome encapsulated doxorubicin, and doxorubicin delivered in temperature sensitive liposomes (Gasselhuber et al. 2012). Howell & Chauhan (2010) developed a whole body PBPK model to assess drug accumulation and toxicity of liposomes. Hendriks et al. (2012) developed a PBPK model that included cellular uptake and pharmacodynamics of liposome transport. Several aspects of the model were validated by comparison of predictions with \textit{in vivo} measurements of pegylated liposomal doxorubicin and conventional doxorubicin uptake in tumours.

While PBPK models have provided a general framework to model liposome transport, the challenge remains in relating exchange rate constants to underlying biophysical transport properties of the tissue. In general, the rate exchange parameters can be related to properties such as TBF and the microvascular permeability; however, linking the parameters to more complicated phenomena such as elevated IFP, an abnormal ECM, etc. is challenging. This is because the PBPK modelling considers each sub-volume of the tumour as an independent unit and is thus not equipped to describe interstitial transport across the tumour volume. As previously described, convection plays an integral role in liposome transport. As such a continuum model approach, where interstitial transport dynamics are continuous across the tumour volume, may provide a more accurate framework for liposome transport, particularly at the tumour periphery where substantial interstitial pressure gradients are known to exist.
Figure 1.9 Schematic of the a physiological based pharmacokinetic compartmental model of liposome transport in tissue proposed by Hendriks et al. (2012). The multi-compartment model describes the biodistribution, delivery and accumulation of liposomal doxorubicin and free doxorubicin. Rate constants are used to describe transport between compartments, cellular uptake, and the release of drug.
A spatially-distributed diffusion-convection continuum model provides an explicit biophysical framework to describe the transport of fluid, macromolecules and nanoparticles across blood vessels and through the interstitial space of solid tumours using parameters that reflect fluid and nanoparticle transport. Furthermore, a spatially-distributed diffusion-convection continuum model is typically comprised of parameters that directly represent biophysical properties of the tissue. Equation 1.5 is a standard convection-diffusion equation for mass transport based on conservation of mass and momentum (Truskey et al. 2004):

$$\frac{\partial C_i}{\partial t} = \frac{J_v}{V} (1 - \sigma) C_p + \frac{PS}{V} (C_p - C_i) - \nabla \cdot (v_i f C_i - D \nabla C_i)$$  

where $C_i$ and $C_p$ are the interstitial and plasma concentrations respectively in (mg/mL), $J_v$ is the rate of transvascular fluid flow (cm$^3$/s), $\sigma$ and $f$ are a reflection and retardation coefficient respectively that account for the ability for nanoparticles to penetrate a porous tissue, $V$ is the volume of tissue (cm$^3$), $P$ is the microvascular permeability diffusion coefficient (cm/s), $D$ is the interstitial diffusion coefficient (cm$^2$/s), $S/V$ is the surface area of blood vessels per unit volume of tissue (cm$^3$), and $v_i$ is the interstitial fluid velocity (cm/s). Assuming steady-state fluid transport, $J_v$ is defined by Equation 1.1 and $v_i$ is defined by Equation 1.2. The first term in Equation 1.5 represents the rate of spatio-temporal accumulation of molecules in the interstitial space. The following two terms account for transvascular convection and diffusion and the remaining two terms account for interstitial convection and diffusion.

Equation 1.5 is a general representation of spatio-temporal transport of molecules in a volume of tissue, where each parameter can be a function of space and time and reflects a biophysical transport process (Table 1.4). This approach has previously been used to mathematically model the diffusive and convective transport of macromolecules in solid tumours, under different physiological conditions (Baxter & Jain 1989; 1990; El-Kareh & Secomb 1997; Welter & Rieger 2013). However, the application of Equation 1.5 to liposome transport in solid tumours has yet to be investigated and validated.
Figure 1.10 (a) Predicted IFP demonstrating that as $\alpha$ increases the relative IFP (defined as IFP/MVP) approaches 1 in the central tumour region and drops precipitously at the periphery. (b) Predicted liposome concentration as a function of distance from the tumour centre demonstrating that as $\alpha$ increases liposome accumulation occurs predominantly in the tumour periphery. (c) Predicted mean liposome concentration over the tumour volume as a function of time, demonstrating that different $\alpha$ values result in markedly different liposome accumulation kinetics. Note the similarity between (c) and quantification of the kinetics of mean liposome accumulation in (d) a cervix cancer mouse xenograft, (e) a lung cancer mouse xenograft, and (f) a syngeneic rabbit carcinoma. Accumulation was assessed using CT imaging of a CT-liposome contrast agent. Further details are given in Chapter 2.
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<th>Description</th>
<th>Relationship to the tumour microenvironment</th>
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</thead>
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<td>Rate of transvascular fluid flow</td>
<td>Vascular hydraulic conductivity, MVP, IFP, osmotic pressure.</td>
</tr>
<tr>
<td>$V$</td>
<td>Volume of tissue</td>
<td>Cellular, interstitial, and vascular volume.</td>
</tr>
<tr>
<td>$L_p$</td>
<td>Vascular hydraulic conductivity</td>
<td>Pore size of endothelial fenestrae and inter-endothelial gaps, basement membrane thickness, fluid viscosity, temperature</td>
</tr>
<tr>
<td>$S/V$</td>
<td>Surface area of functional vessels per unit volume of tissue.</td>
<td>Vascular length, radius and density.</td>
</tr>
<tr>
<td>$P_i$</td>
<td>Interstitial fluid pressure</td>
<td>Vascular/interstitial/lymphatic hydraulic conductivities, MVP, lymphatic pressure, osmotic pressure.</td>
</tr>
<tr>
<td>$P_v$</td>
<td>Microvascular fluid pressure</td>
<td>TBF, viscous and geometric resistance in vessel network, vessel length, vessel diameter.</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Reflection Coefficient</td>
<td>Pore size of endothelial fenestrae and inter-endothelial gaps, basement membrane. Size, shape, and charge of molecule.</td>
</tr>
<tr>
<td>$v_i$</td>
<td>Interstitial fluid velocity</td>
<td>Interstitial hydraulic conductivity, IFP.</td>
</tr>
<tr>
<td>$K$</td>
<td>Interstitial hydraulic conductivity</td>
<td>Collagen fiber arrangement and density, ECM permeability, viscosity.</td>
</tr>
<tr>
<td>$f$</td>
<td>Retardation coefficient</td>
<td>GAG, pore size of interstitial space, size, shape, and charge of molecule.</td>
</tr>
<tr>
<td>$P$</td>
<td>Microvascular diffusion/permeability coefficient</td>
<td>Pore size of endothelial fenestrae and inter-endothelial gaps, basement membrane thickness, fluid viscosity, temperature. Size, shape, and charge of molecule.</td>
</tr>
<tr>
<td>$D$</td>
<td>Interstitial diffusion coefficient</td>
<td>Pore size and structure of interstitial space, fluid viscosity, temperature. Size, shape, and charge of molecule.</td>
</tr>
</tbody>
</table>

Table 1.4 Parameters used to define the spatio-temporal continuum transport model given by Equation 1.5 and their relationship to properties of the tumour microenvironment.
Based on the Péclet analysis, liposomes transport solely by convection. Furthermore, experiments have indicated that osmosis can be neglected in tumour tissue (Stohrer et al. 2000). Therefore Equation 1.5 can be simplified to:

\[
\frac{\partial C_i}{\partial t} = \frac{L_p S}{V}[P_v - P_i](1 - \sigma)C_p(t) - \nabla \cdot [v_i f C_i]
\]

Predicting interstitial concentration of liposomes using Equation 1.6 and different \( \alpha \) values indeed demonstrates that elevated IFP results in predominantly peripheral accumulation of liposomes (Figure 1.10a and b). This is markedly similar to in vivo imaging of the spatial distribution of liposomes shown in Figure 1.2. Furthermore, it is possible to determine the kinetics of liposome accumulation by integrating the interstitial concentration over the tumour volume, giving the mean liposome concentration as a function of time. That is,

\[
\bar{C}_i(t) = \frac{1}{V} \iiint C_i(x, y, z, t) dV
\]

A comparison between the shape of the predicted and measured kinetics of mean liposome accumulation suggests that the inter-tumour variability in in vivo liposome accumulation may be related, in part, to variations in tumour IFP (Figure 1.10). Although heterogeneity in properties of the tumour microenvironment and mechanisms of liposome retention (e.g. cellular uptake) also contribute to the shape of the in vivo kinetic liposome accumulation curves, the marked similarly with predictions is encouraging. Therefore, Equation 1.6 may provide a biophysical framework to mathematically describe the transport of liposomes in solid tumours.

The challenge remains in validating the spatially distributed convection driven mathematical model of liposome transport. Issues such as the number of free parameters, parameter degeneracy (i.e. different combinations of parameters leading to similar predictions), and limited degrees of freedom (i.e. insufficient measurements to accurately inform all model parameters) make accurate predictions of liposome accumulation challenging. A method to alleviate these issues would be to populate model
parameters with independent measurements obtained from imaging. For example, using image-based quantification of liposome pharmacokinetics to populate $C_p(t)$. Therefore, imaging provides a valuable tool to inform the mathematical model, thus improving the ability to accurately predict liposome transport in solid tumours. Furthermore, imaging can spatially and temporally map transport properties, thus providing the ability to incorporate spatio-temporal heterogeneity into the model.

1.4 Hypothesis
The inter- and intra-tumoural accumulation of liposomes can be predicted by using a biophysical mathematical model of fluid and nanoparticle transport. Furthermore, the physiological parameters that comprise the mathematical model can be derived using quantitative imaging methods.

1.5 Significance
A mathematical model of liposome transport used in combination with imaging would provide a powerful tool to predict liposome transport in solid tumours, thus providing a framework to: (1) relate variations in the EPR effect underlying properties of the tumour microenvironment; (2) understand the relationship between fluid dynamics, tumour physiology, and liposome transport in solid tumours; and (3) perform image-guided liposome drug delivery treatment planning.

1.6 Outline of thesis
This thesis is comprised of four experimental chapters on the topics of developing and validating a biophysical transport model for liposome transport, developing robust imaging methods that relate microvascular physiology to the spatio-temporal accumulation of liposomes, and characterising the relationship between IFP, microvascular physiology, and the intra-tumoural distribution of liposomes in solid tumours.

Chapter 2 is entitled ‘A Mathematical Model of the Enhanced Permeability and Retention Effect for Liposome Transport in Solid Tumours’ and was published in the journal PlosONE (Stapleton et al. 2013b). In this chapter, a mathematical model of liposome transport that is reflective of the EPR effect, termed
the intra-tumoural transport model (ITTM), is presented. The model was validated by demonstrating the ability to predict the bulk accumulation of liposomes in solid tumours as a function of time. The ITTM was fit to in vivo measurements of liposome accumulation in three different tumour models and it was demonstrated that the transport model is able to relate the inter-tumoural heterogeneity of liposome transport to variations in IFP. Furthermore, simulations were used to demonstrate that IFP can limit the intra-tumoural accumulation of liposomes, leading to reduced therapeutic efficacy. However, several limitations of the model were noted, including: (1) accurate model parameter estimation; and (2) limitations in the accuracy of predictions of intra-tumoural liposome accumulation based solely on IFP.

Chapter 3 is entitled ‘Tumour Perfusion Imaging Predicts the Intra-Tumoural Accumulation of Liposomes’ and was published in the Journal of Controlled Release (Stapleton et al. 2013a). It was demonstrated that spatial variations in TBF and vascular density play integral roles in heterogeneity of liposome accumulation. Specifically, dynamic contrast enhanced computed tomography (DCE-CT) was used to estimate two parameters that are reflective of TBF ($K_{trans}$ – rate of transvascular transport), and vascular density ($\varepsilon_p$ – plasma volume fraction). It was observed in two tumour models that intra-tumoural regions with higher $K_{trans}$ or $\varepsilon_p$ have significantly higher accumulation of liposomes. These findings demonstrate that DCE-CT measurements of tumour perfusion may be an important technique for selecting patients that are likely to respond to liposome-based and potentially other nanoparticle-based therapies. These results also suggest that incorporating measurements of TBF into the transport model is integral for accurately predicting the intra-tumoural distribution of liposomes. However, one limitation noted was that the parameter $K_{trans}$ reflects a combination of TBF and vascular permeability and as a result it was difficult to isolate the relative contribution that each property plays in liposome transport. Therefore, a novel imaging method to accurately and independently estimate TBF and vascular permeability was developed and is presented in Chapter 4.

Chapter 4 is entitled ‘Volume Fraction Imaging: A New Method for Quantitative Assessment of Tumour Vascularity’ and was recently submitted to the journal Investigative Radiology. In this chapter a novel method, termed Volume Fraction Imaging (VFI), is presented. The methodology is based on the sequential imaging of macromolecular and small molecule CAs, allowing for model independent
quantification of tissue volume fractions that can be used to increase the accuracy of perfusion and microvascular permeability estimates obtained using DCE-CT. Simulations demonstrated that the VFI method can substantially increase the accuracy and precision of plasma and interstitial volume, blood flow, and capillary permeability estimates compared to standard tracer kinetic modelling approaches. It was also observed in vivo in both healthy and tumour tissue that VFI provided more robust estimates of tissue physiology compared to standard tracer kinetics modelling. Therefore, VFI provides accurate and simultaneous quantification of perfusion, permeability, and tissue volume fractions. Furthermore, it may provide a valuable tool to incorporate intra-tumoural transport heterogeneity into the ITTM to improve predictions of the intra-tumoural accumulation of liposomes.

Chapter 5 is entitled ‘Investigating the Intra-Tumoural Relationship between Microcirculation, Interstitial Fluid Pressure, and Liposome Accumulation’. This chapter contains original and unpublished research on the intra-tumoural relationship between tumour microcirculation, IFP, and liposome accumulation in solid tumours. Co-localised point-based mapping of the tumour microcirculation was performed using VFI, IFP using a novel image-guided robotic needle positioning system and the intra-tumoural distribution of liposomes using volumetric micro-CT imaging. In vivo measurements corroborate the findings from Chapter 3 that image derived metrics of tumour perfusion predict for the intra-tumoural accumulation of CT-liposomes. VFI indicated that perfusion and not permeability was the primary mediator of the intra-tumoural accumulation of CT-liposomes. Point-based measurements indicated that tumours with elevated IFP have lower levels of CT-liposome accumulation compared to tumours with low IFP. A correlation between point-based mapping of IFP, metrics of tumour perfusion and CT-liposome accumulation was not observed; however, radial-based analysis indicated a radial dependence between IFP, metrics of tumour perfusion and CT-liposome. It was observed that IFP has a strong negative associate with tumour perfusion and CT-liposome accumulation. These results suggest that both tumour perfusion and elevated IFP play an integral role in mediating the intra-tumoural accumulation of liposomes. Furthermore, this study strengthens the need to account for intra-tumoural heterogeneity in transport properties for accurate predictions of liposome accumulation.

Chapter 6 gives a summary of the main findings presented in this thesis. A general image-guided drug delivery schema is presented, highlighting the importance of both mathematical modelling and imaging
to accurately predict the inter- and intra-tumoural accumulation of liposomes as well as predict for therapeutic response, and ultimately to provide a novel tool to guide the rationale application of liposomes in a clinical setting. A perspective is given on transport heterogeneity and limitations of the EPR effect and several improvements and extensions to the current studies are described.

1.7 References


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Chapter 2
A Mathematical Model of the Enhanced Permeability and Retention Effect for Liposome Transport in Solid Tumours

2.1 Abstract
The discovery of the EPR effect has resulted in the development of nanomedicines, including liposome-based formulations of drugs, as cancer therapies. The use of liposomes has resulted in substantial increases in accumulation of drugs in solid tumours; yet, substantial improvements in therapeutic efficacy have yet to be achieved. Imaging of the tumour accumulation of liposomes has revealed that this poor or variable performance is in part due to heterogeneous inter and intra-tumoural liposome accumulation, which occurs as a result of an abnormal transport microenvironment. A mathematical model that relates liposome accumulation to the underlying transport properties in solid tumours could provide insight into inter and intra-tumoural variations in the EPR effect. In this paper, a theoretical framework to describe liposome transport in solid tumours is presented. The mathematical model is based on biophysical transport equations that describe pressure driven fluid flow across blood vessels and through the tumour interstitium. The model was validated by direct comparison with CT measurements of tumour accumulation of liposomes in three preclinical tumour models. The mathematical model was fit to liposome accumulation curves producing predictions of transport parameters that reflect the tumour microenvironment. Notably, all fits had a high coefficient of determination and predictions of interstitial fluid pressure agreed with previously published independent measurements made in the same tumour type. Furthermore, it was demonstrated that the model attributed inter-tumour heterogeneity in liposome accumulation to variations in peak interstitial fluid pressure. These findings highlight the relationship between transvascular and interstitial flow dynamics and variations in the EPR effect. In conclusion, a theoretical framework is presented that predicts inter- and intra-tumoural variations in the EPR effect based on fundamental properties of the tumour microenvironment and forms the basis for transport modelling of liposome drug delivery.

2.2 Introduction
The discovery of the EPR effect in solid tumours has led to the development of a wide range of nanomedicines, including liposomes, for cancer therapy (Peer et al. 2007). The EPR effect describes the preferential accumulation of nanoparticles at tumour sites due to leaky vasculature (i.e. enhanced permeation) and impaired lymphatic drainage (i.e. enhanced retention), in comparison to normal tissue. Nano-sized delivery systems have been shown to result in significant increases in tumour accumulation of drugs in comparison to that achieved following administration of free drug (Gabizon 1992). Yet, despite demonstrating substantial accumulation of drug in many pre-clinical and human tumours
(Gabizon 1992; Drummond et al. 1999; Harrington et al. 2001), clinically approved liposome formulations, such as Doxil\textregistered/Caelyx\textregistered (pegylated liposomal doxorubicin) and Myocet\textregistered (un pegylated liposomal doxorubicin), have only resulted in a modest increase in anti-tumour efficacy relative to the standard of care (Garcia et al. 1998; Gordon et al. 2001; Halford et al. 2001; Thomas et al. 2001; Solomon & Gabizon 2008). Major limitations of liposome-based drug delivery are: (1) variability in the EPR effect and therefore, total tumour accumulation (Harrington et al. 2001); (2) limited tumour penetration (Yuan et al. 1994); and (3) slow or limited release of hydrophilic/amphiphilic drugs (Laginha et al. 2005; White et al. 2006). While it is clear that the poor performance has been linked to a number of factors, one of the most significant is the inability to achieve consistent inter-subject and intra-tumoural accumulation of liposomes (Simpson-Herren et al. 1988; Wu et al. 1993; Yuan et al. 1994; Harrington et al. 2001).

Heterogeneity in liposome accumulation implies the presence of inter-subject and intra-tumoural variations in the EPR effect. Several studies have indicated that variations in EPR are driven by abnormalities in the tumour microenvironment, including heterogeneity in vascular permeability, and elevated interstitial fluid pressure (Jain 1987a; b; Simpson-Herren et al. 1988; Baxter & Jain 1989; Yuan et al. 1994; Hobbs et al. 1998). Medical imaging has emerged as an important method to non-invasively detect liposome accumulation in vivo, which in turn provides direct visualisation of variations in the EPR effect. In the clinical setting substantial inter-subject variations have been observed using whole body gamma camera imaging of $^{111}$In labeled liposome accumulation in many different solid tumours (Gabizon et al. 1991; Harrington et al. 2001; Karathanasis et al. 2009). Pre-clinical imaging using high resolution CT has shown substantial heterogeneity in the intra-tumoural distribution of liposomes with larger tumours exhibiting predominantly peripheral accumulation (Zheng et al. 2007; Zheng et al. 2009; Dunne et al. 2011). Beyond visualisation of inter-subject and intra-tumoural variations in liposome accumulation, imaging the spatio-temporal distribution of liposomes may also provide information about the underlying transport properties of solid tumours that affect accumulation. This can be accomplished by fitting measurements of liposome accumulation with a biophysical mathematical model that describes liposome transport. The combination of imaging and mathematical modelling of liposome transport then provides the ability to relate inter-subject and intra-tumoural variations in the EPR effect to properties of the tumour microenvironment.
Several modelling approaches, including PBPK, as well as spatially distributed diffusion-convection models, have been proposed to describe the transport of nano-sized vehicles to solid tumours (El-Kareh & Secomb 1997; Harashima et al. 1999; Qin et al. 2009; Liu et al. 2011). PBPK models provide a simple framework to describe the accumulation of macromolecules and nanoparticles in tumours, but are limited in their ability to describe transport properties and neglect intra-tumoural distribution (El-Kareh & Secomb 1997; Liu et al. 2011). In contrast, spatially distributed diffusion-convection models provide an explicit biophysical framework to describe the transport of fluid, macromolecules and nanoparticles across blood vessels and through the interstitial space of solid tumours using parameters that reflect nanoparticle and tumour microenvironment properties. (Baxter & Jain 1989; El-Kareh & Secomb 1997; Liu et al. 2011).

Therefore the aim of this work was to develop and validate a spatially distributed biophysical transport model that can relate measurements of liposome accumulation to inter-subject and intra-tumoural variations in the EPR effect caused by the underlying transport dynamics in a solid tumour. The transport model was tested using an image-based approach that allows for a direct comparison of the predicted and measured accumulation of a CT liposome contrast agent in solid tumours. The contrast agent is comprised of liposomes that encapsulate iohexol, and has been shown to accumulate in solid tumours via the EPR effect (Zheng et al. 2007; Zheng et al. 2009; Dunne et al. 2011). The transport model, hereafter referred to as the ‘Intra-Tumour Transport Model’ or ITTM for short, was developed to describe the inter-subject and intra-tumoural transport of liposomes. However, while the model has been developed to include spatio-temporal variations in liposome accumulation, as an initial step this paper focuses on validation of the ITTM’s ability to describe the mean accumulation of liposomes across the tumour. Therefore, the results presented in this study reflect the inter-subject variations in EPR and liposome transport. The ITTM was validated by comparison to experimentally determined values for mean liposome accumulation (a typical EPR metric) in two xenograft mouse tumour models and a syngeneic rabbit tumour model. When possible, the transport properties obtained through the prediction were compared with previously published measurements obtained in the same tumour model (Jain 1987b; Lunt et al. 2008; Milosevic et al. 2008). Additionally, the syngeneic rabbit model was used to test the ability to scale the model to larger species. Lastly, simulations were performed to understand the limitations of the ITTM and to elucidate the relationship between tumour transport properties and intra-tumoural liposome accumulation.
2.3 Materials and Methods

2.3.1 Intra-Tumoural Transport Model

The ITTM model describes convection driven trans-vascular and interstitial transport of liposomes in a solid tumour. The ITTM was based on convective transport due to the large MW of the agent (~100MDa) and several reports demonstrating that interstitial and transvascular diffusion is negligible compared to convection for macromolecules and liposomes (Swabb et al. 1974; Jain 1987b; Rippe & Haraldsson 1987; Baxter & Jain 1989; El-Kareh & Secomb 1997). The rate of accumulation of liposomes in the interstitial space of tumours is given by,

\[
\frac{\partial C_i}{\partial t} = \frac{L_p S}{V} [P_v - P_i] (1 - \sigma) C_p(t) - \nabla \cdot [v_i f C_i]
\]

\[2.1\]

Figure 2.1. An illustration of convective transport and its relationship to the EPR effect for liposome transport. (a) Tumours experience elevated central IFP due to an increased transvascular fluid transport \((L_p)\), decreased interstitial fluid transport \((K)\), and lack of functional lymphatic vessels. Peri-tumoural lymphatics drain excess fluid at the tumour periphery, resulting in a gradient in IFP. (b) An illustration of the peri-tumoural region where the yellow dashed line indicates the border between tumour and healthy tissue. Trans-vascular \((P_v - P_i)\) and interstitial \((\nabla P_i)\) pressure gradients drive the convective transport across blood vessels and through the tumour interstitium. This process occurs predominantly along the tumour periphery where large trans-vascular and interstitial pressure gradients are present. Convection transports liposomes through large endothelial pores \((\sigma)\) and through the ECM \((f)\) where they accumulate due to a lack of lymphatic clearance. In normal tissue, tight endothelial junctions limit liposome extravasation and functional lymphatics contribute to the clearance of the agent from the interstitium.
The first term on the right of Equation 2.1 represents the trans-vascular convective flux where \( L_p S/V \) denotes the capillary filtration coefficient (CFC), \( L_p \) is the vascular permeability to fluid flow (hydraulic conductivity), \( (P_v - P_i) \) is the difference between the MVP and IFP, \( \sigma \) is the filtration reflection coefficient, and \( C_p \) is the plasma concentration of the nanoparticle. The second term on the right represents the interstitial convective flux where \( f \) represents the fractional rate of liposome transport to fluid flow through the interstitium, \( v_i \) is the interstitial fluid velocity and \( C_i \) is the concentration of nanoparticles in the tumour interstitium. In this study, iodine concentration is used as a surrogate for liposome concentration due to the linear relationship between the two quantities under the assumption that the iohexol is retained within the intact liposomes (Zheng et al. 2007; Zheng et al. 2009).

The parameters that define the ITTM reflect biophysical properties of the tumour microenvironment that mediate liposome transport and the EPR effect. Each of these parameters is directly or indirectly related to factors that are known to influence EPR, including: TBF, vascular organisation, vascular permeability, cell density, and ECM. The ITTM and its relation to the EPR effect are illustrated in Figure 2.1. The principle driving force of fluid and liposome extravasation is the MVP \( (P_v) \), and indirectly relates both vascular organisation and TBF to the rate of extravasation (Jain 1987a). The rate of fluid and liposome extravasation is also determined by the vascular permeability, which are reflected in the parameters \( L_p \) and \( \sigma \), respectively. The rate of extravasation is indirectly related to the rate of TBF through \( P_v \).

The principle driving force for interstitial transport of liposomes is a spatial pressure gradient that can be related to interstitial fluid velocity by Darcy’s law for flow through a porous medium, \( v_i = -K \nabla P_i \). The parameter \( K \) represents the interstitial permeability to fluid flow (hydraulic conductivity) and is reflective of the composition of the ECM including cell density, hyaluronic acid and collagen content (Jain 1987b; Wiig & Swartz 2012). The parameter \( f \) is related to ECM permeability to liposomes and reflects cell density, hyaluronic acid, collagen content, as well as the size and shape of liposomes (Wiig & Swartz 2012). IFP was calculated using the steady-state formula proposed in (Baxter & Jain 1989),

\[
\nabla^2 p_i = \left( \frac{\alpha}{V^{1/3}} \right)^2 (P_i - P_v) \quad 2.2
\]
where \( \alpha = V^{1/3} \sqrt{\left( L_p S - L_{pl} S_L \right) / KV} \) is a unit-less number that represents the ratio of vascular to interstitial permeability to fluid flow. Note that Equation 2.2 is a simplification of Equation 1.4, where \( P_{ss} = P_v \) by assuming the absence of functional lymphatics and a transvascular osmotic pressure gradient. In general, as \( \alpha \) increases the IFP approaches MVP and the spatial gradient in IFP increases at the tumour periphery. The ITTM can describe tumours of arbitrary geometry and with spatio-temporally varying transport properties; however, in this study the model was simplified to a spherically symmetric tumour with uniform transport properties. Under this condition Equation 2.2 can be simplified to the analytic solution given in (Baxter & Jain 1989), with \( \alpha \) ranging from 0.5 to 150 based on previously reported tumour transport properties (Sevick & Jain 1991; Swartz & Fleury 2007; Milosevic et al. 2008). Equation 2.1 was solved using the finite difference method implemented in Matlab (Mathworks, Natick, MA) and the resulting solution was integrated over the tumour volume to get the mean liposome (iodine) concentration as a function of time for comparison to measurements.

### 2.3.2 Validation of the Intra-Tumoural Transport Model

Validation of the ITTM was performed by fitting the CT-based measurements of mean liposome accumulation in two xenograft mouse tumour models and one syngeneic rabbit tumour model. Validation was accomplished by demonstrating that the ITTM: (1) fit the experimental data with an \( r^2 > 0.90 \); (2) produced predictions of peak \( P_i \) and \( K \) that are within the range previously reported for cervical xenograft mouse tumours (Lunt et al. 2008; Milosevic et al. 2008); (3) can describe observed variations in the measured EPR mediated liposome accumulation in the three tumour types; and (4) can scale to larger species. The model parameters, curve fitting technique, and further details of the validation technique are provided in the supplemental information (Appendix 1). The predicted transport properties are reported as the best-fit value and 95% CI. Statistical analysis was performed using the student t-test with a significance threshold of 0.05.

### 2.3.3 Biophysical Modelling of the EPR Effect and its Sensitivity to Parameters

ITTM simulations were performed by varying the transport the parameters \( R, L_p S / V, K, \) and \( P_v \) to determine their effects on tumour IFP, the mean liposome accumulation, the intra-tumoural liposome
distribution. The parameters were varied over a range of accepted values that are shown in Tables A1.1 and A1.4 in Appendix 1. Further details are provided in supplemental information (Appendix 1).

### 2.3.4 Liposome Contrast Agent (CT-liposome) Preparation and Characterisation

The liposome-based CT contrast agent was prepared according to previously described methods (Zheng et al. 2009; Dunne et al. 2011). Briefly, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, MW 734) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol) 2000 (DSPE-PEG_{2000}, MW 2774) were purchased from Genzyme Pharmaceuticals (Cambridge, USA). Cholesterol (CH, MW 387) were obtained from Avanti Lipids Inc. (Alabaster, USA). The lipid components for the CT-liposomes (i.e. DPPC, CH, DSPE-PEG_{2000}) were dissolved in anhydrous ethanol at 70°C at a molar ratio of 55:40:5 DPPC:CH:DSPE-PEG_{2000}. Omnipaque™-300 (300mg/mL iodine, GE healthcare, Mississauga, Canada) was added to the solution with a lipid concentration of 100mM following ethanol removal. The final iodine concentration was approximately 45 mg mL^{-1}. For the rabbit studies gadoteridol was co-encapsulated with iohexol at a concentration of 6.6 mg mL^{-1}. The mean diameter of the liposomes used for all studies was approximately 80 nm, and the calculated MW was ~100 MDa. Detailed preparation and Characterisation procedures can be found in the supplemental information (Appendix 1).

### 2.3.5 Animal Models

All experiments were performed in compliance with the guidelines established by the Canadian Council on Animal Care and the Animals for Research Act of Ontario. The protocol was approved by the University Health Network Institutional Animal Care and Use Committee (Animal Use Protocol #383). Measurements of CT-liposome accumulation were performed in 3 different tumour models: (1) a human cervix carcinoma cell line (ME180) implanted orthotopically (OR) in female SCID mice (n=4); (2) a human non-small cell lung carcinoma cell line (H520) implanted subcutaneously (SC) in male nude mice (n=5); and (3) a syngeneic rabbit carcinoma cell line (VX2) implanted intramuscularly (IM) in male New Zealand white rabbits (n=5). The ME180 and H520 mouse tumour models were employed, as preliminary studies by our group have shown that they result in low and high intra-tumoural liposome accumulation, respectively. The VX2 syngeneic rabbit tumour model was used to evaluate the ability to scale the ITTM to larger species. ME180 tumours were established by suturing a 2–3 mm³ tumour fragment onto the cervix in of female SCID mice (20-25g) (Cairns & Hill 2004). H520 tumours were established by injecting
H520 cells into the subcutaneous tissue of the hind limb of female athymic nude CD-1 mice (20-25g). VX2 tumours were established by injecting VX2 carcinoma cells obtained from 2 donor rabbits into the left lateral quadriceps of male New Zealand White rabbits (2.8-3.2kg). The CT imaging data sets of liposome plasma pharmacokinetics (PK) and tumour accumulation in the H520 and VX2 models were previously published in Dunne et al. (2011) and Zheng et al. (2009), respectively.

2.3.6 CT Imaging of Liposome Accumulation
Longitudinal CT imaging of liposome accumulation was performed once the ME180, H520, and VX2 tumours were approximately 8.3±0.2 mm, 2.3±0.4 mm, and 21.5±4.1 mm in diameter, respectively. All animals were anaesthetised using an isoflurane-oxygen mixture. Each mouse received a bolus of 200 µL of CT-liposomes (~0.400 mg of iodine g\(^{-1}\) and 1.20 mg of total lipid g\(^{-1}\)) via the lateral tail vein. Each rabbit received a bolus of 15 mL of CT-liposomes (0.276 mg of iodine g\(^{-1}\) and 0.83 mg of total lipid g\(^{-1}\)) via the marginal ear vein. CT images were acquired pre-administration and at 5 min, 1 hr, 8 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs and 144 hrs post-administration for ME180 mice. H520 tumour bearing mice underwent the same imaging protocol, with the exception of the 1 hr and 120 hr scans. VX2 tumour bearing rabbits were imaged pre-administration and 30 min, 24 hrs, 48 hrs, 72 hrs, 120 hrs, 168 hrs, 240 hrs and 336 hrs post-administration. Each ME180 mouse received a nominal CT dose of 1.7 Gy over 6 days, each H520 mouse received 1.2 Gy over 6 days, and each VX2 rabbit received a nominal dose of 135 mGy over 14 days. This dosing schedule is likely to have minimal radiation bio-effects (Foster & Ford 2011). Further details of the imaging method can be found in the supplemental information (Appendix 1).

2.3.7 Image-Based Determination of Pharmacokinetics and Tumour Accumulation
The tumour volume and descending aorta were contoured on each CT data set. The mean signal intensity, in Hounsfield units (HU), was determined in each volume of interest at each time point and converted to iodine concentration (in mgI cm\(^{-3}\)) using a calibration factor of 50.1±0.4 HU per mgI cm\(^{-3}\) for the mouse CT scans and 38.0±0.6 HU per mgI cm\(^{-3}\) for the rabbit CT scans. The plasma iodine concentration, \(C_p\), was estimated by adjusting the measured concentration in the aorta for the arterial hematocrit \((Hct_{aorta} = 0.5;\) Cairns & Hill (2004)) and fitting the results to a one compartment PK model. The mean plasma volume fraction \((\epsilon_p)\) of each tumour was estimated by taking the ratio of mean iodine concentration measured in the tumour to that in blood 5 min after injection for mice and 30 minutes post-injection for rabbits. At this early time point the liposomes are assumed to be
predominantly intravascular. The plasma volume fraction was used to subtract the contribution of the plasma compartment from the measured iodine concentration in the tumour. The mean tumour volume was determined from the contours and used to estimate an equivalent radius $R$, representing the radius of a sphere with a volume equal to the contoured tumour. The equivalent radius was used as input to the ITTM. Further details can be found in the supplemental information (Appendix 1).

**Figure 2.2** Micro-CT images of CT-liposome accumulation. Representative images of CT-liposome accumulation are shown for an ME180 mouse OR tumour (a), an H520 mouse SC tumour (b), and a VX2 rabbit IM tumour (c). The arrows indicate the extent of the tumour volume. The transverse images illustrate the intra-tumoural heterogeneity of liposome accumulation; particularly in the ME180 and VX2 tumours which have predominantly peripheral liposome accumulation. Note the difference in scales.
Figure 2.3 Quantification of PK and mean tumour accumulation of CT-liposomes. Quantitative measures of plasma PK and mean tumour accumulation is shown for ME180 mouse OR tumours (a, d), H520 mouse SC tumours (b, e), and the VX2 rabbit IM tumours (c, f). The plasma PK (a-c) and tumour accumulation (d-f) were obtained from the mean concentration of iodine in the blood and tumour volume, respectively. The plasma half-life of the agent was 38±9 hr, 35±6 hr, and 64±6 hr in the ME180, H520, and VX2 models, respectively. Considerable variability in rate and extent of liposome accumulation was observed between the tumour models. Error bars are smaller than the symbols.

2.3.8 Histological Analysis

Tissue sections were processed by a certified medical laboratory technologist at the Applied Molecular Profiling Laboratory (University Health Network, Toronto, ON, Canada) using standard operating procedures (SOPs). The SOPs included evaluation of negative and control sections in order to validate the positive staining of ME180 and H520 tumour tissue sections. Tumour morphology (H&E), vascularity (CD-31), perfusion (Hoechst 33342), and lymphatics (LYVE-1) were assessed in tissue sections of ME180 tumours. This provided an assessment of the transport properties that may deviate from the assumptions of the ITTM. Tumour morphology and vascularity were assessed in tissue sections of H520 tumours. Quantifiable tissue sections were not available for VX2 tumours. Analysis consisted of imaging whole tissue sections from each tumour and quantifying the percentage area of positively stained pixels.
Results were compared between tumours of the same and different types. A detailed description is given in the supplemental information (Appendix 1).

2.4 Results

2.4.1 Measurements of Liposome Accumulation

Figure 2.2 shows the spatio-temporal distribution of the CT-liposomes in the ME180, H520, and VX2 tumour models. Qualitatively, the intra-tumoural distribution of liposomes was primarily along the periphery of the tumour nodules in the ME180-bearing mice and VX2-bearing rabbits. In the H520 tumours, the intra-tumoural distribution appeared heterogeneous throughout the tumour volume for the three small (< 9 mm$^3$) tumours and predominately peripheral in the two larger (> 20 mm$^3$) tumours. The tumours grew in volume from 297±20 mm$^3$ to 498±26 mm$^3$ in ME180 mice, from 7.1±3.2 mm$^3$ to 12.6±8.7 mm$^3$ in H520 mice, and from 5.7±2.9 cm$^3$ to 25.0±4.7 cm$^3$ in VX2 rabbits over the course of the experiments. This corresponded to an increase in equivalent radius of less than 1 mm for ME180 and H520 tumour types, which should theoretically have minimal impact on $\alpha$, IFP, and liposome transport. The VX2 tumours increased on average by 0.7 cm, which likely impacted $\alpha$, IFP, and liposome transport, but these changes were not taken into account in this study.

Figure 2.4 Best fit prediction of mean liposome accumulation. Best fit prediction of CT-liposome accumulation is show for ME180 mouse OR tumours (a), H520 mouse SC tumours (c), and VX2 rabbit IM tumours (d). All fits had an $r^2$>0.9, with the exception of VX2-04 which had an $r^2$=0.85. These results demonstrate that the ITTM can predict variations in liposome accumulation in different tumour types and can be scaled for use in larger species. Note the difference in scales along the axes.
The PK and mean tumour accumulation profiles of the CT-liposomes in the ME180, H520 and VX2 tumour bearing animals are shown in Figure 2.3. The mean peak plasma concentration was 11.2±1.7 mgI cm$^{-3}$, 8.0±1.8 mgI cm$^{-3}$, and 5.1±1.4 mgI cm$^{-3}$ in the ME180, H520, and VX2 tumour models, respectively. The plasma half-life of the agent was 38±9 hr, 35±6 hr, and 64±6 hr in the ME180, H520, and VX2 tumour models, respectively. The half-life of the CT-liposomes in rabbits was significantly longer compared to mice due to recognised inter-species differences (p<0.01). There was no statistically significant difference in the peak tumour accumulation of liposomes between the H520 and VX2 groups (p=0.64). The peak liposome accumulation in tumours and the tumour area under the curve (AUC) in the ME180 group were approximately half that of the values obtained for the H520 group. Liposome accumulation curves were similar in ME180 tumours (Figure 2.3d); however, considerable inter-subject variability was observed in H520 and VX2 tumour groups (Figure 2.3e and f).

### 2.4.2 Validation of the Intra-Tumoural Transport Model

The best fit tumour accumulation curves for each ME180, H520 and VX2 tumour are plotted in Figure 2.4. All fits had an $r^2 >0.90$, with the exception of VX2-04 that had an $r^2=0.85$, when $L_pS/V$, $K$, $P_v$ and $\varepsilon_i$ were constrained to the range of previously published independent measurements (Table A1.1 in Appendix 1). This highlights that the predictions of liposome accumulation are based on realistic and independently measured transport properties in tumours. Predictions of transport parameters $L_pS/V$, $K$, and $P_v$ for each mouse are summarised along with their 95% CI in Table A1.2 and estimates of $\alpha$, and $P_{i,max}$ are summarised in Table A1.3 in Appendix 1. The mean of the predicted $K$ and $P_{i,max}$ in ME180 tumours were $(2.9\pm5.3)x10^{-7}$ cm$^2$ mmHg$^{-1}$ s$^{-1}$ and 4.7±0.9 mmHg, respectively. These values were consistent with previous measurements made in the same tumour model (Lunt et al. 2008; Milosevic et al. 2008). Considering each tumour individually, the 95% CIs for the best-fit values for $K$ and $P_{i,max}$ were also within the range of previously reported measurements. Additionally, the mean of the predicted $P_{i,max}$ in VX2 tumours was 19.8±18.3 mmHg and overlaps with previously published measurements in the same tumour model (Jain 1987b). More recent measurements of $P_{i,max}$ performed in our lab using similar sized IM VX2 tumours were found to be 27.7±11.0 mmHg. There was no statistically significant difference in the predicted $L_pS/V$, $K$ and $P_v$ between tumour types; although VX2 tumours had higher values compared to ME180 and H520 tumours. Predictions of $\varepsilon_i$ were consistent across all tumours.
The mean of the predicted $P_{i,\text{max}}$ was 4.7±0.9 mmHg, 2.9±1.4 mmHg and 19.8±18.3 mmHg in ME180, H520 and VX2 tumours, respectively. There was no statistically significant difference in predictions of $P_{i,\text{max}}$ between tumour types; however, there was an observed trend of higher $P_{i,\text{max}}$ in larger tumours. The predicted $P_{i,\text{max}}$ in individual tumours is consistent with the CT-liposome accumulation curves. Meaning, CT-liposome accumulation curves in ME180 tumours were similar in shape and peak accumulation, and subsequent predictions of $P_{i,\text{max}}$ were also consistent between tumours, having a coefficient of variation of 20%. Conversely, considerable variability in CT-liposome accumulation curves was observed in both the H520 and VX2 tumour models, and was reflected in predictions of $P_{i,\text{max}}$ where the coefficient of variation was 48% for H520 tumours and 92% for VX2 tumours. Therefore, the ITTM suggests that inter-subject variation in CT-liposome accumulation between tumours of the same type was predominantly driven by variations in tumour IFP. These results confirm that the ITTM is able to describe EPR mediated liposome accumulation in three different tumour models. Additionally, the ITTM can be scaled for use in larger species suggesting substantial potential for clinical applicability. Finally, the ITTM attributed inter-subject variations in EPR mediated accumulation of CT-liposomes to variations in IFP between tumours.

### 2.4.3 Histology

ME180 tumours contain patches of necrosis scattered throughout the tumour volume (Figure 2.5a). The necrosis visible on ME180 sections suggests that analysis of intra-tumoural heterogeneity will be more complex for this tumour type and size. No necrosis was observed in the H520 tumour sections (Figure 2.5d). Lymphatic staining in ME180 tumour sections was minimal (% positive LYVE-1 was 2.7±0.6 %) and limited to the periphery (Figure 2.5b). The ME180 tumour sections were largely avascular at the end of the study (% positive CD-31 was 4.1±1.6 %) which agreed with previous findings (Lunt et al. 2008). The vascular regions were isolated to viable tissue and only 40±8 % of CD-31 positive vessels were perfused (Figure 2.5c). The % perfused over the whole tumour section was 13±5 % indicating limited perfusion at the end of the study for ME180 tumours. H520 tumour sections appeared uniformly vascularised (% positive CD-31 was 10±2 %), and due to their small size are believed to be well perfused. The H520 tumours had a twofold higher % positive CD-31 staining than ME180 tumours (p-value=0.004), which may be a contributing factor to the two fold increase in peak tumour accumulation and tumour AUC observed from imaging.
Figure 2.5 Representative histology sections for H520 and ME180 tumour tissue. (a) H&E and (b) LYVE1 sections for ME180 tumours showing patches of necrosis, and predominantly peripheral lymphatic vessels. (c) A representative section showing heterogeneous perfusion (blue) and blood vessels distribution (red) in an ME180 tumour. (d) H&E and (e) CD31 sections for H520 tumours showing no necrosis and a largely homogeneously vascularised tumour. (f) Quantitative analysis demonstrating the mean percent positive staining for the H520 tumour sections and ME180 tumour sections. Error bars represent the standard deviation.

2.4.4 Biophysical Transport Modelling of the EPR effect and Sensitivity Analysis

Simulations showed that an increase in $\alpha$ results in elevated tumour IFP in the center that drops precipitously at the periphery, and a correspondingly predominate peripheral liposome accumulation. Figure 2.6 and Figure A1.1 in Appendix 1 demonstrate the sensitivity of the model to the biophysical transport properties ($R, L_pS/V, K, P_p$) and how these parameters influence tumour IFP, the intra-tumoural accumulation of liposomes and the mean liposome accumulation. The model predicts that a faster rate and higher peak in liposome accumulation occurs in low IFP tumours that have a relatively homogeneous intra-tumoural distribution of liposomes. Simulations suggest that an $\alpha < 3$ is optimal for
liposome therapeutics. Under these conditions, tumour IFP is consistently lower than the MVP (ratio of IFP to MVP is less than 0.9) leading to a uniform distribution of liposomes in the tumour volume.

The simulations suggest that this transport environment exists in small tumours ($R < 0.5$ cm) or those that have a relatively low CFC ($L_pS/V < 2.5 \times 10^{-5}$ mmHg$^{-1}$ s$^{-1}$) or high interstitial hydraulic conductivity ($K > 6.9 \times 10^{-7}$ cm$^2$ mmHg$^{-1}$ s$^{-1}$). The transition from low to high relative IFP occurs for $\alpha > 3$, and the corresponding intra-tumoural distribution of liposomes becomes predominantly peripheral. This transition can occur when either the tumour radius is larger, $L_pS/V$ is higher, or $K$ is lower than the aforementioned values. The rate of liposome accumulation and peak liposome concentration was most sensitive to tumour size and MVP. Small tumours or those with high MVP have the highest rate of liposome accumulation and peak liposome concentration, and exhibit a more homogeneous intra-tumoural liposome distribution. Increasing $L_pS/V$ or $K$ only moderately influences the shape of the liposome accumulation curves.

Figure 2.6 Biophysical modelling of the relationship between mean liposome concentration (relative to plasma), the intra-tumoural distribution of liposomes, and tumour IFP. As $\alpha$ increases (equivalent to an increase in IFP) the ITTM model predicts a decrease in the mean concentration of liposomes in the tumour volume (a) and the transitions from uniform to periphery-dominated, non-uniform intra-tumoural liposome accumulation (b).
These results suggest that a potentially advantageous strategy to improve liposome accumulation is to increase the MVP or to reduce tumour IFP by reducing tumour size, modulating the vascular permeability, or increasing the interstitial hydraulic conductivity. Indeed, several studies have demonstrated that modulating each of these parameters improves the accumulation and intra-tumoural distribution of nanoparticles (Heldin et al. 2004; Chauhan et al. 2011; Marcucci & Corti 2012).

2.5 Discussion

A theoretical framework was developed to describe the transport and accumulation of liposomes in solid tumours. The ITTM is based on biophysical transport equations that describe pressure driven fluid flow across blood vessels and through the tumour interstitium. It was demonstrated that the ITTM can predict the mean temporal liposome accumulation in three pre-clinical tumour models with fitted parameters that reflect accepted independent measurements of the tumour microenvironment. These results highlight that the ITTM can relate the inter-subject heterogeneity in liposome accumulation to the biophysical properties of the tumour microenvironment. Specifically, it was found that substantial inter-subject heterogeneity in liposome accumulation can be caused by variations in peak tumour IFP. Furthermore, it was demonstrated that the ITTM is scalable between species due to the ability to directly measure the plasma PK using imaging and to input this measurement into the mathematical model.

An important implication of the ITTM is its ability to relate the intra-tumoural variations in the EPR effect to the convective transport of liposomes in a solid tumour. As demonstrated in this study, the application of the ITTM to imaging of intra-tumoural liposome accumulation provides information about the spatial distribution of convective transport and tumour IFP. This has been attempted previously in a limited manner using invasive point-based mapping of radial IFP profiles in animal tumours (Boucher et al. 1990). However, this approach is not feasible in patients. Little is known about the underlying intrinsic tumour parameters that influence fluid and macromolecule flux in human malignancies. A non-invasive imaging approach to measure IFP and/or convective transport would provide valuable new clinical insight into human tumour pathophysiology and allow the prognostic and predictive effects of transport parameters to be evaluated on a large scale. For example, this approach could be used to identify convective transport factors limiting the intra-tumoural accumulation of liposomes and to identify strategies to modulate these factors to improve accumulation. However, accomplishing this
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requires maturing the ITTM to include the additional factors that influence intra-tumoural heterogeneity (e.g. necrosis and perfusion).

A major limitation of the study was neglecting the intra-tumoural distribution when fitting to measurements of the mean CT liposome enhancement over the tumour volume. This approach was taken as an initial simple validation of the ITTM using experimental data in a manner that is consistent with previously reported EPR measurements (Gabizon 1992; Drummond et al. 1999; Harrington et al. 2001; Maeda 2001; Karathanasis et al. 2009; Zheng et al. 2009; Dunne et al. 2011); however, it ignores the wealth of information available on intra-tumoural liposome distribution. As shown in this work, the intra-tumoural distribution of liposomes is influenced by tumour IFP; however, the underlying spatial varying characteristics of the tumour microenvironment, including: microvascular density, vascular permeability, interstitial composition and tumour necrosis also play an important role. Applying the ITTM to the spatial measurements of liposome accumulation at a single or multiple time points may provide improvements in predicting transport properties; however, fitting the ITTM to spatial measurements of liposome distribution requires incorporating measurements of spatial variations in tumour transport properties. There have been several approaches to characterise the spatially variable transport properties of tumours (Zhao et al. 2007; Gasselhuber et al. 2010). The use of imaging techniques, such as DCE-CT and diffusion weighted magnetic resonance imaging, may provide quantitative spatial measurements of tumour microenvironment properties, such as: TBF, vascular permeability, necrosis, and cell density. These independent measurements could be used as input into the ITTM and would strengthen predictions of the intra-tumoural heterogeneity in liposome transport and the EPR effect.

Moving forward, the ITTM forms the basis for transport modelling of drug delivery using imaging data. In this study a relatively high lipid dose was used for CT-liposome imaging compared to standard therapeutic doses reported for other lipid nanoparticles, such as DOXIL®, Lipoplatin™, and SPI-77. The ITTM compensates for alterations that lipid dose may have on PK as the image-derived measure of plasma kinetics is used as direct input to the model. There are several limitations of the presented ITTM, including: (1) accurately modelling the physico-chemical properties of liposomes in relation to transvascular and interstitial transport; (2) incorporating the release kinetics of the encapsulated drug; and (3) cellular uptake of liposomes by tumour cells and mononuclear phagocyte cells. Modelling of liposome
properties has been limited to morphological properties that contribute to size exclusion by trans-
endothelial pores and the ECM. Several other physico-chemical properties of liposomes are known to
influence transport, including surface properties such as charge and hydrophilicity, as well as the
presence of targeting moieties (Drummond et al. 1999). Incorporating these properties into the model is
integral to understanding and optimising the effects of liposome properties on intra-tumoural transport
and predicting therapeutic response. Previous work has explored modelling the release kinetics of drugs
from conventional and triggered-release liposomes, that could easily be incorporated into the ITTM and
would allow for predictions of drug bioavailability using the ITTM (Gasselhuber et al. 2010). Finally,
liposomes have been shown to be internalised by tumour associated macrophages (Drummond et al.
1999), which can make up a substantial population in tumours (Caillou et al. 2011). Cell uptake of
liposome can alter the retention kinetics of liposomes within a tumour. In the present study, cell uptake
likely made a negligible contribution to variability between tumour accumulation in mice with the same
tumour type; yet, it may have had a considerable impact on the variability observed in tumour
accumulation between tumour types. Future work will focus on integrating cell uptake into the ITTM.

CT was chosen for this study as it provides a simple tool to assess the concentration and spatio-temporal
distribution of liposomes in a solid tumour. The CT-liposome formulation used in this study is stable for
weeks (Zheng et al. 2007) allowing for longitudinal assessment of liposome PK and intra-tumoural
accumulation. The ITTM is not restricted to CT, and several liposome formulations have been developed
for MRI, PET and optical imaging (Yuan et al. 1994; Zheng et al. 2007; Qin et al. 2009). These techniques
may improve detection sensitivity allowing for administration of a decreased lipid dose of the agent, but
are limited by either spatial resolution or quantitative ability. Multimodal liposomes may provide a
suitable imaging platform that takes advantage of the strengths of each imaging modality (Huang et al.
2013). For example, multimodal (e.g. optical and CT) imaging would allow for macroscopic and
microscopic assessment of intra-tumoural liposome distribution in the same tumour. Combining these
imaging approaches with the proposed ITTM provides a powerful tool to further understand the
mechanisms that lead to intra-tumoural heterogeneity in EPR mediated liposome accumulation.
2.6 Conclusion
We have developed a biophysical transport model to describe the total tumour and intra-tumoural accumulation of liposomes. The ITTM was validated by comparison of predicted values to measurements of EPR mediated accumulation of liposomes in multi-species pre-clinical tumour models. The ITTM reveals the critical link between the EPR effect and IFP, and demonstrates that biophysical properties of the tumour microenvironment that influence fluid transport dynamics play an integral role in liposome accumulation. The ITTM also offers the potential for development of a quantitative, image-based approach to non-invasively estimate parameters related to IFP. Such a method could be used to guide the application of nanomedicine in a clinical setting. Applying the ITTM to the spatial measurements of liposome accumulation will enable improved predictions of transport properties, further validating the model, and bringing an image-based approach to assess nanomedicine closer to reality. In conclusion, the ITTM provides a theoretical framework that links intra and inter-subject variations in EPR to the underlying transport properties of solid tumours.

2.7 Acknowledgements
The authors would like to thank Dr. Naz Chaudary and Dr. Richard P. Hill for providing the ME180 xenograft mice. S. Stapleton is grateful to the Natural Science and Engineering Research Postgraduate Scholarships Program and the Terry Fox Foundation Strategic Initiative for Excellence in Radiation Research for the 21st Century (EIRR21) at CIHR. This study was supported by grants from the Terry Fox New Frontiers Program and the Canadian Institutes of Health Research.

2.8 References


Chapter 3

Tumour Perfusion Imaging Predicts the Intra-Tumoural Accumulation of Liposomes

3.1 Abstract

Liposomes have proven to be a viable drug delivery strategy resulting in substantial increases in tumour accumulation of drugs via exploitation of the EPR effect. However, considerable variability has been observed in their bulk tumour accumulation and intra-tumoural distribution. The heterogeneous accumulation of liposomes in solid tumours is largely believed to result from the chaotic morphology and physiology of tumour blood vessels. Thus, tumour perfusion imaging may provide a novel method to predict the accumulation and resulting therapeutic effect of liposome formulations. In this study, DCE-CT was employed to estimate the intra-tumoural distribution of perfusion and anatomical CT was used to map the spatio-temporal accumulation of a CT-liposome contrast agent. A statistically significant positive correlation was found between quantitative and semi-quantitative measures of tumour perfusion (i.e. $K_{\text{trans}}$, $\varepsilon_p$ and $AUC_{\text{t}0\text{x}}$) and liposome accumulation ($AUC_{\text{t}ipo}$ and $C_{\text{peak}}$) in a mouse xenograft model of human cervical cancer implanted in two different tissues. Specifically, it was found that regions with higher $K_{\text{trans}}$, $\varepsilon_p$ and $AUC_{\text{t}0\text{x}}$ had greater liposome accumulation. These findings demonstrate that DCE-CT measurements of tumour perfusion may be an important technique for selecting patients that are likely to respond to liposome and potentially other nanoparticle-based therapies. The results also suggest that incorporating measurements of TBF into the intra-tumoural transport model is integral for accurately predicting the intra-tumoural distribution of liposomes. However, one limitation noted was that the parameter $K_{\text{trans}}$ reflects a combination of perfusion and vascular permeability, and it was thus difficult to isolate their relative contribution to liposome transport.

3.2 Introduction

Encapsulation of chemotherapeutic agents in liposomes can result in substantial increases in their tumour accumulation relative to administration of drug in conventional formulations (Drummond et al. 1999). However, pre-clinical and clinical studies have shown that the degree of tumour accumulation of liposomes can vary substantially, both within and between tumour types (Gabizon 1992; Harrington et al. 2001; Zheng et al. 2009; Dunne et al. 2011). Effective response to chemotherapy depends on the delivery of drugs to cancer cells at cytotoxic concentrations and therefore heterogeneity in both total tumour accumulation and intra-tumoural distribution remain major challenges for liposome-based therapeutics (Garcia et al. 1998; Gordon et al. 2000; O’Brien et al. 2004). Indeed, several pre-clinical studies have shown that achieving both an adequate concentration and consistent intra-tumoural
distribution of cytotoxic drugs is crucial to the successful outcome of liposome-based chemotherapy (Huang et al. 1994; Kong & Dewhirst 1999; Kong et al. 2000; Ponce et al. 2007; Seynhaeve et al. 2007).

Direct incorporation of CAs into liposomal delivery vehicles has been used for both diagnostic and theranostic applications. This approach has shown potential for visualising the drug delivery process (Gabizon 1992; Harrington et al. 2001; Zheng et al. 2009; Lammers et al. 2010; Dunne et al. 2011); however, the development and application of diagnostic liposome formulations require careful consideration of many factors including: imaging resolution, detection sensitivity, additional complexity of synthesis and cost for each liposome formulation and potential regulatory hurdles (Cheng et al. 2012). Furthermore, even though diagnostic and theranostic liposome formulations enable direct visualisation of liposome transport kinetics longitudinally, there has yet to be a framework to link variability in accumulation to properties of the tumour microenvironment. Therefore, there may be added benefit to directly measure microenvironmental properties that are known to determine liposome accumulation and distribution using readily accessible clinical imaging methods. This strategy would provide complementary information that can be used in conjunction with direct visualisation of liposomes to elucidate underlying mechanisms responsible for variable accumulation and therapeutic efficacy. Such a strategy could also be used to select patients who are likely to respond to nanomedicine-based therapies and to guide the application of potential pharmacological agents that have been shown to reduce transport barriers and improve accumulation of nanomedicine-based therapies, including liposomes (Goldacre & Sylven 1962; Suzuki et al. 1981; Jirtle 1988; Sagar et al. 1993; Heldin et al. 2004; Hattori et al. 2011).

There are several physiological barriers that influence the intra-tumoural distribution of liposomes. In general, liposomes have been shown to largely accumulate in well perfused tumour regions with liposome extravasation limited by elevated IFP and vessel permeability (Baxter & Jain 1989; Yuan et al. 1994). The intra-tumoural penetration of liposomes is also hindered by the tumour stroma and the ECM (Chauhan et al. 2011). There are currently no established techniques to image interstitial fluid pressure; however, DCE-CT or dynamic contrast enhanced magnetic resonance imaging (DCE-MRI), allows for direct assessment of interstitial and vessel properties of the tumour microenvironment (O’Connor et al. 2011). This is accomplished by measuring the kinetics of a low-MW contrast agent as it flows through
tumour blood vessels, extravasates from the vessels and penetrates into the interstitial space. Fitting the measured kinetic curves using an established pharmacokinetic model provides quantitative estimates of the interstitial volume fraction (a surrogate of contrast agent penetration), the plasma volume fraction and $K_{trans}$ which reflects vessel permeability/blood flow (Tofts et al. 1999). This method provides the ability to relate the interstitial and vascular properties of tumours to the intra-tumoural accumulation of liposomes. Therefore, the ability to predict the intra-tumoural accumulation of liposomes using DCE imaging of tumour perfusion and a clinically approved small MW contrast agent is investigated.

DCE-CT was used to measure the intra-tumoural distribution of perfusion and permeability and anatomical CT was used to map the spatio-temporal accumulation of a CT-liposome contrast agent. CT was chosen for this proof of principle study since it provides simple and accurate quantitative measurements of both tumour perfusion and liposome accumulation (Zheng et al. 2009; O’Connor et al. 2011); however, other perfusion imaging strategies such as DCE-MRI may also be used. Analysis was performed by dividing the tumour into sub-volumes based on a curvilinear coordinate system. This approach was taken to minimise the deleterious effects of image noise and deformation errors while observing the intra-tumoural pattern of CT-liposome accumulation. A statistically significant positive correlation was found between quantitative measures of tumour perfusion and liposome accumulation. This demonstrates that quantitative, image-based measures of tumour perfusion can predict the intra-tumoural accumulation of liposomes, suggesting that DCE-CT may be an important technique for selecting patients that are likely to respond to liposome-based therapies.

3.3 Materials and methods

3.3.1 Animals

All experiments were performed under a protocol approved by the University Health Network Institutional Animal Care and Use Committee. The experiment was performed using a ME180 cervix carcinoma tumour model implanted in an OR site and an IM site. The OR ME180 tumours were established by suturing a 2–3 mm³ tumour fragment onto the cervix of female SCID mice (Cairns and Hill 2004). The IM ME180 tumours were established by injecting approximately $1 \times 10^6$ ME180 cells into the
left gastrocnemius muscle tissue of female SCID mice. In total 16 OR and 10 IM tumour bearing mice were used for this study. The 16 OR mice were a conglomerate of 3 independent experiments and the 10 IM mice were a conglomerate of 2 independent experiments. The imaging studies commenced 3 weeks post inoculation. The mean of the tumour volumes at the start of each study were 267±45 mm$^3$ and 196±138 mm$^3$ for the OR and IM cohorts, respectively. Tumour volume was measured using micro-CT (eXplore Ultra, GE Healthcare, London, Canada).

### 3.3.2 CT-liposome Preparation and Characterisation

Imaging the intra-tumoural accumulation of liposomes was accomplished by encapsulating a clinically approved CT contrast agent (iohexol) within the aqueous interval volume of the liposomes (Zheng et al. 2009; Dunne et al. 2011; Huang et al. 2013). The CT-liposome contrast agent was prepared according to previously described methods (Zheng et al. 2009). The liposomes were composed of DPPC, cholesterol and a polyethylene glycol derivatised lipid (DSPE-PEG$_{2000}$). The CT contrast agent, iohexol (Omnipaque™, 300 mg/mL of iodine, GE Healthcare) was encapsulated within the lipid vesicles and concentrated to a final iodine concentration of approximately 45 mg mL$^{-1}$. The CT liposomes were approximately 90 to 100 nm in diameter as measured using a 90Plus particle size analyzer (Brookhaven, Holtsville, NY). The iodine concentration in the CT-liposomes was determined by rupturing liposomes using a 10-fold excess of ethanol, diluting in HBS and measuring the ultraviolet (UV) absorbance at a wavelength of 245 nm using a Cary 50 UV-visible spectrophotometer (Varian, Palo Alto, CA). Previous studies have confirmed good retention of the CT contrast agent within liposomes in vivo (Zheng et al. 2006; Zheng et al. 2007; Dunne et al. 2011).

### 3.3.3 DCE-CT and CT-liposome Imaging

Mouse imaging was performed using a micro-CT system (80 kV, 70 mA). The reconstructed voxel size was 0.153x0.153x0.153 mm$^3$ for anatomical and 0.153x0.153x0.462 mm$^3$ for DCE-CT scans. A laser positioning system was used to approximately orient the mice/tumours in the same position between scans and manual rigid registration was performed during post-processing to spatially align the data sets. An intravascular bolus of CT-liposomes equivalent to roughly 1240 mg lipid kg$^{-1}$ and 400 mg iodine kg$^{-1}$ was administered using an injection pump (Harvard Apparatus, Canada) at a rate of 10 µL per second through a 27G butterfly catheter inserted into the lateral tail vein. Volumetric anatomical images were
acquired pre-administration of the CT-liposome formulation and at 5 min, 8 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs and 144 hrs post-administration. The DCE-CT measurements were accomplished by injecting approximately 90 µL of free iohexol (Omnipaque™-300, GE Healthcare, New Jersey, USA) mixed with 10 µL of saline through the tail vein catheter using the same injection pump settings. Volumetric images were acquired continuously every second for the first 30 seconds and then intermittently every 10 seconds for 4.5 minutes to capture the rapid wash-in and slow wash-out kinetics typical of free-iohexol.

![Figure 3.1](image.png)

**Figure 3.1** A schematic of the sub-volume sectioning of an OR tumour. (a) A transverse CT image of an OR cervix tumour xenograft (dashed contour) at 72hrs post liposome injection. (b) The tumour contour was divided into 4 concentric rings and a core volume. The rings were further sub-divided to generate a total of 91 sub-volumes. The parameters $\varepsilon_i$, $\varepsilon_p$, $K_{\text{trans}}$, $AUC_{\text{iox}}$, $F_{\text{lip}}$, $AUC_{\text{lip}}$ and $C_{\text{peak}}$ were determined in each sub-volume.

### 3.3.4 Data Analysis

The DCE-CT data was spatially re-sampled using the nearest neighbour criteria to match the voxel dimensions of the anatomical data sets. The CT-liposome and DCE-CT data sets were contoured and three-dimensional volumes of interest (VOI) were generated of each tumour at each time point. The VOIs were made using a semi-automatic approach that combined manual contouring and threshold refinement. A custom algorithm was implemented in Matlab (Mathworks, Natick, MA) to divide each tumour VOI into 4 concentric rings of roughly equal radius surrounding a tumour core region (Figure 3.1). The concentric rings were divided into sub-volumes using a spherical coordinate system. This was accomplished by defining the center of mass (CM) of the VOI as the origin for the coordinate system, defining the sagittal, coronal and transverse planes as the $xz$, $xy$ and $yz$ planes respectively, and using the
spherical coordinates $r$, $\theta$, and $\varphi$ to define the tumour coordinate system. The 4 rings and core region were defined by dividing the tumour radius (defined as the radius of a sphere with a volume equivalent to the VOI), by 5. The sub-volumes were determined by dividing each ring into small $dr$, $d\theta$ and $d\varphi$ segments, where $dr$, $d\theta$ and $d\varphi$ were chosen to give roughly equal sub-volume sizes. Overall, the tumour was divided into a total of 91 sub-volumes. The number of sub-volumes and their geometry was chosen to: (1) reduce the effects of co-localisation errors; (2) reduce the effects of tumour growth during the period of liposome imaging; (3) provide sufficient contrast signal for both DCE-CT and anatomical liposome quantification; and (4) to track the radial pattern of CT-liposome accumulation observed from imaging. A similar segmentation approach has previously been used to investigate the intra-tumoural heterogeneity in perfusion using DCE-CT (Cao et al. 2009). The tumour concentration of free iohexol was determined in each region by calculating the mean HU and converting to iodine concentration by subtracting the mean HU obtained prior to contrast administration and scaling by the calibration factor of 50.1±0.4 HU per mgI cm$^3$. The plasma concentration for the free iohexol was determined in the descending aorta and adjusted for the arterial hematocrit ($Hct_{aorta} = 0.5$; (Cairns & Hill 2004)).

The modified Tofts model (Tofts et al. 1999) was fit to the DCE-CT derived time intensity curves (TIC) measured in each sub-volume using the Levenberg-Marquardt non-linear least squares algorithm implemented in Matlab. Multiple initial conditions were used to fit each TIC in an attempt to determine the global minimum of the residual fitting metric. The modified Tofts model gave estimates of the plasma volume fraction ($e_p$), extra-cellular extra-vascular (interstitial) volume fraction ($e_i$) and the rate of extravasation ($K_{trans}$). The rate of extravasation $K_{trans}$ has been shown to be related to both vascular permeability and TBF (Tofts et al. 1999). Additionally, the area under the curve of the free iohexol TICs ($AUC_{iox}$), normalised to the arterial plasma AUC, was calculated for each sub-volume by integrating the TIC over the duration of the scan (i.e. from 0 to 300 seconds).

The plasma concentration for CT-liposomes was determined in the descending aorta and adjusted for the arterial hematocrit. An estimate of the percent injected dose (%ID) for CT-liposomes in the tumour was obtained by dividing the tumour concentration by the initial plasma concentration of CT-liposomes and multiplying by 100%. The mean plasma volume fraction ($e_p^{lipo}$) of each tumour was also estimated using early time point imaging of CT-liposomes. At an early time point the CT-liposomes are assumed to
be predominantly intravascular. \( e_p^{lipo} \) was determined by taking the ratio of mean CT-liposome iodine concentration measured in each tumour sub-volume to that in plasma 5 min after injection. The plasma volume fraction was used to subtract the contribution of the vascular CT-liposomes from the measured tumour concentration of liposomes, providing an estimate of the interstitial concentration of CT-liposomes in the tumour. The area under the curve over the total scan time (\( \text{AUC}_{lipo} \)) and the peak concentration (\( C_{peak} \)) of the CT-liposome concentration curves were determined in each sub-volume and used as metrics of CT-liposome accumulation. To account for inter-mouse variability in pharmacokinetics, \( \text{AUC}_{lipo} \) was normalised to the AUC of the arterial plasma concentration and \( C_{peak} \) was normalised to the peak plasma concentration.

### 3.3.5 Statistical Analysis

A linear mixed effect (LME) model was used to determine if the four DCE-CT derived parameters \( e_p, e_i, K_{trans} \) and \( \text{AUC}_{iox} \) and the CT-liposome estimate \( e_p^{lipo} \) were predictors of CT-liposome accumulation defined by the two response variables, \( \text{AUC}_{lipo} \) and \( C_{peak} \). Previous studies have demonstrated that estimating the plasma volume fraction using a low MW contrast agent may not be accurate, due in part to a high extraction fraction of the agent during first pass, and is better characterised using a macromolecular blood pool agent (Brasch 1991). Therefore, the CT-liposome estimate \( e_p^{lipo} \) was included as a predictor of liposome accumulation. Finally, each predictor was tested against the CT-liposome concentration at each time point. The predictor variables were considered random and modeled independently. Measured parameters were rejected from the analysis if they were less than zero or outside of the range of reported physiological values. All the predictors tested successfully for normality by observing that no bias or trend existed in the residuals of the best fit. A p-value<0.05 was considered statistically significant. Due to the exploratory nature of this study, multiple testing correction was not performed (Eliaz et al. 2004).

### 3.1 Results

Representative images of the free iohexol and CT-liposome enhancement patterns in an OR tumour at 300s and 72 hrs post-injection, respectively, are shown in Figure 3.2. In general, there was a strong visual concordance between the free-iohexol enhancement and CT-liposome enhancement patterns.
Strong peripheral DCE-CT enhancement was observed in most IM and OR tumours; however, the peripheral enhancement was heterogeneous. The core region was perfused in both tumour models and perfusion increased towards the periphery. The mean of the peak enhancement for free iohexol at the tumour periphery (ring 4) was on average 1.8 and 2-fold higher compared to the core in IM and OR tumours, respectively (Figure 3.3 a and b). The grand median estimates of $\varepsilon_p$, $\varepsilon_i$, $K_{\text{trans}}$ and $AUC_{i\text{ox}}$ are given in Table 3.1. There was considerably greater intra-tumoural compared to inter-tumoural variation for each parameter, indicating substantial heterogeneity in perfusion across the tumour volume. Perfusion appeared most heterogeneous along the tumour periphery, as indicated by substantial variation in DCE-CT parameter estimates in ring 4 compared to the other ring and core regions. In general, there was little difference in the DCE-CT derived parameters between tumour types suggesting similar levels of perfusion.

**Figure 3.2** (a) A representative DCE-CT at 300s post-injection of free iohexol in an OR tumour. (b) A similar slice obtained from CT-liposome imaging at 72 hrs post-injection of liposomes. (c) A representative parametric map of the DCE-CT derived parameter $K_{\text{trans}}$ (min$^{-1}$) and (d) CT-liposome accumulation parameter $AUC_{\text{lip}}$ (%).

Heterogeneous peripheral CT-liposome enhancement was also observed in many of the OR and IM tumours, which is consistent with observations of heterogeneous perfusion (Figure 3.2). The peak CT-
liposome enhancement at the tumour periphery (ring 4) was on average 1.3 fold higher compared to the core in both IM and OR tumours (Figure 3.3 c and d). The CT-liposome enhancement was similar in the core and intermediate ring regions (i.e. rings 1 to 3) in IM tumours and OR tumours. The grand median estimates of \( \varepsilon_p^{lipo} \), \( AUC_{lipo} \) and \( C_{peak} \) are given in Table 3.1. These results indicate that IM tumours had more intra-tumoural heterogeneity in CT-liposome enhancement compared to OR tumours. On average, there was a moderately higher peak level of CT-liposome accumulation in OR compared to IM tumours, which is also demonstrated in Figure 3.3 b and d. Given that perfusion metrics were similar between the two tumour types, this may be a consequence of reduced transvascular convection in the IM tumours caused by elevated IFP, which in turn limits the transport of liposomes. Indeed, IM ME180 tumours are known to have significantly higher IFP compared to OR ME180 tumours with no significant difference in vascular density (Lunt et al. 2008). The vascular half-life of the CT-liposome agent, as measured by CT-imaging, was on average 44±14 hrs.

LME analysis showed a statistically significant linear relationship between \( \varepsilon_p^{lipo} \) and \( \varepsilon_p \) in both OR tumours (slope=0.38±0.11; p-value<0.001) and IM tumours (slope=0.18±0.06; p-value=0.001). This indicates that both parameters provide estimates of the plasma volume fraction; however, there was not a direct one to one relationship between the two parameters. This may be due in part to the high extraction fraction of iohexol during first pass through the tumour. This has been reported to reduce the accuracy of \( \varepsilon_p \) estimates (Brasch 1991). Tumour growth was negligible for both tumour types. On average the OR and IM tumour volumes increased by approximately 1.5 fold, which was equivalent to a 0.5 mm increase in tumour radius over the course of the study.

The LME results showed that the DCE-CT derived parameters \( \varepsilon_p \), \( K_{trans} \) and \( AUC_{iox} \) independently predicted for \( AUC_{lipo} \) in IM and OR tumours (Table 3.2). The same parameters were found to be predictors of \( C_{peak} \) in both tumour types, with the exception of \( K_{trans} \) in IM tumours. Additionally, \( \varepsilon_p^{lipo} \) was also found to be an predictor of CT-liposome accumulation in both tumour types. The interstitial volume fraction (\( \varepsilon_i \)) was the only parameter that did not predict for CT-liposome accumulation. An example of the linear relationship between the predictors \( \varepsilon_p \), \( K_{trans} \), \( AUC_{iox} \), \( \varepsilon_p^{lipo} \) and the response variable \( AUC_{lipo} \) in a single tumour is given in Figure 3.4.
Figure 3.3 Average DCE-CT enhancement in a given ring, over all tumours, for OR (a) and IM (b) tumours. Tumours were well perfused, with perfusion increasing towards the tumour periphery (ring 4). Average CT-liposome accumulation in OR (c) and IM (d) tumours. There was a strong peripheral CT-liposome accumulation (ring 4) and a similar level of central tumour accumulation (core and rings 1 to 3) in both tumour types. Error bars represent standard error.

We also investigated how predictions of CT-liposome accumulation varied as a function of time. It was found that $\varepsilon_p^{tipo}$, $\varepsilon_p$, $K_{trans}$ and $AUC_{lox}$ independently predicted for CT-liposome accumulation in OR tumours between the 8 hr and 144 hr time points, with the exception of $\varepsilon_p^{tipo}$ at the 8 hr time point. In IM tumours $AUC_{lox}$ predicted for CT-liposome accumulation between 24 and 144 hrs and $\varepsilon_p^{tipo}$ predicted for CT-liposome accumulation from 48 to 144 hrs. There were only two time points (96 hr and 144 hr) where $\varepsilon_p$ and $K_{trans}$ predicted for CT-liposome accumulation in IM tumours.
Finally, given the large variability in IM tumour volume it was investigated if there was a relationship between tumour volume, tumour perfusion and liposome accumulation. IM tumours were separated into a small tumour group (86±39 mm$^3$; n=5) and a large tumour group (372±87 mm$^3$; n=5). In general, there was a trend towards higher perfusion and CT-liposome accumulation in small compared to large IM tumours. Specifically, small IM tumours had higher $AUC_{\text{iox}}$, $\varepsilon_i$ and $\varepsilon_p$ compared to larger tumours ($p$-value=0.012, 0.015, 0.056 respectively; two-sample t-test with equal variance). There was a trend towards lower $K_{\text{trans}}$ in larger tumours, but was not statistically significant ($p=0.34$). A statistically significant decrease in peak CT-liposome accumulation ($p=0.002$) and $AUC_{\text{lipo}}$ ($p=0.010$; after the removal of 1 outlier) was observed in larger compared to smaller IM tumours ($p=0.002$). Overall these results show that decreased tumour perfusion results in reduced CT-liposome accumulation in a tumour volume dependent manner. Furthermore, these results demonstrate the ability of tumour perfusion imaging in predicting liposome accumulation.

### 3.1 Discussion

In this study, it was demonstrated that quantitative measures of tumour perfusion predict for the intra-tumoural accumulation of liposomes in OR and IM ME180 cervix tumours. The tumour implantation sites were chosen based on known differences in tumour interstitial fluid pressure (Lunt et al. 2008). Given that MVP is the principle driving force for elevated IFP pressure (Boucher & Jain 1992) and that changes in MVP are reflected by changes in vascular morphology and TBF (Jain 1988), it was hypothesised that IM and OR ME180 tumours will show differences in the intra-tumoural accumulation of the CT-liposomes. Only small differences in the accumulation of CT-liposome were observed and no significant differences in $\varepsilon_p$ and $K_{\text{trans}}$ between the two tumour models were observed. However, as discussed above, small IM tumours had increased tumour perfusion and CT-liposome accumulation compared to large IM tumours. This further validated the use of tumour perfusion imaging to predict the intra-tumoural liposome accumulation.
Table 3.1 Summary of values obtained for the predictor and response variables in each region of the tumour in the two mouse xenograft models. Values reported are the mean over all mice for a given region (whole tumour, rings, or core). The range of values obtained is provided in brackets.
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Table 3.2: Summary of LME results for the predictor (across) and response (down) parameters. Values represent the fixed effect slope, the standard error of the fixed effect slope and the $p$-value (* denotes statistical significance).
The plasma volume fraction was found to be a good predictor for liposome accumulation, whether estimated using DCE-CT or CT-liposomes. A simple biophysical explanation for this relationship is that tumour blood vessels serve as the source of liposome delivery to tumour tissue. Given that liposomes typically remain in the peri-vascular space, an estimate of $e_p$ should be representative of liposome accumulation (Yuan et al. 1994). $K_{trans}$ was also a strong predictor of liposome accumulation. $K_{trans}$ represents the rate of trans-vascular liposome transport which depends on vascular permeability, trans-vascular fluid flow and blood flow (Tofts et al. 1999; Sarntinoranont et al. 2003; Haider et al. 2007). These properties of the tumour microenvironment have been shown to directly or indirectly influence the delivery of liposomes to tissue (Yuan et al. 1994; Heldin et al. 2004; Chauhan et al. 2012). Given that $AUC_{iox}$ is reflective of a combination of tumour perfusion, vascular permeability and the interstitial volume it is not surprising that it is also a predictor of liposome accumulation. The interstitial volume fraction, $e_i$, represents the fraction of tissue available for agent accumulation and was the only parameter that did not predict for liposome accumulation. The lack of correlation between $e_i$ and liposome accumulation may be attributed to the dense interstitial matrix which acts as a barrier, limiting liposome penetration (Chauhan et al. 2012). Conversely, the available fraction of interstitial volume is much greater for the small molecule iohexol. This result suggests that the perfusion imaging using a low MW contrast agent is insufficient to predict for liposome penetration.

A method of dividing the tumour into sub-volumes was used to probe the spatial relationship between perfusion and liposome accumulation. While performing the analysis on a voxel-by-voxel basis would further elucidate the spatial relationship between perfusion and liposome accumulation and strengthen the predictive capacity of the method, uncertainty in co-registration of data sets due to tumour growth, misalignment and deformation over the course of the study makes accurate tracking on a voxel-by-voxel basis difficult. Therefore, the analysis method used in this study represents a realistic framework to compensate for the aforementioned uncertainties. The framework was based on dividing the tumour into rings based on the contoured surface of the tumour and each ring was further divided into smaller sub-volumes using a curvilinear coordinate system. The results presented in this study are based on dividing the tumour into 91 sub-volumes; however, the analysis was repeated using 33 to 91 sub-volumes and similar results were obtained. Given that reproducible results were achieved for different numbers of sub-volumes tested, dividing the tumour into smaller sub-volumes was not explored.
Figure 3.4 Representative scatter plots demonstrating the relationships between the parameters $\varepsilon_p$ (a), $\varepsilon_{lip}$ (b), $K_{trans}$ (c), $AUC_{iox}$ (d) and $AUC_{lipo}$. Each point represents a measurement obtained from a sub-volume of an individual tumour. The solid lines represent the fixed effect slopes obtained from linear mixed-effects modelling and demonstrate that each of these parameters predict for tumour accumulation of CT-liposomes.

The inter- and intra-tumoural accumulation of liposomes is known to be heterogeneous (Harrington et al. 2001; Zheng et al. 2009; Dunne et al. 2011), limiting the bioavailability of drug and reducing overall therapeutic effect (Huang et al. 1994; Kong & Dewhirst 1999; Kong et al. 2000; Minchinton & Tannock 2006; Ponce et al. 2007). The heterogeneous accumulation of liposomes is believed to be a consequence of several factors including spatio-temporally transient perfusion (Zlotecki et al. 1993; Hattori et al. 2011) and heterogeneous vascular permeability (Yuan et al. 1994). In this study, it was shown in a mouse model of human cervix cancer implanted in two different sites, that tumour perfusion imaging using
DCE-CT predicted for the intra-tumoural accumulation of CT-liposomes but did not predict for their intra-tumoural penetration. The ability to predict the accumulation of liposomes demonstrates that DCE-CT measurements of perfusion could be a powerful technique to select patients that are likely to respond to liposome-based, and potentially other nanoparticle-based, therapies. Extending on this concept, DCE-CT could be used to guide the application of “modulating” agents that render the tumour microenvironment more conducive to the delivery of liposomes. These modulating agents include several clinically approved therapies such as angiotensin II, radiation, hyperthermia and low dose TNF-α. These agents have been found to improve TBF, and/or modulate vascular permeability and in turn improve the accumulation and intra-tumoural distribution of liposomes (Kong et al. 2000; Ten Hagen et al. 2000; Davies et al. 2004; Hattori et al. 2011).

Direct imaging of liposome-based CAs and ‘theranostic’ liposomes have been used to monitor their spatio-temporal distribution in solid tumours. Several studies have demonstrated that image-based methods can provide quantitative information on the inter- and intra-tumoural heterogeneity in liposome accumulation (Gabizon et al. 1991; Harrington et al. 2001; Zheng et al. 2009). Indeed, there is considerable interest in the development of therapeutic-diagnostic pairs wherein the diagnostic formulation is an image-able surrogate of the therapeutic. In a study by Karathanasis et al. (2009) quantitative imaging of the rate and extent of liposome accumulation prior to the administration of liposomal doxorubicin was strongly correlated with a slower tumour growth rate in response to the liposomal therapy. This demonstrates the utility of an image-based technique to provide information on the degree of liposome accumulation and subsequent therapeutic response. However, there are challenges associated with direct imaging of liposomes, including the additional complexity of synthesis and cost for each liposome formulation and potential regulatory hurdles (Cheng et al. 2012). Also, due to the extended circulation lifetime of liposomes and relatively slow rate of tumour accumulation, images need to be acquired several hours or even days following contrast agent administration. This complicates the work flow due to the requirement for additional patient visits. This may not be feasible due to patient and scanner availability, increased cost associated with imaging and increased radiation exposure (in the case of radiographic imaging techniques).
The use of DCE perfusion imaging can be used to complement direct imaging of liposome accumulation. While the proof of principle presented in this study was based on DCE-CT other perfusion imaging modalities such as DCE-MRI could be used. Indeed both techniques provide a quantitative method to assess tumour perfusion in the clinical setting. The DCE perfusion imaging methodology requires only a single imaging session prior to liposome administration to assess the state of tumour perfusion and permeability which can then be used to make a clinical decision as to the potential benefit of liposomal therapy prior to administration. Tumours exhibiting low $K_{trans}$ could be pre-treated with a “modulator” that improves TBF or vascular permeability and leads to improved liposome accumulation. Furthermore, DCE-CT imaging can be used to guide the rationale application such EPR modulating agents.

We have also demonstrated that direct imaging of CT-liposomes can be used to quantify the plasma volume fraction, $\varepsilon_p^{lip}$ and predict liposome accumulation. The plasma volume fraction can be estimated immediately after imaging and obviates the need to wait for sufficient tumour accumulation and plasma clearance of liposomes. Alternatively, the use of macromolecular CAs could also be used as a proxy to estimate the plasma volume fraction with higher sensitivity, higher accuracy and faster clearance than what may be achieved using diagnostic or theranostic liposomes. There are several macromolecular imaging agents available for SPECT, MRI and PET that may be suitable for this application (Brasch 1991; Harrington et al. 2001; Barrett et al. 2006; Qin et al. 2009).

There are still some fundamental questions that would help facilitate clinical translation of the proposed technique. For instance, what effect does the altered accumulation kinetics of liposomes in humans compared to mice have on the predictive power of DCE-CT measurements of tumour perfusion? Differences in tumour growth rate and the tumour microenvironment have been observed in human tumours compared to xenograft mouse cancer models. Do these issues reduce the ability of DCE-CT to predict liposome accumulation in the clinical setting? How should $K_{trans}$, $\varepsilon_p$ and $AUC_{iax}$ be interpreted such that accurate identification of potential non-responders to liposome treatment is possible.
3.2 Conclusions

In this study, the capacity of tumour perfusion imaging to predict intra-tumoural accumulation of liposomes was demonstrated in a mouse xenograft tumour models of cervical cancer implanted in two different tissue sites. It was found that regions with higher $K_{\text{trans}}$, $\varepsilon_p$ and $AUC_{\text{iox}}$ have a greater degree of liposome accumulation. This suggests that DCE-CT measurements of perfusion could provide a powerful technique for the selection of patients that are likely to respond to liposome-based and potentially other nanoparticle-based, therapies. Furthermore, these results have translational significance owing to the fact that DCE-CT is already a clinically approved procedure and there are several liposome-based therapies in clinical use. In summary, the DCE-CT technique allows for estimates of tumour perfusion, predicts for liposome accumulation and is readily accessible in the clinical environment.

3.3 Acknowledgements

The authors would like to thank Dr. Naz Chaudary and Dr. Richard P. Hill for providing mice bearing the ME180 xenografts and Lin Yu Fan for preparing the CT-liposomes. Shawn Stapleton is grateful to have received funding from the Natural Science and Engineering Research Postgraduate Scholarships Program and the Terry Fox Foundation Strategic Initiative for Excellence in Radiation Research for the 21st Century (EIRR21) at CIHR. This study was supported by grants from the Terry Fox New Frontiers Program and the Canadian Institutes of Health Research.

3.4 References


Chapter 4

Volume Fraction Imaging: A New Method for Quantitative Assessment of Tumour Vascularity

4.1 Abstract

DCE-CT and DCE-MRI are employed to non-invasively characterise the abnormal morphology and physiology of tumour microcirculation caused by unregulated angiogenesis. Quantification is achieved by fitting a tracer kinetic model (TKM) to the imaging data to obtain estimates of plasma and interstitial volume, blood flow, and capillary permeability. The computational complexity, lack of reproducibility and limitations of TKMs have hindered the widespread use of DCE imaging. A dual CA imaging strategy, termed Volume Fraction Imaging or VFI for short, is proposed as a means to improve quantification of morphology and physiology of solid tumours. The VFI methodology is based on the sequential injection of intravascular and freely diffusible CAs, allowing for model independent estimates of tissue volume fractions that can subsequently be used to increase the accuracy of TKM. In this study, the ability of VFI to improve image-based quantification of tissue volume fractions, perfusion, and microvascular permeability of different tissues was evaluated. Quantification of tissue volume fractions was performed by taking the ratio of tissue to arterial TIC for the intravascular and freely diffusible CA respectively. Perfusion and permeability was then estimated using a two-compartment model where the tissue volume fractions are fixed parameters. The accuracy of the method was determined using simulated TICs with a range of noise and physiological values, generated using an established physiologically relevant indicator dilution model. Additionally, in vivo VFI was performed in brain, muscle, and tumour tissue. CT-liposomes and free-ihexol were the intravascular and freely diffusible CA respectively. It was demonstrated in silico that the VFI method substantially improves the accuracy and precision of plasma and interstitial volume, blood flow, and capillary permeability estimates compared to the standard two-compartment model. It was also demonstrated that VFI can substantially improve the robustness of tumour perfusion imaging in vivo in brain, muscle, and tumour tissue. Therefore, Volume Fraction Imaging can substantially improve the accuracy and robustness quantitative tissue perfusion imaging. Thus, allowing for an improved understanding of the relative importance between perfusion and vascular permeability in mediating the intra-tumoural accumulation of CT-liposomes.
4.2 Introduction

There is a long standing interest in using imaging to non-invasively characterise the abnormal physiology of the tumour microcirculation that arises due to unregulated angiogenesis (Miller et al. 2005). Clinically relevant imaging methods include DCE-CT and DCE-MRI (O’Connor et al. 2011). DCE imaging is typically performed by detecting the temporal dynamics of a freely diffusible low molecular weight CA after injection into the blood stream. The temporal changes in the imaging signal, or TIC, obtained following the administration of the CA is a function of the local blood supply, trans-vascular rate of transport, interstitial space of the tissue region of interest (ROI), and physicochemical properties of the agent (Zierler 1962). Quantification is achieved by fitting a pharmacokinetic model to the measured TICs to obtain estimates of several physiological parameters including plasma and interstitial volume, blood flow, and capillary permeability (Sourbron & Buckley 2012). Quantitative DCE imaging of tumour perfusion has shown potential in both the pre-clinical and clinical research settings for disease assessment and monitoring of treatment response, providing an important tool to non-invasively quantify physiologically relevant parameters (Dugdale et al. 1999; Miles 2002; Padhani & Miles 2010; Chung et al. 2012).

Several mathematical approaches have been proposed for quantification of DCE imaging data (Sourbron & Buckley 2012) and all are based on two fundamental and related theories: (i) indicator dilution theory (Zierler 1962; Peters 1998; Sourbron & Buckley 2012) and (ii) PK compartmental modelling (Tofts et al. 1999; Brix et al. 2010; Sourbron & Buckley 2012). Based on the complexity of the mathematical approach (Johnson & Wilson 1966; St Lawrence & Lee 1998; Brix et al. 2010), it is possible to obtain estimates of several physiological parameters, including: the plasma volume fraction ($\varepsilon_p$), interstitial volume fraction ($\varepsilon_i$), regional vascular permeability surface area product ($PS/V$), regional tissue perfusion ($F_p/V$), and regional mean capillary transit time ($t_c$). However, the computational complexity as well as limited reproducibility and accuracy have hindered the widespread use of DCE imaging for absolute quantification of tissue morphology and physiology (Buckley 2002; Brix et al. 2009). Of notable difficulty is the application of complex models that have many free parameters, leading to over-fitting and estimate bias. This issue is compounded by noise in the detection system, and by high extraction fractions of freely diffusible CA during the first pass that can result in a TIC with minimal information regarding the vascular characteristics of the tissue and leading to bias in estimates of both $\varepsilon_p$ and $F_p/V$ (Brasch 1991; Brasch & Turetschek 2000).
We propose a simple strategy, termed Volume Fraction Imaging or VFI for short, as a means to reduce the complexity of TKM by allowing for model independent estimates of $\epsilon_p$ and $\epsilon_i$, in a manner that does not require imaging the dynamics of the CAs over time. The VFI methodology is based on the differential transport nature of intravascular and freely diffusible CAs. As demonstrated in this study, the sequential injection of an intravascular CA followed by a freely diffusible CA can be used to quantify $\epsilon_p$ and $\epsilon_i$ using only three static scans. A similar framework was proposed by Weissleder et al. (1998) and extended by Kim et al. (2004). However, their study was based on DCE-MRI imaging where absolute estimates of both parameters are strongly dependent on the MRI acquisition protocol, with considerable variability observed. In this study several improvements were made, including: (1) framing the method in an imaging modality independent manner; (2) demonstrating that accurate model independent quantification of $\epsilon_p$ and $\epsilon_i$ can be made with only three static scans; (3) estimating the accuracy of VFI for determination of $\epsilon_p$ and $\epsilon_i$ using an *in silico* approach; and (4) demonstrating that using model independent estimates of $\epsilon_p$ and $\epsilon_i$ can reduce the complexity of TKM-based quantification using DCE imaging, resulting in improved accuracy of $F_p/V$ and $PS/V$ determination.

Overall, this study demonstrates that the VFI method provides a simple, TKM independent, and robust method to quantify two key properties of the tumour microenvironment, $\epsilon_p$ and $\epsilon_i$, that are important biomarkers of treatment response (Dugdale et al. 1999; Miles 2002; Kambadakone & Sahani 2009). Furthermore, model independent estimates of $\epsilon_p$ and $\epsilon_i$ can be used to increase the accuracy and precision of TKM estimates of $PS/V$, and $F_p/V$. The reduced complexity and increased accuracy may help facilitate the adoption of quantitative physiological imaging in the clinic. In this study, the VFI theory is presented, the accuracy and precision of model independent estimates $\hat{\epsilon}_p$ and $\hat{\epsilon}_i$, explored and the ability to use the VFI method to improve TKM of DCE imaging data assessed.

4.3 Materials and methods

4.3.1 VFI Theory

The general framework for quantitative VFI is presented in Figure 4.1. In this framework the sequential injection of an intravascular CA followed by a freely diffusible CA can be used to quantify $\epsilon_p$ and $\epsilon_i$ without the need of a TKM. Pre- and post-injection detection of the intravascular CA as well as and post–
injection detection of the freely diffusible CA provides sufficient information to quantify $\varepsilon_p$ and $\varepsilon_i$ within a tissue ROI. The signal enhancement arising from a CA within the tissue ROI is the sum of the signal enhancement originating from the vascular (or plasma), interstitial, and cellular compartments. Assuming that a hydrophilic CA, such as ioxehol, remains extracellular and a linear relationship between image signal intensity and CA concentration (e.g. using CT), one can then formulate an expression relating the image derived CA concentration to the weighted sum of CA concentrations in each compartment.

$$C_{roi}(t) = \varepsilon_p \cdot C_p(t) + \varepsilon_i \cdot C_i(t)$$ \hspace{1cm} 4.1

where $C_{roi}(t)$ is the measured CA concentration in the ROI as a function of time (t), $\varepsilon_p$ and $\varepsilon_i$ are the plasma and interstitial volume fractions respectively [ml ml$^{-1}$], and $C_p(t)$ and $C_i(t)$ are the plasma and interstitial CA concentrations, respectively.

The plasma volume fraction, $\varepsilon_p$ can be quantified using a macro-molecular (i.e. strictly intravascular) CA (i.e. $C_i(t) = 0$). Furthermore, steady-state between the feeding artery plasma and tissue plasma concentrations must be reached. Under these conditions, Equation 4.1 can be simplified and rearranged to give:

$$\varepsilon_p = \frac{C_{roi}^M(T_{ss}^M)}{C_a^M(T_{ss}^M)/(1 - Hct_{aorta})}$$ \hspace{1cm} 4.2

where the superscript $M$ denotes the use of a macro-molecular intravascular CA. It is assumed that at the steady-state time, $T_{ss}^M$, the tissue plasma concentration can be approximated by $C_p^M(T_{ss}^M) \equiv C_a^M(T_{ss}^M)/(1 - Hct_{aorta})$ where $C_a^M$ is the macro-molecular CA concentration in a large arterial vessel, and $Hct_{aorta}$ is the arterial hematocrit. $Hct_{aorta}$ ranges between 0.38 to 0.54 (Grabowski & Tortora 2003), is relatively invariant between species (Schmidt-Nielsen 1984), and is approximated as 0.5 in this study based on previous measurements performed in SCID mice (Cairns & Hill 2004).
Once $\varepsilon_p$ has been estimated $\varepsilon_i$ can be quantified using the TIC for a small molecule CA that rapidly diffuses from the vascular to the interstitial compartment. The CA concentration in the ROI is a sum of both the macro-molecular and small molecule agent concentrations (Figure 4.1), that is $C_{roi}(t) = C_{roi}^M(t) + C_{roi}^m(t)$ where the superscript $m$ denotes the small molecule agent. Assuming that the clearance of the macro-molecular agent is negligible compared to the time required to acquire the imaging data of the small molecule agent, then $C_{roi}^M = const$ and the contribution of the macro-molecular CA can be removed by a simple baseline subtraction (i.e. subtracting $C_{roi}^M$). Additionally, assuming the plasma and interstitial CA concentration is in steady-state, then Equation 4.1 can be rearranged to solve for $\varepsilon_i$,

$$\varepsilon_i = \frac{C_{roi}^m(T_{ss}^m)}{C_a^m(T_{ss}^m)/(1 - Hct_{aorta})} - \varepsilon_p$$

where $\varepsilon_p$ is obtained from Equation 4.2, $C_{roi}^m(T_{ss}^m)$ is the measured tissue concentration of the small molecule CA at the steady-state time $T_{ss}^m$, and $C_a^m(T_{ss}^m) \equiv C_p^m(T_{ss}^m) \equiv C_a^m(T_{ss}^m)/(1 - Hct_{aorta})$.

### 4.3.2 Simplification of Tracer Kinetic Modelling using VFI

After both $\varepsilon_p$ and $\varepsilon_i$ have been determined, a simplified approach can be used to obtain estimates of tissue perfusion ($F_p/V$), and the permeability surface area per unit volume ($PS/V$). Estimates of $F_p/V$, and $PS/V$ are possible by fitting the TIC for the freely diffusible CA with different types of TKM including: a distributed parameter model, tissue homogeneity model, or compartmental exchange model (Sourbron & Buckley 2012). Alternatively, a simplified indicator dilution theory approach can be used such as the ‘steepest slope’ method (Miles 1991) and Patlak analysis (Patlak et al. 1983). In this study, the two-compartment exchange TKM is used to illustrate the ability to reduce the complexity and improve the accuracy of $F_p/V$, and $PS/V$ estimates using the VFI methodology.
Figure 4.1 (a) A schematic representation of the concentration of a macro-molecular CA (green line) and small molecule CA (blue line) in a ROI placed in tissue (solid line) and in a large vessel (dashed line). The sequential injection of an intravascular, macro-molecular weight CA, followed by a small molecule, freely diffusible CA can be used to determine the $\varepsilon_p$ and $\varepsilon_i$, once steady-state has been reached. Subsequently capillary perfusion ($F_p/V$) and permeability ($PS/V$) can be estimated by fitting the freely diffusible CA TIC using a TKM where both $\varepsilon_p$ and $\varepsilon_i$ are known parameters. (b) Further illustration of the VFI theory in relation to imaging the tissue distribution of the macro-molecular and small molecule CAs. Three static scans pre- and post-injection of the macro-molecular and small molecule CAs can be used to determine $\varepsilon_p$ and $\varepsilon_i$, where the post-injection scans are performed once the plasma and interstitial concentrations have reached steady state.

The two-compartment exchange TKM allows for estimates of $F_p/V$, $PS/V$, $\varepsilon_p$ and $\varepsilon_i$. The general form of the two-compartment exchange model is:

$$\varepsilon_p \cdot \frac{dC_p(t)}{dt} = \frac{F_A}{V} \left( \frac{C_a(t - t_d)}{1 - Hct_{aorta}} - C_p(t) \right) + \frac{PS}{V} \cdot \left( C_i(t) - C_p(t) \right) \tag{4.4}$$

$$\varepsilon_i \cdot \frac{dC_i(t)}{dt} = \frac{PS}{V} \cdot \left( C_p(t) - C_i(t) \right) \tag{4.5}$$

where $V$ is the volume of tissue encompassed by the ROI [cm$^3$], $F_A/V$ is the apparent perfusion in [mL·min$^{-1}$·mL$^{-1}$], $PS/V$ is the capillary permeability surface area product per unit volume in [mL·min$^{-1}$·mL$^{-1}$], and $t_d$ represents the delay in time between the measured arterial plasma CA and the tissue ROI.
in [min]. Note that for TKM, $C_p(t) \neq C_a(t)/(1 - Hct_{aorta})$. For simplicity it was assumed that the density of the tissue of interest is 1 mg·cm$^{-3}$. The apparent plasma flow rate is based on a weak assumption that the concentration of CA at the draining venous outlet is equal to the spatially averaged capillary CA concentration. Under this condition $F_A = R \cdot F_p$, where $R = E/(1 - E \cdot F_A/PS)$ and $E = (1 - e^{-PS/F_A})$. This set of equations can then be used to approximate the true plasma flow rate (Brix et al. 2009). For the VFI methodology, each of the parameters in Equations 4.4 and 4.5 are known except for $F_p/V, PS/V$, and $t_d$. These parameters can be determined by fitting the solution to Equations 4.4 and 4.5 to the TICs obtained from a DCE scan of a small molecule freely diffusible CA.

### 4.3.3 Simulating Tissue Kinetic Curves using MMID4

A realistic physiological model incorporating multiple parallel vascular pathways and heterogeneous flow was used to simulate TICs of a realistic nature to which simplified VFI and a two-compartment TKM were fitted. The experiment was designed to assess the improvement in accuracy achievable using the VFI method compared to a standard two-compartment model. Realistic TICs were simulated using the multiple indicator, multiple path, indicator Dilution 4 region model (MMID4) and the JSim software package (JSim 2013). MMID4 is based on multiple indicator dilution theory and describes convection driven plasma flow and trans-vascular diffusion of a tracer in tissue. Tracer transport is modeled in three sequential tissue regions: the tracer enters the tissue region through an arterial vessel, and subsequently flows through multiple parallel pathways that consist of non-exchange vessels (arterioles and venules) and exchange vessels (capillaries), and then flows out of the tissue through a common vein. The transport process is described by several physiological parameters and all were set to zero with the exception of $F_p/V, PS/V, \varepsilon_p$ and $\varepsilon_i$. The multiple parallel exchange pathways provide the ability to incorporate regional flow heterogeneity and the transvascular transport is determined using a Lagrangian sliding fluid element algorithm. The MMID4 has been describe in further detail elsewhere and has also previously been used to validate different TKM approaches for quantifying DCE-CT and DCE-MRI imaging data (Brix et al. 2009; Brix et al. 2010).

The tissue of interest was modeled to consist of only exchange vessels (capillaries), delay and dispersion of the plasma input function were not considered (i.e. $t_d = 0$), and flow heterogeneity was modeled by including 20 parallel exchange vessels with a right skewed lagged normal density flow distribution.
(RD=0.55 and Skewness = 1.3). The mean $F_p/V$, $PS/V$, $\varepsilon_p$ and $\varepsilon_i$ were chosen to represent most normal and tumour tissue values and are shown in Table 4.1 (Brix et al. 2009; Brix et al. 2010). The input plasma concentration curves ($C_a(t)$) for both the intra-vascular and freely diffusible CA were obtained from in vivo DCE-CT measurements made in the descending aorta of a mouse – see description in the in vivo imaging methods below. The measured plasma concentration curves were smoothed using a lowess algorithm and resampled to a temporal resolution of 1 second (Matlab, Mathworks, Natick, MA). In total 40 TICs (i.e. $C^M_{ROI}(t)$ and $C^m_{ROI}(t)$) were generated using the intra-vascular and freely diffusible $C_a(t)$ data in combinations with the physiological parameters in Table 4.1. For the simulated CT-liposome tissue curves $PS/V$ was set to zero.

<table>
<thead>
<tr>
<th>$F_p/V$ (mL·mL⁻¹·min⁻¹)</th>
<th>$PS/V$ (mL·mL⁻¹·min⁻¹)</th>
<th>$\varepsilon_p$</th>
<th>$\varepsilon_i$</th>
<th>Noise $\sigma$ (A.U.)</th>
<th>Sampling Scheme</th>
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</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0</td>
<td>(S1) 1:300</td>
</tr>
<tr>
<td>0.2</td>
<td>0.05</td>
<td>0.02</td>
<td>0.04</td>
<td>0.5</td>
<td>(S2) 1:30 / 10:270</td>
</tr>
<tr>
<td><strong>0.3</strong></td>
<td><strong>0.1</strong></td>
<td><strong>0.03</strong></td>
<td><strong>0.06</strong></td>
<td>1</td>
<td>(T3) 1:60 / 10:210</td>
</tr>
<tr>
<td>0.4</td>
<td><strong>0.2</strong></td>
<td>0.04</td>
<td>0.08</td>
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<tr>
<td>0.5</td>
<td>0.3</td>
<td><strong>0.05</strong></td>
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</tr>
<tr>
<td>0.6</td>
<td>0.4</td>
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<td>0.15</td>
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<tr>
<td>1.0</td>
<td>1.0</td>
<td>0.10</td>
<td>0.35</td>
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</tr>
</tbody>
</table>

Table 4.1 Parameters used for MMID4 simulation of intravascular and freely diffusible agent TICs.

The simulated TICs also encompassed a variety of noise conditions and temporal sampling schemes that are typical in the pre-clinical and clinical setting (Table 4.1). Gaussian-distributed system noise of different levels was created using a random number generator (Matlab, Mathworks, Natick, MA) with a given standard deviation and added to the simulated TICs. Noise levels were chosen to be representative of the low and high noise levels representative of clinical and pre-clinical CT imaging systems. The temporal sampling schemes were chosen based on typical DCE-CT acquisitions and included: (S1) an ideal sampling scheme of 1 sample per second for 300 seconds for a total of 300 samples; (S2) an accepted pre-clinical sampling scheme of 1 sample per second for 30 seconds followed by 1 sample per...
10 seconds for 270 seconds for a total of 57 samples (Stapleton et al. 2013a); and (S3) an accepted clinical sampling scheme of 1 sample per seconds for 60 seconds followed by 1 sample per 10 seconds for 210 seconds for a total of 81 samples (Miles et al. 2012).

### 4.3.4 Validating the Steady-State Assumption

The requirement for an accurate model independent estimate of $\varepsilon_p$ is a steady-state between the feeding artery plasma and tissue plasma concentrations (i.e. $C_p^m(T_{ss}^M) \equiv C_a^m(T_{ss}^M)/(1 - Hct_{aorta})$). Similarly, an accurate model independent estimate of $\varepsilon_i$ requires that there is a steady state between the arterial plasma, tissue plasma and interstitial compartments (i.e. $C_i^m(T_{ss}^M) \equiv C_p^m(T_{ss}^m) \equiv C_a^m(T_{ss}^m)/(1 - Hct_{aorta})$). These assumptions are approximately valid after sufficient time when: (1) the input and output plasma concentrations have equilibrated; and (2) the plasma and interstitial concentrations are changing very slowly. Furthermore, the validity of $C_i^m(T_{ss}^M) \equiv C_p^m(T_{ss}^M)$ requires that the partition coefficient of the CA between plasma and interstitial space is unity and was suggested by Tofts & Kermode (1991) to be likely given that the solvent is plasma in both compartments. Steady-state may occur for a bolus injection at late time points when the slow phase of CA systemic clearance or by infusing the small molecule CA such that a steady-state concentration is reached in the plasma.

The MMID4 simulations were used to determine if the steady state conditions are achievable for the pharmacokinetics, range of physiological properties, and duration of the DCE scan used in this study. The time to reach steady state concentration for the macro-molecular agent is denoted by $T_{ss}^M$, and was defined as the minimum time where the percent difference between the inflow and outflow plasma concentrations was less than 5% for at least 30 seconds. The time to reach steady state concentration for the macro-molecular agent was denoted by $T_{ss}^m$ and was defined as the minimum time where the percent difference between the plasma and interstitial concentration was less than 5% for at least 30 seconds.
4.3.5 Establishing the VFI Method

We estimated the theoretical accuracy and precision of the VFI based quantification of $\varepsilon_p$ and $\varepsilon_i$ by applying Equations 4.2 and 4.3 to the MMID4 simulated TICs. In total 2400 TICs were generated and comprised a combination of 40 TICs generated using the range of physiological parameters given in Table 4.1, 6 noise levels, and 10 trials. The accuracy and precision of a parameter estimate (e.g. $\varepsilon_p$) at a given noise level was defined by the median relative error and the interquartile range of the solution set obtained from repeated trials, and for different combination of the other physiological parameters (e.g. $\varepsilon_1, F_p/V, PS/V$).

4.3.6 Improving the Accuracy of Tracer Kinetic Modelling using VFI

The ability to improve the accuracy and precision of TKM estimates of $F_p/V$ and $PS/V$ when $\varepsilon_p$ and $\varepsilon_i$ were known, was established by comparison to a standard two-compartment model approach. Estimates of physiologic parameters were obtained by fitting the freely diffusible CA TIC using the following approaches: (1) fixing $\varepsilon_p$ and $\varepsilon_i$ and fitting the TIC using a two-compartment model resulting in estimates of $F_p/V$ and $PS/V$, termed VFI-1; (2) bounding $\varepsilon_p$ and $\varepsilon_i$ by $\pm20\%$ of their estimated value and fitting the TIC using a two-compartment model resulting in estimates of $F_p/V$ and $PS/V$, termed VFI-2; and (3) keeping all parameters free and fitting the TIC using a two-compartment model resulting in estimates of $F_p/V$, $PS/V$, $\varepsilon_p$ and $\varepsilon_i$, termed 2CM. For the VFI-1 method model independent estimates of $\varepsilon_p$ and $\varepsilon_i$ were used as the fixed parameters. The bounds used for the VFI-2 method were determined based on simulation results that suggested uncertainties in model independent estimates of $\varepsilon_p$ and $\varepsilon_i$ are in the range of $\pm20\%$. Additionally, the free parameters for each method were bounded during the fitting process as follows: $F_p/V$ was bounded between 0 and 5 mL·min$^{-1}$·mL$^{-1}$, $PS/V$ was bounded between 0 and 2 mL·min$^{-1}$·mL$^{-1}$, both $\varepsilon_p$ and $\varepsilon_i$ were bounded between 0 and 1 mL·mL$^{-1}$. Curve fitting was performed using a trust-region nonlinear least squares algorithm using Matlab and multiple initial conditions were used in order to obtain a global minimum of the residual function. A comparison between the accuracy and precision of estimated physiological parameters obtained using the three methods (VFI-1, VFI-2, and 2CM) were performed, where each parameter estimate was repeated 10 times. The investigation also included evaluating the effects of noise and the sampling scheme on the accuracy and precision of the VFI method compared to the 2CM method.
4.3.7 The Computed Tomography VFI Protocol

As previously mentioned, a model independent assessment of $\varepsilon_p$ and $\varepsilon_i$ requires only three static scans: (1) a baseline scan prior to the injection of the intra-vascular CA; (2) a scan post-injection of the intra-vascular CA at time $T_{ss}^M$, but pre-injection of the freely diffusible CA; and (3) a scan post-injection of the freely diffusible CA at a time greater than $T_{ss}^M$. Taking the difference between the signal enhancement from scans 2 and 1 in a major artery and in the tissue ROI can be used to estimate of $C_{a}^m(T_{ss}^M)$ and $C_{roi}^m(T_{ss}^M)$ can be used to calculate $\varepsilon_p$ through Equation 4.2. Taking the difference between Scan 3 and 2 gives an estimate of $C_{a}^m(T_{ss}^M)$ and $C_{roi}^m(T_{ss}^M)$ that can be used to calculate $\varepsilon_i$ through Equation 4.3.

For quantification of $F_p/V$ and $PS/V$ the VFI protocol was modified as follows: (1) a baseline scan prior to the injection of the intra-vascular CA; (2) a scan post-injection of the intra-vascular CA at time $T_{ss}^M$, but pre-injection of the freely diffusible CA; and (3) an approximately 5 minute DCE scan during the bolus injection of the freely diffusible CA. Scans 1 and 2 can be used to calculate $\varepsilon_p$ as previously described. Scan 3 can be used to find a length of time (e.g. 30 seconds) when the freely diffusible CA is in steady state. At this time point the mean signal intensity can be calculated and used to estimate $\varepsilon_i$ as described above. Then either the VFI-1 or VFI-2 methods (or a combination of both) can be used to obtain an accurate estimate of $F_p/V$ and $PS/V$. In this study $t_d$ was a free parameter during the fitting process.

4.3.8 In Vivo Application of VFI

All in vivo experiments were performed under a protocol approved by the University Health Network Institutional Animal Care and Use Committee. VFI measurements were performed in two human xenograft model in mice: (1) a patient derived orthotopic cervix carcinoma tumour model (OCICx26); and (2) an orthotopic metastatic breast adenocarcinoma tumour model (MDA-MB-231 or MDA231 for short). The OCICx26 tumours were established by suturing a 2–3 mm$^3$ tumour fragment onto the cervix of female SCID mice (n=9) and the model has been previously described elsewhere (Chaudary et al. 2012). The MDA231 were established by injecting $2 \times 10^6$ cells in the lower abdominal mammary fat pad of female SCID mice (n=9). VFI was performed once MDA231 tumours reached $249\pm 79$ mm$^3$ and OCICx26 tumours reached $121\pm 33$ mm$^3$. 
VFI was performed using a micro-CT system (80 kV, 70 mA). The reconstructed voxel size was 0.153x0.153x0.153 mm\(^3\) for anatomical and 0.153x0.153x0.462 mm\(^3\) for DCE-CT scans. The macro-molecular CA employed in this study consisted of liposomes carrying the CT contrast agent iohexol, termed CT-liposomes, and the formulation details have been previously described (Chapters 2 and 3). An intravascular bolus of CT-liposomes equivalent to roughly 1240 mg lipid kg\(^{-1}\) and 400 mg iodine kg\(^{-1}\) was administered using an injection pump (Harvard Apparatus, Canada) at a rate of 10 µL per second through a 27G butterfly catheter inserted into the lateral tail vein. Volumetric anatomical images were acquired pre- and 5 minutes post administration of the CT-liposome. A 5 minute DCE-CT scan was performed during the bolus administration of CT-liposomes and used to derive the CT-liposome arterial input function for simulations. The DCE-CT imaging protocol consisted of acquiring volumetric images continuously every second for the first 30 seconds and then intermittently every 10 seconds for 4.5. Immediately following the 5 minute post-injection CT-liposome scan, a DCE-CT scan of the freely diffusible tracer iohexol (Omnipaque\textsuperscript{TM}-300, GE Healthcare, New Jersey, USA) was performed. The DCE-CT scan consisted of injecting approximately 90 µL of free iohexol mixed with 10 µL of saline using the same injection method as with the CT-liposomes. Following the free-iohexol DCE-CT scan, a final static CT scan was performed at approximately 5 minutes after the administration of Omnipaque\textsuperscript{TM}. The VFI protocol described above was used to determine \(\varepsilon_p\), \(\varepsilon_i\), \(F_p/V\) and \(PS/V\) for gastrocnemius muscle, brain, and tumour tissue. The field of view for the DCE-CT scans only allowed for imaging of tumour and muscle in the same field of view, thus precluding the ability to estimate \(F_p/V\) and \(PS/V\) in the brain. The estimated parameters were also compared to literature values.

4.3.9 Statistical Analysis

Statistical analysis was performed using OriginPro 9.0 (OriginLabs, USA). A one-way ANOVA and Tukeys Test were used to determine statistical significance between in vivo VFI and 2CM parameter estimates for different tissues. Tukeys test includes a correction for multiple testing. Normality was confirmed using the Shapiro-Wilk test. The null hypothesis was rejected at a p-value less than 0.05.
Figure 4.2 Time to reach steady-state conditions for both the intravascular CA ($T_{ss}^M$) and the freely diffusible CA ($T_{ss}^M$) as a function of (a) $F_p/V$, (b) $PS/V$, (c) $\varepsilon_p$ and (d) $\varepsilon_i$. The intravascular CA reaches steady-state in less than 1 minute for all physiological parameters. On average the freely diffusible agent reaches steady-state within 1.2 minutes, but can take up to 4.2 minutes for low $PS/V$ or high $\varepsilon_i$.

4.4 Results

4.4.1 Establishing Validity of Steady-state Assumptions

The MMID4 simulations indicated that in general it is possible to achieve steady state for a bolus injection of both the intra-vascular and freely diffusible CAs, range of physiological properties, and scan duration used in this study (Table 4.1). For the intra-vascular CA the arterial and tissue plasma concentrations reached steady state with a median $T_{ss}^M$ =0.4 min (range: 0.4 to 1.2 min) for all physiologic parameters used in the simulation. In general, $T_{ss}^M$ increased with decreasing $F_p/V$ or
increasing $\varepsilon_p$. Once $T_{ss}^M$ was reached it was possible to obtain a model independent estimate of $\varepsilon_p$ with an uncertainty of at most 5%. This level of accuracy was maintained for up to 5 minutes, the duration of the simulated intra-vascular CA TICs.

For the freely diffusible CA the interstitial and tissue plasma concentrations reached steady with a median $T_{ss}^m = 1.20$ min (range: 0.47 to 4.20 min). Steady state interstitial and tissue plasma concentrations were not achievable within 5 minutes for both $PS/V = 0.01$ and $\varepsilon_i = 0.35$, indicating that a substantially longer imaging time is needed for tissue regions that have extremely low vessel permeability or high interstitial volume fraction. In general, $T_{ss}^m$ increased with decreasing $F_p/V$, decreasing $PS/V$, increasing $\varepsilon_p$, or increasing $\varepsilon_i$. Once the steady state times $T_{ss}^m$ was reached it was possible to obtain a model independent estimate of $\varepsilon_i$ with an uncertainty of at most 5%. This level of accuracy was also maintained for up to 5 minutes.

These results established that a model independent estimates of $\varepsilon_p$ and $\varepsilon_i$ can be obtained within a few minutes post-injection of the intra-vascular CA, freely diffusible CA and the range of physiological parameters tested in this study. These results are CA agent dependent and thus specific the arterial plasma TICs used for the simulations presented in this study. Translation to other CA still requires further investigation.

### 4.4.2 Establishing the VFI Method for Model Independent Estimates of Volume Fractions

Overall, the VFI method provided more accurate and precise estimates of $\varepsilon_p$ and $\varepsilon_i$ compared to the 2CM method for all noise levels (Figure 4.3). VFI estimates of $\varepsilon_p$ are within -0.2% and $\varepsilon_i$ are within 1.5% of their true value over the range of simulated physiological parameters, when no noise is present (Figure 4.3a and b). Some inaccuracies were observed for VFI estimates of $\varepsilon_i$ for large values of true $\varepsilon_i$ (>0.3) or extremely low values of true $PS/V$ ($\leq 0.01$ mL mL$^{-1}$ min$^{-1}$). As demonstrated in the previous section, this is due to 5 minutes being insufficient for the freely diffusible CA to reach steady-state under these conditions. Alternatively, the accuracy of 2CM estimates of $\varepsilon_p$ and $\varepsilon_i$ with no noise present was -22% and 9% respectively. Additionally, the 2CM method demonstrated poor precision whereby the accuracy of a given parameter at a fixed level (e.g. true $\varepsilon_p = 0.05$ or true $\varepsilon_i = 0.1$) varied considerably
as function of the other physiological parameters (Figure 4.3a and b). The presence of noise did not substantially affect the accuracy of $\varepsilon_p$ or $\varepsilon_i$ using the VFI method; however, the precision of $\varepsilon_p$ and $\varepsilon_i$ decreased as noise increased (Figure 4.3c and d). Overall, the accuracy and precision of the VFI estimates of $\varepsilon_p$ and $\varepsilon_i$ were considerably improved compared to the 2CM method, demonstrating the ability to improve the accuracy of both parameters using the model independent VFI strategy.

**Figure 4.3** The VFI method substantially improves the accuracy and precision of $\varepsilon_p$ and $\varepsilon_i$ compared to the 2CM method. Each point in (a) and (b) represent the mean and standard deviation (error bars are within the size of the symbol) of the estimated parameters under the condition of no noise. Considerable variability in the relative error was observed for 2CM estimates of $\varepsilon_p$ and $\varepsilon_i$ at 0.05 and 0.1 respectively and demonstrates the lack of precision of 2CM method. The accuracy (median relative error) and precision (interquartile range) of the parameters are shown as a function of noise in (c) and (d).
4.4.3 Improving the Accuracy of Tracer Kinetic Modelling using VFI

The MMID4 simulations indicated that the VFI method substantially improves the accuracy of TKM estimates of $F_p/V$ and $PS/V$ compared to the 2CM method (Figure 4.4). At low noise levels ($\sigma \leq 4$ HU), the VFI-1 estimates of $F_p/V$ had an accuracy of less than 1%, compared to the 2CM method whose accuracy ranged between 22% and 37% (Figure 4.4a). At high noise levels ($\sigma > 4$ HU), the VFI-1 method maintained an accuracy between 6% and 26%, while the 2CM method had an accuracy between 88% and 160%. A similar trend was observed for the precision.

The VFI-2 produced the most accurate and precise estimates $PS/V$ (Figure 4.4b). In fact, the VFI-1 method produced the least accurate and precise estimates of $PS/V$. Furthermore, it was observed that obtaining an accurate estimate of $PS/V$ using either the VFI-2 or 2CM methods results a decrease in accuracy of $F_p/V$, $\varepsilon_p$, and $\varepsilon_i$ (Figure 4.4a, c, and d). This highlights the inability of the two-compartment model to accurately account for the spatial distributed nature of trans-vascular exchange for a freely diffusible molecule, a feature that is accounted for in the MMID4 model. Therefore, the two-compartment method may not be an ideal TKM to accurately estimate all four physiological parameters simultaneously. This issue has been previously observed (Buckley 2002) and in the current study the combination of VFI-1 and VFI-2 methods was shown to provide the most accurate assessment of all four physiological parameters. The VFI-2 method resulted in similar, if not modest, improvements in the accuracy and precision of $PS/V$ compared to the 2CM method. Specifically, at low noise levels ($\sigma \leq 4$ HU), the VFI-2 method had an accuracy of between -8% and -12%, while the 2CM method had an accuracy of between -8% and -13%. At high noise levels ($\sigma > 4$ HU), the accuracy of the VFI-2 method decreased to -20%, while the accuracy of the 2CM method decreased to -32%. The precision was similar between the VFI-2 and 2CM methods.

4.4.4 Investigating the Effects of Sampling Methods

Figure 4.5 demonstrates the accuracy and precision of the estimated perfusion parameters for different samplings strategies, for a $\sigma=4$HU, and nominal values for $F_p/V$, $PS/V$, $\varepsilon_p$ and $\varepsilon_i$. The accuracy and precision for VFI-1 and VFI-2 estimates of $PS/V$, $\varepsilon_p$ and $\varepsilon_i$ were similar for the S1, S2 and S3 sampling schemes, with minimal variations at a fixed noise level. On the contrary, substantial variations in the accuracy and precision of 2CM method based estimates of physiological parameters were observed.
across all noise levels. Therefore, the VFI method is able to provide robust and accurate estimates of physiological parameters for relevant DCE-CT imaging protocols.

Figure 4.4 The effects of noise on the accuracy and precision of (a) $F_p/V$, (b) $PS/V$, (c) $\varepsilon_p$, and (d) $\varepsilon_i$ estimated using the VFI-1 (vertical pattern), VFI-2 (horizontal pattern) and 2CM (diagonal pattern) methods as a function of noise. The VFI-1 method produced the most accurate and precise estimates of $F_p/V$, while VFI-2 produced the most accurate and precise estimates $PS/V$. The VFI-1 method has the highest accuracy and precision for $\varepsilon_p$ and $\varepsilon_i$ estimates.
Figure 4.5 The accuracy and precision of (a) $F_p/V$, (b) $PS/V$, (c) $\varepsilon_p$, and (d) $\varepsilon_i$ estimated using the VFI-1 (vertical pattern), VFI-2 (horizontal pattern) and 2CM (diagonal pattern) methods as a function of sampling scheme and for $\sigma=4$HU. The sampling scheme had negligible effects on the accuracy and precision of VFI based estimates.

4.4.5 In Vivo Application of VFI

The VFI method was applied to in vivo CT imaging of tumour bearing SCID mice. For in vivo experiments, the VFI method that gave the most accurate estimates of a given parameter, as shown by simulations, is reported. An example of whole body parametric imaging of $\varepsilon_p$ and $\varepsilon_i$, determined from applying the VFI method to 3 static CT scans of an OCICx26 tumour bearing mouse, is shown in Figure 4.6. In this example a median filter was applied to each static CT scan to obtain a noise level of $\sigma \approx 7$, resulting in an anticipated accuracy of $\varepsilon_p$ and $\varepsilon_i$ estimates were $0.3\% \pm 10\%$ and $2.2\% \pm 7.5\%$ respectively (Figure 4.3c and d). It was determined that parametric imaging of $\varepsilon_p$ can be used to visualise the plasma volume fraction of different tissues including large vessels such as the descending aorta and inferior vena cava.
and small vessels such as the microvasculature of muscle tissue, tumour tissue, and several organs. Similarly, parametric imaging of $\varepsilon_i$ can be used to visualise the interstitial volume fraction of different tissues. However, there are several tissues where the VFI based method gave erroneous results, including: (1) the gastrointestinal tract and lungs, due to both tissue and gas motion; (2) the kidneys due to the rapid clearance of iohexol; and (3) the brain due to the blood brain barrier.

VFI estimates of $\varepsilon_p$, $\varepsilon_i$, $F_p/V$ and $PS/V$ were evaluated in different tissues including the brain, gastrocnemius muscle, orthotopic MDA231 tumours, and orthotopic OCICx26 tumours. Figure 4.7 shows the whole tissue mean values for each parameter. Overall, the VFI based quantification was more robust at estimating $\varepsilon_p$, $\varepsilon_i$, $F_p/V$ and $PS/V$ compared to 2CM. In agreement with simulations, a trade-off in accuracy of parameter estimates obtained using the 2CM method was apparent. Estimates of $F_p/V$ and $PS/V$ were consistent, but at the sacrifice of $\varepsilon_p$ and $\varepsilon_i$ parameter estimates. In 4 of the 16 muscle measurements the 2CM method was not able to estimate $F_p/V$. Furthermore, 2CM estimates of $PS/V$ were approximately zero in 10 muscle and 2 tumour measurements. 2CM estimates of $\varepsilon_p$ showed a high variability in tumour tissues and no statistical difference between muscle and grouped tumour ($p=0.08$). Additionally, 2CM estimates of $\varepsilon_i$ resulted in several values close to zero in both muscle and MDA231 tumour tissue.

VFI quantification at the whole tissue level resulted in robust estimates of $\varepsilon_p$ and $\varepsilon_i$ that are consistent with values reported in the literature, providing confidence in the VFI method (Jain 1987b; a; Wiig & Swartz 2012). In brain tissue VFI estimates of $\varepsilon_p$ were greater than muscle and estimates of $\varepsilon_i$ were close to zero due to the blood brain barrier. Motion was also observed in 3 of the 9 brain measurements, making registration and VFI analysis problematic. Therefore, only 6 of 9 brain estimates of $\varepsilon_p$ and $\varepsilon_i$ were included. Both $\varepsilon_p$ and $\varepsilon_i$ were elevated in tumour compared to muscle tissue (Figure 4.7a and b). VFI estimates of $F_p/V$ indicated that perfusion was similar in muscle and MDA231 tissue, while perfusion was increased in OCICx26 tumours compared to both muscle and MDA231 tissue ($p=0.0003$). VFI estimates of $F_p/V$ in tumour tissue were in the range of previously reported values (Vaupel et al. 2009). In muscle, VFI estimates of $PS/V$ were highly variable, but still within the range of previously reported values for highly permeable vasculature assessed using small molecules (MW=821 Da) (Gerlowski & Jain
1986; Jain 1987a; El-Kareh & Secomb 1997). VFI estimates of $P S / V$ in tumours were also highly variable and were not differentiable from muscle ($p=0.17$).

**Figure 4.6** Representative volume rendered parametric maps of whole body plasma and interstitial volume fractions calculated using the VFI method applied to 3 static CT scans. Image processing included applying a 3x3x3 median filter to the three CT scans to improve the signal to noise ratio, calculating the volume fractions using the VFI method, removal of volume fractions less than zero, and segmenting and displaying the tissues of interest in Amira (Visage Imaging, San Diego, CA). Note that for clarity the intestinal tract has been omitted in both images and the bladder and kidneys have been omitted from the interstitial volume fraction image.
4.5 Discussion
In this study, a model independent method, termed VFI, to estimate the plasma and interstitial volume fractions of tissue without the need for DCE imaging or TKM analysis was reported. The VFI method takes advantage of the differential transport of a macro-molecular CA that remains intravascular at early post-injection time points and a small molecule CA that rapidly diffuses out of blood vessels. The VFI allows for quantification of $\varepsilon_p$ and $\varepsilon_i$ using only three static CT scans, reducing the complexity of data acquisition and analysis, and potentially reducing the radiation dose compared to standard DCE-CT imaging; however, the amount of dose reduction depends on the desired resolution, field of view, and other scanning parameters. The simplicity in acquisition and accuracy of the VFI method facilitates integration into any clinical setting that has CT available, thus expanding access to improved diagnosis and treatment monitoring tools based on quantitative $\varepsilon_p$ and $\varepsilon_i$ imaging. Indeed, several studies have demonstrated that $\varepsilon_p$ and $\varepsilon_i$ are important biomarkers for the identification and classification of some malignant tumours, and for monitoring therapeutic response to radiation, chemotherapy, and anti-angiogenic therapy (Dugdale et al. 1999; Miles 2002; Kambadakone & Sahani 2009). In the future, quantitative whole body imaging of $\varepsilon_p$ and $\varepsilon_i$ imaging may facilitate improved monitoring of the pharmacokinetics and pharmacodynamics of small molecule chemotherapy and drugs delivered in nanoparticles to healthy and diseased tissues, allowing for non-invasive assessment of toxicity and treatment response.

It was also demonstrated that the VFI based quantification of $\varepsilon_p$ and $\varepsilon_i$ can be used to improve the accuracy and precision of $F_p/V$ and $PS/V$ estimates obtained from standard TKM and DCE-CT imaging. The improvement stems from using a priori estimates of $\varepsilon_p$ and $\varepsilon_i$ to reduce the number of free parameters, reduce the degrees of freedom in the fitting process, reduce the correlation between parameter estimates, and improve the ability to generate a unique solution to the TKM. A few studies have suggested that the combination of intra-vascular and freely diffusible CAs would help improve the accuracy of TKM quantification (Bassingthwaighte & Goresky 1984; Jerosch-Herold et al. 1999); however, this is the first study to investigate the accuracy and precision of the VFI method using quantitative simulations and in vivo imaging.
Figure 4.7 In vivo VFI estimates of (a) $\varepsilon_p$, (b) $\varepsilon_I$, (c) $F_p/V$ and (d) $PS/V$ in the brain ($N=5$), gastrocnemius muscle ($N=18$), MD231 tumours ($N=9$), and OCICx26 tumours ($N=9$). Each point represents the mean value across the whole tissue for a given parameter and the lines represent the mean of the ensemble. Colored circles represent VFI estimates and grey diamonds represent 2CM estimates. Note estimates in the brain were only performed in a subset of OCICx26 mice due to tissue motion and DCE-CT imaging limitations.

The improvement in accuracy using the VFI method was assessed using a simulation approach. Typically, the accuracy of a new imaging method is established by comparison with a “gold standard” measurement; however, there is a paucity of suitable techniques for measurements of tissue volume fractions, blood flow, and vascular permeability. PET is widely regarded as a “gold standard” method of blood flow, but the TKM used to quantify PET measurements are similar to the simple two-compartment TKM used here and suffer from the same limitations (Xu et al. 2012). The MMID4 model used in this study is a realistic and established model that is able to generate TICs that closely match experimental data (Buckley 2002). Therefore, TICs were generated using known physiological variables that were
independent of the VFI and two-compartment model used to interpret them. In this way, it was possible to assess the ability of the VFI method to accurately quantify tissue volume fractions, TBF, and vascular permeability. Furthermore, it was possible to determine the improvement in accuracy of the VFI method compared to a standard two-compartment model.

The VFI methodology was compared to a standard 2CM approach; yet, this approach can be generalised to all TKMs. More physiologically relevant TKMs exist including the distributed tissue homogeneity model proposed by Johnson & Wilson (1966) and refined by St Lawrence & Lee (1998). The tissue homogeneity model more closely resembles the MMID4 model in that both account for spatially varying trans-vascular exchange within the ROI, leading to a closer representation of the underlying true tissue physiology and thus may result in an improvement in the ability to estimate all four physiological parameters simultaneously. However, the tissue homogeneity model also suffers from the uniqueness of model solution (Cenic et al. 1999; Jerosch-Herold et al. 1999; Buckley 2002), and therefore the VFI method could also improve the absolute quantification of $F_p/V$ and $PS/V$. It is believed that the combination would further improve absolute quantification of all four physiological parameters compared to what was observed using the 2CM method in this study. Therefore, a future endeavour will be to repeat the current study using the tissue homogeneity model.

A key assumption of the VFI method is that a steady-state is obtained between the arterial and tissue plasma concentrations for both the intravascular and freely diffusible CAs and between the tissue plasma and interstitial concentrations of the freely diffusible CA. Simulations demonstrated that it is possible to reach steady-state conditions in under 2 minutes for a bolus administration of both CAs and for the majority of physiological tissues properties used in this study. However, the time to reach steady-state is specific to the properties of the CA and mode of administration. The agent half-life and ability to reach a slowly changing tissue concentration is a requirement for accurate VFI estimates of tissue volume fractions. This may not be possible for all CAs or all modes of administration, and requires further investigation. Although reaching steady-state is possible by infusing the CAs, this limits the ability to assess capillary perfusion and permeability.
In this study CT is proposed and validated as an ideal method for the VFI method. CT provides a simple, rapid, high resolution and quantitative method to measure CA concentrations in different tissues simultaneously. Absolute quantification of all parameters requires the estimate of an arterial input function (AIF). In general, there is sufficient spatial resolution and signal to identify a large vessel and measure an AIF using CT imaging. The challenge of performing dual tracer imaging using MRI was previous investigated (Kim et al. 2004). The authors found the accuracy of vascular and interstitial volume estimates using dual tracer DCE-MRI imaging depended strongly on the CA concentration, imaging parameters (flip angle, repetition time - TR, and echo time - TE), and the ratio between blood and interstitial volume. Considerable optimisation was required to generate estimates that agree with reported literature values for muscle and tumour tissue. Furthermore, identifying an AIF can be problematic using MRI and quantification of agent concentration is limited by several MRI specific acquisition issues (O'Connor et al. 2011). As a result, VFI using DCE-MRI is far more technically challenging compared to CT. An added benefit of the VFI based quantification $\varepsilon_p$ and $\varepsilon_i$ is that once a steady-state plasma concentration has been reached, the input function can be determined using either the arterial plasma or venous plasma CA concentration. Typically, it is easier to measure the in a venous vessel due to its size. However, if further quantification of $F_p/V$ and $PS/V$ is required, then the arterial plasma concentration must be used for the input function.

Currently, the VFI method is based on the sequential injection of two different MW tracers. This presents an additional complexity compared to standard DCE imaging using a single tracer. However, in the future it may be possible to use a single tracer that has the properties of both a macro-molecular and small molecule CA. For example, a triggered liposome system that stably encapsulates a small molecule CA could be used as an alternative. Upon intravascular administration the liposome system acts as a macro-molecular CA and can be used to estimate $\varepsilon_p$ in a given tissue region. After reaching steady-state in the tissue, external application of heat could be used to trigger the release of the small molecule CA from the liposome and facilitate the estimate of $\varepsilon_i$. The use of a triggered liposome system may also allow for the quantification of capillary perfusion and vascular permeability through the measured kinetics of the locally released freely diffusible agent. However, considerable modification of the VFI theory presented here would be required. The triggered liposome approach is attractive as it has been studied extensively in the context of drug and CA release in the pre-clinical and to a more limited extent in the clinical setting (de Smet et al. 2010; Koning et al. 2010). In this space, the VFI approach could potentially be used
to provide novel quantitative biomarkers for treatment planning and treatment assessment of triggered liposome-based drug delivery. The feasibility of a single triggered release CA and its application to heat activated liposome drug delivery is a topic for future investigation.

A final important consideration of the VFI method is the half-life of the macro-molecular agent in relation to performing repeat VFI measurements. The CT-liposome CA used in this study has a half-life of between 30 to 40 hrs in mice and exhibits substantial tissue accumulation between 8 to 120 hrs post-injection (Stapleton et al. 2013b). The long half-life and interstitial accumulation renders repeat measurements within a few hours to weeks problematic. Therefore, in addition to considerations of the time to achieve steady-state previously described, further optimisation of the macro-molecular CA in terms of half-life and repeat measurements is required. Given that the half-life of liposomes can be easily tailor based on their lipid composition (Drummond et al. 1999), they may still represent a viable CA for the VFI method. Additionally, one important consideration in moving forward is clinical approval of macro-molecular agents. Clinical approval of liposome-based imaging agents for CT and MRI are still pending. There are a few clinically approved macro-molecular agents that could serve as substitutes for the CT-liposome CA; however, none are available for CT imaging and few are approved for MRI (Bremerich et al. 2007). It is clear that further optimisation and clinical approval of an intra-vascular CA are two key requirements for the success of the VFI method.

4.6 Conclusions

The proposed VFI method provides a novel framework for simple and accurate quantification of tissue volume fractions using only three static scans. Furthermore, the VFI method provides the ability to substantially improve the quantification of blood flow and capillary permeability. Based on the simplicity of the VFI method, the possibility of rapid adoption to the clinic setting is envisioned. However, the adoption of VFI still faces several hurdles, including contrast agent optimisation, clinical approval, and validation as a biomarker for disease assessment and treatment response to chemotherapy, radiotherapy, and anti-angiogenic therapy. Each of these hurdles is surmountable and the VFI method has the potential to usher in a new era of simple quantitative assessment of the morphological and physiological properties of the tumour microenvironment.
4.7 Acknowledgements

The authors would like to thank Dr. Naz Chaudary, Dr. Richard P. Hill, Dr Anthony Fyles, and Dr. Michael Milosevic for the OClCy26 xenografts, Dr Javed Mahmood for the MDA-231 xenografts and Lin Yu Fan for preparing the CT-liposomes. Shawn Stapleton is grateful to have received funding from the Natural Science and Engineering Research Postgraduate Scholarships Program and the Terry Fox Foundation Strategic Initiative for Excellence in Radiation Research for the 21st Century (EIRR21) at CIHR. This study was supported by grants from the Terry Fox New Frontiers Program and the Canadian Institutes of Health Research.

4.8 References


Chapter 5
Investigating the Intra-Tumoural Relationship between Microcirculation, Interstitial Fluid Pressure and Liposome Accumulation
5.1 Abstract
The heterogeneous intra-tumoural accumulation of liposomes has been linked to both the chaotic tumour microcirculation and to elevated IFP. Mathematical modelling suggests that TBF and IFP are intrinsically coupled, and that their complex interplay drives heterogeneity in liposome accumulation. There is a paucity of experimental evidence to support this theory. Therefore, in this study the relationship between tumour microcirculation, IFP, and the intra-tumoural accumulation of liposomes is further explored experimentally. Co-localised measurements of the tumour microcirculation using Volume Fraction Imaging, IFP using a novel image-guided robotic needle positioning system, and the intra-tumoural distribution of liposomes using volumetric micro-CT imaging were performed. Measurements made in mice bearing MDA231 tumours indicated that image derived metrics of tumour perfusion predict for the intra-tumoural accumulation of CT-liposomes. Tumour blood flow and not vascular permeability was found to be the primary mediator of the intra-tumoural accumulation of CT-liposomes. No statistically significant relationship was found between co-localised measurements of IFP, metrics of tumour perfusion, and the intra-tumoural accumulation of liposomes; however, a strong relationship was observed when investigating the radial patterns. Specifically, tumours with elevated central IFP that decreased at the periphery had low perfusion and low levels of CT-liposome accumulation that increased towards the periphery. Conversely, tumours with low and radially uniform IFP exhibited higher levels of tumour perfusion and CT-liposome accumulation. These results suggest that both tumour perfusion and elevated IFP play an integral role in mediating the intra-tumoural accumulation of liposomes, and strengthen the need to account for intra-tumoural heterogeneity in transport properties for accurate predictions of liposome accumulation.

5.2 Introduction
Tumour microcirculation and elevated IFP are key determinants of the intra-tumoural accumulation of liposomes in solid tumours. This concept is supported by several studies which have shown that modulating the tumour microcirculation (e.g. TBF) can dramatically alter the micro-regional and bulk accumulation of macromolecules, including liposomes, in solid tumours (Gaber et al. 1996; Lu et al. 2007; Seki et al. 2009; Hattori et al. 2011). Indeed, it was shown in Chapter 3 that tumour perfusion is strongly associated with heterogeneity in liposome accumulation. Additionally, several studies have shown that decreasing IFP or modulating the transvascular pressure gradient can also dramatically alter micro-regional and bulk accumulation (Eikenes et al. 2004; Eikenes et al. 2005; Giustini et al. 2012).
Furthermore, as reviewed in Stapleton & Milosevic (2013), these studies and others have noted that modulating the tumour microenvironment is often associated with changes to both TBF and IFP, and the coupling of these properties contributes to heterogeneity in liposome accumulation. Mathematical modelling has supported the theory that TBF and IFP are intrinsically coupled (Netti et al. 1996; Baish et al. 1997; Milosevic et al. 1999), and has also demonstrated that their complex interplay drives the heterogeneous intra-tumoural distribution of liposomes (Zhao et al. 2007; Shipley & Chapman 2010; Welter & Rieger 2013). Currently, there is a paucity of direct experimental evidence that supports this theory. Establishing the relationship between the tumour microcirculation, elevated IFP, and the intra-tumoural accumulation of liposomes is imperative for accurate modelling of liposomal transport and for proper interpretation of liposome imaging data.

Only one study has experimentally investigated the relationship between the tumour microcirculation, IFP, and the accumulation of nanoparticles (Torosean et al. 2013). Using three different tumour types that differed in terms of vascular density and IFP characteristics, the authors observed that the bulk tumour accumulation of 40 nm fluorescent beads was highest in the most vascularised tumours and tumours with the lowest IFP. However, these results are not sufficient to establish a causal relationship as other tumour specific properties, such as size, ECM composition and vascular permeability, may have also contributed to the observed changes in liposome accumulation. Therefore performing intra-tumoural analysis could reduce the effects of between tumour differences.

In the present study the intra-tumoural relationship between the tumour microcirculation, elevated IFP, and liposome accumulation is investigated. This is accomplished by performing co-localised measurements of tumour microcirculation using VFI, tumour IFP using a novel image-guided robotic needle positioning, and intra-tumoural liposome accumulation using CT-liposomes. The relationship between the aforementioned properties was established in a model of human breast cancer implanted either SC or OR in mice.
5.3 Methods

5.3.1 Animal Models

All in vivo experiments were performed under a protocol approved by the University Health Network Institutional Animal Care and Use Committee. Experiments were performed using the MDA231 metastatic breast adenocarcinoma tumour model. The MDA231 tumours were established by injecting 2x10⁶ cells orthotopically in the lower abdominal mammary fat pad of female SCID mice (n=8) or subcutaneously in the hind limb (n=4). Measurements were performed once the OR and SC tumours reached a volume of 258±86 mm³ and 214±56 mm³ respectively. Tumour volume was measured using micro-CT (eXplore Ultra, GE Healthcare, London, Canada).

5.3.2 CT-liposome Preparation and Characterisation

Imaging the intra-tumoural accumulation of liposomes was accomplished by encapsulating a clinically approved CT contrast agent (iohexol) within the aqueous interval volume of the liposomes. The CT-liposome contrast agent was prepared according to previously described methods (Chapters 2 to 4). The liposomes were composed of DPPC, cholesterol and a polyethylene glycol derivatised lipid (DSPE-PEG₂₀₀₀). The CT contrast agent, iohexol (Omnipaque™, 300 mg/mL of iodine, GE Healthcare) was encapsulated within the lipid vesicles and concentrated to a final iodine concentration of approximately 45 mg mL⁻¹. The CT liposomes were approximately 90 to 100 nm in diameter as measured using a 90Plus particle size analyzer (Brookhaven, Holtsville, NY). The iodine concentration in the CT-liposomes was determined by rupturing liposomes using a 10-fold excess of ethanol, diluting in HBS and measuring the UV absorbance at a wavelength of 245 nm using a Cary 50 UV-visible spectrophotometer (Varian, Palo Alto, CA).

5.3.3 Quantitative Imaging of the Tumour Microcirculation and Liposome Accumulation

Quantitative imaging of the tumour microcirculation was performed using a similar protocol to that described in Chapter 4. Briefly, volumetric static micro-CT scans (80 kV, 70 mA) were acquired pre-, 5 min post-, 10 min post-, 24 hrs post, 48 hrs post-, and 72 hrs post- administration of the CT-liposome contrast agent. The CT-liposomes were administered at a dose of roughly 400 mg iodine kg⁻¹ (1240 mg lipid kg⁻¹) using an injection pump (Harvard Apparatus, Canada) at a rate of 10 µL per second through a
27G butterfly catheter inserted into the lateral tail vein. Immediately following the 5 min post-injection CT-liposome scan, a DCE-CT measurement of the freely diffusible tracer iohexol (Omnipaque™-300, GE Healthcare, New Jersey, USA) was performed. The DCE-CT scan consisted of injecting 100 µL of free-iohexol mixed with saline (9:1 ratio by volume) using the same injection protocol as with the CT-liposomes. Volumetric images were acquired continuously every second for the first 30 seconds and then intermittently every 10 seconds for 4.5 minutes. The reconstructed voxel size was 0.153x0.153x0.153 mm³ for anatomical and 0.153x0.153x0.462 mm³ for DCE-CT scans.

5.3.4 Measurements of Interstitial Fluid Pressure

Interstitial fluid pressure was measured using a modified wick-in-needle technique at either 48hrs (n=8) or 72 hrs (n=4) post-injection of liposomes. Measurements were made with a pen tip 25Gx3.5” Whitacre needle (BD, Franklin Lakes, NJ, USA) containing a single open port 0.75 mm in length, situated approximately 1.25 mm from the needle tip. The needle was connected to a pressure transducer (Model P23XL, Harvard Apparatus, Canada) and to a data acquisition system (PowerLab 4/35 with LabChart Pro, ADInstruments Pty Ltd., USA) through 50 cm of PE20 polyethylene tubing (Becton Dickinson, Franklin Lakes, NJ, USA). The entire system was flushed with a heparin sulphate/saline solution (1:10). No wick was placed in the needle. Spatial IFP measurements were made under image guidance using a robotic needle placement system connected to the micro-CT scanner (Figure 5.1). It was previously shown that the image-guided robotic needle position system has the ability to position the needle with an accuracy of approximately 200 µm in all directions (Bax et al. 2013). A micro-CT scan was performed prior the needle insertion, the tumour identified on the volumetric CT scan, and a series of positions (5 to 8) were manually selected within the tumour volume. The needle was then robotically inserted to the target and IFP recorded. A volumetric CT scan was performed with the needle in place and used to determine the radial position of the needle port. The relative radial position was calculated by measuring the position of the needle port relative the tumours centre of mass and dividing by the length of a line from the centre of mass to the tumour boundary that intersects the needle port.
Figure 5.1 Representative images of robotic based IFP measurements in an orthotopic MDA231 tumour. (a) Volumetric scan of the intra-tumoural distribution of CT-liposomes at 48hr after injection. (b) Post-insertion of IFP needle showing the position of the port within the tumour volume. (c) A pre-insertion 3D volume rendering showing the robot relative to the mouse and tumour. (d) A post-insertion 3D volume rendering shown the IFP needle penetrating the tumour volume.

5.3.5 Data Analysis

The relationship between tumour microcirculation, IFP, and CT-liposome accumulation was evaluated using three methods: (1) Point-based spatial mapping where co-localised measurements of tumour perfusion, IFP, and CT-liposome accumulation obtained at each needle port position were correlated; (2) Sub-volume spatial mapping in a spherical coordinate system where tumour perfusion and CT-liposome accumulation was determined in multiple sub-volumes and correlated using LME; and (3) Radial-based mapping analysis where spatial measurements of tumour perfusion, IFP, and CT-liposome accumulation were collapsed along a radial profile for comparison. For the point-based spatial mapping analysis, all volumetric and DCE-CT imaging data sets were co-registered and a spherical VOI with a radius of 0.5 mm centred at the needle port position was used to determine metrics of tumour perfusion and CT-liposome
enhancement. For the sub-volume spatial mapping analysis, the segmentation method described in Chapters 3 was used. Briefly, all volumetric and DCE-CT imaging data sets were co-registered, contoured and three-dimensional VOIs were generated for each tumour at each time point using the software Amira (v5.2.2, Visage Imaging Inc.). The VOIs were divided into 9 concentric rings of roughly equal radius surrounding a tumour core region. The concentric rings were divided into sub-volumes using a spherical coordinate system. Overall, the tumour was divided into a total of 316 radial sub-volumes. The large number of rings/sub-volumes, compared to Chapter 3, was used to capture the high spatial heterogeneity in CT-liposome enhancement observed in the OR MDA231 tumours. The spatial relationship between metrics of tumour perfusion and CT-liposomes was determined using the LME method described in Chapter 3. For radial-based mapping analysis, the sub-volume measurements of tumour perfusion and CT-liposome accumulation where collapsed along a radial profile defined by the 9 rings and core. This was accomplished by taking the median values of tumour perfusion metrics and CT-liposome enhancement in each ring for an individual tumour and averaging the median values across all OR and SC tumours. Additionally, all of the spatial IFP measurements for each tumour was collapsed along a radial profile and fit using the analytic solution to Equation 2.2 described by Baxter & Jain (1989):

\[
p_i = p_{ss} \left( 1 - \frac{R \sinh(\alpha \cdot r/R)}{r \sinh(\alpha)} \right)
\]

where \( p_{ss} \) is the steady state pressure and was fixed at 15.6 mmHg, \( r/R \) is the normalised radial position, and \( \alpha \) was a free parameter that represents the potential for fluid accumulation in the interstitial space. The fitted IFP data established a hypothetical continuous relationship between radial position and IFP in both OR and SC tumours. The radial-based mapping method was used because it: (1) provides a unified coordinate system that captures the considerable radial variations observed using both measurements and modeling of IFP, measurements of tumour perfusion, and measurements of CT-liposome enhancement; and (2) provides the ability to amalgamate inter- and intra-tumoural IFP measurements to improve the spatial comparison with tumour perfusion and CT-liposome accumulation.
Quantification of tumour microcirculation was accomplished using the VFI methodology described in Chapter 4. The tumour concentration of free-iohexol was determined in each region by calculating the mean HU and converting to iodine concentration by subtracting the mean HU obtained prior to contrast administration and scaling by the calibration factor of 50.1±0.4 HU per mgI cm$^{-3}$. The arterial plasma concentration for the free-iohexol was determined in the descending aorta and adjusted for the arterial hematocrit ($Hct_{aorta} = 0.5$). The VFI method was applied to the volumetric data and DCE-CT derived TICs measured in each sub-volume giving estimates of $F_p/V$, $PS/V$, $\epsilon_p$ and $\epsilon_i$. Additionally, the flow/permeability product was calculated using the equation $K_{trans} = F_p \left(1 - e^{-PS/F_p}\right)$. The area under the curve for the free-iohexol TICs ($AUC_{iox}$), normalised to the arterial plasma AUC, was also calculated for each sub-volume by integrating the TIC over the duration of the scan (i.e. from 0 to 300 seconds).

The arterial plasma concentration for CT-liposomes was determined in the descending aorta and adjusted for the arterial hematocrit. An estimate of the percent injected dose (%ID) for CT-liposomes in the tumour was obtained by dividing the tumour concentration by the initial plasma concentration of CT-liposomes and multiplying by 100%. The plasma volume fraction, obtained using the VFI method, was used to subtract the contribution of the vascular CT-liposomes from the measured tumour concentration of liposomes, providing an estimate of the interstitial concentration of CT-liposomes in the tumour. The area under the curve over the total scan time ($AUC_{lipo}$) and the peak concentration ($C_{peak}$) of the CT-liposome concentration curves were determined in each sub-volume and used as metrics of CT-liposome accumulation. To account for inter-mouse variability in pharmacokinetics, $AUC_{lipo}$ was normalised to the AUC of the arterial plasma concentration and $C_{peak}$ was normalised to the peak plasma concentration.

5.3.6 Statistical Analysis

A LME model was used to determine if the DCE-CT derived metrics of tumour perfusion ($F_p/V$, $PS/V$, $\epsilon_p$, $\epsilon_i$, $K_{trans}$ and $AUC_{iox}$) were predictors of CT-liposome accumulation defined by the two response variables, $AUC_{lipo}$ and $C_{peak}$. The predictor variables were considered random and modeled independently. Separate analyses were performed for co-localised measurements and radial sub-volume measurements. Measured parameters were rejected from the analysis if they were less than zero or
outside of the range of reported physiological values. All the predictors were tested for normality by observing that no bias or trend existed in the residuals of the best fit. A p-value<0.05 was considered to indicate a statistically significant relationship between predictor and response values. Due to the exploratory nature of this study, multiple testing correction was not performed (Eliaz et al. 2004).

Figure 5.2 Representative images showing point-based measurements of IFP overlaid on the intra-tumoural distribution of CT-liposomes in an OR (top) and a SC tumour (bottom). The colored circles and corresponding numbers represent the ROI locations, ROI size used for point-based analysis, and measured IFP. Predominantly peripheral CT-liposome enhancement was observed, with some heterogeneous accumulation within the central tumour region of OR tumours. Metrics of perfusion were spatially heterogeneous in both tumour types, but tended to increase towards the tumour periphery.

5.4 Results

5.4.7 Imaging Intra-Tumoural Transport and IFP

Example images showing spatial measurements of IFP, tumour perfusion, and CT-liposome accumulation are shown in Figure 5.2. Strong peripheral CT-liposome enhancement was observed in SC tumours, while heterogeneous intra-tumoural CT-liposome enhancement was observed in OR tumours. Image-guided robotic needle insertion resulted in stable measurements of tumour IFP and the ability to spatially localise IFP measurements within the tumour volume. Some tissue deformation was observed during needle insertion and resulted in deviations between the intended and actual target positions. In general, good co-localisation was observed between IFP measurements with CT-liposome accumulation obtained.
from pre-insertion images. Co-localisation of IFP measurements with metrics of tumour perfusion obtained from VFI was challenging in OR tumours due to a large increase in tumour volume (258±80 mm$^3$ to 552±98 mm$^3$) over the course of the study. This made registration of pre-insertion needle scans to VFI measurements, which were performed 48 to 72 hrs earlier, challenging. Conversely, SC tumour growth over the course of the study was minimal (214±48 mm$^3$ to 242±64 mm$^3$) and accurate registration was possible. This allowed for improved co-localisation between IFP and VFI in SC tumours.

![Figure 5.3](image)

**Figure 5.3** Point-based mapping of IFP, perfusion, and CT-liposomes accumulation in both OR and SC tumours. (a) IFP is elevated in the central tumour region and decreases at the tumour periphery. Central IFP appeared higher in SC compared to OR tumours. (b) A higher level of perfusion was observed towards the tumour periphery; however, there was considerable heterogeneity. (c) The accumulation of CT-liposomes was lower in SC compared to OR tumours. (d-f) No spatial relationship was observed between perfusion, IFP, and CT-liposome accumulation using the point-based analysis method. Each point represents an individual measurement made at the needle port position in OR and SC tumours.

### 5.4.8 Point-Based Analysis of IFP, Tumour Microcirculation and CT-liposome Accumulation

In total, 56 and 23 point-based measurements of IFP, metrics of tumour perfusion, and CT-liposome enhancement were made in OR and SC tumours respectively. The mean IFP was 3.7±3.4 mmHg (range: -
5.6 to 11.0 mmHg) in OR tumours and 7.9±9.2 mmHg (range: -1.6 to 34.2 mmHg) in SC tumours. In general, IFP was elevated in the central tumour region and decreased towards the tumour periphery in both OR and SC tumours; however, considerable variability in central IFP was observed in both OR and SC tumours (Figure 5.3a). Tumour perfusion, as defined by AUC_{tlox}, was heterogeneous across the tumour volume in OR tumours and appeared to increased towards the periphery in SC tumours (Figure 5.3b). CT-liposomes accumulation was higher in OR tumours compared the SC tumours and no trend in spatial distribution was observed (Figure 5.3c). No spatial relationship was observed between IFP, metrics of tumour perfusion, and CT-liposome enhancement in both OR and SC tumours using the point-based mapping method (Figure 5.3d to f).

<table>
<thead>
<tr>
<th></th>
<th>Orthotopic Tumours</th>
<th>Subcutaneous Tumours</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AUC_{tupo}</td>
<td>C_{peak}</td>
</tr>
<tr>
<td>ε_p</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ε_i</td>
<td>-</td>
<td>13.4±4.4 (p-value=0.0020)</td>
</tr>
<tr>
<td>F_p/V</td>
<td>9.2±4.1 (p-value=0.0254)</td>
<td>19.9±7.2 (p-value=0.0057)</td>
</tr>
<tr>
<td>PS/V</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K_{trans}</td>
<td>18.0±7.2 (p-value=0.0131)</td>
<td>31.8±10.3 (p-value=0.0020)</td>
</tr>
<tr>
<td>AUC_{tlox}</td>
<td>-</td>
<td>0.20±0.09 (p-value=0.0184)</td>
</tr>
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Table 5.1 Summary of LME results for the predictor (down) and response (across) parameters. Values represent the fixed effect slope, the standard error and the p-value (in brackets). The p-value indicates a statistically significant relationship between metrics of perfusion (first column) and CT-liposome accumulation (second row).
Figure 5.4 Representative scatter plots demonstrating the relationships between the tumour perfusion metrics: (a) $AUC_{iox}$, (b) $\varepsilon_p$, (c) $\varepsilon_i$, (d) $K_{trans}$, (e) $F_p/V$, (f) $PS/V$ and the accumulation of liposomes represented by $AUC_{lipe}$. Each point represents a measurement obtained from a sub-volume of an individual tumour. The solid lines represent fixed effect slopes obtained from linear mixed-effects modelling where a statistically significant correlation was observed.

5.4.9 Tumour Perfusion Image Predicts for CT-liposome Accumulation

Using the same method described in Chapter 3, the ability to predict the intra-tumoural accumulation of CT-liposomes using tumour perfusion imaging was determined in OR and SC MDA231 tumours. LME modelling indicated that all image derived metrics of tumour perfusion ($AUC_{iox}$, $\varepsilon_p$, $\varepsilon_i$, $K_{trans}$, and $F_p/V$), with the exception of $PS/V$, predicted for the intra-tumoural accumulation of CT-liposomes ($AUC_{lipe}$ or $C_{peak}$) in SC tumours. All correlations were positive and the statistically significant fixed effect slopes, standard errors, and p-values are reported in Table 5.1. Additionally, the metrics $AUC_{iox}$, $\varepsilon_i$, $K_{trans}$, and $F_p/V$ predicted for the intra-tumoural accumulation of CT-liposomes ($AUC_{lipe}$ or $C_{peak}$) in OR tumours. Representative scatter plots for each metric of tumour perfusion in relation to CT-liposome accumulation are shown in Figure 5.4.
In both SC and OR tumours $\varepsilon_i$ was positively correlated with $AUC_{tipo}$, which is different to results obtained in Chapter 3 and shows variability in the relationship between metrics of perfusion and CT-liposome accumulation in different tumour models. The lack of correlation observed between $\varepsilon_i$ and $AUC_{tipo}$ in ME180 tumours was believed to be due to the limited penetration of CT-liposomes caused by a dense ECM (Yuan et al. 1994; McKee et al. 2006). This suggests that both SC and OR MDA231 tumours may have a less dense ECM and better liposome penetration; however, this has yet to be experimentally confirmed. Additionally, it was also previously shown in Chapter 3 that the blood flow/permeability product ($K_{trans}$), predicted for the intra-tumoural accumulation of CT-liposomes. In the present study $K_{trans}$ predicted for the intra-tumoural accumulation of CT-liposomes in both SC and OR MDA231 tumours. In this study VFI was used to independently quantify perfusion ($F_p/V$) and permeability ($PS/V$). It was observed that is $F_p/V$ and not $PS/V$ predicted for the intra-tumoural accumulation of CT-liposomes. This suggests that transvascular transport of the CT-liposomes is not permeability limited and the intra-tumoural distribution of CT-liposomes is predominantly driven by $F_p/V$ in OR and SC MDA231 tumours.

![Figure 5.5](image.png)

**Figure 5.5** Fitting of IFP measurements gave hypothetical continuous radial distributions of IFP in SC and OR tumours. The dashed line represents the line of best fit and the shaded regions represent the 95% confidence intervals of the fit.
5.4.10 Radial-based Analysis of IFP, Tumour Microcirculation and CT-liposome Accumulation

The radial-based analysis provided the ability to further explore the intra-tumoural relationship between IFP, tumour microcirculation and CT-liposome accumulation. Radial fits of IFP measurements demonstrated that IFP is elevated in the central region, is higher in OR compared to SC tumours, and drops precipitously towards the tumour periphery (Figure 5.5). Estimates of $\alpha$ obtained from fitting IFP data were 2.0±0.5 and 3.1±1.4 for OR and SC tumours respectively, and suggests a greater resistance to fluid flow in SC compared to OR tumours. Metrics of tumour perfusion and CT-liposome accumulation were collapsed onto a single radial profile and averaged across tumours (Figure 5.6). The low central IFP observed in OR tumours corresponded to elevated levels of $F_p/V$, $\varepsilon_i$, and CT-liposome accumulation. Conversely, the high central IFP observed in SC tumours corresponded to decreased levels of $F_p/V$, $\varepsilon_i$, and CT-liposome accumulation. Furthermore, low IFP observed at the tumour periphery or SC tumours corresponded with increased levels of $F_p/V$, $\varepsilon_i$, and CT-liposome accumulation. These results suggest that indeed a relationship exists between radial profiles of IFP, microvascular physiology, and CT-liposome accumulation.

Figure 5.6 Radial analysis of (a) IFP, (b,c,e,f) metrics of tumour perfusion, (d) CT-liposome accumulation. Each point represents the mean across all tumours of the median value for a given parameter at given radial position. Error bars represent the standard deviation.
Figure 5.7 The relationship between IFP, the accumulation of liposomes (a), and metrics of perfusion (b-e) determined using the radial-based analysis method.

The spatial relationship between IFP, metrics of tumour perfusion and CT-liposome accumulation was investigated by plotting radial estimates of IFP against metrics of tumour perfusion and CT-liposome accumulation (Figure 5.7). Regions with elevated IFP tended to have lower accumulation of CT-liposomes and regions of low IFP (<6 mmHg) had high CT-liposome accumulation (Figure 5.7a). A negative relationship was observed between IFP and all metrics of tumour perfusion ($\varepsilon_p$, $\varepsilon_i$, $F_p/V$, and $PS/V$). The radial-based analysis suggests a coupling between IFP, tumour microcirculation and CT-liposome accumulation.

5.5 Discussion
In this study the intra-tumoural relationship between tumour microcirculation, IFP, and liposome accumulation was investigated. It was demonstrated that image derived metrics of tumour perfusion predicts for the intra-tumoural accumulation of CT-liposomes in SC and OR MDA231 tumours. These results corroborate the findings from Chapter 3 and further strengthen the applicability of tumour perfusion imaging to guide liposome drug delivery. Point-based mapping of IFP did not predict for
tumour perfusion or CT-liposome accumulation and was likely due to the limited number of point-based measurements made in OR and SC tumours. However, a relationship was observed between idealised radial profiles of IFP, radial measurements of tumour perfusion and radial measurements of CT-liposome accumulation. Specifically, tumours with elevated central IFP exhibited low metrics of perfusion and low levels of CT-liposome accumulation. Conversely, tumours with low and relatively uniform IFP exhibited higher and spatially uniform metrics of tumour perfusion and CT-liposome accumulation. Additionally, a negative relationship was observed between IFP, all metrics of tumour perfusion, and CT-liposome accumulation. These results suggest that indeed elevated IFP plays an integral role in mediating the intra-tumoural accumulation of liposomes through a complex relationship with TBF.

The point-based measurement and analysis method suffered from under-sampling where only 5 to 7 spatial measurements were made in each tumour. The limited number of measurements in an individual tumour made it challenging to establish a relationship between tumour microcirculation, IFP, and CT-liposome accumulation given the variability of each measured quantity. According to mathematical modeling, IFP is elevated in the central tumour region and drop precipitously at the tumour periphery (Chapter 2). While this trend was generally observed using point-based mapping in OR and SC tumours, a high variability was observed in the central tumour region. Furthermore, only a few measurements of IFP were made at the tumour periphery. At the bulk level, a comparison between point-based measurements made in SC versus OR tumours indicated that high intra-tumoural IFP corresponds to lower CT-liposome accumulation and low intra-tumoural IFP corresponds to high CT-liposome accumulation; however, the limited number of point-based IFP measurements made at the tumour periphery made it difficult to elucidate the relationship between spatial IFP gradients and CT-liposome accumulation. Overall, point-based mapping was sufficient to demonstrate the presence of intra-tumoural variability in IFP, tumour perfusion, and CT-liposome accumulation.

A potential limitation of the study was that measurements of tumour perfusion, IFP, and CT-liposome accumulation was undertaken at single time points separated by 48 or 72 hrs. Measurements of perfusion and IFP represent a snapshot in time of a dynamically evolving system, and thus may not fully capture the complex relationship between these properties and CT-liposome accumulation. The rapid tumour growth may be related to spatio-temporally evolving angiogenesis and ECM remodelling. This in
turn would lead to spatio-temporal fluctuations in IFP, tumour microcirculation, and ultimately a heterogeneous accumulation of CT-liposomes. Therefore, measurements of tumour perfusion and IFP made at a single time point may not be completely reflective of the intra-tumoural distribution of CT-liposome. Conversely, slow growing tumours tend to have less aggressive angiogenesis and ECM remodelling, and thus a more stable evolution of IFP and tumour perfusion. Measurements of tumour perfusion and IFP made at single time points are more likely representative of the intra-tumoural distribution of liposomes. This may help explain the substantial spatio-temporal heterogeneity and higher levels of CT-liposome observed in fast growing OR tumours compared to the slow growing SC tumours. Furthermore, it may also help to explain the lack of spatial-correlation observed between point-based measurements of IFP, metrics of tumour perfusion and CT-liposome accumulation. Indeed, performing measurements of tumour perfusion, IFP and CT-liposome accumulation at the same time point would significantly improve co-localisation. Unfortunately, the invasive nature of IFP measurements limit the number of times the measurement can be performed and non-invasive methods still need to be developed.

The radial segmentation scheme used in this study may reduce the effects of spatio-temporal heterogeneity in transport. As discussed in Chapter 2, the spatial integration provides a bulk estimate of liposome accumulation that incorporates the effects of spatio-temporal heterogeneity in transport within the region of integration. Therefore, tumour perfusion and CT-liposome accumulation measurements were integrated within radial sub-volumes and the mean was taken across all tumours allowing for assessment of bulk radial transport. Additionally, the radial-segmentation scheme is amenable with the radial based mathematical model of tumour IFP proposed by Baxter & Jain (1989), thus allowing for hypothetical steady-state radial IFP curves to be derived from point-based measurements of IFP. The radial-based analysis method indeed showed a strong relationship between idealised estimates of IFP, bulk estimates of tumour perfusion, and bulk estimates of CT-liposome accumulation as a function of radial position within the tumour volume. A significant limitation of the radial-based analysis method is neglecting the substantial heterogeneity observed within each radial sub-volume. Therefore, the findings of this study reflect only the bulk radial relationship between tumour microcirculation, IFP, and CT-liposome accumulation.
A strong negative relationship between IFP and metrics of tumour perfusion was observed when performing the radial-based analysis and was in general agreement with previous studies. It was previously observed that IFP is negatively correlated to microvascular density in a human melanoma (A-07) window chamber tumour model (Rofstad et al. 2009) and is consistent with the findings of this study; however, it has been shown that the relationship between IFP and microvascular density is tumour model dependent (Simonsen et al. 2012). The negative relationship between $P_p/V$ and IFP observed in this study is also supported by simulations showing that elevated IFP can lead to a bulk reduction in TBF (Netti et al. 1996; Baish et al. 1997; Milosevic et al. 1999). These results are also supported by the study of Haider et al. (2007) where the permeability/blood flow product ($K_{trans}$), quantified using DCE-MRI, was found to be negatively correlated with IFP. A negative relationship was also observed between $\varepsilon_i$ and IFP which is counterintuitive. It is generally believed that the interstitial space in tumours is larger than normal tissue due to distention caused by interstitial fluid accumulation (Vaupel et al. 2009) and thus it seems logical that high $\varepsilon_i$ is associated with elevated IFP; however, $\varepsilon_i$ is also linearly related to the interstitial hydraulic conductivity (Swartz & Fleury 2007). Accordingly, mathematical modeling suggests that elevated $\varepsilon_i$ reduces the $\alpha$-value, leading to increased interstitial fluid flow and ultimately reduced IFP. This is in agreement with the experimental findings presented here. In summary, the radial relationship between IFP and metrics of tumour perfusion are in agreement with mathematical modeling of fluid transport and experimental measurements.

5.6 Conclusions

In this study the complex relationship between intra-tumoural tumour perfusion, IFP, and liposome accumulation was investigated. Quantitative metrics of tumour perfusion were strongly associated with the accumulation and spatial distribution of CT-liposomes. The point-based measurement method employed in this study was not sensitive enough to detect the intra-tumoural relationship between the microcirculation, IFP and CT-liposome accumulation. However, the radial-based analysis method was able to quantify the radial relationship between the tumour microcirculation, IFP and CT-liposome accumulation. Tumours with elevated central IFP had low perfusion and low levels of CT-liposome accumulation, while tumours with low and relatively uniform IFP exhibited higher levels of tumour perfusion and CT-liposome accumulation. Additionally, a strong negative relationship was observed between an idealised estimate of radial IFP, metrics of tumour perfusion, and CT-liposome
accumulation. These results suggest that IFP plays an integral role in mediating the intra-tumoural accumulation of liposomes through a complex relationship with the tumour microcirculation.

5.7 Acknowledgements

The authors would like to thank Dr Javed Mahmood for the MDA-MDA-231 xenografts, Lin Yu Fan for preparing the CT-liposomes, and Daniel Mirmilshteyn for assisting with the image-guided robotic needle placement studies. Shawn Stapleton is grateful for funding from the Natural Science and Engineering Research Postgraduate Scholarships Program and the Terry Fox Foundation Strategic Initiative for Excellence in Radiation Research for the 21st Century (EIRR21) at CIHR. This study was supported by grants from the Terry Fox New Frontiers Program and the Canadian Institutes of Health Research.

5.8 References


Chapter 6
Summary and Future Directions
The goal of this thesis was to develop and validate a mathematical framework that describes liposome transport in solid tumours. In Chapter 2, a biophysical mathematical model based on convection driven transport, termed the intra-tumoural transport model or ITTM for short, was presented to describe the inter- and intra-tumoural accumulation of liposomes in solid tumours. The ITTM was validated using in vivo measurements of bulk accumulation of a CT-liposome contrast agent made in three different tumour models. The ITTM attributed inter-tumoural heterogeneity of liposome accumulation to variations in IFP; however, several limitations were noted including: (1) considerable variability in parameter estimation; and (2) limitations in the accuracy of intra-tumoural liposome accumulation predictions based solely on IFP. It was recognised from imaging data that tumour microcirculation plays an important role in mediating intra-tumoural liposome accumulation. Therefore, in Chapter 3 the relationship between the tumour microcirculation (e.g. rate of transvascular transport, plasma volume fraction and interstitial volume fraction) and the intra-tumoural accumulation of liposomes was investigated. It was established in a cervical carcinoma xenograft tumour model, implanted in two different tissues (IM and SC), that the rate of transvascular transport and the plasma volume fraction predict for the intra-tumoural heterogeneity in liposome accumulation. These results demonstrate the importance of accounting for properties of the microcirculation in ITTM. However, it was conjectured that incorporating image-based metrics of tumour perfusion into the ITTM would benefit by isolating the relative contributions of TBF and microvascular permeability. Therefore, in Chapter 4 a novel method, termed Volume Fraction Imaging or VFI for short, was developed to accurately and simultaneously quantify perfusion, vascular permeability, interstitial volume fraction, and plasma volume fraction. It was demonstrated that VFI provides the ability to independently quantify microvascular permeability and perfusion, thus allowing for an improved understanding of the relative importance of each parameter in mediating the intra-tumoural accumulation of CT-liposomes. In Chapter 5, the intra-tumoural relationship between tumour microcirculation, tumour IFP, and liposome accumulation was investigated. In vivo measurements indicated that image derived metrics of tumour perfusion predict for the intra-tumoural accumulation of CT-liposomes in a tumour xenograft model of metastatic breast cancer implanted OR or SC, corroborating the findings from Chapter 3. VFI indicated that perfusion and not vascular permeability was the primary mediator of intra-tumoural accumulation of CT-liposomes. No statically significant relationship was found between point-based measurements of tumour IFP, metrics of tumour perfusion, and the intra-tumoural distribution of CT-liposomes; however, it was observed that tumours with elevated central IFP that decreased at the periphery had low central perfusion and low central CT-liposome accumulation that increased towards the tumour periphery.
Tumours with low and relatively uniform IFP exhibited higher levels of tumour perfusion and CT-liposome accumulation. Furthermore, radial-based analysis indicated that a strong negative relationship exists between radial measurements of IFP, metrics of tumour perfusion, and CT-liposome accumulation. These results suggest that both tumour perfusion and elevated IFP play an integral role in mediating the intra-tumoural accumulation of liposomes, further supporting the importance of convection driven liposome transport and strengthening the need to account for transport heterogeneity in the ITTM. In summary, the results presented in this thesis provide a framework where imaging is used to inform a biophysical mathematical model of transport to accurately predict the inter- and intra-tumoural accumulation of liposomes.

One of the strengths of combining imaging and mathematical modelling is that it provides a foundation for characterising heterogeneity in the EPR effect. The EPR effect is often overstated or misrepresented as the gold standard rationale for developing and using nanoparticles in cancer therapy; however, considerable inter- and intra-tumoural heterogeneity in EPR has been observed in pre-clinical tumour models (Chapters 2 to 5) and in a clinical setting (Harrington et al. 2001). Heterogeneity in EPR is driven by many barriers of the tumour microenvironment, including vascular permeability, perfusion, IFP, and a dense ECM (Chauhan et al. 2011). Diagnostic and theranostic nanoparticles, including liposomes, can be used to monitor tumour accumulation, thus providing a tool to visualise highly heterogeneous EPR. This strategy has the potential to pre-select patients that are more likely to respond to nanoparticle therapy (i.e. patients with a sufficient concentration and homogeneous distribution of drug). The question remains as to what factors contribute to the poor accumulation that imaging nanoparticle accumulation alone is unable to reveal. The ability to perform image-based characterisation of transport barriers could be used to guide the rational use of pharmacologic (e.g. Angiotensin II), heat based, and radiation based agents that have known abilities to enhanced delivery, extravasation, and penetration (Stapleton & Milosevic 2013). As presented in this thesis, the combination of quantitative imaging methods, such as VFI, and mathematical modelling of transport, such as the ITTM, can be used to assess heterogeneity in EPR by relating specific transport barriers to the intra-tumoural accumulation of nanoparticles. Therefore, the combination of imaging and mathematical modelling can be used for quantitative assessment of variability in EPR, allowing for the rational use of nanomedicines on a patient by patient basis.
The intra-tumoural transport framework presented in this thesis provides a pivotal piece of the image-guided drug delivery schema whereby a combination of imaging and mathematical modelling can be used to personalise liposome drug delivery and assess treatment response (Figure 6.1). The concept of mathematical modelling of drug delivery and imaging nanoparticle accumulation has been around for several decades (Gabizon et al. 1991; Sanga et al. 2006; Lammers et al. 2010); however, integrating quantitative imaging data with mathematical models of drug delivery is relatively new. Under the image-guided drug delivery schema, it is envisioned that drug delivery can be personalised on a patient by patient basis whereby quantitative imaging is used to derive patent specific information on drug pharmacokinetics, biodistribution, intra-tumoural transport, biological targets, and mechanisms of resistance. Image derived information is then input into a collection of mathematical models to provide an accurate prediction of toxicity, intra-tumoural drug delivery, pharmacodynamics, and treatment response for a given nanoparticle. Finally, optimisation methods are then be used to determine optimal dosing and scheduling of nanomedicine treatments. The image-guided drug delivery schema provides a novel tool for personalised and adaptive treatment of solid tumours using nanoparticle drug delivery technologies. This presents a major leap forward compared to the convectional chemotherapeutic strategies where dosing and scheduling is prescribed based on empirical data derived from clinical trials and clinical experience. Additionally, the image-guided drug delivery schema may provide an efficient and cost effective method to identify drug candidates and to identify optimal treatment schedules and dosing, thereby maximising the benefit to the patient and the healthcare system.

The mathematical modelling and imaging work presented in this thesis provides a foundation that requires further development before reaching a state suitable for clinical use. There are still several exciting research opportunities to improve both the robustness of the ITTM and ability to integrate imaging derived metrics of transport. These including addressing limitations and assumptions of the ITTM and developing novel imaging methods to integrate spatio-temporally heterogeneous transport properties into the ITTM.
Figure 6.1 Schematic of the proposed image-guided drug delivery framework. Above the dashed horizontal line each box represents a set of imaging methods to quantify patient specific properties that affect drug delivery and treatment response. For example, imaging pharmacokinetics using diagnostic drug mimics, imaging the barriers to intra-tumoural drug transport, and imaging markers which affect treatment response and resistance. Below the dashed horizontal line each box represents a set of mathematical algorithms (or modules) that use the quantitative image data as input to allow for patient specific modeling of pharmacokinetics, biodistribution, pharmacodynamics, and response for a given drug. Optimisation algorithms can be used to determine a favourable patient specific dose scheduling. In this thesis the focus was placed on quantitative imaging of transport properties to be used as input into a mathematical framework to predict the intra-tumoural accumulation of nanoparticle drug delivery vehicles.
6.1 Addressing Limitations of the Intra-Tumoural Transport Model

The ITTM presented in Chapter 2 establishes a framework to describe the intra-tumoural accumulation of liposomes. However, there are several fundamental assumptions and limitations of the mathematical model that need to be addressed in order to improve the accuracy of the transport predictions. These include: (1) the assumption of spherical symmetry; (2) incorporating independent measures of the spatial variations of transport properties; (3) mathematical modelling of cellular uptake, active targeting, and other retention mechanisms; and (4) mathematical modelling of drug release.

6.1.1 Spherical Symmetry

In Chapter 2, the assumption of a spherically symmetric tumour was used to simplify the numerical approach used to solve the ITTM, in turn giving predictions of the spatio-temporal liposome accumulation relatively quickly. Rapid calculation of solutions is important when curve fitting experimental data, where the ITTM is solved hundreds of times in order to find an optimal solution to the least squares minimisation problem. However, a few studies have indicated that tumour shape and size may substantially affect IFP and spatial heterogeneity in transport suggesting that the assumption of spherical symmetry may lead to inaccurate ITTM predictions (El-Kareh & Secomb 1995; Soltani & Chen 2012). Under the assumption of a uniform microenvironment, shape does not substantially affect tumour IFP, intra-tumoural liposome distribution, and mean liposome accumulation for tumours that are several hundred mm$^3$ in volume (Figure 6.2, unpublished data). There is no loss in the accuracy of ITTM solutions by reducing the 3D tumour to a 1D spherically symmetric tumour of equal volume, if the assumption of a homogeneous transport microenvironment is valid. However, solid tumours have considerable spatio-temporal heterogeneity in transport properties and thus modelling the tumour as a 1D spherically symmetric entity with homogeneous transport properties has limited applicability. Incorporating transport heterogeneity into the ITTM requires accounting for the size and shape of tumours. CT and MRI enables measurement of tumour shape and size, and it is possible to generate accurate 3D solutions to the ITTM using finite element analysis and established software packages (e.g. Comsol Multiphysics). The challenge then becomes the ability to obtain quantitative image-based measurements of intra-tumoural transport heterogeneity that can be incorporated into the ITTM.
Figure 6.2 ITTM simulations of IFP and intra-tumoural liposome accumulation in a realistic 3D tumour geometry obtained from a micro-CT scan of an OR ME180 tumour (Chapter 2) using different transport properties (a-c). The finite element method (Comsol Inc, USA) was used to solve ITTM in 3D, while the finite difference method was used to obtain 1D spherically symmetric solutions. The tumour was assumed to have homogeneous transport properties. The 3D simulations of IFP (d) and liposome accumulation (e) agreed with 1D simulations in a spherically symmetric tumour of an equivalent volume with uniform transport properties. This indicates fluid and liposome transport in large tumours with uniform transport properties can be modeled as spherically symmetric without a loss in accuracy.

6.1.2 Image Based Quantification of Intra-tumoural Transport Properties

The ITTM is comprised of several biophysical parameters that represent properties of the transport microenvironment, including: the vascular and interstitial hydraulic conductivities ($L_p$, $K$), surface area of blood of vessels ($S$), tumour volume ($V$), microvascular pressure ($P_m$), interstitial fluid pressure ($P_i$), nanoparticle reflection and retardation coefficients ($\sigma, f$), the plasma and interstitial concentrations ($C_p, C_i$), and tissue volume fractions ($\epsilon_i, \epsilon_p$). Accurate predictions of the intra-tumoural accumulation of liposomes (i.e. $C_i$) requires knowledge of each of the remaining eleven parameters. Imaging can play an
important role in providing a priori estimates of these transport properties. For example, VFI can be used to quantify tissue volume fractions, volumetric CT or MRI imaging can be used to measure tumour shape and volume, and image-able nanoparticles, such as CT-liposomes, can be used to quantify the plasma pharmacokinetics. Image-based assessment of the other parameters ($L_p, S, P_v, P_i, \sigma$ and $f$) is considerably more challenging, as no established methods have been identified.

In Chapters 3 and 5 it was observed that perfusion predicts for the intra-tumoural accumulation of liposomes; however, directly incorporating perfusion into the ITTM is challenging as TBF is linked to several of the parameters, including $L_p, S, P_v,$ and $P_i$ (Netti et al. 1996; Gentile et al. 2008). Using mathematical modelling, it may be possible to use the image derived measurements of tumour perfusion to estimate the transvascular fluid flow. The continuity equation relating axial blood flow ($Q_p$) to the rate of transmural fluid flow along a straight permeable vessel is (Netti et al. 1996):

$$\frac{dQ_p}{d\xi} = -L_pS[P_v(x) - P_i(x)]$$  \hspace{1cm} (6.1)

where $dQ_p/d\xi$ represents the drop in axial blood flow over the normalised length of the vessel (i.e $\xi = x/L$ where $L$ is the length of the vessel). Extending Equation 6.1 to the macroscopic scale by assuming that the transmural flux in a macroscopic tissue region (i.e. length scale on the order of hundreds of microns) can be approximate by the spatial gradient of blood flow across that region gives:

$$\gamma \cdot \nabla \frac{F_p}{V} = -\frac{L_pS}{V}[P_v - P_i]$$  \hspace{1cm} (6.2)

where $\gamma$ is the linear dimension of a voxel, $\nabla F_p/V$ represents the spatial gradient in perfusion and $L_pS/V[P_v - P_i]$ represents the transvascular fluid flux given in Equation 2.1. Therefore, Equation 6.2 used in combination with intra-tumoural measurements of perfusion using the VFI method may provide an estimate of the rate of transvascular convection, allowing for a simple method to incorporate intra-tumoural measurements of heterogeneity in transvascular transport directly into the ITTM.
Imaging may also provide the ability to quantify intra-tumoural heterogeneity in interstitial transport parameters. The interstitial hydraulic conductivity is related to several properties of the ECM by:

\[
K = \frac{\varepsilon_i r_b^2}{G}
\]

where \(\varepsilon_i\) is the interstitial volume fraction, \(r_b\) is the size of the pores of the ECM, and \(G\) the specific geometry of fibrillar components (e.g. collagen) such as fiber spacing, orientation, and diameter (Swartz & Fleury 2007). It is possible to quantify \(\varepsilon_i\) using VFI and to estimate both \(r_b\) and \(G\) using diffusion tensor magnetic resonance imaging (DTI). The later stems from the ability of DTI to spatially map anisotropy in the water diffusion coefficient thereby providing information about the fiber structure and porosity of the tissue (Le Bihan et al. 2001). Alternatively, DTI could be used to estimate \(K\) by creating a calibrated spatial map of interstitial hydraulic conductivity using DTI imaging to scale literature reported values of \(K\). This approach has had some success in predicting the local pressure fields and intra-tumoural drug transport in brain and spinal cord tissue (Sarttinoranont et al. 2006; Linninger et al. 2008). Finally, it may be possible to directly image the interstitial fluid velocity using DCE-MRI using the method developed by Hompland et al. (2012). Mapping the outward motion of a high-signal-intensity rim in the tumour periphery immediately after gadolinium diethylene-triamine penta-acetic acid (Gd-DTPA) administration provides an estimate of interstitial fluid velocity at the tumour surface. Currently, this method is limited to measuring interstitial fluid velocity at the tumour surface.

The reflection and retardation coefficients \((\sigma, f)\) are challenging metrics to measure as they depend on steric and electrostatic hindrance (Wiig & Swartz 2012); the later may not be accessible by imaging. It may be possible to estimate steric hindrance across blood vessels through image derived quantification of vessel pore size. Microvascular pore size could be estimated by taking the ratio of the effective permeability coefficient estimated using VFI to that of diffusion in free solution (e.g. water) (Truskey et al. 2004). Once the pore size is known, the simple steric hindrance model presented in Chapter 2 can be used in conjunction with the particle size to determine to determine \(\sigma\) (Anderson & Malone 1974). However, the mechanism of transvascular transport of the small molecule CAs may be different from nanoparticles (Jain 1987) and may not produce accurate predictions of \(\sigma\). A similar approach could be used to estimate \(f\), where DTI imaging is used to directly estimate interstitial pore size \((r_p)\). In general,
image-based quantification of the reflection and retardation coefficients is challenging and requires further investigation. Further considerations include the shape, surface charge and presence of molecularly targeted surface ligands are known to affect the transvascular transport and interstitial penetration of liposomes (Drummond et al. 1999; Miles et al. 2006; Metz et al. 2010; Dunne et al. 2011). In general, an accurate and validated theoretical framework that relates the physicochemical properties of nanoparticles, such as liposomes, to their ability to extravasate and penetrate tumour tissue remains to be developed.

In conclusion, there are several image-based approaches available to quantify ITTM parameters. It is challenging to estimate each transport parameter individually, but a potential alternative is to estimate bulk transport parameters (e.g. transvascular and interstitial fluid flow rates) using established imaging methods such as VFI and DTI. So far diffusion has been neglected in the discussion of liposome transport; however, it is know that several therapeutic agents can substantially increase the rate of diffusion of macromolecules and nanoparticles in tumour tissue (Ramanujan et al. 2002; Brown et al. 2004; Nagano et al. 2008). Therefore, diffusion may be an important transport mechanism in the context of therapy. As discussed in Chapter 1, diffusive transport can be readily integrated into the ITTM; however, further development of image-based methods to quantify diffusive transport, independent of convection, need to be investigated.

6.1.3 Retention, Cellular Uptake and Active Targeting

Once liposomes have extravasated from tumour capillaries, they remain in the tumour compartment for an extended period of time (Chapter 2). Their retention may be due to several factors including: (1) the lack of functional lymphatic vessels, which are primarily responsible for the clearance of macromolecules; (2) uptake by tumour cells and tumour associated macrophages; and (3) nonspecific or specific binding. Currently, the ITTM accounts for the retention based solely on the lack of functional lymphatic vessels; however, cellular uptake by tumour cells and tumour associated macrophages, and nonspecific interactions with the ECM affect retention. Furthermore, there is a long standing interest in incorporating molecularly targeted surface ligands that also alter retention (Dunne et al. 2011).
Several approaches to mathematical modelling of retention and cell uptake using standard and molecularly targeted nanoparticles have been proposed (Harashima et al. 1999; Eliaz et al. 2004; Liu et al. 2011; Albanese et al. 2012; Hendriks et al. 2012; Xu et al. 2012). The complexity of the mathematical models vary depending on the level of detail regarding the cell uptake, non-specific binding and specific binding, physicochemical properties of the nanoparticle, and properties of the tumour microenvironment. Incorporating a mathematical model of liposome retention into the ITTM adds considerable complexity and increases the need for quantitative imaging to characterise retention kinetics.

6.1.4 Drug Release

In order for liposome drug delivery to be efficacious, a cytotoxic concentration of the drug must be released from the carrier. Currently the ITTM only considers the intra-tumoural transport and retention of the liposome carrier. Accounting for the release of drug from the carrier will allow for predictions of the intra-tumoural concentration of cytotoxic agent and the assessment of treatment efficacy. Several mathematical models exist to describe the release of drug from nanocarriers, including liposomes (Harashima et al. 1999; Eliaz et al. 2004; Arifin et al. 2006). The simplest is the reservoir model based on Fick’s second law which describes diffusion across a semi-permeable membrane, where no outside factors influence the release of agent from the carrier. Under this framework, the release profile of drug from the nanocarrier can be determined in vitro and directly incorporated into the model without the need for image derived parameters. In a more complicated scenario, the release of agent can be modified through cell uptake (Drummond et al. 1999) or triggered release mechanisms (Gaber et al. 1996). The mathematical description of release can be modified accordingly (Gasselhuber et al. 2010), and imaging may be required to quantify factors that influence the release of the drug (e.g. MR thermometry).

6.2 Predicting the Intra-tumoural Distribution of Liposomes and Treatment Response

It was demonstrated in Chapters 3 and 5 that image derived metrics of tumour perfusion predicted for the intra-tumoural accumulation of liposomes in solid tumours. Therefore, tumour perfusion imaging may be a useful tool to predict liposome treatment efficacy and monitor treatment response. The rationale relies on observations that a more homogeneous intra-tumoural distribution of liposomes will result in more efficacious treatment (Huang et al. 1994; Kong & Dewhirst 1999; Kong et al. 2000;
Primeau et al. 2005; Minchinton & Tannock 2006; Ponce et al. 2007; Patel et al. 2013). However, the tumour microenvironment is comprised of a heterogeneous population of tumour epithelial and stromal cells with limited vascular access for cytotoxic agents to reach cancer cells (Lehmann et al. 2009). For example, Chaudary et al. (2012) observed that both human and patient derived xenograft primary cervical tumours have a substantial proportion of stromal tissue (10 to 60%), and that tumour blood vessels were primarily found intermingled with the stromal cells (Figure 6.3). Given the limited penetration of liposomes (30 µm) and low MW cytotoxic agents, such as doxorubicin (50 µm), (Yuan et al. 1994; Primeau et al. 2005), the majority of liposomes are not expected to penetrate through the stromal compartment. In this scenario the delivery of the cytotoxic agent is largely limited to the stromal compartment, and anti-cancer effects are anticipated to be minimal. Therefore, even though tumour perfusion imaging may predict liposome accumulation in such a setting, it may not predict for therapeutic response in all tumours. Perfusion imaging would likely benefit from a companion imaging method that can characterise the perfused cellular compartment.

**Figure 6.3** Representative immunohistochemical (IHC) stained sections of a patient-derived OR cervix cancer xenograft. The hematoxylin and eosin (H&E) stained section shows the tumour epithelial cells in dark pink and stromal cells in light pink. The CD31 stained section shows that blood vessels are predominantly found in the stromal cell compartment. IHC sections were generously provided by Dr. Naz Chaudary.
The combination of magnetisation transfer and DCE-MRI has shown promise in quantifying the stromal density in pancreatic xenografts (Sakata et al. 2006). Using a combination of VFI and magnetisation transfer MRI it may be possible to predict the intra-tumoural accumulation of liposomes and assess the bioavailability of cytotoxic agent with respect to the stromal and tumour cell compartments. Furthermore, anti-cancer agents are usually administered over several courses, which may improve penetration through tumour tissue, providing access to distal tumour cells as those proximal to blood vessels are removed (Lehmann et al. 2009). VFI and MRI methods may play a substantial role whereby vascular, interstitial, and cellular properties can be assessed over the course of therapy allowing for improved understanding of drug delivery and therapeutic response that could ultimately be used to determine optimal dosing and treatment scheduling independent of complex transport models.

**6.3 Monitoring Therapeutic Changes using Volume Fraction Imaging**

In Chapter 4, the VFI method was presented as a means to improve the simultaneous quantification of perfusion, vascular permeability, plasma volume fraction, and interstitial volume fraction. It was shown through simulations and *in vivo* experiments that the VFI method can substantially improve quantification of morphologic and physiologic properties of tissue, and thus may provide a sensitive method to detect changes in tumour microcirculation over the course of therapy. Preliminary experiments were performed to assess the ability of the VFI method to monitor morphological and physiological changes cause by multi-tyrosine inhibition using Sorafenib (BAY 43-9006, Nexavar, USA), and mild hyperthermia (using a custom laser based heating system). Figure 6.4 shows the results of VFI in five control, two Sorafenib treated (4 mg/kg daily for 3 days, administered IP) MDA231 breast OR tumours and five control, five heated (42°C for 20 minutes) ME180 SC tumours.

A decrease in all VFI derived metrics of perfusion was observed at 48 hrs after Sorafenib treatment compared to control tumours. With only two Sorafenib treated tumours it is difficult to interpret the validity of these results; however, they are consistent with previous reports that Sorafenib decreases vascularity and perfusion (Mayer et al. 2004). In comparison, no changes in perfusion metrics were observed using the standard two-compartment TKM method. The VFI method detected an increase in interstitial volume fraction and perfusion in SC ME180 tumours after mild hyperthermia and is consistent with previous reports (Airley et al. 2003; Song et al. 2005). In comparison, the standard two-
compartment exchange method was only able to detect an increase in the interstitial volume fraction. Furthermore, intra-tumoural analysis using the VFI was more robust in quantifying noisy TICs and able to produce consistent estimates of tumour perfusion parameters. Conversely, the two-compartment method produced heterogeneous estimates with many outliers (Figure 6.5). Therefore, the preliminary results demonstrate that the VFI is sensitive to modulations of the tumour microenvironment using Sorafenib and mild hyperthermia, is robust in the presence of signal noise, and further strengthens the benefit of using VFI over standard tracer kinetic modelling.

Figure 6.4 Preliminary results showing VFI of control and Sorafenib treated MDA231 orthotopic tumours, as well as control and heated (42°C) ME180 SC tumours. The colored circles represent bulk (whole tumour mean) estimates obtained from VFI, while the grey diamonds represent estimates obtained using the standard two-compartment exchange model. A decrease in estimates of perfusion (a), interstitial volume fraction (b), perfusion (c) and permeability (d) was detected 48 hrs post Sorafenib treatment using the VFI method. An increase in both interstitial volume fraction and perfusion was detected in ME180 tumours heated for 20 min. The two-compartment exchange method was not always consistent with VFI results and was not able to detect most therapeutic changes observed using VFI.
Figure 6.5 Representative intra-tumoural measurements of perfusion in control and heated ME180 SC tumour. VFI imaging resulted in robust and consistent estimates of perfusion (a) and interstitial volume fraction (b) with minimal outliers. Conversely, the two-compartment exchange method resulted in estimates of perfusion (c) and interstitial volume fraction (d) that exhibited considerable variability and outliers. These results are representative of intra-tumoural estimates of all tumour perfusion metrics. Boxplots represent the intra-tumoural distribution of perfusion metrics, error bars represent the 95% confidence interval. Circles represent mean whole tumour perfusion metrics.

6.4 Metastatic Disease

This thesis focused on transport of fluid and nanoparticles in a single solid tumour mass derived from primary or metastatic cancer cells and implanted either intramuscularly, subcutaneously, or orthotopically. This setting is useful for testing and validation, but is not a common clinical scenario. In many cases metastatic disease is already present by the time primary cancer is detected. As highlighted by Lammers et al. (2012), treatment of local versus metastatic disease using chemotherapy are substantially different problems that may require different image-guided drug delivery approaches. Chemotherapy is principally used to treat metastatic disease as either an adjuvant therapy to
prevent/treat occult metastatic disease, or as a primary therapy to treat cancers that have spread to lymph nodes or other organs. Furthermore, metastatic disease can be found in several different organs each having a unique transport environment (Figure 6.6). Therefore, image-guided mathematical modeling of nanoparticle transport needs to be framed in the context of treating either occult or apparent metastatic disease that is present in several different sites, including: the lung, lymph nodes, and bones.

**Figure 6.6** Metastatic Spread to different organs. Percentages refer to the relative incidence of metastatic spread to a specific organ for a given primary cancer. Adapted from (Schroeder et al. 2011).
Image-guided drug delivery for the treatment of metastatic disease is challenging due to limitations in imaging. Currently established clinical imaging techniques (i.e. anatomical imaging) have a detection threshold of approximately $1 \text{ cm}^3 (\sim 10^9 \text{ cells})$, which is approximately 3 orders of magnitude below the $1000 \text{ cm}^3 (\sim 10^{12} \text{ cells})$ size threshold that is linked to patient death (Frangioni 2008). Anatomical imaging is unable to detect malignant lesions with fewer than $10^9 \text{ cells}$, limiting the ability to treat a potentially considerable number of cancers using image-guided drug delivery. The development of novel imaging methods that can characterise physiological, metabolic, and molecular cancer signatures may reduce the detection threshold and provide an opportunity for image-based assessment of nanoparticle transport. Indeed, several novel imaging techniques, such as the VFI method presented in Chapter 4, are being developed to detect early markers of cancer including: angiogenesis, glucose metabolism, hypoxia, and overexpressed proteins (Frangioni 2008). Sensitive imaging methods that allow for early detection will open the door for image-guided drug delivery to both small and large metastatic tumours.

Primary tumours metastasize to several different organs, including: brain, lung, breast, liver, pancreas, kidney, colorectal region, and bone metastases (Figure 6.6). The majority of these sites are accessible to intravenously administered agents and thus the ITTM presented in this thesis remains applicable. Each site has a unique tumour microenvironment that requires image-based Characterisation of transport for accurate predictions of drug delivery using the ITTM. For example, the blood brain barrier can substantially limit the transvascular transport of nanoparticles into brain tumours. The rigid structure and porosity of bone metastases affects the interstitial transport of nanoparticles. Lymph node metastases present a considerable challenge for intravascularly administered nanoparticles, due to the requirement of nanoparticle extravasation into a tissue drained by the malignant lymph node, interstitial transport, and lymphatic uptake. Variability in EPR and limited interstitial transport can substantially hinder nanoparticle based drug delivery to lymph nodes. Several strategies have been employed to overcome these limitations, including: active targeting, surface charge, and interstitial administration (Schroeder et al. 2011). Substantial developments in both quantitative imaging methods and the ITTM are required in order to accurately model drug delivery to malignant lymph nodes. In summary, there are several challenges in detecting and treating metastatic disease; however, it is possible to adapt and improve the image-guided drug delivery framework presented in this thesis to overcome these challenges.
6.5 Conclusion & Perspectives on the Future of Image-guided Drug Delivery

The application of nanomedicines in oncology has seen exciting developments that have resulted in the acceptance of therapeutic nanocarriers, including liposome and albumin based nanoparticles, in the clinical setting. The initial excitement was based on pre-clinical evidence that nanoparticles tend to accumulate in substantial concentrations at the disease site via EPR, while largely bypassing healthy tissue. Unfortunately, the rapid progress was stymied by the less than exciting clinical findings that liposomal drug delivery treatments, such as Doxil, have a negligible impact on treatment outcome. The promise of nanomedicines ability to translate to meaningful results was clearly premature.

Future success relies on understanding past failures and recognising novel methods to move forward. Mathematical modelling is an underutilised tool for understanding nanomedicines, but has the potential for substantial impact on comprehending, guiding, and ultimately improving nanoparticle based treatments. In this thesis, a novel schema using a combination of imaging and mathematical modelling is presented as a powerful tool to predict the intra-tumoural transport of liposomes in solid tumours. Through the use of mathematical modelling, quantitative imaging, and experimental observation a complete understanding of nanoparticle drug delivery will emerge, leading to an improved understanding of their limitations and, most importantly, insight on the rational use of nanotechnology-based therapies. Indeed, the image-guided drug delivery schema presented in this thesis has substantial potential to unlock the ability to perform patient specific nanoparticle treatment planning and response assessment. Currently, only a small group of researchers are active in the area of combined mathematical modelling and quantitative imaging of nanoparticle transport and we continue to demonstrate the power that physics provide in understanding the complex interactions between nanoparticles, biological transport and the tumour microenvironment. In the future it is envisioned that the state of art treatment in medical oncology will encompass a combination of imaging and mathematical modeling to reveal the biophysical properties of cancer, determine an optimal treatment plan, and guide the application of nanoparticle drug delivery systems. It is through our continued effort that this framework will become a reality and substantial improvements in nanoparticle treatments and patient outcomes will emerge. I am one of the many who are excited to contribute to this promising future.
6.6 References


Appendix 1
Supplement Material for Chapter 2

A1.1 Methods and Materials

A1.1.1 CT-liposome Preparation
1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, MW 734) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol) 2000 (DSPE-PEG2000, MW 2774) were purchased from Genzyme Pharmaceuticals (Cambridge, USA). Cholesterol (CH, MW 387) were obtained from Avanti Lipids Inc. (Alabaster, USA). Omnipaque™, a commercially available iodinated CT contrast agent that contains iohexol (M.W. 821.14) at 300 mg/mL iodine, was purchased from GE Healthcare (New Jersey, USA). The method used for preparation of the CT-liposomes was adapted from a previously published report. Briefly, lipid components for the CT-liposomes (i.e. DPPC, CH, DSPE-PEG2000) were dissolved in anhydrous ethanol at 70°C at a molar ratio of 55:40:5 DPPC:CH:DSPE-PEG2000. Omnipaque™-300 (300mg/mL iodine, GE healthcare, Mississauga, Canada) was added to the solution with a lipid concentration of 100mM following ethanol removal. The solution was kept at 70°C for 4 h with intermittent vortexing. Unilamellar vesicles were formed via extrusion at 70°C using a 10-mL Lipex Extruder (Northern Lipids Inc, Vancouver, Canada) with 5 passages through two stacked 200 nm pore size Track-Etch polycarbonate membranes (Whatman Inc., Clifton, NJ) followed by 5 passages through two stacked 80 nm membranes. The unincorporated contrast agent was removed by 16 h of dialysis (MWCO 8kDa) against a 250-fold volume excess of 0.02mM HEPES-buffered saline solution (HBS, pH 7.4). The liposome formulation was then concentrated to a final iodine concentration of approximately 45 mg mL⁻¹.

A1.1.2 CT-Liposome Characterisation
The size distribution of the liposomes was measured by dynamic light scattering at an angle of 90° and a temperature of 25°C using a 90Plus particle size analyzer (Brookhaven, Holtsville, NY). Samples were prepared by diluting liposome formulations with HBS to a final lipid concentration of 0.25 mM. The
iodine concentration in the CT-liposomes was determined by rupturing liposomes using a 10-fold excess of ethanol, diluting in HBS, and measuring ultraviolet absorbance at 245 nm using a Cary 50 UV-visible spectrophotometer (Varian, Palo Alto, CA). The MW was estimated by summing the weights of the lipids, PEG, cholesterol and iohexol molecules.

A1.1.3 CT Imaging of Liposome Accumulation

Mouse CT imaging was performed using a micro-CT system (eXplore Ultra, GE Healthcare, London, Canada). The reconstructed voxel size was 0.154x0.154x0.154 mm$^3$ for the anatomical images and 0.154x0.154x0.462 mm$^3$ for the DCE-CT images. Rabbit CT imaging was performed using a clinical PET/CT system (GE Discovery ST, General Electric Medical Systems, Milwaukee, WI, USA). The reconstructed voxel size was 0.430x0.430x0.625 mm$^3$. CT Liposome injections were performed using an injected pump (Harvard Apparatus, Canada) and a 27G butterfly catheter inserted into the lateral tail vein for M180 tumour bearing mice. Injections were performed manually for H520 tumour bearing mice and VX2 tumour bearing rabbits.

A1.1.4 Measurements of Liposome Pharmacokinetics and Accumulation

The CT data sets for each time point and mouse were imported into MicroView 2.2 (GE Healthcare) and three-dimensional volumes of interest analysis for the tumour and descending aorta were generated. The contours were made using a semi-automatic approach that combined manual contouring and threshold refinement. The tumour volume of interest was used to estimate an equivalent radius $R$, representing the radius of a sphere with an equivalent volume, at each time point. The equivalent radius was then averaged over time and used as input to the ITTM (eqns. 1-3). The signal enhancement in the tumour and descending aorta were estimated by computing the temporal change in the mean HU in each compartment relative to the mean HU measured pre-liposome administration. The iodine concentration (in mgI cm$^{-3}$) was determined by scaling the relative signal enhancement by a calibration factor of 50.1±0.4 HU per mgI cm$^{-3}$ for the mouse CT scans and 38.0±0.6HU for the rabbit CT scans. The calibration factor was obtained by CT scanning 5 syringes with increasing concentrations (1.4, 2.8, 5.6, 11.3, 22.5, and 45 mgI mL$^{-1}$) of the CT-liposomes and determining the slope of a HU vs CT-Liposome concentration curve.
The PK for each mouse were estimated by fitting the plasma concentration to a one compartment PK model and used as input to the ITTM (Equation 2.1). The mean plasma volume fraction ($\varepsilon_p$) of each tumour was estimated by taking the ratio of mean iodine concentration measured in the tumour to that in blood 5 min after injection in mice and 30 min in rabbits. The plasma volume fraction was used to subtract the contribution of the plasma compartment from the measured iodine concentration in the tumour, leaving an estimate of the apparent interstitial concentration. That is $\varepsilon_iC_i(t) = C_i(t) - \varepsilon_pC_p(t)$, where the subscript $t$, $i$ and $p$ denote the tumour, interstitial and plasma compartments and $\varepsilon_i$ is the interstitial volume fraction. The plasma blood pool concentration is given by $C_p(t) = C_a(t)/(1 - Hct_{aorta})$ where $Hct_{aorta} = 0.5$ based on previous estimations (Cairns & Hill 2004). The ITTM described in this paper was fit to the measurement of $C_i(t)$, where $\varepsilon_i$ was a free parameter determined by the curve fitting process.

**A1.1.5 Histological Analysis**

Tumour morphology, vascularity, perfusion, and lymphatics were assessed in each ME180 tumour bearing mouse. Tumour perfusion was assessed at the end of the experiment by i.v. injection of the fluorescent dye Hoechst 33342 (40 mg kg$^{-1}$ dissolved in PBS, Sigma-Aldrich, MW=561.93 Da) immediately following the last imaging time point. Sixty seconds after injection the animal was sacrificed by cervical dislocation and the tumours were surgically resected. Each tumour was divided into two equal hemispheres along a plane parallel to the CT imaging slice. One hemisphere was fixed in 10% formalin for 48 hours, dehydrated in 70% ethanol, mounted in paraffin and then cut into 4 µm sections. The second hemisphere was rapidly frozen over liquid nitrogen in Tissue-Tek optimum cutting temperature compound (Sakura Finetek Inc., Torrance, CA, USA) and cut into 5 µm sections. The paraffin-embedded tissue was stained for morphology using H&E, blood vessels using goat derived anti-mouse CD31 and for lymphatics using rabbit derived anti-mouse LYVE-1 (1/200 Abcam, Canada). The frozen sections were stained for blood vessels using a rat derived anti-mouse CD31 and cyanine-3 (Cy3)-donkey anti-rat secondary. Tumour morphology and vascularity for H520 tumours was assessed in rapidly frozen tissue sections that were stained for morphology using haematoxylin and eosin (H&E) and blood vessels using goat derived anti-mouse CD31.
The stained paraffin-embedded sections were imaged in their entirety using a digital microscope (ScanScope CD, Aperio Technologies, Vista, CA, USA) and 40x magnification. For H520 tumour sections the total area of positive CD-31 staining was quantified using the positive pixel algorithm provided by the Aperio analysis software ScanScope. The same technique was used to quantify the total area of positive lymphatic staining in ME180 tissue. The settings were standardised for each section and the % positive staining was assessed by taking the ratio of positive pixels to the total number of pixels in the tissue section. The stained frozen ME180 tumour sections were imaged in their entirety using a wide field fluorescent tiling microscope (Olympus BX50, Centre Valley, PA, USA) with a 10x objective (N.A. =0.4, W.D.=3.1, F.N. 26.5, Olympus, USA). Separate images were obtained for Hoechst 33342 staining and CD31 staining. Analyses were performed in Matlab 2009b (Mathworks Inc., Natick, MA) and the % perfused area and the % vascular area were calculated by taking the ratio of the number of positively stained pixels to the total number of pixels in the image. Additionally, the % perfused vasculature was calculated by taking the ratio of the number of positively staining CD31 pixels that co-localised with positively stained Hoechst 33342 pixels to the total number of pixels in the image.

A1.2 Curve Fitting of CT-Liposome Measurements

A1.2.1 Curve Fitting and Analysis
The prediction of the mean accumulation of liposomes was calculated by integrating the solution to Equation 2.1 over the tumour volume, giving:

\[
\overline{C}_i(t) = \frac{1}{V} \iiint C_i(r, t) \cdot dV
\]  

(A1.1)

In the case of spherical symmetry, the interstitial concentration is given by \(C_i(r, t)\), where \(r\) is the radial position. Equation A1.1 was fit to the measured mean liposome accumulation curves (Chapter 2, Figure 2.3d to f) using a nonlinear least-squares minimisation method (Matlab 2009a, Mathworks, Natick, MA). The fitted (i.e. free) parameters were \(L_p, S/V, K, P_v\) and \(\varepsilon_i\) with the remainder of the ITTM parameters fixed (Table A1.1). The free parameters were constrained within the range of previously published values. An attempt to find the global minimum of the least-squares objective function was performed by
Summary

using multiple initial conditions in the fitting process. The initial conditions were chosen by stepping evenly through the range of transport parameters. The coefficient of determination ($r^2$) was used as the goodness of fit metric and the solution with the lowest residuals was considered the best fit. The 95% confidence interval of each free parameter was assessed using the Monte Carlo Method described by Motulsky and Christopoulos (Motulsky & Christopoulos 2004).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tissue</th>
<th>Type</th>
<th>Bounds \ Value</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\frac{L_p S}{V}$ (mmHg$^{-1}$ s$^{-1}$)</td>
<td>Tumour</td>
<td>Free</td>
<td>1x10$^{-7}$ to 1x10$^{-3}$</td>
<td>(Baxter &amp; Jain 1989; Sevick &amp; Jain 1991; Milosevic et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Fixed</td>
<td>2.5x10$^{-6}$</td>
<td>(Baxter &amp; Jain 1989)</td>
</tr>
<tr>
<td>$K$ (cm$^2$ mmHg$^{-1}$ s$^{-1}$)</td>
<td>Tumour</td>
<td>Free</td>
<td>1x10$^{-8}$ to 1x10$^{-4}$</td>
<td>(Baxter &amp; Jain 1989; Sevick &amp; Jain 1991; Milosevic et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Fixed</td>
<td>8.53x10$^{-9}$</td>
<td>(Baxter &amp; Jain 1989)</td>
</tr>
<tr>
<td>$P_v$ (mmHg)</td>
<td>Tumour</td>
<td>Free</td>
<td>5 to 50</td>
<td>(Jain 1988)</td>
</tr>
<tr>
<td>$\varepsilon_i$</td>
<td>Tumour</td>
<td>Free</td>
<td>0.1 to 0.6</td>
<td>(Baxter &amp; Jain 1989)</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Tumour</td>
<td>Fixed</td>
<td>0.19</td>
<td>(Anderson &amp; Malone 1974)</td>
</tr>
<tr>
<td>$f$</td>
<td>Tumour</td>
<td>Fixed</td>
<td>0.5</td>
<td>(Swabb et al. 1974)</td>
</tr>
<tr>
<td>$R$ (cm)</td>
<td>Tumour</td>
<td>Fixed</td>
<td>Measured using CT</td>
<td></td>
</tr>
<tr>
<td>$C_p(t)$ (mg/l cm$^{-3}$)</td>
<td>Tumour</td>
<td>Fixed</td>
<td>Measured using CT</td>
<td></td>
</tr>
</tbody>
</table>

Table A1.1 Parameters used to populate the ITTM.

The reflection coefficient $\sigma$ can be determined using the cylindrical pore model developed by Anderson & Malone (1974) is given by $\sigma = \left(1 - \frac{r_s}{r_p}\right)^2$, where $r_s$ and $r_p$ are the liposome and pore radii respectively. The pore radius of tumour blood vessels ranges from 200 to 1200 nm (Hobbs et al. 1998). Assuming a pore radius of 200 nm as a worst case approximation and mean hydrodynamic radius of 50 nm for liposomes, the cylindrical pore model gives $\sigma = 0.2$. As the pore radius gets larger, $\sigma$ gets smaller indicating minimal sieving of liposomes by the endothelium of tumour capillaries. The retardation coefficient $f$ can be estimated using the empirical relationship derived by Swabb et al. (1974). The estimated retardation coefficient for 100nm liposomes is $f = 0.5$, assuming a liposome MW of 100 MDa and a tissue glycosaminoglycan concentration of 0.01 g GAG/100g.
A1.2.2 Results of fitting ITTM to Liposome Accumulation Data

The best fit parameters and the estimated $\alpha$ and $P_{i,max}$ for each mouse tumour are given in Table A1.2 and Table A1.3 respectively. There was a high variability and large 95% CI for each parameter between tumours of the same type and difference types. As such there is no statistically significant difference was found between any of the transport properties. The variability for a given transport parameter between the individual mice and rabbits may be reflective of the underlying biological variability; however, the large confidence interval suggest the dominant issue was insufficient measurement information to precisely estimate the transport parameters. The high variability and large 95% CI are likely reflective of not accounting for the spatio-temporal heterogeneity in transport microenvironment and the limited degrees of freedom in the fitting process. Taking measurements at more time points would provide a better representation of the shape of the liposome accumulation curves, and may improve the precision of predicted parameters; however, issues with necrosis and changes in vascular morphology, permeability, perfusion, etc. over time will affect the accuracy of predictions. A potentially more robust method would be to fit Equation 2.1 to the intra-tumoural distribution of liposomes at a given time point. The high spatial resolution of CT imaging provides ample data for fitting, and more importantly, as shown in Figure 2.2, the spatial distribution of liposomes may be related to the spatial distribution of tumour IFP. Due to the distinct relationship between tumour IFP and liposome accumulation, fitting the spatial distribution may provide a more precise estimate of transport properties. In addition to increasing the precision of predictions, this method would allow for estimates of transport parameters, such as IFP, at multiple time points. The challenge, however, remains accounting for other factor that influence the intra-tumoural transport of liposomes such as: heterogeneity in vascular density, vascular permeability, blood flow, and tumour necrosis. Currently, the ITTM assumes the transport microenvironment is a uniform and static system, when in reality there are spatio-temporal variations in $L_pS/V, K, P_v, \sigma$ and $f$.

A1.3 Biophysical Transport Modelling of the EPR Effect and Sensitivity Analysis

A1.3.1 Methods

Biophysical transport modelling simulations were performed by varying the parameters $R$, $L_pS/V$, $K$ and $P_v$ in order to elucidate their effects on IFP, the intra-tumoural distribution of liposomes and mean liposome accumulation. The range of values for each parameter is given in Table A1.3. The nominal values for $L_pS/V$, $K$, and $P_v$ were chosen based on previously published data. Each parameter was
changed while fixing the remaining at their nominal value. For $R$, $L_p S/V$ and $K$ the range of values were chosen to produce the same $\alpha$.

### A1.3.2 Results

The ITTM simulations demonstrated how the underlying biophysical transport properties ($R$, $L_p S/V$, $K$ and $P_v$) influences tumour IFP, the mean liposome accumulation, and the intra-tumoural liposome distribution, and (Figure A1.1). In general, the simulations showed that as $\alpha$ increases, tumour IFP increases, the rate of accumulation and peak liposome concentration decreases, and the intra-tumoural distribution of liposomes become predominantly peripheral. The ITTM predictions of liposome accumulation is most sensitive to the transport properties $R$ and $P_v$.

These results indicate that it is more efficacious to treat tumours with lower IFP. Given that most solid tumours have elevated IFP, efficient liposome drug delivery would benefit from pre-treating tumours with agents that lower tumour IFP. As the biophysical transport modelling indicates this would improve the rate of accumulation, peak accumulation and intra-tumoural distribution of liposomes. Some studies have shown that modifiers such as vasoactive agents (Zlotecki et al. 1993; Zlotecki et al. 1995; Seki et al. 2009), molecularly targeted agents (Tong et al. 2004; Vlahovic et al. 2007), and radiation therapy (Znati et al. 1996) can reduce tumour IFP and improve the delivery of macromolecules to the solid tumours. While, these studies did not look at improvement in intra-tumour distribution, the reported mechanism of action in reducing IFP is in line with the ITTM framework presented in this study (Zlotecki et al. 1995; Tong et al. 2004; Jain et al. 2007).
<table>
<thead>
<tr>
<th></th>
<th>( L_p S/V \times 10^{-7} ) (mmHg(^{-1}) ( \cdot ) s(^{-1}))</th>
<th>( K \times 10^{-7} ) (cm(^2) ( \cdot ) mmHg(^{-1}) ( \cdot ) s(^{-1}))</th>
<th>( P_v ) (mmHg)</th>
<th>( \varepsilon_t )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME180-01</td>
<td>25.1 (19.5, 35.7)</td>
<td>0.35 (0.21, 5.89)</td>
<td>5.0 (5.0, 5.3)</td>
<td>0.25 (0.18, 0.27)</td>
</tr>
<tr>
<td>ME180-02</td>
<td>46.5 (37.2, 63.1)</td>
<td>0.15 (0.12, 0.34)</td>
<td>5.0 (5.0, 5.3)</td>
<td>0.17 (0.14, 0.18)</td>
</tr>
<tr>
<td>ME180-03</td>
<td>28.1 (20.6, 47.2)</td>
<td>0.15 (0.12, 1.75)</td>
<td>5.8 (5.2, 6.5)</td>
<td>0.28 (0.18, 0.30)</td>
</tr>
<tr>
<td>ME180-04</td>
<td>6.3 (2.05, 19.9)</td>
<td>10.8 (0.48, 824)</td>
<td>9.2 (6.4, 12.6)</td>
<td>0.23 (0.19, 0.33)</td>
</tr>
</tbody>
</table>

**Average**

|        | 26.5±16.4 | 2.9±5.3 | 6.2±2.0 | 0.23±0.05 |

|        | H520-01  | 13.5 (9.5, 20.0) | 995 (2, 1000) | 11.2 (8.4, 14.1) | 0.13 (0.12, 0.14) |
|        | H520-02  | 11.7 (9.5, 13.5) | 999 (31, 1000) | 11.9 (10.7, 14.0) | 0.37 (0.36, 0.38) |
|        | H520-03  | 93.4 (68.2, 115) | 0.12 (0.1, 0.4) | 5.2 (5.0, 5.4) | 0.35 (0.30, 0.36) |
| H520-04 | 46.9 (27.8, 70.6) | 0.13 (0.1, 6.8) | 5.1 (5.0, 5.7) | 0.22 (0.19, 0.24) |
| H520-05 | 28.4 (22.0, 36.7) | 249 (3.28, 1000) | 5.6 (5.0, 6.5) | 0.27 (0.25, 0.28) |

**Average**

|        | 38.8±33.6 | 448±510 | 7.8±3.5 | 0.27±0.10 |

|        | VX2-01  | 4405 (748, 8434) | 651 (417, 959) | 42.4 (35.4, 50.0) | 0.13 (0.12, 0.15) |
|        | VX2-02  | 70.8 (8.7, 6605) | 998 (200, 1000) | 38.1 (32.3, 50.0) | 0.14 (0.13, 0.17) |
|        | VX2-03  | 1.4 (1.0, 2.1) | 541 (23, 1000) | 17.5 (14.0, 19.7) | 0.26 (0.22, 0.31) |
|        | VX2-04  | 1.1 (1.0, 1.6) | 988 (19, 1000) | 26.3 (20.2, 29.4) | 0.50 (0.43, 0.56) |
|        | VX2-05  | 963 (287, 1839) | 133 (70, 540) | 9.8 (8.7, 11.0) | 0.54 (0.47, 0.60) |

**Average**

|        | 1088±1898 | 662±358 | 26.8±13.7 | 0.31±0.19 |

**Table A1.2** Best fit parameters obtained by fitting the ITTM to the tumour accumulation curves measured in each mouse. The 95% CI of the best fit predictions are shown in brackets. The mean and standard deviation for each parameter was calculated using the best fit values.
Summary

Table A1.3 The transport properties $\alpha$ and the maximum IFP ($P_{i,max}$) were calculated using the best fit values from given in Table A1.2. The 95% CI are shown in brackets. The mean and standard deviation for each tumour type was also determined from the best-fit values. There is no statistically significant difference between the averaged parameters when comparing ME180, H520 and VX2 tumours.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nominal Value</th>
<th>Range for Simulations</th>
<th>Alpha (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R$ (cm)</td>
<td>0.5</td>
<td>0.17 to 4.2</td>
<td>1 to 25</td>
</tr>
<tr>
<td>$L_pS/V$ (mmHg$^{-1} \cdot s^{-1}$)</td>
<td>2.5x10$^{-5}$</td>
<td>(0.28 to 180) x 10$^{-5}$</td>
<td></td>
</tr>
<tr>
<td>$K$ (cm$^2 \cdot$ mmHg$^{-1} \cdot s^{-1}$)</td>
<td>7.0x10$^{-7}$</td>
<td>(63 to 0.1) x 10$^{-7}$</td>
<td></td>
</tr>
<tr>
<td>$P_v$ (mmHg)</td>
<td>10</td>
<td>5 to 30</td>
<td>3</td>
</tr>
</tbody>
</table>

Table A1.4 Values used for the biophysical transport modelling and sensitivity analysis.
Figure A1.1 Biophysical modelling results that demonstrates how the underlying transport properties ($R$, $L_pS/V$, $K$ and $P_v$) of the tumour influences tumour IFP, mean liposome accumulation, and the intra-tumoural distribution of liposomes. For $R$, $L_pS/V$ and $K$ the symbols denote different $\alpha$ values obtained by varying the respective parameter while keeping the remaining parameters fixed (see Table A1.4). For $P_v$ the symbols denote different MVPs. The y-axis represents the % of liposomes that have accumulated.
in the tumour, relative to the initial plasma concentration, and represents the mean value over the tumour volume (column 1) or at a given spatial location within the tumour (column 2).

A1.4 References