Microsphere Kinetics in Chronic Total Occlusions

by

Ashley R. Fraser

A thesis submitted in conformity with the requirements for the degree of Master’s of Health Science in Clinical Biomedical Engineering
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

Chronic total occlusions are a common problem in patients with coronary artery disease. The primary barrier to successful percutaneous coronary intervention is inability to cross the lesion with a guidewire. We seek to characterize polymer microspheres as a controlled delivery mechanism for collagenase and VEGF, novel intralesional therapies being investigated to alter CTO structural properties.

Release profiles for protein-loaded PLGA [poly(lactic-co-glycolic acid)] microspheres showed sustained BSA and VEGF release over eight and 48 hours respectively. Polymer degradation products had no impact on endothelial cell growth and protein bioactivity was maintained post-release. In vivo localization of microsphere-released collagenase was not possible due to low concentrations remaining at the site. Histology confirmed microspheres remained in the collagen-dense, proximal 15 mm of the lesion, likely altering the structural integrity of the plaque.
Acknowledgments

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<td>Chronic Total Occlusion</td>
</tr>
<tr>
<td>IEL</td>
<td>Inner Elastic Lamina</td>
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<tr>
<td>EEL</td>
<td>External Elastic Lamina</td>
</tr>
<tr>
<td>PFC</td>
<td>Proximal Fibrous Cap</td>
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<tr>
<td>PCI</td>
<td>Percutaneous Coronary Intervention</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>Gly</td>
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<td>Leu</td>
<td>Leucine</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>W/O/W</td>
<td>Water-in-Oil-in-Water</td>
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<tr>
<td>O/W/O</td>
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<td>S/O/W</td>
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<td>SA</td>
<td>Surface Area</td>
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<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>SNR</td>
<td>Signal to Noise Ratio</td>
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<td>External Magnetic Field</td>
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<tr>
<td>B₁</td>
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<td>γ</td>
<td>Gyromagnetic Ratio</td>
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<tr>
<td>EMF</td>
<td>Electromagnetic Force</td>
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<tr>
<td>T₁</td>
<td>Longitudinal Relaxation Time</td>
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<tr>
<td>CA</td>
<td>Contrast Agent</td>
</tr>
<tr>
<td>Gd</td>
<td>Gadolinium</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetic acid</td>
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<tr>
<td>BAL</td>
<td>British antilewisite</td>
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<tr>
<td>DMSA</td>
<td>dimercaptosuccinic acid</td>
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<tr>
<td>DTPA</td>
<td>Diethylenetriaminepentaacetic Acid</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>T₂</td>
<td>Transverse Relaxation Time</td>
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<td>DOTA</td>
<td>1,4,7,10-Tetraazacyclododecane-1,4,7,10-Tetraacetic acid</td>
</tr>
<tr>
<td>IR</td>
<td>Inversion Recovery</td>
</tr>
<tr>
<td>TI</td>
<td>Inversion Time</td>
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<td>Longitudinal Relaxation Rate</td>
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<tr>
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<td>Repetition Time</td>
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<td>Spoiled Gradient Recalled</td>
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<td>S_SPGR</td>
<td>Signal Intensity</td>
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<td>M₀</td>
<td>Equilibrium Longitudinal Magnetization</td>
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<tr>
<td>PVA</td>
<td>Polyvinyl Acetate</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>OD</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>RCCA</td>
<td>Right Common Carotid Artery</td>
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<tr>
<td>OTWB</td>
<td>Over-the-Wire-Balloon</td>
</tr>
<tr>
<td>TE</td>
<td>Echo Time</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; Eosin</td>
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<td>SD</td>
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<td>ROI</td>
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<td>Fibroblast Growth Factor</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
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<tr>
<td>Flt-1</td>
<td>fms-like tyrosine kinase</td>
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<td>KDR</td>
<td>kinase domain region</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
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Chapter 1: General Introduction

1.1 Chronic Total Occlusions

Coronary artery disease is the leading cause of mortality in the western world\(^1\). Arterial Chronic Total Occlusions (CTOs) are a common and clinically relevant problem in patients with coronary artery disease. As many as 30% of patients undergoing coronary artery angiography present with at least one CTO\(^2\), quite often, without suffering from myocardial infarction\(^1\).

The definition of a CTO, as it has been described, is a vascular occlusion greater than one month old\(^1\), however when defining a CTO it is important to also describe the state of the narrowed lumen and the antegrade blood flow in addition to the age\(^3\). One of the problems surrounding the definition of a CTO relates to the discrepancy of when an occlusion is considered chronic. For the sake of consistency, it has been published that an occlusion greater than three months can be termed chronic\(^3\).

There are two types of CTOs, functional total occlusions and true total occlusions. Functional occlusions show minimal contrast penetration through the occlusion during arteriography suggesting the presence of a narrowed microchannel through the lesion, whereas true total occlusions exhibit complete interruption of blood flow\(^3\).

Currently, coronary CTOs are treated with medication, and when revascularization is necessary there are two treatment options: bypass surgery or Percutaneous Coronary Intervention (PCI)\(^1\). PCI involves advancing a small guidewire through the diseased portion of the artery and inserting a metal stent to keep the artery open. The stent is placed using balloon angioplasty, where the deflated balloon (that is inside of the stent) is advanced percutaneously to the site of the thrombus and inflated to compress the plaque against the vessel wall. It is then deflated, leaving the stent in its place to protect the vessel from restenosis (Figure 1).
Figure 1: Demonstration of balloon angioplasty, whereby (A) a balloon catheter is advanced percutaneously through the lesion. (B) demonstrates the inflated balloon compressing the plaque and (C) shows the stent that remains in the vessel to prevent restenosis of the artery.

The success rate of PCI for CTOs is between 50% and 70%, with the main source of failure being inability to cross the lesion with the guidewire. Although CTOs are quite prevalent, PCI is reportedly only attempted in 8-15% of cases, due to the difficulty of crossing a lesion of this density with a guidewire.

1.1.1 Composition of CTOs

Arterial anatomy is presented in Figure 2 for clarification of terms used in the following section.

Figure 2: Arterial anatomy described. A. Tunica Intima; B. Inner Elastic Lamina (IEL); C. Tunica Media; D. External Elastic Lamina (EEL); E. Tunica Externa. The lumen is where blood flows, internal to the tunica intima.

CTOs are formed as a result of an acute atherosclerotic plaque rupturing that causes bidirectional thrombus formation. Initially, the thrombus contains a fibrin matrix that encapsulates platelets and erythrocytes, leading to an inflammatory response, which is
characterized by macrophage infiltration, foam cells and lymphocytes. The inflammatory response is considered the early stage (in the first few weeks) of the CTO development. It predominantly occurs in the intima, but can also become present in the media and adventitia layers. Endothelial cells (cells that line the lumen) also migrate into the thrombus to aid in the formation of microvessels through the lesion. The initial extracellular matrix that is formed at this early stage is composed of proteoglycan-rich tissue with relatively low collagen density.

After six weeks, the CTO has reached the intermediate stage and disruption to the inner elastic lamina (IEL) is evident, as is intraluminal neovascularization. Our experimental work showed marked negative remodeling at this point in development, which is characterized by a decrease in EEL size. By 12 weeks, perfusion through the vessel is markedly decreased, which continues into the advanced stages. With time, the lipid content of the thrombus is replaced with collagen and calcium, which is particularly dense at the proximal and distal ends of the occlusion. The proximal end of the CTO is the segment adjacent to the patent portion of the artery at the entry to the lesion. The area of more densely packed collagen is known as the proximal fibrous cap and is located at the entrance to the CTO. Figure 3 illustrates the relative location of the proximal fibrous cap through a longitudinal histology section.

**Figure 3:** (A) Longitudinal section of a 12-week-old rabbit femoral chronic total arterial occlusion stained with elastic tri-chrome. Proximal fibrous cap (PFC) is located at the entrance to the CTO next to the lumen (L) of the patent vessel. Arrows point to fragmented internal elastic lamina (IEL). External elastic lamina (EEL) is shown for reference. (B) Greater magnification of the PFC. Taken from Jaffe et al.

### 1.1.2 Microvasculature through the CTO

It has been demonstrated clinically that CTO guidewire crossings have greater success in the first six weeks of CTO development, when the lesion is considered “soft”. Soft lesions are
composed of loose fibrous tissue with neovascular channels\textsuperscript{3}. Conversely, hard plaques are found in more mature CTOs and are composed of dense fibrous tissue interspersed with highly calcified regions, with minimal to no neovascular microchannels\textsuperscript{3}. The mature CTOs thus present a greater challenge during PCI due to the increase in calcium and collagen content particularly in the proximal and distal caps, which act as barriers to guidewire passage. Previous experimental work has shown that the intraluminal microvascular networks in CTOs are greatest at six weeks of age. Histological images of CTOs at different ages of development in the rabbit femoral artery model are presented in Figure 4 to compare the presence of microchannels.

![Image A: Movat-stained histological cross-sections of CTOs at different stages of maturity highlighting the greater number of microvessels (*) present at the six week time point. (L=lumen; M=media; Ad=adventitia). Adapted from Jaffe et al\textsuperscript{6}.

The relationship between the decrease in microvessels and the decline in CTO crossing success after six weeks of age is evident, suggesting that the presence of microvessels throughout the lesion may facilitate guidewire crossing\textsuperscript{6}. Carlino et al. have shown that injection of contrast through the wire port of an over-the-wire balloon catheter to the proximal end of the CTO facilitates guidewire crossing, likely by expanding preexisting microchannels\textsuperscript{7}. 

![Figure 4: Movat-stained histological cross-sections of CTOs at different stages of maturity highlighting the greater number of microvessels (*) present at the six week time point. (L=lumen; M=media; Ad=adventitia). Adapted from Jaffe et al\textsuperscript{6}.](image-url)
1.1.2.1 Endothelium

Endothelium is the single layer of endothelial cells that line the interior surface of the body’s entire vasculature system, providing an interface between the circulating blood in the lumen and the remainder of the blood vessel wall. Endothelial cells act as an anticoagulant barrier, in part due to the heparin-like glycosaminoglycan receptors on the surface that bind thrombin via antithrombin and thrombomodulin to prevent clotting. In addition to being a semipermeable membrane, endothelial cells are also involved in the regulation of thrombosis, thrombolysis, platelet adherence, vascular tone, blood flow, angiogenesis and immune and lymphocyte interactions with the vessel wall.

1.1.2.2 Angiogenesis

Blood vessel formation is a result of many different processes. Vasculogenesis describes vessel growth during the early stages of development. It involves endothelial cell differentiation, proliferation and eventual coalescence to form a tubule network in previously avascular tissue. Angiogenic sprouting is the term describing the principle mechanism of vessel growth in an adult and involves the sprouting of new vessels from the parent vasculature. These immature vessels develop through endothelial cell integration with the surrounding matrix and the supporting cells, principally smooth muscle cells and pericytes, which are undifferentiated connective tissue cells. Angiogenesis is naturally involved in many conditions, including wound repair, but is also the force driving tumor growth.

Vascular endothelial growth factor (VEGF) is a pro-angiogenic growth factor and a key regulator in new vessel formation. VEGF is produced and released by cells in close proximity to endothelial cells and, upon binding to the VEGF-receptor on the surface of endothelial cells, induces their growth, migration, survival and assembly into an organized, connected network. In addition to VEGF, angiogenesis is also induced by placental growth factor (PIGF), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), angiopoietins, chemokines and interleukins. VEGFA, which is commonly referenced as VEGF, was one of the first pro-angiogenic proteins identified. Since then, the VEGF family has grown to include VEGFA, B, C and D and also PIGF. All of these exist as multiple isoforms. For example, VEGFA consists of six spliced isoforms including VEGF121, VEGF145, VEGF165, VEGF183, VEGF189 and VEGF206.

VEGF binding sites exist on the surface of endothelial cells. Lower-affinity receptors are present, but are isoform-specific (eg. VEGF164, but not VEGF121). Two high-affinity VEGF
binding sites are located on the surface of endothelial cells, specifically on the surface of large and small vessels confirming that vascular endothelium is the main target of VEGF action\textsuperscript{15}. When VEGF binds to these two high-affinity receptors, known as Flt-1 (\textit{fms}-like tyrosine kinase) and KDR (kinase domain region, also known as VEGFR2)\textsuperscript{15}, phosphorylation of 11 proteins results\textsuperscript{15}. VEGF induces endothelial cell growth by activating the Raf-Mek-Erk pathway\textsuperscript{16}, a signal transduction pathway that binds growth factors to cell surface receptors, and ultimately yields cell division. Figure 5 illustrates the pathway with VEGFR2 as the principle cell surface receptor involved in VEGF-induced angiogenesis. When the growth factor binds to the endothelial cell membrane receptor it phosphorylates GDP (guanosine diphosphate) to GTP (guanosine triphosphate), which activates Ras through phosphorylation\textsuperscript{17}. Activated Ras binds to Raf kinases, translocating them to the membrane where they can be activated\textsuperscript{17}. Raf activates MEK which activates ERK through phosphorylation leading to phosphorylation of the transcription factor Elk\textsuperscript{17}. Transcription is the synthesis of a complementary, antiparallel strand of RNA from a DNA template. Transcription is the first step of gene expression, which in this case leads to cell proliferation.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Figure5.png}
\caption{VEGF receptor binding and the subsequent protein cascade that is activated, yielding endothelial cell growth\textsuperscript{17}.}
\end{figure}

Angiogenesis is the result of a very complex process involving the co-ordinated activation of a number of receptors by many ligands\textsuperscript{16}. Delivery of growth factors capable of inducing angiogenesis to a region of endothelial cell growth results in its uptake by the VEGFR2
surface receptors and the cascade of events in the Raf-Mek-Erk pathway leading to cell proliferation.

1.1.2.3 Angiogenic Therapies

Angiogenic therapies are treatments administered to treat acute and chronic vascular insufficiencies resulting in reperfusion to an ischemic area. Many angiogenic factors have been investigated for their induction of neovascularization. Angiogenic agents studied in the treatment of stroke ischemia include: VEGF, transforming growth factor (TGF)-β, FGF-2 and platelet-derived growth factor (PDGF)\textsuperscript{14}. Loges and Carmeliet recommend the local administration of VEGF-B or PlGF as alternative growth factors, with impressive safety profiles, for the treatment of ischemia\textsuperscript{13}. Treatment of ischemic heart disease is focused on re-establishing blood flow through neovascularization, which is achieved with administration of angiogenic growth factors including FGF-1, FGF-2, VEGF and HGF\textsuperscript{18}.

1.1.3 Therapeutic Treatments for CTOs

There are several biological approaches for altering the composition of a CTO to facilitate guidewire crossing. Growth factor treatment to induce microvessel formation through the PFC is one method and local delivery of bacterial collagenase to degrade the interstitial collagen in the PFC is another.

The intravascular delivery of VEGF to the proximal end of the CTO has demonstrated effects of activating the pathway of angiogenesis and generating microvessels at the PFC. The presence of microvessels alters the structural density of the cap while at the same time provides channels of entry through the CTO to facilitate guidewire crossing. A histological image in Figure 6 demonstrates the effect of local VEGF delivery on the stimulation of microvessel formation.
**Figure 6:** Cross-sectional, movat-stained histology slides of 15 week-old CTOs. A. VEGF-treated CTO with multiple microvessels (*) traversing the lesion and B. Control, Bovine serum albumin (BSA)-treated CTO with much fewer and smaller microvessels present.

### 1.1.3.1 Collagenase

Collagenase is a zinc matrix metalloproteinase (MMP) that is isolated and purified from the bacterium, *Clostridium histolyticum*\(^{19}\). It degrades multiple types of collagen and gelatin\(^{20}\) and requires tightly bound zinc and loosely bound calcium for activity\(^{19}\). Bacterial collagenase has broad substrate specificity, meaning it is more active and potent than vertebral collagenases\(^{20}\). It cleaves collagen at the Glycine amino acid resulting in many small, residual peptides\(^{5}\). For instance, in a sequence resembling GLY-PRO-X-GLY-PRO-Y, cleavage occurs between X-Gly, where X and Y are any amino acid\(^{21}\). It has been shown that bacterial collagenases have many forms ranging in molecular weight from 68 to 130 kDa\(^{20}\).

### 1.2 Controlled Release Pharmaceuticals

With advances in the fields of protein sequencing and genetic engineering, protein activity has become much more precise and the use of protein therapies in the treatment of illnesses has become more feasible\(^{22,23}\). However, proteins have short plasma half-lives and must be injected frequently due to their low oral bioavailability\(^{23}\). Parenteral administration of protein therapeutic agents is necessary due to the unstable nature of proteins in the stomach and intestines\(^{24}\). As such, there is often poor patient compliance because multiple injections are painful, expensive and result in fluctuating blood-drug concentrations\(^{22,23,25}\). Controlled release drug delivery is an emerging treatment system that targets these problems by delivering the desired molecule in a protective casing that prevents it from being metabolized or excreted\(^{26}\), and releasing it at a predetermined rate to satisfy the target concentration levels. The objective
behind controlled drug release systems is to achieve a constant blood-drug concentration over an extended period of time for drugs with short half-lives. This will eliminate the need for multiple injections and as a result, improve patient compliance and the drug administration process\textsuperscript{27}.

### 1.2.1 Microsphere Drug Delivery Systems

With the advantages associated with controlled drug delivery systems, there are considerable research efforts being invested in the development of biodegradable polymeric microspheres/nanospheres for drug delivery\textsuperscript{28, 29}. Polymers have proven to be excellent carriers of macromolecules and due to their biocompatibility and biodegradability they are an optimal choice for controlled release\textsuperscript{23, 25, 27, 30}. PLGA [poly(lactic-co-glycolic acid)] is the most commonly used polymer for injectable, protein-loaded microspheres, because it is approved by the Food and Drug Administration (FDA) for use in humans and has already been successfully implemented as a biodegradable suture material\textsuperscript{27, 31}. As PLGA degrades, it is hydrolyzed into lactic and glycolic acids, which are incorporated into the Kreb’s cycle\textsuperscript{31} as CO\textsubscript{2} and in urine as water\textsuperscript{32}, without impacting the biological system.

When biodegradable polymers are used for controlled drug release systems, it is generally in the form of a microsphere (also referred to as a microparticle or microbead), which is a sphere ranging in diameter from 50 nm to 2 mm\textsuperscript{29} that encapsulates a specific drug to be eluted over time.

PLGA microspheres are prepared using emulsion techniques that are modified to accommodate the type of drug being used. By mixing together immiscible liquids (oil and water) in an appropriate order and volume, the repelling nature of the hydrophobic and hydrophilic components produces an emulsion, which is stabilized by introducing a surfactant. The term surfactant is short for surface active agent. It has a polar hydrophilic head and a hydrophobic hydrocarbon tail. When added to water, a surfactant orient itself such that the hydrophilic head has the most contact with the water and the hydrocarbon tail the minimum\textsuperscript{33}. Figure 7 shows an individual surfactant and an assembled surfactant in the presence of water.
Figure 7: A. Cone-shaped surfactant that results in B. normal micelles in the presence of water. Adapted from Pileni, 2003\textsuperscript{33}.

In the case of PLGA microsphere formation, the surfactant is introduced to stabilize the spheres because it acts as a bridge between the polymer and the water, reducing the surface energy between the two. Figure 8 shows the surfactant position on the hydrophobic (oil) sphere and the surrounding aqueous fluid.

Figure 8: Surfactant bridging the oil/water interface because the hydrophobic tail dissolves in the oil sphere and the hydrophilic head dissolves in the water, thus maintaining emulsion stability\textsuperscript{34}.

The possible emulsion techniques for microsphere production include water-in-oil-in-water (W/O/W); oil-in-water-in-oil (O/W/O); oil-in-water (O/W); or solid-in-oil-in-water (S/O/W).

The W/O/W technique is best suited to encapsulate drugs that are hydrophilic\textsuperscript{23,29}. The drug is initially dissolved in an aqueous solution and is then added to a hydrophobic solution causing an emulsion of the dissolved, aqueous drug droplets in the larger, organic phase. This first emulsion is well-mixed, such that the drug is well-dispersed throughout the organic phase. This means that when the first emulsion is added to a second aqueous solution, the oil droplets encapsulate the drug in spheres within the larger, aqueous phase. In PLGA microspheres, the polymer must be dissolved in an organic solvent, which is often a halogenated solvent that can be toxic to both the body and the environment\textsuperscript{35}. As such, the solvent must be evaporated or
extracted, causing the polymer to precipitate into spherical microparticles that enclose the aqueous, dissolved drug. The O/W emulsion solvent evaporation is the most commonly used technique in the production of PLGA microspheres, because it is used when working with hydrophobic drugs. This process involves dissolving the hydrophobic drugs in the polymer-organic solvent mix before emulsification in an aqueous solution. The evaporation of the volatile agent results in the formation of microparticles that encapsulate the drug.

Proteins and peptide therapeutic agents are typically administered via multiple injections to the patient due to their rapid loss of activity in vivo. Controlled drug release systems are ideal when administering these agents to decrease the frequency of injections to one single bolus shot. However, a drawback associated with the use of microspheres is difficulty controlling the drug release rate. Drugs elute from polymer microspheres primarily by permeating through the degrading polymer barrier to the external surroundings; however, some proteins are able to diffuse through the polymer as well. When first exposed to an aqueous surrounding, there is an initial burst of drug released from the spheres. This is followed by subsequent, controlled protein release, which is well-documented in the literature but poorly understood. A possible explanation has been attributed to the complex nature of the mass transport mechanism.

1.2.2 Microsphere Drug Release Mechanisms

Once immersed in an aqueous environment (i.e., the blood stream) the polymer microspheres experience rapid water uptake. The PLGA is broken down through hydrolysis reactions at the ester linkages, resulting in the formation of many smaller acidic chains.

Figure 9: PLGA structure where hydrolysis occurs at the ester linkages (arrow) resulting in the release of the original monomers (lactic and glycolic acid). (X = number of lactic acid units; Y = number of glycolic acid units).

These acidic chains are lactic and glycolic acid monomers and they diffuse out of the sphere and are neutralized by the surrounding aqueous fluid. However, the acid degradation products are formed more rapidly than they are neutralized resulting in a lower pH within the
microsphere\textsuperscript{38}. The acidic byproducts of PLGA degradation further catalyze the polymer erosion process, which is known as the “autocatalytic effect.”

The rate of polymer hydrolysis and subsequent protein release is dependent on a number of factors, namely the lactic acid to glycolic acid ratio, the reaction temperature and the physical properties of the microsphere structure (including the surface to volume ratio, the surface porosity of the sphere, the density, the drug encapsulation efficiency, the drug distribution and the molecular weight of the polymer employed)\textsuperscript{23, 24, 27, 37}. Tailoring the release profile of protein (ie. the cumulative amount released as a function of time) is the most challenging aspect of a microsphere controlled-release drug delivery system\textsuperscript{24, 28}. Yang et al.\textsuperscript{24} assessed the effect of preparation temperature on drug release profiles of microspheres through its effect on sphere morphology. They found that preparation of microspheres at low temperatures (5°C, 15°C, 22°C) yielded smaller microspheres that released over an extended period of time, with low initial release. Conversely, microspheres manufactured at higher temperatures (38°C, 42°C) resulted in larger microspheres, which also exhibited minimal initial release\textsuperscript{24}. Interestingly, microspheres prepared at 33°C exhibited the largest initial burst release\textsuperscript{24}. Burst release is usually attributed to poor protein entrapment, which is controlled by the polymer’s ability to encapsulate the protein. The amount of polymer used and its molecular mass both influence the speed at which the polymer solidifies and faster solidification corresponds to better protein entrapment, i.e. lower initial release\textsuperscript{24}. Alternatively, microspheres with porous surface geometry also have an increased drug release rate. Microspheres fabricated at higher temperatures exhibit a rougher exterior, which corresponds to their lower overall release\textsuperscript{24}. Often divalent cations, such as zinc salts, are incorporated into PLGA microspheres to control the pH or stabilize the encapsulated proteins; however it has been shown that zinc salt increases the porosity of the spheres, which interferes with the timed release\textsuperscript{25}.

In addition to structural properties, the release of protein from the microspheres is also impacted by the stability of the protein. Incomplete release of the protein can occur as a result of protein-polymer interaction. It has been reported that the hydrophobic interactions between the encapsulated protein and the PLGA can lead to incomplete release due to adsorption\textsuperscript{27}. Similarly, the PLGA hydrolysis reactions can drop the internal pH to as low as 1.5-4.7\textsuperscript{27} meaning that the protein may also become denatured prior to release. The acidic microenvironment can also cause hydrolysis reactions within the drug complex and the PLGA byproducts can undergo acylation reactions with the amines (mostly primary amines) of the protein\textsuperscript{38}. The type of protein being encapsulated can also impact its release rate from PLGA microspheres. For instance,
Klose et al.\textsuperscript{38} report that the encapsulation of highly water-soluble drugs can lead to excessive water penetration that can in turn lead to a porous polymer. In contrast, the encapsulation of lipophilic drugs can slow polymer degradation due to the decrease in water absorption\textsuperscript{38}.

1.2.2.1 Effect of Microsphere Size on Drug Release

The effect of diffusion pathway on drug release rates has been demonstrated previously. Therefore, to achieve consistent and repeatable drug release rates, the size of the microspheres must be relatively constant. Ribeiro-Costa et al.\textsuperscript{41} have reported that using controlled micromixer speeds during the preparation of PLGA microspheres, specifically during the emulsion production and solvent evaporation steps, results in well-controlled microsphere diameters. They showed that larger microspheres released more protein than smaller microspheres\textsuperscript{41}. Berchane et al.\textsuperscript{28} also reported on the impact of microsphere diameter on drug release profiles. Using sieves, they were able to control microsphere diameter and showed that smaller microspheres (diameter < 20µm) released a greater fraction of their total in the first 15 days of the trial before reaching peak release after approximately 30 days under physiological conditions. Conversely, larger microspheres (diameter > 40µm) exhibited a sigmoidal release profile, with an initial burst release that slows down before reaching a second rapid release phase at the 30 day mark prior to tapering off\textsuperscript{28}. Smaller microspheres (< 20µm diameter) tend to exhibit concave downward release profile trends that are characteristic of diffusion-dependent controlled release\textsuperscript{42}. However, as demonstrated by Berchane et al.\textsuperscript{28}, large microspheres exhibit sigmoidal release patterns that cannot be explained by drug diffusion alone\textsuperscript{42}. Berkland et al.\textsuperscript{42}, studied the effect of microsphere size on diffusion characteristics and reported that several other factors in addition to diffusion through the polymer matrix contribute to the release kinetics of polymer microspheres: desorption of the drug from the surface of the polymer, erosion of the polymer matrix, polymer degradation and drug distribution. These factors are also influenced by microsphere diameter\textsuperscript{42}.

1.2.2.1.1 Polymer Degradation

As PLGA is hydrolyzed, there is a decrease in the overall molecular weight of the polymer present, which corresponds to an increase in the diffusion coefficient of drugs exiting the microspheres. This phenomenon gives rise to the importance of surface area to volume ratio and its effect on microsphere degradation. From the relationships given in [Eq.1], as
microsphere radius \((r)\) increases, the surface area \((SA)\) and volume \((V)\) increase at different rates. Therefore, larger microspheres have a small surface area to volume ratio meaning that the polymer is degrading at a slower rate.

\[
SA = 4\pi r^2
\]
\[
V = \frac{4}{3}\pi r^3
\]

[Eq.1]

That being said, larger microspheres have been shown to degrade at a more rapid rate, which has been contributed to the greater buildup of acidic byproducts and the autocatalytic effect resulting from polymer degradation\(^{28, 43}\).

1.2.2.1.2 Drug Distribution

Drug distribution within the microspheres also impacts release rate as demonstrated by Berkland et al.\(^{42}\) using Rhodamine and Piroxicam as the loaded drugs. Using confocal fluorescence microscopy, they showed that as microsphere size increased, Rhodamine tended to distribute itself at the surface, whereas Piroxicam behaved oppositely, distributing itself to the interior of the larger microspheres\(^{42}\). Interestingly, the smaller microspheres exhibited more uniform distribution for the two drugs. Drugs that locate at the periphery of microspheres have a shorter diffusion distance compared to the drugs that position themselves at the core, suggesting that drug distribution impacts microsphere release profiles. Since this phenomenon appears to be characteristic of large microspheres, which are also impacted by polymer degradation and the internalization of acidic byproducts, the effects on the release profiles may be muted depending on how the drug is distributed. The sigmoidal nature of the release profiles for large microspheres is likely due to a combination of these properties.

1.2.2.1.3 Initial Burst Release

There has been extensive discussion to explain the basis for the initial burst of protein in the release profile, which is demonstrated in Figure 10.
Since the initial burst occurs for such a small amount of time over the entire drug release, it is dealt with in many different ways. Some researchers ignore it completely, others research it specifically, and still others use it to deliver a high dose of drug immediately as part of the drug administration process\(^4^4\). That being said, the amount of drug released in the initial burst is often non-reproducible suggesting that it cannot be reliably used for drug administration. In addition, the initial burst can cause local or systemic toxicity, both acutely and over long-term release because it can exceed the toxic dose\(^3^7,4^4\). Choy et al.\(^3^7\) discuss mitigation of the initial burst by coating the microsphere surface. However this method requires additional processes and potentially toxic chemical additives to the drug carriers. The burst effect is generally explained by protein that becomes trapped on the surface of the polymer matrix during the manufacturing process which is released immediately upon exposure to the releasing medium\(^4^4\).

Small microspheres may be responsible for the initial burst release\(^4^2\). Berkland et al.\(^4^2\) compared release profiles for uniform and non-uniform microspheres and found that the profiles for non-uniform exhibited the initial burst release, whereas uniform microspheres showed “regular” release profiles characteristic of their size. Since traditional fabrication techniques tend to produce an extensive number of small microspheres (diameter 5-10 µm), this hypothesis remains plausible.

Although PLGA microspheres have been shown to be effective when used as the vehicle for controlled drug delivery systems, very little information is available in the literature to characterize the release patterns for different drugs. Studies that discuss microsphere drug delivery tend to characterize the release of peptides or BSA, not the therapeutic proteins (VEGF or collagenase) of interest in our laboratory’s study of CTOs.
1.3 Magnetic Resonance Imaging

Magnetic Resonance (MR) imaging is an imaging modality that provides excellent soft tissue contrast with high spatial resolution\(^45\). MR imaging uses a strong magnet (0.1–3 T) to align magnetic dipoles. More recently, 3T magnets have become more widely used in the clinical setting due to a reduction in their cost and their improved signal to noise ratio (SNR) compared to 1.5T scanners\(^46\). Atoms with an odd total number of protons and neutrons have intrinsic magnetic moments, causing them to align with an applied magnetic field\(^47\). The hydrogen atom, which has a nucleus composed of one proton, is the most naturally abundant atom for MR since biological systems are primarily composed of water molecules and is the principle atom used in MR imaging\(^47\).

Prior to the presence of an applied magnetic field, the protons’ magnetic moments (or “spins”) are randomly oriented with a net magnetic moment of zero. However, with an applied external magnetic field \(B_0\), the majority of spins align in its direction (referred to as the \(z\)-direction or longitudinal direction) creating a net magnetic moment\(^47\). The spins also rotate about the \(z\)-axis at a well-defined frequency known as the Larmor frequency \(\omega_0\), which is dependent on the atom’s gyromagnetic ratio \(\gamma\), which is the ratio of magnetic dipole moment to angular momentum for the tissue, and the \(B_0\) through the following relationship:

\[
\omega = B_0 \gamma
\]

[Eq.2]

Once the spins are aligned with the \(B_0\), they resonate at their Larmor frequency. In order for a signal to be generated, the spins must be excited out of equilibrium with the application of a radiofrequency (RF) magnetic pulse \(B_1\) in the transverse \((xy)\) plane. The RF pulse is usually only a few milliseconds in length, but it is sufficient to cause the spins to oscillate about the \(z\)-axis (precess) towards the \(xy\) plane at a predetermined flip angle. Once the RF magnetic pulse \(B_1\) stops, the spins continue to precess in the \(xy\) plane. Since these are essentially rotating magnetic fields, they generate an electromotive force that is detected by an RF receiver\(^47\). In the absence of the RF pulse, the spins return to their initial position in line with the external magnetic field \(B_0\). The time required for the protons to realign with the external magnetic field is known as \(T_1\), or longitudinal relaxation time. Alternatively, the rate of decay of the signal that results from the dephasing of signals between tissues with different precessional frequencies is known as \(T_2\), or transverse relaxation time\(^48\).
Different tissues vary in water composition, meaning they have different $T_1$ and $T_2$ values, making it possible to distinguish between them using MR. In general, human tissue $T_1$ values range from 100–1500 ms and $T_2$ values from 20–300 ms. Table 1 provides published $T_1$ values for common tissue and blood measured at 3T.

Table 1: $T_1$ values for select tissues and substances at 3T

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$T_1$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous fat</td>
<td>365 ± 9.046</td>
</tr>
<tr>
<td>Cartilage</td>
<td>1240 ± 10746</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>1420 ± 3849</td>
</tr>
<tr>
<td>Blood</td>
<td>1932 ± 8550</td>
</tr>
</tbody>
</table>

In 25% of MRI scans, contrast agents (CAs) are used to enhance the image contrast between diseased and healthy tissues\textsuperscript{51}. CAs are able to alter the $T_1$ and $T_2$ relaxation times of the hydrogen nuclei located nearby\textsuperscript{51}.

1.4 Gadolinium and Chelation Chemistry

The most commonly employed MRI CAs that are approved for clinical use are low-molecular weight Gadolinium(III) chelates\textsuperscript{52,53}. Gadolinium (Gd) is a lanthanide element with atomic number 64 and a molecular weight of 157.25 Da. The Gd used in MRI is in the form of the Gd$^{3+}$ ion which, when not bound to another molecule, is toxic. Gd$^{3+}$ has an ionic radius of 0.99 Å, which is almost equal in size to that of Ca$^{2+}$ ion\textsuperscript{54}. Due to this characteristic, Gd is toxic to biological systems because it competes with calcium ions and binds to calcium substrates with a much higher affinity\textsuperscript{54}. Free Gd$^{3+}$ can cause splenic degeneration, liver necrosis, hematological abnormalities, enzyme inhibition and/or blocking of calcium channels. Thus, Gd$^{3+}$ must remain firmly bound to a chelating molecule when introduced to a biological system\textsuperscript{53}.

Chelation is the formation of three or more bonds between a single ligand, which can be either linear or cyclic, and a central atom, usually a metal. Because chelating molecules have multiple bonding locations, they tend to have a very strong affinity for metal ions, which have multiple unpaired electrons in their outermost orbitals. For instance, the Gd$^{3+}$ ion is most commonly in the 3+ oxidation state, which means that it has seven unpaired electrons in its outermost f-orbital, making it an ideal candidate to bond with chelating agents that can provide as many bonds. Chelation chemistry first became popular in the 1950s due to trace element research\textsuperscript{55}. Now, chelating molecules are commonly used to pre-concentrate metal ion contaminants in both environmental and food samples\textsuperscript{56,57}, prior to performing additional
technical analysis that requires the metals to be at specific concentration levels. Chelation therapy also uses chelating molecules to competitively bind with metal contaminants to detoxify blood that has been contaminated with poisonous metal compounds, such as mercury, arsenic or lead\textsuperscript{58}. In these cases, chelators such as EDTA, (ethylene diamino tetraacetic acid), BAL (British antilewisite), dimercaptosuccinic acid (DMSA) or D-penicillamine bind with the metal contaminants and are removed from the system via excretion through the renal tract\textsuperscript{59}.

Seven extracellular Gd CAs are available for clinical use and are presented in Table 2. Each of these MRI contrast agents uses chelation chemistry to bind the Gd\textsuperscript{3+} to a molecule to ensure it is not released \textit{in vivo}. The chelators listed in Table 2 are the most common chelation molecules used in Gd CAs.

**Table 2: The seven clinically-approved Gd contrast agents, adapted from Morcos\textsuperscript{53}**.

<table>
<thead>
<tr>
<th>Extracellular Gd-CA</th>
<th>Chelator</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gadoversetamide</td>
<td>DTPA</td>
<td>Non-ionic; linear</td>
</tr>
<tr>
<td>Gadodiamide</td>
<td>DTPA</td>
<td>Non-ionic; linear</td>
</tr>
<tr>
<td>Gadobutrol</td>
<td>DO3A</td>
<td>Non-ionic; cyclic</td>
</tr>
<tr>
<td>Gadoteridol</td>
<td>DO3A</td>
<td>Non-ionic; cyclic</td>
</tr>
<tr>
<td>Gadopentetate</td>
<td>DTPA</td>
<td>Ionic; linear</td>
</tr>
<tr>
<td>Gadobenate</td>
<td>BOPTA</td>
<td>Ionic; linear</td>
</tr>
<tr>
<td>Gadoterate</td>
<td>DOTA</td>
<td>Ionic; cyclic</td>
</tr>
</tbody>
</table>

The cyclic agents provide a more stable bond to the lanthanide metal ion because they form five or six bonds with the metal. Therefore, to release Gd\textsuperscript{3+}, these five or six bonds must be broken simultaneously, which is less likely\textsuperscript{53}.

Gadolinium has nine co-ordination sites with which it may bind to a chelator molecule\textsuperscript{53}. DTPA (diethylenetriaminepentaacetic acid) is a linear chelating molecule that binds Gd\textsuperscript{3+} through: five carboxylate-oxygen groups, three amine-nitrogen atoms and one water-oxygen molecule\textsuperscript{53}. DOTA (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid), a circular agent, binds Gd\textsuperscript{3+} through: four carboxylate-oxygen atoms; four amine-nitrogen groups and one water-oxygen group\textsuperscript{52}. The configurations of these two structures are shown with and without Gadolinium in Figure 11 and Figure 12.
Although DTPA and other chelators provide a strong bond to Gd$^{3+}$, these complexes, when used as CAs, are rapidly excreted from the vasculature and taken up by the tissue. Consequently, high doses or multiple doses of CA are required to obtain a proper MR image. The use of macromolecular Gd CAs has shown promise of a prolonged effect in imaging, which is primarily due to the globular structure of the complex$^{60}$. However, the slower excretion time corresponds to an increased risk of Gd$^{3+}$ leaking from the structure. By bonding a cyclic chelator such as DOTA with Gd$^{3+}$, and in turn conjugating this stable structure to a macromolecule such as a protein, the retention time of the CA may increase. This could make it possible to lower the dose, and in turn, the possible toxic effects of Gd$^{3+}$ release$^{60}$. By selectively conjugating specific proteins to the DOTA-Gd complex, it is possible to deliver therapeutic agents to a target site, in addition to increasing retention time.

Therapeutic radiopharmaceuticals are an established application of biomolecular conjugation. In this example, the target biomolecule is attached to a bifunctional chelator to deliver a high dose of metallic radionuclide to a target site$^{61}$. Positron Emission Tomography (PET) is a non-invasive imaging modality that quantitatively detects radiotracer accumulation$^{62}$. This means that these conjugated biomolecules can be used in PET to label the target site (e.g.
tumour) for diagnostic purposes\textsuperscript{62,63}. For instance, indium-111 (\textsuperscript{111}In), gallium 67/68 (\textsuperscript{67/68}Ga), yttrium-86 (\textsuperscript{86}Y), yttrium-90 (\textsuperscript{90}Y – used in targeted radiotherapy) and lutetium-177 (\textsuperscript{177}Lu) are all trivalent radionuclides employed in radioisotope imaging\textsuperscript{64}. These radionuclides can be conjugated to biomolecules that are able to target, diagnose or treat diseases via chelating molecules. DOTA or DTPA bind to the radiometals through the amino and carboxylic acid groups, with one or more of the carboxy groups providing the reaction site for the biomolecule conjugation. Since lysine side chains are polar and hydrophilic in nature, they are often located on the surface of the protein, making their amino groups the primary site of conjugation\textsuperscript{65}. If the biomolecule is a peptide, then the preparation conditions can be more flexible than if it is a protein. For instance, DOTA conjugation is more efficient at higher temperatures compared with DTPA and must be at a pH less than 6. However, proteins are readily denatured at high temperatures and have very narrow ranges of stable pH, making this an ineffective reaction mechanism for macromolecules. A structurally-modified DOTA molecule is often used for conjugation to ensure it readily reacts with the desired protein. For instance, the addition of an activated ester chain can improve its reactivity, such as the DOTA-NHS ester or the tris-\textit{tert}-butyl ester.

\subsection{1.5 $T_1$ Mapping}

The longitudinal relaxation time $T_1$ is the time required for the MR signal to recover to its initial state under the external magnetic field ($B_0$) following precession about the $z$-axis due to the RF pulse. Protons oscillate at their Larmor frequency [Eq.2], which is unique to each tissue through the gyromagnetic ratio ($\gamma$). Because each tissue has different water content, and therefore proton density, its $T_1$ is unique. $T_1$ maps are images generated using MR images that show the $T_1$ value for each voxel rather than signal intensity that is characteristic of MR images. Quantification of longitudinal relaxation time is beneficial in the study of biochemical and structural changes resulting from disease progression\textsuperscript{66}. For instance, $T_1$ changes have been involved in many neurological conditions including Parkinson’s disease, epilepsy and brain tumour, and in some cases $T_1$ changes are indicative of these states\textsuperscript{66}.

\subsection{1.5.1 Rapid $T_1$ Determination – DESPOT1}

Accurately measuring $T_1$ in a timely manner has been a challenge, which has limited its clinical application\textsuperscript{67-69}. The gold standard method of $T_1$ mapping uses the inversion recovery
(IR) pulse sequence that applies a 180° RF pulse initially, and then varies inversion time (\(T_I\)) before applying a 90° RF pulse. In this manner, transverse magnetization is measured for each \(T_I\) and a curve of signal vs. \(T_I\) is generated and fit to an exponential curve. \(T_I\) is found using the relationship shown in [Eq.3]. Inversion recovery \(T_I\) mapping is accurate, but cannot be performed in a clinically relevant period of time, which limits its applicability.

\[
y = e^{-\frac{T_I}{T_1}}
\]

[Eq.3]

Another method of \(T_1\) determination is known as saturation recovery (SR). SR determines longitudinal relaxation rate \((R_1)\), which is related to \(T_1\) by the relationship given in [Eq.4].

\[
R_1 = \frac{1}{T_1}
\]

[Eq.4]

While running the SR sequence the longitudinal magnetization \((M_L)\) is set to zero and then measured after a given period of recovery time, \(\tau\). By altering the recovery time, a curve is fit to the corresponding longitudinal magnetization measurements that allows for the determination of \(R_1\) from [Eq.5] (where \(M_{eq}\) is longitudinal magnetization at thermal equilibrium).

\[
M(\tau) = M_{eq}(1 - e^{-\tau R_1})
\]

[Eq.5]

A benefit of SR is that full magnetization recovery is not necessary prior to the next repetition, which decreases the scan time. Similar to IR, it provides good SNR; however this method is also designed to find specific \(T_1\) values, meaning it is still not sufficient when detecting ranges of \(T_1\) values.

Alternative methods for the determination of \(T_1\) and \(T_2\) have been reported and although they accurately determine the relaxation time in a timely manner, their low SNR, long reconstruction time and hardware requirements render them less clinically relevant. Deoni et al. have described a technique, known as the variable nutation method, which was first introduced by Christensen et al. in 1974. This method is capable of determining \(T_1\) as accurately as IR and SR, but in a shorter timeframe. This requires that a spoiled steady state first be established prior to the collection of the spoiled gradient-recalled echo images (SPGR) for a series of flip angles \((\alpha)\), while holding repetition time (TR) constant. For each voxel of the MRI, a plot of Signal Amplitude \((S_i)\) per sin \(\alpha\) versus \(S_i\) per tan \(\alpha\) is generated, which is described by the linear equation \((Y = mX + b)\) given by [Eq.6].

\[
y = mX + b
\]

[Eq.6]
An example of this linear plot is presented in Figure 13. $T_1$ is extracted from the slope of the line ($m$) using [Eq.7]^{69}.

\[
\frac{S_{SPGR}}{\sin \alpha} = g \frac{TR_{T1}}{\tan \alpha} \left( \frac{S_{SPGR}}{\sin \alpha} \right) - M_0 \left( 1 - g \frac{TR_{T1}}{\tan \alpha} \right)
\]  

[Eq.6]

Figure 13: Example of the plot described by [Eq.6], where $\alpha_H$ refers to the higher flip angle and $\alpha_L$ refers to the lower flip angle data points. This plot demonstrates the error in $T_1$ that results when the slope is overestimated (dashed line) or underestimated (dotted line)\textsuperscript{67}.

\[T_1 = -\frac{TR_{T1}}{m_L m_H}\]  

[Eq.7]

The number of flip angles selected impacts the plot generated. Selecting the appropriate flip angles to ensure $T_1$ accuracy is dependent on the TR/$T_1$ ratio\textsuperscript{72}. When a range of $T_1$ values is anticipated, the DESPOT1 algorithm and flip angle determination methods reported by Deoni et al.\textsuperscript{72} are well-suited because the choice of flip angles is dependent on the $T_1$ value of interest.

Previous methods used to determine the ideal flip angles for select $T_1$ meant plotting the dynamic range of the regression line as a function of signal fraction ($f$) and searching for the maximum. This time-consuming process was simplified by Deoni et al.\textsuperscript{69} when they showed that the maximum occurs when [Eq.8] is satisfied.

\[f = \frac{S_{\alpha_H}}{S_{\alpha_E}} = \frac{S_{\alpha_L}}{S_{\alpha_E}}\]  

[Eq.8]

In recognizing this relationship it was determined that the maximum occurred when the signal fraction equaled 0.71. [Eq.9] is the equation used to solve for optimum flip angles for the $T_1$ values of interest.
DESPOT1 is the name coined by Deoni et al.\(^69\) to describe their optimized variable nutation method that determines \(T_1\) using just two flip angles.

Rapid determination of \(T_1\) on a voxel-by-voxel basis is useful when locating areas of specific \(T_1\), which may be the result of a CA. Using the DESPOT1 algorithm, \(T_1\) maps can be generated from the SPGR MR images at the ideal flip angles to localize the treatment. Some inaccuracies do exist with these methods, the most prominent of which is inaccurate knowledge of the flip angle\(^69\). Flip angle errors either arise due to \(B_1\) field inhomogeneities or due to slice profile errors\(^69\). Slice profile errors are reduced towards the centre of the 3D slab due to the 3D nature of the sequence. However errors resulting from patient-induced \(B_1\) inhomogeneities are much more challenging to correct and appear to be more prevalent at the higher field strengths (> 1.5T)\(^69\). To accommodate for this error, \(B_1\) mapping correction can be done as a calibration step and the flip angle correction can be applied to the \(T_1\) maps. Random noise in the signal can also impact the calculated \(T_1\) value causing the slope to be over or underestimated on the linear plot of \(S_i/\sin \alpha\) versus \(S_i/\tan \alpha\) (see Figure 13). If slope equals one, then \(T_1\) is undefined, based on [Eq.7], so noise impacts the determination of \(T_1\). Introducing a third flip angle into the equation can alleviate some of the error associated with noise bias. Emphasizing the high SNR points through weighted least-squares analysis may also help\(^67\).

1.6 Study Rationale

Percutaneous intervention remains an important, yet underutilized technique for the treatment of chronic total occlusions. Although successful in some instances, its principle limitation is due to the inability to cross the lesion with a guidewire. Bacterial collagenase, being the initial mediator of interstitial collagen degradation, has proven itself effective as a softening agent to the dense collagen structure of the proximal fibrous cap\(^5\). However, the most effective vehicle for its delivery and its kinetics following local administration at the proximal end of the CTO remain unknown. There exists the need for a controlled-release macromolecular delivery system that unleashes collagenase at the proximal end of the CTO without inhibiting its enzymatic activity. A tracking system that monitors the collagenase movement following its administration is necessary to detect its post-release behaviour and its residency within the CTO.
1.7 Research Objectives

The objectives of this thesis are to characterize the biologic behaviour of a PLGA microsphere delivery system that has been specifically designed for application in CTOs. The PLGA microspheres have been synthesized to deliver two separate therapeutic agents that act on specific targets in the CTO: collagen in the proximal fibrous cap and the microvascular network through the lesion.
Chapter 2: Release kinetics of PLGA Microsphere-Encapsulated Proteins and Biological Impact on Cultured Endothelial Cells

2.1 Study 1: PLGA Microsphere Release Kinetics

2.1.1 Study Rationale and Objectives

PLGA microspheres degrade when exposed to aqueous solution through hydrolysis reactions at the ester linkages. This results in the formation of shorter, acidic chains. As the PLGA degrades, encapsulated protein is slowly released into the surroundings. As the literature reports, this release is characterized by an initial burst that may or may not be followed by subsequent bursts prior to reaching maximum release. The objective of the microsphere decay study is to determine the release profile for PLGA microspheres encapsulating BSA and VEGF, and to quantify the amount of protein being released.

2.1.2 Study Hypothesis

We hypothesized that protein release would be characterized by an initial burst in the first day of sample collection, followed by subsequent, steady release. It was also hypothesized that because BSA is larger than VEGF and encapsulated at a higher concentration, it would release over a longer period of time.

2.1.3 Introduction

The release kinetics of PLGA microspheres have been widely documented for BSA (m.w. 66 kDa), but to our knowledge, encapsulation of either VEGF (m.w. 45 kDa) or Collagenase (m.w. 107 kDa) has not been shown. In order to evaluate these release profiles, two PLGA microsphere formulations were prepared for the BSA and VEGF-loaded microspheres according to Table 3.

2.1.4 Methods

2.1.4.1 Microsphere Preparation

A water-in-oil-in-water (W/O/W) double emulsion technique was used to fabricate biodegradable microspheres. Initially, the desired protein combination (Table 3) was vortexed at a constant speed in 5% 50:50 Poly(DL-lactide-co-glycolide) acid (Lactel Absorbable Polymers, Pelham, AL) solution in chloroform. This emulsion was added to a solution of 1% polyvinyl
acetate (PVA) (Sigma-Aldrich Canada, Oakville, ON) in 7.5% dextrose (Sigma-Aldrich Canada, Oakville, ON) and vortexed at constant speed before finally mixing in 0.3% PVA solution in 7.5% dextrose to further stabilize the microspheres. The final double emulsion was mixed at room temperature for two hours to ensure chloroform evaporation. Following evaporation, the emulsion was centrifuged at 2000 RPM for 20 minutes at room temperature to collect the microspheres into a single pellet. The microsphere precipitate was frozen at -80°C with 5 ml of supernatant for one hour before lyophilizing over night. Microspheres were separated into 16 mg aliquots and stored at -20°C until further use. Scanning electron microscopy confirmed the mean microsphere diameter as approximately 30 µm (Figure 14).

Table 3: Proteins dissolved in 200 µl double distilled water and encapsulated in PLGA microspheres.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass of Protein</th>
<th>Mass of BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>-</td>
<td>10 mg</td>
</tr>
<tr>
<td>VEGF</td>
<td>5 µg</td>
<td>10 mg</td>
</tr>
<tr>
<td>Collagenase</td>
<td>1 mg</td>
<td>5 mg</td>
</tr>
<tr>
<td>Collagenase-Gd</td>
<td>1 mg</td>
<td>5 mg</td>
</tr>
</tbody>
</table>

Figure 14: SEM of PLGA microspheres encapsulating VEGF at 300x magnification. The average diameter of microspheres is approximately 30µm.
2.1.4.2 Quantification of VEGF Release

The *in vitro* VEGF release profile from PLGA microspheres was determined using the Quantikine Mouse VEGF Immunoassay Kit (R&D Systems, Burlington, ON). Briefly, 18 mg of PLGA microspheres containing roughly 1 µg VEGF were reconstituted in 2 ml of phosphate buffer (0.1 M, pH 7.4) in 15 ml Falcon tubes. The tubes were incubated at 37°C and agitated at 225 RPM. At pre-determined time points the samples were centrifuged at 2000 RPM for 10 minutes at room temperature and the 2 ml supernatant collected and stored at -20°C until analysis. Microspheres were re-suspended in 2 ml of fresh buffer and agitated at 37°C until the subsequent time point. The cumulative amount of VEGF released from the microspheres over 167 hours was measured following the Mouse VEGF Immunoassay kit protocol.

2.1.4.3 Quantification of BSA Release

The release profile for bovine serum albumin (BSA) incorporated into PLGA microspheres followed a similar sampling protocol however protein concentration was determined using the Bradford dye-binding technique and the Bio-Rad Protein Assay (Bio-Rad Laboratories, Mississauga, ON). Samples of known protein concentration were used to plot a standard optical density (OD) versus BSA concentration curve. Briefly, 100 µl of samples and standards were mixed with 25 µl of Bio-Rad Protein Assay Buffer and allowed to equilibrate five to ten minutes prior to analysis. 100 µl of the prepared sample was read at 595 nm using a spectrophotometer (Fisher Scientific Ultrospec 3100 pro).

2.1.5 Results: PLGA Microsphere Release Profiles

2.1.5.1 BSA Release

Due to the large molecular size of BSA and its insolubility in polymers, the BSA release rate from PLGA microspheres is thought to be initially dependent on its diffusion through the polymer pores that are formed by swelling due to water uptake\(^2\). Subsequent release becomes dependent on the rate of polymer degradation\(^7\). The formulations used for the other protein microspheres often include BSA in the protein mix because it increases the encapsulated protein mass and therefore, increases the effective delivery rates for the smaller proteins of interest\(^7\). The experimentally-determined release profiles for five individual trials made from two separate batches of microspheres are presented in Figure 15.
The theoretical mass of BSA incorporated into the spheres was approximately 2 mg for each trial. This means that 10-20% of the total incorporated protein is being released.

The BSA release profiles show a biphasic release pattern, characterized by an initial burst release over the first hour followed by subsequent controlled release that reaches a plateau by day three. The pH of the supernatant at the time of collection was neutral, but this likely did not detect the micro-pH changes occurring at the polymer level, changes that could impact protein stability.

2.1.5.2 VEGF Release

VEGF-encapsulated microspheres also contained BSA, in a much higher concentration (5 x 10^{-4} VEGF to one BSA) to ensure that the VEGF was able to release from the microspheres in a clinically-relevant timeframe. The experimentally measured release profile for five trials of VEGF microspheres is presented in Figure 16.
Figure 16: Release profiles for five trials of VEGF encapsulated PLGA microspheres, from two separate batches. The approximate mass of VEGF per trial was 1.0 µg.

The five separate trials of VEGF release show a similar triphasic release pattern that was characterized by an initial burst in the first hour, followed by sustained, diffusion-controlled release that begins to plateau near day four. The difference across trials is the total mass of VEGF being released. The mass of VEGF per sample of microspheres is approximately 1.0 µg, meaning that the VEGF being released is approximately 1-2.5% of the total mass incorporated. The mass of VEGF present in the discarded supernatant following the washing and collection of the microspheres was found to be approximately 160 ng, which accounts for another 16% of the missing protein.

2.1.6 Discussion: Microsphere Release Profiles

Findings of the release quantification study for PLGA microspheres confirm that BSA is released over a period of eight hours in a biphasic pattern, whereas VEGF is released over a period of 24-48 hours following the triphasic release characteristic to PLGA degradation. Both profiles released only a fraction of the theoretically-encapsulated protein.

BSA and VEGF-encapsulated microspheres were the proteins selected for release characterization. BSA is co-encapsulated with VEGF to ensure its release is over a clinically-relevant period of time due to the small encapsulated dose. Total mass of released VEGF is crucial when evaluating its impact as an angiogenic agent in the treatment of CTOs.

PLGA microsphere release profiles tend to exhibit triphasic patterns\textsuperscript{75}. They are characterized by an initial burst release, followed by a lag phase and conclude with a secondary
The BSA release profile presented in Figure 15 is characteristic of a biphasic release, where there is essentially no lag phase. Conversely, the VEGF release follows the triphasic pattern. It is thought that the initial burst release is likely due to protein that is attached to the inner and outer polymer surfaces due to poor encapsulation. In our BSA and VEGF microspheres, the burst release phenomenon describes the release occurring in the first hour of sample collection. For the BSA microspheres, the protein collected in the first two hours accounts for 56-69% of the total mass released. This is much higher than reported values which tend to be in the range of 30%. Similarly, the mass of VEGF collected in the first two hours accounts for 18-31% of the total mass released. The mass of VEGF not encapsulated in the microspheres (i.e. remaining in the discarded supernatant) was 160 ng, which corresponds to 16% of the unaccounted protein.

Sample collection for the release profiles was done over a period of seven or eight days, therefore the protein released subsequent to the burst is likely a result of a different release mechanism. It has been proposed that this “steady release” is characterized by two phenomena: diffusion and degradation. Likely both of these mechanisms of release are at work because the two proteins of different size exhibit different release patterns. For the BSA spheres, the second phase of release occurs over a period of 12 hours and for VEGF, 48 hours. It is thought that the steady release phase is governed by diffusion of the protein that is located near the surface of the microspheres through water filled pores. Alternatively, the final linear release can likely be attributed to the protein released from the core of the microspheres via polymer degradation due to hydrolysis.

Variability is evident across the five trials of BSA and VEGF microspheres. The BSA microspheres were made from two separate batches. The highest and lowest protein-releasing curves were from the same batch and the three curves with medium release were from another batch. The release difference between the five trials is approximately 200 µg, which may be due to variability in the mass of microspheres actually included in the sample. During manufacturing, the pellet of microspheres is separated from the lyophilized buffer manually and divided up based on weight. Masses were approximately 16 mg but some variation could exist if some of the lyophilized buffer was included in the mass. In this case the recorded mass is not a direct reflection of the mass of microspheres releasing. This alone would not have a significant impact on the release profile of the BSA spheres, but could in conjunction with other factors.

The three VEGF trials showing lower total release all came from the same batch, whereas the two trials showing greater release were from a separate one. Although the batches were
fabricated following an identical protocol, the release profiles, while still exhibiting the same patterns, release different quantities of protein. This type of variability is not surprising when working with biological systems, however the impact on the total protein released is substantial (range of VEGF is 13 ng versus 22 ng). With differences in released protein of this magnitude, the resulting endothelial cell proliferation is impacted. The profiles begin to differ after the first 24 hours of release during the linear phase where the release is due to diffusion. This phase is impacted by the size of the microspheres. Between the different days of manufacturing, the size of the microspheres may have changed due to variability in the vortex speed and ambient conditions in the laboratory. As microsphere size increases, the diffusion distance increases, resulting in greater chances of polymer-protein interaction prior to release. Further to this point, size can also impact protein distribution within the spheres, which can slow the release if protein concentrates in the centre of the polymer matrix.

The visual trends of the release profiles are similar to other published studies, however it is difficult to compare whether the mass of released protein is similar because it is usually reported as a percent cumulative release, rather than as a mass. One study, which employed 65:35 (polylactic acid:glycolic acid) microspheres released 70% of the theoretically encapsulated BSA and a second study that used 75:25 and 65:35 PLG microspheres released 70-80% of the encapsulated protein. Comparison to these studies confirms the low overall protein release we are seeing in our studies.

The total protein released in the profiles accounts for 10-20% of the BSA and 1-2.5% of the VEGF encapsulated. Some of this loss of protein is likely due to denaturing at some point throughout the preparation process. Van de Weert and colleagues determined potential causes of protein instability in PLGA microspheres by examining different stages of the preparation process. They determined that protein loading and microsphere formation are both vulnerable stages for protein degradation. We employ the W/O/W double emulsion method of microsphere formation, which exposes released protein to two water-oil interfaces prior to reaching the surroundings. These interfaces can be destabilizers for proteins because they have high interfacial tension which can lead to protein adsorption at the surface and ultimately to less protein available for release. In our microsphere protocol the first emulsion is created by vortexing the dissolved protein with the organic polymer solvent (chloroform). Although it has been shown that the use of a vortex can cause sufficient shear forces to denature protein, it is its interaction with chloroform (a protein extractor) that results in greater protein instability. Further to that, our microsphere protocol follows the solvent evaporation method, whereby
chloroform is evaporated from the double emulsion causing the PLGA to precipitate as spherical particles encapsulating the protein. Little is actually known about the denaturing that can occur during this step, however it is likely that interfacial stresses are felt by some of the proteins located at the surface of the microparticles\textsuperscript{27}.

Outside of microsphere preparation, the most potent interactions influencing the amount of protein released from the microspheres is the PLGA-protein interaction\textsuperscript{27}. Both hydrophobic and electrostatic mechanisms result in the adsorption of protein to the polymer via the carboxyl end groups on the PLGA and the protein surface amino acids. Covalent or non-covalent aggregation, hydrolysis and/or non-specific adsorption to the PLGA surface are all examples of this interaction\textsuperscript{27}. Another aspect affecting protein stability is the acidic microenvironment created as PLGA degrades into its acidic monomers. Protein has very particular conditions of activity, namely pH and temperature. As such, a significant drop in pH can negatively impact the activity of the protein being released from the spheres. The pH of the supernatant was neutral, however the acidity at the polymer surface was not detectable using these methods.

The sum of potential factors leading to protein instability during microsphere formation makes this a likely explanation for the low protein yields detected in the release profiles. With the additional likelihood that some protein also adsorbs to the polymer surface and still more is denatured by the acidity and the interfacial stresses. The high percentage of protein released during the burst release for all microsphere trials suggests that there is ample protein that is not encapsulated that remains on the surfaces of the spheres. These interactions are characteristic of \textit{in vitro} behaviour, but direct extrapolation to \textit{in vivo} conditions is not straightforward\textsuperscript{27} because biological fluids may compete for interaction with the protein.

Comparison of BSA (m.w. 66 kDa) release characteristics with those of VEGF (m.w. 45 kDa) suggest that microsphere release of larger proteins does not require a longer timeframe. In fact, the BSA released over a shorter period of time than the VEGF. A direct comparison is not possible because the mass of encapsulated protein is vastly different between the trials (on the order of $10^4$). Published work by Chen et al. confirm that this impacts release because quite often BSA is co-encapsulated with smaller masses of the desired protein simply to increase the amount of protein present in the spheres to deliver the protein of interest in a reasonable period of time\textsuperscript{74}.

Although protein release was rather low for our microsphere trials, there are methods that can be applied to the formulation to target these problems. Altering the molecular weight of the polymer can influence the amount of protein released. Zolnik et al. report that increasing the
molecular weight of the polymer can decrease the amount of protein released during the initial burst and prolong the release during the lag phase, due to the increased degradation time\textsuperscript{75}. Buket Basmanav et al. successfully applied this theory by using 4% polymer microspheres for drugs requiring early stage release and 10% polymer microspheres for the drugs requiring long term release\textsuperscript{26}.

Similarly, altering preparation temperature of the first emulsion can also impact the release patterns, bearing in mind that protein denatures at high temperatures. Yang et al. determined that for microsphere-encapsulated BSA, the lowest initial burst occurred at the lower and higher preparation temperatures (5°C and 42°C), whereas medium temperatures (33°C) yielded the highest burst release\textsuperscript{24}. In our case, both emulsions were performed at room temperature, which may explain the relatively large initial burst we see in the release profiles. Additionally, it has been demonstrated that microsphere size has a significant effect on release rates\textsuperscript{28}. The mean diameter of our microspheres was approximately 30 µm. Berchane et al. report that initial burst release decreases with increasing microsphere diameter, which corresponds to Fick’s Laws of diffusion\textsuperscript{28}. Berchane et al. report that microspheres in the range of 30 µm tend to exhibit sigmoidal release patterns, which are characterized by a large initial burst, followed by slower release and then rapid release once more before leveling off\textsuperscript{28}. Our microspheres did not show this second rapid release pattern, which is likely due to the assortment of microsphere sizes in our samples.

Other groups have reported the co-encapsulation of buffer salts or protein stabilizers to mitigate the effects of an acidic pH\textsuperscript{27,32}. The water-soluble inorganic basic salts that have been proposed include magnesium hydroxide, calcium carbonate or sodium bicarbonate\textsuperscript{27}. In most cases the weak bases increase the stability of the protein \textit{in vitro}, however if a stronger base such as calcium hydroxide is employed the protein may form aggregates\textsuperscript{27}. In the case of BSA, the weak salts would have no noticeable effect on the protein, however collagenase is less forgiving to changes in pH, so incorporation of basic salts must be monitored.

Changing any one of these parameters will not have a single effect on the release patterns of the PLGA microspheres. It is a fine balance across all of these factors that provides optimal release for the system in question. Depending on the protein, initial burst release is required and for others it can be toxic. We seek to develop a formulation that provides sustained protein release rather than a large initial burst. Increasing the molecular weight of the polymer and using a sieve to ensure that microsphere diameter is in the range of 20-40 µm should ensure more gradual release over a longer period of time.
2.2 Study 2: Biological Impact of Microsphere Degradation

2.2.1 Study Rationale and Objectives

In addition to the protein activity at the time of release, the impact of microsphere degradation products on the endothelial cell line was monitored. The objective of this study was to determine whether the products released during or the conditions resulting from microsphere degradation had a negative impact on cell growth.

2.2.2 Study Hypotheses

The hypothesis for this experiment was that cell growth would be uninhibited by the PLGA breakdown given that PLGA is currently employed in many biological systems.

2.2.3 Introduction

PLGA breakdown occurs as a result of hydrolysis reactions at the ester linkages that break the polymer into its acidic monomers. These acidic chains can cause the pH at the polymer surface to drop to as low as two. Endothelium has optimum growth at physiological pH, meaning that exposure to these conditions may negatively impact cell growth.

2.2.4 Methods

Similar to the protein biological activity study, MS1 endothelial cells were cultured to passage and plated in 96-well tissue culture plates at a density of 3.0 x 10³ cells/well. Cells were serum-starved and plated in 100µl, 0.25% FBS supplemented DMEM and allowed to grow for 24 hours at 37°C, 5% CO₂. Cells were then treated with 100µl of the 0.1M phosphate buffer (pH 7.4) that was collected as supernatant from the BSA microsphere release trial. This treatment was supplemented with FBS to maintain serum concentration at 0.25%. Cell growth was measured by absorbance at 450 nm using the Powerwave X340-1 plate reader (Bio-Tek Instruments Inc., Winooski, VT) after 1 hr incubation with WST-1 proliferation reagent (Roche Diagnostics, Laval, QC). Higher absorbance measurements correlate to greater cell count.

2.2.5 Results: Impact of PLGA Degradation Products on Endothelial Cell Growth

Endothelial cells that were serum-starved for maximum treatment impact were treated with the release products of BSA-encapsulated PLGA microspheres to determine its impact on
cell growth. A WST-1 proliferation assay was used to detect cell growth, which directly correlates to absorbance at 450 nm. Figure 17 shows cell proliferation for the BSA microsphere treatment compared to the control, plated in 0.25% DMEM.

![Figure 17](image)

**Figure 17:** Cell proliferation following treatment with byproducts of PLGA degradation. Error bars show standard deviation (n=3).

The insignificance of these two treatments was confirmed using a student’s t-test. This confirms that PLGA degradation had insignificant impact on endothelial cell growth. Measurement of pH of the cell treatment 24 hours post-administration was found to be neutral.

### 2.2.6 Discussion: Polymer Degradation and Cell Growth

Findings of this study confirm that PLGA degradation does not impact endothelial cell growth patterns compared with a serum-starved control treatment.

One of the benefits of using PLGA as a vehicle for macromolecular delivery is that it is already FDA-approved for clinical use as a resorbable suture material. As it is hydrolyzed the polymer is broken into its monomer constituents, which are released from the body through the Kreb’s cycle. This suggests that in the form of a microsphere it would also have negligible impact on cell growth. Figure 17 displays the results of the cell proliferation assay that evaluated the impact of PLGA degradation of BSA microspheres on endothelial cell growth. Although the biocompatibility of PLGA is still somewhat controversial\(^36\), the evidence from this plot confirms...
that treatment of endothelial cells with the release products of BSA microspheres had no effect on cell growth when compared with the control treatment (0.25% DMEM).

2.3 Study 3: Biological Activity of Protein Following Release from Microspheres

2.3.1 Study Rationale and Objectives

The formation of acidic side chains during the breakdown of PLGA can cause the pH at the surface of the microspheres to drop to as low as two\textsuperscript{77}. Due to the specific environment that proteins require, they can become denatured in these acidic conditions. The objective of this study is to determine whether or not the released protein, in this case VEGF, is still active upon its release from microspheres by determining its effect on endothelial cell growth, compared to direct treatment with VEGF and a control receiving no treatment.

2.3.2 Study Hypotheses

It is hypothesized that VEGF released from the PLGA microspheres will have similar effect on cell growth as the same mass of VEGF delivered independent of microspheres. Therefore, it is hypothesized that VEGF activity will remain the same and that the acidic environment surrounding PLGA degradation will not impact the bioactivity of the VEGF being released.

2.3.3 Introduction

PLGA microsphere delivery systems appear quite promising for controlled delivery of macromolecular therapies, but the processing conditions and exposure to polymer and water-oil interfaces remain a problem for protein stability. Although some protein is denatured throughout microsphere preparation, the bioactivity of the encapsulated protein must be confirmed for our double emulsion protocol.

2.3.4 Methods

The bioactivity of the VEGF incorporated into, and released from, the biodegradable microspheres was determined by testing its ability to stimulate growth in a cultured mouse endothelial cell line (MS1). Prior to use, MS1 cells were cultured to passage in Dulbecco’s Modified Eagle’s Medium (DMEM) (Thermo Scientific, HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Ottawa, ON) and 1% penicillin-
streptomycin (Wisent, St. Bruno, QC). At the time of testing, cells were plated at a density of 3.0 x 10^3 cells per well on 96-well flat-bottom tissue culture plates (Sarstedt, Newton, NC) and serum-starved in 100 µl 0.25% Fetal Bovine Serum (FBS) in DMEM. Cells were allowed to grow for 24 hours at 37°C, 5% CO₂, and then were treated with different concentrations of VEGF in 0.25% FBS DMEM. This allowed for the generation of a growth response curve, which was used to evaluate cell growth resulting from treatment with VEGF released from biodegradable microspheres. Cell growth was measured using a WST-1 assay that measures colorimetrically the cleavage of a tetrazolium salt to formazan by metabolically active cells. Therefore, absorbance corresponds directly to cell growth. Absorbance was measured at 450 nm using the Powerwave X340-1 plate reader (Bio-Tek Instruments Inc., Winooski, VT) after 1 hr incubation with WST-1 proliferation reagent (Roche Diagnostics, Laval, QC). Higher absorbance measurements correlate to greater cell count.

2.3.5 Results: Biological Activity of Microsphere-Released Protein

Cell proliferation of MS1 endothelial cells was measured following treatment with varying quantities of recombinant mouse (rm) VEGF164 from the manufacturer’s vial and rmVEGF164 released from PLGA microspheres. The 10% FBS-supplemented serum is presented for reference. The results of this experiment are presented in Figure 18, where cell proliferation corresponds directly to absorbance at 450 nm.
Figure 18: Endothelial cell proliferation (correlated directly to absorbance) as a function of treatment. Error bars show standard deviation (n=3, except t, n=2). * corresponds to statistically significant growth as compared to the 0% control, p<0.05. Note: masses refer to VEGF treatment and percentages refer to % FBS in media.

Endothelial cell growth patterns presented in Figure 18 demonstrate no significant difference across VEGF treatments as compared with the 0.25% DMEM control. Significant differences were confirmed for the 0.3 and 0.5 ng VEGF treatments as well as the microsphere-released VEGF treatment (mass 0.1 ng) when compared with the 0% DMEM control.

2.3.6 Discussion: Bioactivity of Released Protein

The findings of this study confirm that VEGF released from PLGA microspheres is capable of inducing endothelial cell growth in significant quantities when compared with a serum-starved control.

Due to the opportunities for protein denaturing that are involved in microsphere formation and protein release, we have tested the bioactivity of VEGF following its release from microspheres. Figure 18 shows endothelial cell growth following treatment with different concentrations of VEGF (0.1 – 1.2 ng) directly from the manufacturer’s vial and also with VEGF released from PLGA microspheres (0.1 ng). The endothelial cell growth patterns are not significantly different across VEGF treatments, likely because the two high-affinity and the few low-affinity receptors are already exhausted at the lowest dose. As such, maximum cell division is already occurring through the Raf-Mek-Erk signal transduction pathway. Statistical significance was measured using a Dunnett t-test, which is used when comparing experimental means to one control group. The Dunnett t-test showed no significant difference for the treatments against the 0.25% DMEM control, but did confirm significance in the growth patterns of the 0.3 and 0.5 ng VEGF treatments and the VEGF microsphere treatments compared with the 0% DMEM control. The means of the 0% and 0.25% DMEM controls are similar, but the true control (0.25% DMEM) had too few observations to demonstrate the effect. Increasing the sample size of both controls and samples would likely result in significant differences across the treatments due to more refined results.

The purpose of VEGF treatment in our case is to maximize microvessel growth through the CTO to facilitate guidewire crossing. Therefore, sustained release of VEGF in masses that are sufficient to promote angiogenesis is the goal of the protein bioactivity study. Silva and Mooney determined that gradually decreasing the dose of VEGF actually leads to greater
endothelial cell growth\textsuperscript{78}, an ideal condition when examining our release profile and the decrease in VEGF released following the initial burst. The mass of VEGF released from the microspheres at the 24 hr time point is in the vicinity of 13 ng; a mass that has been shown to induce endothelial cell growth\textsuperscript{78}. According to work done by Silva and Mooney, doses of VEGF in the range of 4-16 ng showed an increase in endothelial cell growth of 23\%. With a delivery of 24 ng, cell growth increased significantly to 42\%\textsuperscript{78}. The experimental conditions such as cell density during plating were different for this study, which is likely why their cells could withstand a greater dose of VEGF.

Although VEGF masses are low at each time point of the release profile, the combination of the initial burst and the accumulation of VEGF from the second phases of release provide a total VEGF mass of approximately 13 ng. The in vitro studies confirm that this released VEGF is bioactive following microsphere release and capable of inducing endothelial cell proliferation when compared with the 0\% DMEM control.

2.4 Study Contributions

I performed all endothelial cell culture, proliferation assays and microsphere production, for the in vitro studies. Analysis was also performed by me, with aid in the statistical significance of the studies by John Sparkes of Biostat Consulting. The scanning electron microscopy images of the microspheres were performed by Chuen Lo of Dr. Michael Sefton’s laboratory at the University of Toronto.
Chapter 3: Investigation of Collagenase Residency in the CTO

3.1 Study Rationale and Objectives

Local administration of bacterial collagenase has shown softening effects that facilitate guidewire crossing for the CTO, however the kinetics of the enzyme are unknown following administration. The objective of this study is to determine the migration pattern of collagenase when delivered intra-arterially with and without microspheres. The second objective is to determine the state of the microspheres following protein release in vivo.

3.2 Study Hypothesis

The hypothesis of this study was that although some protein may become denatured during the gadolinium conjugation to collagenase, sufficient labeling should occur such that the $T_1$ shortening effects of gadolinium are detectable using $T_1$ mapping. It is thought that the treatment, when delivered locally to the proximal end of the CTO will primarily be localized in the proximal end of the occlusion in the histological analysis and $T_1$ map images. By the 24 hour time point following the treatment, it is thought that the lower $T_1$ effects will be found external to the CTO due to the capillary network branching from the femoral artery to the surrounding tissue.

The microsphere drug delivery system is meant to control the protein release over the first 24-48 hours therefore; we hypothesize that although the microspheres will be found in the proximal end of the CTO, the labeled collagenase will likely have a smaller radius of effect because of its delayed release from the microspheres.

3.3 Introduction

The dense collagen structure of the proximal fibrous cap of CTOs acts as a barrier to guidewire crossing during PCI. Previous work has shown that local delivery of bacterial collagenase to the proximal end of a CTO softens the collagen-dense cap sufficiently to facilitate guidewire crossing during PCI. Since protein therapies are becoming increasingly available for treatment of illnesses, delivery methods must be optimized to accommodate their short in vivo half lives. Intravascular delivery of protein therapies remains the access point of choice, however, a biocompatible vehicle to ensure the collagenase remains at the site of delivery actively cleaving collagen for a period of 24 hours remains to be studied. The purpose of this in vivo study is to evaluate the effectiveness of a PLGA microsphere delivery system that steadily
releases collagenase to the PFC of the CTO. Some initial *in vitro* work to establish the behaviour of the contrast agent is presented.

### 3.4 Methods

#### 3.4.1 CTO Model Creation

Approval for all procedures was obtained by the Animal Care Committee at Sunnybrook Health Sciences Centre. The Chronic Total Occlusion (CTO) model was created in the femoral arteries of New Zealand White rabbits weighing 3.0 to 3.5 kg (Charles River Laboratories Canada, Saint Constant, QC). Rabbits were sedated with an intramuscular injection of Ketamine (50 mg/kg) (Bioniche, Belleville, ON), Xylazine (10 mg/kg) (Bayer, Toronto, ON) and maintained at surgical plane with 2-3% Isoflurane and Oxygen (2 L/min). Bovine thrombin (1000 U) (Millipore, Kankakee, IL) was reconstituted in 1.0 ml of 0.9% sodium chloride and a volume of 0.1 ml of the diluted thrombin was injected into a 2 cm femoral artery segment that was isolated with tourniquets. The proximal tourniquet was removed immediately following injection, whereas the distal ligature was maintained for 60 min. This technique prevents the thrombin from washing away and allows interaction between the blood and thrombin to induce intra-arterial coagulation. The muscle and skin were sutured with absorbable 2-0 vicryl. A pre-emptive dose of Duplocillin (2 ml) (Intervet Canada Ltd., Kirkland, QC) was given intramuscularly to prevent infection at the incision site. Upon recovery, rabbits received a subcutaneous injection of Buprenorphine Hydrochloride (0.3 mg/kg for three days) (Schering-Plough, Hertfordshire, UK). Three to four weeks following the CTO induction surgery, rabbits were sedated for transcutaneous doppler ultrasound to confirm the presence of a CTO. The absence of blood flow and pulse to that region were markers of diagnosis.

#### 3.4.2 Collagenase Conjugation to Gadolinium

The two-day conjugation (Figure 19) began with the DOTA-gadolinium chelation. DOTA (M-140, Macrocyclics, Dallas, TX) was reacted with excess Gadolinium(III) chloride hexahydrate (Sigma-Aldrich Canada, Oakville, ON) in 300 µl of 5 mM sodium acetate buffer (pH 5.3). Mixed constantly at room temperature for 24 hours, this chelation formed a stable, cyclic complex. Following chelation, 3.2 µl of *N*(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) (Sigma-Aldrich Canada, Oakville, ON), which is an ester linker, was
added to react with the carboxy groups of the DOTA molecule to generate ester linkages at the carboxylic side chains. In a separate vial, 8 mg of bacterial collagenase (Sigma-Aldrich Canada, Oakville, ON) was reacted with 3.1 mg of 4-(N Maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxy-succinimide ester sodium salt (Sigma-Aldrich Canada, Oakville, ON) for one hour at room temperature in 5 ml 0.1 M phosphate buffer (pH 7.4). The addition of the succinimide ester activated the primary amines of the collagenase, likely the lysine side chains located on the surface of the enzyme, producing a new maleimide protein. Maleimide functional groups readily react with cysteamine (Sigma-Aldrich Canada, Oakville, ON), to produce a thiol-modified product. The thiol-modified collagenase was then added to the ester-enhanced DOTA-Gd complex with additional phosphate buffer to maintain a neutral pH. The reaction was continued for two hours at room temperature before passing the final product through 6 kDa size exclusion chromatography columns (Bio-Rad Laboratories, Hercules, CA) and then performing centrifugal filtration at 30 kDa (Millipore, Billerica, MA) to ensure free Gadolinium ions and unreacted DOTA-Gd complexes were removed from the mixture. Samples were divided into seven vials of 200 µl each and then lyophilized overnight and stored in -20°C until ready for use.

**Reaction 1:**

![Chemical structure of Reagent 1](image1)

**Reaction 2:**

![Chemical structure of Reagent 2](image2)

**Product A**
Figure 19: Reaction mechanism describing collagenase conjugation to gadolinium via the DOTA chelating molecule.

3.4.3 Collagenase-Gd Relaxation Testing

Prior to the *in vivo* studies, the conjugated collagenase was measured at 10, 100 and 1000 μM concentrations to determine its relaxation properties on a 1.5 T MR scanner (GE Signa HDx,
The samples were contained in 1.5 ml microcentrifuge tubes (Eppendorf Canada, Mississauga, ON) and held in a 5% w/w agar gel. Briefly, water was heated to ~80°C in a plastic specimen container and 12.5 g of agar (Sigma-Aldrich Canada, Oakville, ON) was added to make a 5% w/w gel. A 1.5 mL microcentrifuge tube was pressed into the center of the agar with the top of the tube flush with the agar surface. The agar was allowed to solidify in this mold overnight to form wells for sample testing. The samples were placed in these wells and scanned using the following parameters: a 6” ø receive-only surface coil was placed under the container, and spin echo sequences with TE = 14 ms, TR = 17, 25, 50, 100, 200, 400, and 800 ms, α = 90°, N_freq = 128, N_phase = 128, FOV = 8 × 8 cm and slice thickness = 2 mm.

The second in vitro experiment done was to evaluate the behaviour of the collagenase-Gd when released from PLGA microspheres. The same MR hardware and imaging sequence mentioned previously was used. Briefly, 16 mg of microspheres containing Collagenase-Gd (approximate concentration of Collagenase-Gd in microspheres was 3 µM) were reconstituted with 300 µl of double distilled water and injected into an empty agar well mentioned previously. The sample was imaged 22, 80 and 200 minutes following introduction into the agar and correction for receiver sensitivity falloff was done using ImageJ 1.38x (U.S. National Institutes of Health, Bethesda, MD).

### 3.4.4 Intravascular Intervention

Six weeks following the CTO induction, rabbit femoral artery CTOs were treated with one of three treatments: sterile water (control); collagenase bound to gadolinium (Gd) or collagenase bound to Gd and encapsulated in PLGA microspheres. Rabbits were prepared for surgery and maintained under general anesthesia as previously described in Section 3.4.1 CTO Model Creation. Standard angioplasty was performed in order to access the proximal end of the CTO. Briefly, a neck incision was made to surgically expose the right common carotid artery (RCCA). Two ligatures, 2 cm apart, were sutured onto the RCCA to mitigate bleeding. A 5 Fr arterial sheath (Arrow International Inc., Reading, PA) was inserted into the RCCA. 1,000 units of heparin sodium (Pharmaceutical Partners of Canada Inc., Richmond Hill, ON) dissolved in 2 ml of saline was injected intra-arterially via the sheath introducer to prevent perioperative clot formation. Under fluoroscopic guidance, using the Cardiac-C-arm OEC 9800 Plug (GE OEC Medical Systems, Inc., Salt Lake City, UT) a 5 Fr guiding catheter (Medtronic, Minneapolis, MN) was then advanced over a 0.014” Hi-Torque Balance Guide wire (Abbott Vascular Devices, Aichi, Japan) to the Th12-L2 level. 3 ml of Omnïpaque™ (Iohexol, GE Healthcare,
Mississauga, ON) diluted 1:2 with normal saline was injected to create a “road map” of the arteries for the angioplasty procedure. Angiograms were performed three times throughout the procedure with the first being prior to guidewire placement; the second at balloon inflation and the last following balloon deflation to ensure integrity of the CTO. A 1.5-2.5 mm diameter, over-the-wire (OTW) angioplasty balloon catheter (Voyager OTW, Abbott Vascular, Abbott Park, IL) was advanced into the femoral artery to the proximal end of the CTO, leaving a distance of a few millimeters to the PFC. The balloon was inflated with 4-7 atmospheres of hydraulic pressure. The infusate was reconstituted to a total volume of 0.5 ml in sterile water and administered through the wire port of the balloon catheter after removal of the guidewire. Following the protein injection, the catheter was flushed with 0.3 ml of sterile water. The balloon inflation was maintained for 40 minutes, allowing the infusate access to the proximal CTO without competing blood interference. After balloon deflation, the angioplasty catheter was withdrawn and muscle and skin were sutured with absorbable 2-0 Vicryl. Rabbits received postoperative pain management as previously mentioned. The animal was euthanized following the 24 hour imaging time point using 0.2 ml intravenous injection of euthanyl pentobarbital (Bimeda-MTC, Cambridge, ON).

3.4.5 MRI Protocol

To assess the degree of collagenase residency within the CTO following treatment, the animal was transferred to the 3.0 T MR scanner (GE Discovery MR750, GE Healthcare, Waukesha, WI) and maintained under general anesthesia as previously described, with remote vitals monitoring. The animal was placed supine, feet first into the magnet with the CTO at the isocentre to minimize $B_1$ inhomogeneities. A custom, receive-only surface coil (3 × 5 cm) was placed over the occluded portion of the femoral artery with the proximal end of the coil placed at the rabbit’s centre line and fixed parallel to the artery.

Following localization, the first scan done was a 2D SPGR sequence (TE = 7 ms; TR = 18 ms; $\alpha$ = 30°; $N_{\text{freq}}$ = 256; $N_{\text{phase}}$ = 256; FOV = 6 × 6 cm; bandwidth = 31.25 kHz; slice thickness = 3 mm (no space between slices); number of slices = 28; averages = 2), which was used to visualize the location of the PFC of the CTO. Once located, the scan range prescription was adjusted to ensure the PFC became slice 14. This was done to mitigate the impact of 3D slice profile errors that can interfere with the DESPOT1 algorithm used for the $T_1$ mapping analysis. Subsequent
images were then obtained using 3D SPGR sequences (TE = 7 ms; TR = 18 ms; $\alpha = 5, 16, 36^\circ$; $N_{freq} = 256$; $N_{phase} = 256$; FOV = $6 \times 6$ cm; bandwidth = 31.25 kHz; slice thickness = 3 mm (no space between slices); number of slices = 28; averages = 1.5). Using similar parameters but with TR = 4 s, two 2D fast images were acquired ($\alpha = 120^\circ, 60^\circ$) to perform $B_1$ mapping to correct for flip angle errors, which are the main source of error when using the DESPOT1 algorithm of $T_1$ determination. After all scans for the day had been performed, $T_1$ weighted images were obtained before and 30 s following intravenous injection of an intravascular iron-based contrast agent using a dose of 0.05 ml/kg. All of these scans were performed one week prior to surgery to generate a baseline reading and again 1.5, 2 and 24 hours post collagenase administration. The animal was allowed to recover between the 2- and 24-hour time points. $T_1$ maps were generated using MATLAB (The Mathworks, Natick, MA) which was programmed using the DESPOT1 algorithm with $B_1$ mapping correction factors. DICOM-format MR images for each flip angle ($\alpha = 5, 16, 36^\circ$) were read and $T_1$ maps for each time point were generated.

3.4.6 In Vivo Study Analysis

Each imaging modality employed in the surgical process was used to piece together different aspects of the puzzle. Initially, fluoroscopic angiography files were qualitatively examined to determine the vascular network proximal to the CTO and locate any side branches that had formed post-CTO induction. Secondly, the 3D SPGR MRI ($\alpha = 45^\circ$) taken following contrast injection was used to localize the femoral artery cross section proximal to the CTO. The region of interest (ROI) placed on the MRI was translated directly to the $T_1$ map. However, localizing the vessel inside of the CTO proved to be technically challenging, therefore, the $T_1$-weighted Maximum Intensity Projection (MIP) image generated using the pre- and post-contrast (Clariscan) images was used to determine the planar movement of the CTO from the proximal to the distal cap to ensure movement of the artery was accounted for while moving the ROI in the CTO. $T_1$ maps were generated for MRI slices 12-17 (occlusion primarily began at slice 14) and analysis was performed on the proximal 6 mm of the occlusion.

3.4.7 Histology Preparation

The arterial specimens were dissected and fixed in 10% neutral buffered formalin (VWR, West Chester, PA) over three days and maintained in 70% ethanol until ready for processing at UHN Pathology Research Program (PRP) (Toronto General Hospital, Toronto, ON). Tissue was
sectioned using a slice thickness of approximately 5 mm. In paraffin embedded specimens, 5-µ thick cross-sections of the femoral artery were stained with Haematoxylin and Eosin (H&E) and Movat. Serial slides were analyzed to determine the depth of treatment penetration. Slices were scanned using a Leica Microscope (Leica Microsystems, Richmond Hill, ON) and the MiraxScan (Carl Zeiss, Toronto, ON). H&E histology slides were analyzed qualitatively by examining molecular composition of the CTO. Movat stained slides were used to evaluate penetration depth of microspheres and structural disruption in the CTO as a result of treatment.

3.5 Results

3.5.1 Results: In Vitro Characterization of the Collagenase-Gadolinium Complex

Prior to in vivo administration of the collagenase-Gd treatment, relaxivity testing was done on the 1.5 T magnet to determine the magnetic properties of the complex. The $T_1$ values for the sample at different concentrations were determined with reference to other known agents, such as Magnevist, a gadolinium contrast agent. The results, which are presented in Table 4, yielded an $R_1$ value (mean ± SD) of 14 ± 3 s⁻¹mM⁻¹.

Table 4: $T_1$ values for the collagenase-gadolinium complex at different concentrations using the 1.5 T magnet.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T_1$ Value (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM Collagenase-Gd</td>
<td>64</td>
</tr>
<tr>
<td>100 µM Collagenase-Gd</td>
<td>666</td>
</tr>
<tr>
<td>10 µM Collagenase-Gd</td>
<td>1717</td>
</tr>
<tr>
<td>10 mM Magnevist</td>
<td>12</td>
</tr>
<tr>
<td>10 µM Collagenase</td>
<td>2432</td>
</tr>
</tbody>
</table>

Subsequent to this experiment, ~16 mg of the collagenase-Gd infused microspheres were injected into an agar filled container and imaged using MR at 22, 80 and 200 minutes post-injection. Figure 20 shows a sagittally-reformatted spin echo image of the microsphere-encapsulated compound diffusing through the agar at the select time points.
From this image, it appears that the area of greater signal intensity is migrating lowering into the agar, suggesting that the microspheres are either diffusing through the agar themselves or they are degrading and the collagenase-Gd complex is diffusing through the agar.

3.5.2 Phantom $T_1$ mapping

The custom, receive-only surface coil was fixed to a container holding agar of known $T_1$. The agar was scanned with the same parameters used in the animal study to simulate the in vivo conditions to validate the $T_1$ map algorithm. It was found that the first slice yielded accurate $T_1$ values (1500 ms), with slight variability immediately adjacent to the coil, however $T_1$ values increased with slice depth (up to 1800 ms).

3.5.3 In Vivo Collagenase Residency in the CTO

Five subjects were treated with the collagenase-Gd complex, four with the collagenase-gadolinium in microspheres and one with saline as the control. Only one subject for each of the treatments is presented in the subsequent sections.

Local delivery of the treatment through the wire port of an over-the-wire balloon catheter was quite pressurized due to the confined volume of space receiving the 500 µl injection. This was evidenced by the difficulty associated with injection and the vessel perforation that occurred in two of the subjects (not included in the study). 3D SPGR images ($\alpha = 45^\circ$) following contrast injection for each time point (pre, 1.5, 2 and 24 hours post-treatment) are presented in Table 5. A MRI displaying the patent vessel, proximal to the occlusion, is shown as well as the MRI of the first slice of the proximal fibrous cap of the CTO. Both images are presented to show that vessel recognition in the slice proximal to the CTO was quite effortless, and it was this slice that
was used to locate the occlusion on the $T_1$ maps. The large ROI is used to identify the region of the CTO because exact localization was challenging.
Table 5: MR images showing the patent vessel and the proximal fibrous cap (PFC) of the CTO for the four imaging time points (pre, 1.5 h, 2 h and 24 h post-treatment). The femoral artery is at the centre of the ROI.

<table>
<thead>
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<th>PFC</th>
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Signal generated by the gadolinium component of the collagenase complex is too low to detect using MRI. Signal visible is due to blood flow in a microvessel or side branch. Detection of these smaller changes in longitudinal relaxation time is theoretically possible using the $T_1$ maps generated using the DESPOT1 algorithm. Maps for three of the subjects are shown in Table 6 however examination of the proximal 6 mm of the occlusion does not show consistent areas of lower $T_1$ in the CTO or the region surrounding it. $T_1$ values in the region of the femoral artery and the CTO are in the range of 100 to 2000 ms for all treatments, including the control.

Following the 24 hour MRI and euthanization femoral arterial tissue was removed for histological staining. Table 7 presents the histology images for three of the subjects. Histology slides are 5 mm apart with a slice thickness of 5 µm and are especially useful in evaluating the depth of penetration of microspheres, the presence of microvessels and the verification of lumen dissection by the guidewire.
Table 6: $T_1$ maps for the first 6 mm of the CTO for R152 Control; R147 Collagenase-Gd and R154 Microspheres (M/S). Vessel is located in the centre of the ROI and colour scale is in milliseconds.

<table>
<thead>
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<th>Subj.</th>
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<th>6 mm</th>
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<td>PFC</td>
<td>3 mm</td>
<td>6 mm</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
<td>-----</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Pre</td>
<td></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>1.5 h</td>
<td></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>R154 M/S</td>
<td>2 h</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Table 7: Histological samples stained for H&E and Movat. Slices are 5 mm apart. Unmarked scale bars are 200 µm, images labeled with * have a scale bar of 500 µm.

<table>
<thead>
<tr>
<th></th>
<th>H&amp;E</th>
<th>Movat</th>
</tr>
</thead>
<tbody>
<tr>
<td>R152 Ctrl</td>
<td><img src="image1" alt="H&amp;E" /></td>
<td><img src="image2" alt="Movat" /></td>
</tr>
<tr>
<td>CTO</td>
<td><img src="image3" alt="H&amp;E" /></td>
<td><img src="image4" alt="Movat" /></td>
</tr>
<tr>
<td>Distal</td>
<td><img src="image5" alt="H&amp;E" /></td>
<td><img src="image6" alt="Movat" /></td>
</tr>
</tbody>
</table>

* Unmarked scale bars are 200 µm, images labeled with * have a scale bar of 500 µm.
<table>
<thead>
<tr>
<th></th>
<th>H&amp;E</th>
<th>Movat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>147 Coll-Gd</strong></td>
<td><img src="image1" alt="H&amp;E Image" /> <img src="image2" alt="Movat Image" /></td>
<td></td>
</tr>
<tr>
<td><strong>Prox.</strong></td>
<td><img src="image3" alt="H&amp;E Image" /> <img src="image4" alt="Movat Image" /></td>
<td></td>
</tr>
<tr>
<td><strong>CTO</strong></td>
<td><img src="image5" alt="H&amp;E Image" /> <img src="image6" alt="Movat Image" /></td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image7" alt="H&amp;E Image" /> <img src="image8" alt="Movat Image" /></td>
<td></td>
</tr>
<tr>
<td><strong>Distal</strong></td>
<td><img src="image9" alt="H&amp;E Image" /> <img src="image10" alt="Movat Image" /></td>
<td></td>
</tr>
<tr>
<td>R154 M/S</td>
<td>Prox.</td>
<td>*</td>
</tr>
<tr>
<td>----------</td>
<td>-------</td>
<td>---</td>
</tr>
<tr>
<td>CTO</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The movat stains easily identify microchannels through the CTO and provide better contrast for identifying microspheres. The chart presented in Table 8 identifies the penetration depth of the microspheres into the CTO.

**Table 8: Subjects treated with microsphere-encapsulated collagenase. Microsphere penetration depth through the CTO is noted. Extravascular signifies that the microspheres are located external to the IEL; open refers to the patent vessel, distal to the occlusion.**

<table>
<thead>
<tr>
<th>Subj.</th>
<th>0-5 mm</th>
<th>5-10 mm</th>
<th>10-15 mm</th>
<th>15-20 mm</th>
<th>20-25 mm</th>
<th>25-30 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>137</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>Open</td>
<td>Open</td>
<td>Open</td>
</tr>
<tr>
<td>74965</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Open</td>
<td>Open</td>
<td>Open</td>
</tr>
<tr>
<td>74962</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Extravasc.</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>154</td>
<td>Extravasc.</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Open</td>
<td>Open</td>
</tr>
</tbody>
</table>

**3.6 Discussion: Collagenase Residency in the CTO**

We have developed a method of tracking collagenase through gadolinium labels using MRI. Although the complex generates signal *in vitro*, concentrations remaining at the CTO in our *in vivo* model appear to be too low for our detection. Polymer microspheres as a vehicle for collagenase delivery in our CTO model appear to alter the architecture of the occlusion, likely due to the pressure of injection and also because they displace some of the plaque and integrate themselves within the lesion.

**3.6.1 Relaxivity Testing of the Collagenase-Gd Complex**

Prior to the *in vivo* studies, longitudinal relaxation times on the 1.5 T magnet were determined for the collagenase-Gd complex as well as the collagenase-Gd complex in microspheres. Collagenase-Gd at a concentration of approximately 100 µM yields a $T_1$ in the vicinity of $6.7 \times 10^2$ ms. Extrapolating from the exponential curve that was developed using serial dilutions of the collagenase-Gd complex yields a theoretical longitudinal relaxation time of approximately $9.4 \times 10^2$ ms for the 45 µM collagenase-Gd complex on the 1.5 T. The 16.5 mg pellet of microspheres containing collagenase-Gd at a concentration of roughly 45 µM yields a $T_1$ of $2.8 \times 10^2$ ms. Interestingly, collagenase-Gd microspheres that were injected directly into the agar (not pelleted) had a much higher $T_1$ ($7.0 \times 10^2$ ms), likely due to a decrease in concentration. This suggests that when the microspheres or the collagenase-Gd complex is not
highly concentrated, the overall signal decreases substantially, which impacts its visibility in the $T_1$ maps.

Although relaxivity testing was done on the 1.5 T magnet, conjugation protocols performed after this testing increased the number of reaction sites on the collagenase for gadolinium marking by up to ten times, thereby permitting greater signal at the site. Increasing the concentration of gadolinium in the collagenase complex is beneficial for signal, but it comes at the cost of enzyme activity. It has been noted that as larger masses of gadolinium salts are added to the collagenase, precipitate appears. A protein gel was performed to confirm the presence of collagenase following conjugation, and although it was present, it was less concentrated than anticipated. Therefore, doses that are theoretically 1 mg collagenase are much less in reality. Strauss et al. confirmed that collagenase treatment of the CTO in a dose of 100 µg and 450 µg resulted in effective treatment and successful guidewire crossing, however the lower of the two was deemed safer for the subject as larger doses are associated with subcutaneous bruising1,5. Due to the denaturing process that occurs during conjugation with gadolinium, a 1 mg dose likely falls in the 450 µg range. When collagenase doses greater than 1 mg were administered, collagenase impact on the tissue was evident as subcutaneous bruising during arterial dissection following sacrifice.

The microsphere infused agar (Figure 20) was scanned at different time points following injection to determine whether or not the signal changed with time. It appears as though the bulk of the signal migrates deeper into the agar by the 200 minute time point. The brighter signal likely corresponds to the protein released during the initial burst. This protein is either coating the surface of the microspheres or poorly-encapsulated and is released within the first hour of reconstitution. The downward migration pattern of the MR signal suggests that the collagenase-Gd complex released during the initial burst collects and diffuses with gravity through the agar.

3.6.2 In Vivo Collagenase Kinetics

Although both VEGF and collagenase are investigated therapeutic agents for the treatment of CTOs, the use of collagenase in this aspect of the study was due to the structural availability of the lysine side chains for conjugation to Gd.

Six-week-old rabbit CTO models were used for the in vivo studies. The rabbit femoral artery is an established CTO model because it exhibits many features of human CTO maturity. For instance, it has demonstrated the earlier thrombus and proteoglycan-rich properties as well as the dense collagen formulation of the mature occlusion. It has also exhibited the presence of a
PFC, the main barrier to PCI. Six-week-old CTOs are primarily composed of lipids, calcium and collagen, interspersed with microchannels. Histology slices for all of the vessels examined shows that the primary components of CTOs are blood and neutrophils due to the inflammatory reaction as well as collagen and lipids. Due to this menagerie of molecules in the CTO and the inconsistency of CTO composition across samples, the longitudinal relaxation times of the CTO are unknown.

Examination of the proximal 6 mm of the CTO in the $T_1$ maps confirms values for all treatments are in the range of 100-2000 ms. This suggests that the collagenase-Gd complex is not residing in the occlusion in concentrations sufficient for detection. The mechanism seen here is likely similar to the in vitro studies where relaxivity testing showed significantly longer $T_1$ values for the diffusing microsphere infusate than the pelleted microspheres. As the collagenase diffuses through the occlusion and into the surrounding tissue, the concentration significantly decreases making detection from surrounding tissue impossible.

A very likely explanation behind the low detection of the collagenase treatment at the CTO is due to administration into side branches. Although fluoroscopy confirmed that balloon placement blocked large vascular branches proximal to the occlusion it is possible that smaller branches were not detected or smaller branches existed too close to the entrance of the occlusion to be blocked. For instance, R74965 was treated with collagenase-Gd in microspheres. The fluoroscopy image confirmed treatment administration was directly into the occlusion, with all side branches blocked. The MRIs confirm the presence of a side branch 3-6 mm proximal to the occlusion. It is possible that the vessel was blocked during surgery; however, the histological slices of the occlusion exhibit minimal evidence of the microsphere treatment with the only proof of microspheres being in the most distal slice of the occlusion (Figure 21). This suggests that the injection travelled through collateral branches past the lesion to reside in the distal end of the occlusion.
Figure 21: H&E stained cross-section of the CTO distal to the occlusion. Microspheres (diameter ~ 30 µm) are the small white circles in the lumen of the vessel. Scale bar is 200 µm.

A similar circumstance is visible in the pre-scan MRI of R154 (Table 5). The image shows a side branch at the lower left of the artery, just proximal to the CTO making exclusive access to the CTO quite difficult. That being said, some microspheres are evident in the proximal slices of the CTO suggesting that when two pathways exist for treatment delivery, some of the treatment goes each way.

In addition to side branches, some treatment may travel extravascularly due to dissection of the vessel wall by the pressure of the injection. Fluoroscopy was done following balloon deflation to confirm that the vessel was not perforated, but during examination of the histology it became evident that in some cases the perforation occurred to the IEL, causing blood flow between the elastic lamina. Figure 22 provides an example.
Vessel perforation misleads treatment delivery. In a separate instance, the IEL was perforated and the microsphere treatment was delivered between the elastic lamina, which means that the CTO was not treated. This example is presented in Figure 23.

Figure 22: Movat-stained cross-section of the CTO proximal to the occlusion. Blood (dark pink) external to the lumen (L) suggests IEL perforation proximal to this slice. Scale bar is 200 µm.

Figure 23: H&E stained histological slide showing microspheres (black arrows) present external to the IEL and the occlusion due to guidewire perforation proximal to this slice. Damaged IEL is labeled with a green arrow. Scale bar is 200 µm.
Following balloon deflation, the proximal end of the CTO is no longer protected from blood flow. The treatment that remains at the PFC because it did not enter the microvessels or the occlusion is not quantified and is likely washed away. This remains a problem for detection of the collagenase-Gd complex, for which signal is concentration-dependent. The \textit{in vitro} studies were performed under controlled conditions where the mass of administered protein and gadolinium was constant. In controlled situations it is possible to determine the $T_1$ value of the complex and visualize it because it is compared to known background values. The dynamic \textit{in vivo} setting and the unique anatomy of each subject makes translation of the laboratory data very difficult due to the factors influencing the residence of the treatment at or in the CTO.

Histological samples for each of the treatments taken 24 hours post-administration confirmed the presence of neutrophils in the vessel due to the inflammatory response to the occlusion. Also present are the collagen matrix and in some cases, fibrin strands. Across treatments, the evidence of microspheres within the lesion is the only difference between the histology slides. Of the microsphere treatments, fifty percent of the samples showed evidence of microspheres within the CTO.

3.6.3 Microspheres as a Drug Delivery System

Microsphere delivery of collagenase did not exhibit any differences in the $T_1$ maps when compared with no microsphere delivery, presumably because of the low concentration of collagenase (and therefore signal) remaining at the site. However, the histology images proved that the local delivery of microspheres to the lesion had impact on its structural integrity.

Local administration of collagenase-Gd microspheres reconstituted in sterile water is quite pressurized and at times physically difficult due to the small injection port and the narrow volume of space receiving the injection. Although this pressure is not quantified, histology confirms some vessel perforation, primarily dissection of the inner elastic lamina. This is not unique to the microsphere treatment because perforation is also seen with the collagenase injection. Carlino et al. have shown that injection of imaging contrast agent to the proximal end of the CTO facilitates guidewire crossing, likely by expanding preexisting microchannels as a result of the pressurized injection\textsuperscript{7}. Although no correlation has been made with the number of microchannels present following treatment administration, plaque disruption is quite evident as a result of the injection. It is this pressure of injection that dislodges the plaque from the edges of
the lumen (Figure 24) forcing channel formation and ultimately delivering collagenase treatment deep within the CTO.

![Figure 24: Movat-stained cross-section displaying evidence of plaque disruption from treatment (microsphere) injection. Arrow depicts where the plaque has separated from the IEL and created a channel for treatment entry into the CTO. Scale bar is 200 µm.](image)

Figure 24: Movat-stained cross-section displaying evidence of plaque disruption from treatment (microsphere) injection. Arrow depicts where the plaque has separated from the IEL and created a channel for treatment entry into the CTO. Scale bar is 200 µm.

The structural contrast in the Movat stained histology slides provides excellent visibility of the microspheres in the CTO. Although structurally similar to fat molecules, microspheres are much smaller in size (diameter ~ 30 µm) and more circular in nature. The depth of penetration of the microspheres is presented in Table 8. In the samples where the tissue was not dissected by the advancing guidewire, the microspheres were evident in the first 10-15 mm of the CTO, where the collagen and fibrin matrix is most dense. Recognizing that the histology slide is just a 5 µm thick slice of a 5 mm thick cross-section emphasizes that the presence of the microspheres is much greater than the small volume visible in the slides. This positioning suggests that the collagenase released from the microspheres has a better opportunity of contributing to successful guidewire crossings due to its softening impact on the dense collagen cap. Histology also confirmed the presence of microspheres in the microchannels deep within the CTO suggesting that the softening effects of the released collagenase is not limited to the proximal end of the CTO. Figure 25 demonstrates this effect.
While microspheres are lodged within the CTO, their polymer exterior begins to degrade as a result of the surrounding conditions and they elute their drug over a period of time, likely 24 hours. *In vitro* characterization of microsphere release exhibits an initial burst, followed by a lag phase and concluding with a linear release pattern. However, correlation between *in vitro* and *in vivo* experimentation is not absolute and it has been observed that the overall release pattern occurs over a shorter period of time, due to the lack of a lag phase\(^7\). It is speculated that this faster release is also a result of the *in vivo* conditions such as local pH, the presence of additional enzymes and the interstitial fluid volume\(^7\). Strauss et al. note that a 24 hour waiting period following administration of collagenase to the lesion is sufficient before PCI due to the collagenase impact on the proximal fibrous cap\(^1\). Because the *in vivo* release profile of collagenase-encapsulated microspheres is not defined, it remains to be seen whether or not the released collagenase has sufficient time to cleave the collagen within the CTO. Crossings were not performed following treatment with these subjects, although the outcome could provide an additional avenue of analysis that would be worthwhile pursuing. Crossing the vessels would allow for an idea of the quick-acting nature of the collagenase released from the microspheres when compared with non-microsphere collagenase administration and also the impact that microsphere disruption has on the overall density of the lesion.

It is possible that microsphere distribution following administration is different from that of collagenase due to the pressure of the injection and the size of the microsphere compared with that of the protein. The identification of microspheres within the CTO suggests that the collagenase treatment also enters the CTO due to the identical administration process. During
some of the dissections, subcutaneous bruising and the damaged appearance of the tissue surrounding the vessel confirms the collagenase treatment enters the capillaries that branch from the femoral artery, degrading the collagen in those areas as well.

*In vitro* characterization of microsphere release is easier due to the static conditions surrounding analysis. The concentration of collagenase released by microspheres was not sufficient for detection via $T_1$ mapping, but histology confirms the disruptive nature of microsphere injection by the dislodged plaque and penetration of microspheres within the CTO.

### 3.7 Study Contributions

I developed a novel conjugation protocol binding gadolinium to collagenase for detection using MRI. The *in vitro* characterization of the collagenase-Gd complex was performed with Wilfred Lam of Dr. Charles Cunningham’s research group at Sunnybrook Health Sciences Centre. Wilfred Lam also coded the DESPOT1 algorithm in Matlab and, along with Xiuling Qi of Dr. Graham Wright’s lab at Sunnybrook Health Sciences Centre, drove the 3T MRI for most of the animal studies.

CTO induction surgery, confirmation of vessel occlusion and administration of the intravascular therapies were performed by Dr. Beiping Qiang and Michelle Ladouceur-Wodzak. I performed vessel dissection and fixation following the 24 hour MRI time point, but histology was performed by the University Health Network Pathology Research Program (PRP) at Toronto General Hospital. Rebecca Zendel and Maxwell Weisbrod assisted me with scanning of the histology slides. I performed all of the analysis in the *in vivo* study.
Chapter 4: Summary and Future Work

4.1 In Vitro Study Conclusions

The *in vitro* studies characterized the release profiles for protein-loaded microspheres, examined the bioactivity of the released protein and looked at the effects of PLGA degradation on endothelial cell growth. It was confirmed that BSA-encapsulated microspheres released a maximum of 20% of the encapsulated protein over a period of eight hours, 56-69% of which was released in the initial burst. VEGF microspheres released 1-2.5% of the theoretically-encapsulated protein in a triphasic pattern over 24-48 hours. Although proliferation assays confirmed that the released VEGF is active and able to induce endothelial cell growth, the quantity released is much less than that which was encapsulated.

The lack of protein released from the microspheres is primarily contributed to protein denaturing that occurs throughout the manufacturing process. Protein adsorption as a result of hydrophobic and electrostatic interactions with the amino acids is possible as the protein crosses the water-oil interfaces and the polymer barrier. Exposure to chloroform, the organic solvent involved in microsphere formation, and also the acidic microenvironment created during PLGA degradation can both denature the released protein. It was shown, however, that the products of PLGA degradation did not impact endothelial cell growth.

Altering the preparation conditions for the microspheres does influence protein release, primarily with regards to the initial burst. Preparing microspheres at low or high temperatures, decreasing the polymer molecular weight, and using larger microspheres are all methods of decreasing the initial burst and sustaining release over a greater period of time.

4.2 In Vivo Study Conclusions

Collagenase conjugated to gadolinium generates signal on the MRI when tested *in vitro*, but the concentration of collagenase that remains in the CTO following local administration is inadequate to provide sufficient signal for detection on the $T_1$ map. Collagenase and microspheres delivered to the proximal end of the CTO can be lost extravascularly if the vessel is perforated by the guidewire, through side branch vessels if they are close to the proximal end of the CTO or by being washed away post-balloon deflation.

Histology confirmed the presence of microspheres in the proximal 15 mm of the CTO, which not only validates the administration process, but confirms that treatment delivered locally
targets the dense collagen and fibrin-rich matrix of the proximal fibrous cap, that is the barrier to crossing.

4.3 Study Limitations

Quantification of the $T_1$ shortening effects of Gd proved to be a limitation of this study. A contributing factor is the difficulty associated with the localization of the occlusion once inside of the CTO using MRI. The narrowed lumen and the lack of blood flow in the occlusion render the vessel boundaries unclear for ROI designation.

The resolution of the $T_1$ maps and of the MRIs proved to be a limitation of this study. Scan times were minimized due to the subject’s reaction to extended anesthetization. Lower resolution further impeded the localization of the proximal fibrous cap. Time constraints contributed to the fact that there are still skewed values generated by the $T_1$ mapping program on phantoms of known value. In addition, quantification of $T_1$ in the CTO is difficult due to the extreme $T_1$ voxels resulting from a poor linear fit. These outlier values distort the $T_1$ measured in the ROI leading to a qualitative study.

Limitations also existed through unavoidable avenues of uncertainty. Ideally, pre and post MR scans have identical coil placement so the images can be properly compared. However, with live subject testing this is not feasible and comparisons across time points other than the 1.5 and 2 hr scans were subject to imprecision.

4.4 Future Work

Further *in vitro* characterization of the collagenase-Gd complex is necessary using the 3T magnet for both Collagenase-Gd formulations used in this work. The relaxivity values determined from this analysis will allow for optimization of the conjugation protocol to determine whether additional Gd can and should be added to facilitate *in vivo* localization.

The experiment determining the release profile of collagenase-Gd from microspheres using MR should also be re-performed using pure collagenase microspheres as a control to determine whether or not the signal is a result of the Gd on the collagenase or a function of the microsphere degradation process. An increase in the number of time points would also be beneficial for this characterization.

If further microsphere work is of interest, more controls and a greater sample size to evaluate cell proliferation from microsphere-released protein is necessary. *In vitro – in vivo*
correlation studies are also recommended to better understand the behaviour of the system under true biological conditions.
Chapter 5: References


46. Han EG, G; Stainsby, J; Wright, G; Beaulieu, C; Brittain, J. In-Vivo T1 and T2 Measurements of Muskuloskeletal Tissue at 3T and 1.5T. Paper presented at: International Society for Magnetic Resonance in Medicine (ISMRM), 2003; Toronto, ON.


57. Cancela S, Yebra MC. Flow-injection flame atomic absorption spectrometric determination of trace amounts of cadmium in solid and semisolid milk products
coupling a continuous ultrasound-assisted extraction system with the online preconcentration on a chelating aminomethylphosphoric acid resin. *Journal of AOAC International*. 2006;89(1):185-191.


