Development of in situ Forming Hydrogels for Intra-articular Drug Delivery

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Hydrogels are 3-dimensional crosslinked polymer networks that can absorb significant amounts of water. The physical properties associated with hydrogels affords them resemblance to biological tissues making them good candidates for biomedical applications. Many pharmaceuticals, specifically non-steroidal anti-inflammatory drugs (NSAIDs), have poor aqueous solubility, which limits their bioavailability and efficacy. People suffering from chronic osteoarthritis (OA) are required to frequently take large doses to mitigate pain, which can lead to serious side effects. Hydrogels are good strategies to deliver NSAIDs via articular injection because they can form solid gels in situ. This thesis describes the synthesis, formulation, mechanical testing, in vitro and in vivo trials of triblock copolymer (PCLA-PEG-PCLA) hydrogels. We observed that drug-loading can have a negative impact the gelation behaviour. Block lengths were tuned, and we found that using PEG$_{2000}$ was better to maintain gelation integrity upon drug-loading. We further looked to improve mechanical properties by investigating a redox initiated crosslinking system with methacrylated end-caps. These gels were able to maintain or improve gelation upon loading of various drugs. The optimized chemically crosslinked formulation was able to provide a sustained release of celecoxib in horses for up to 32 d. These results are significant because currently there is no curative or pain relief option for OA patients beyond 16 d while limiting systemic drug concentration.

Keywords
Hydrogel, micelles, triblock copolymer, drug release, drug delivery, thermo-responsive, osteoarthritis, rheology, compression, syneresis,
Co-Authorship Statement

The work described in this thesis was performed by the author as well as contributions from co-workers Mr. Ian Villamagna, Dr. Amir Rabiee Kinaree, Dr. Cameron Hopkins, Prof. John de Bruyn,

Chapter 1 was written by the author and edited by Dr. Elizabeth Gillies

Chapter 2 describes the synthesis, characterization, mechanical testing and drug release studies of physically crosslinked hydrogels. The author was the primary experimentalist. Mr. Ian Villamagna assisted with experimental planning and design. The manuscript was written by the author and edited by Dr. Elizabeth Gillies and Dr. John de Bruyn,

Chapter 3 describes the synthesis, characterization, mechanical testing and in vivo drug release studies using horses. The author was the primary experimentalist. Mr. Ian Villamagna assisted with experimental planning and design. Ms. Jennifer Proulx and Dr. Mark Hurtig performed clinical administration and sampling of bodily fluids for in vivo drug release trials. Dr. Hurtig also provided the information for the experimental section 3.2.12. Ms. Aneta Borecki assisted with analytical method development for celecoxib analysis and performed HPLC analysis and chromatogram processing. The chapter was written by the author and edited by Dr. Elizabeth Gillies.

Chapter 4 describes the conclusions and suggested future work and was written by the author and edited by Dr. Elizabeth Gillies.
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Table of Contents

Abstract ..................................................................................................................... i
Co-Authorship Statement......................................................................................... ii
Acknowledgments .................................................................................................. iii
Table of Contents .................................................................................................. iv
List of Tables .......................................................................................................... viii
List of Figures ........................................................................................................ ix
List of Schemes ..................................................................................................... xii
List of Abbreviations ............................................................................................. xiii
Chapter 1 .............................................................................................................. 1
  1.1 Classes of drug delivery systems ................................................................. 2
      1.1.1 Polymer microparticles ................................................................. 2
      1.1.2 Polymer nanoparticles ................................................................. 3
      1.1.3 Polymer micelles ........................................................................ 4
      1.1.4 Hydrogels ..................................................................................... 6
  1.2 Intra-articular drug delivery systems ........................................................... 16
  1.3 Mechanical properties for IA drug delivery ................................................ 18
      1.3.1 Syneresis ..................................................................................... 19
      1.3.2 Rheology ..................................................................................... 19
      1.3.3 Compression testing ................................................................. 21
  1.4 Thesis Scope ............................................................................................... 22
  1.5 References .................................................................................................. 23
Chapter 2 ............................................................................................................. 29
  2 Effect of Drug Loading on the Properties of Temperature-responsive Polyester-poly(ethylene glycol)-polyester Hydrogels ......................................................... 29
2.1 Introduction .............................................................................................................. 29

2.2 Experimental ........................................................................................................... 32
    2.2.1 General materials and procedures ................................................................. 32
    2.2.2 Synthesis of PCLA-PEG_{1500}-PCLA (1.5k) ................................................. 33
    2.2.3 Synthesis of PCLA-PEG_{2000}-PCLA (2k) ................................................. 33
    2.2.4 Synthesis of PCLA-PEG_{3000}-PCLA (3k) ................................................. 34
    2.2.5 Hydrogel Preparation ...................................................................................... 34
    2.2.6 Rheometry ....................................................................................................... 35
    2.2.7 In Vitro CXB Release ...................................................................................... 35
    2.2.8 Syneresis Measurements .............................................................................. 36
    2.2.9 Compression Testing ..................................................................................... 36

2.3 Results and Discussion .......................................................................................... 37
    2.3.1 Synthesis and Characterization of PCLA-PEG-PCLA Triblock Copolymers ...... 37
    2.3.2 Thermo-responsive Gelation ......................................................................... 39
    2.3.3 Rheological Properties of Drug-free and CXB-loaded Hydrogels ................. 40
    2.3.4 In Vitro Release of CXB From the Hydrogels ............................................... 45
    2.3.5 Syneresis of 15\% 2k Hydrogels Loaded with Different Drugs ................. 47
    2.3.6 Rheology of 15\% 2k Hydrogels Loaded with Different Drugs ................. 49
    2.3.7 Compression Testing of Drug-loaded 15\% 2k Hydrogels ............................. 51

2.4 Conclusions ........................................................................................................... 52

2.5 References ............................................................................................................. 54

3 Covalently crosslinkable thermo-responsive hydrogels for intra-articular drug delivery

3.1 Introduction ............................................................................................................ 58

3.2 Experimental ......................................................................................................... 61
    3.2.1 General materials and procedures ............................................................... 61
    3.2.2 Synthesis of MA-PCLA-PEG_{1500}-PCLA-MA (1.5k_{3150}-MA) ............... 62
3.2.3 Synthesis of MA-PCLA-PEG\textsubscript{1000}-PCLA-MA (\textbf{1k\textsubscript{2080}-MA}) ..................... 63

3.2.4 Synthesis of MA-PCLA-PEG\textsubscript{1000}-PCLA-MA (\textbf{1k\textsubscript{2840}-MA}) ..................... 64

3.2.5 Synthesis of MA-PCLA-PEG\textsubscript{1500}-PCLA-MA (\textbf{1.5k\textsubscript{4190}-MA}) ..................... 64

3.2.6 Synthesis of MA-PCLA-PEG\textsubscript{2000}-PCLA-MA (\textbf{2k\textsubscript{4600}-MA}) ..................... 65

3.2.7 Hydrogel Preparation ........................................................................................................ 65

3.2.8 Rheometry .......................................................................................................................... 66

3.2.9 Syneresis Measurements .................................................................................................... 66

3.2.10 Compression Testing ......................................................................................................... 67

3.2.11 In Vitro CXB Release from 20\% \textbf{1k\textsubscript{3150}-MA} and 15\% \textbf{2k} ......................... 67

3.2.12 Intra-articular injection of the CXB-loaded hydrogels and synovial fluid sampling in horses ............................................................................................................................................................................. 68

3.2.13 Determination of celecoxib concentration in synovial fluid.......................... 69

3.3 Results and Discussion ........................................................................................................ 70

3.3.1 Synthesis and Characterization of Methacrylate Capped PCLA-PEG-PCLA Triblock Copolymers ............................................................................................................................................................................. 70

3.3.2 Feasibility Screening of Methacrylated Hydrogels .................................................... 72

3.3.3 Rheological Properties of \textbf{1.5k\textsubscript{3150}-MA} ................................................................. 74

3.3.4 Syneresis of 20 wt\% \textbf{1.5k\textsubscript{3150}-MA} Hydrogels Loaded with Different Drugs 75

3.3.5 Rheology of 20 wt\% \textbf{1.5k\textsubscript{3150}-MA} Hydrogels Loaded with Different Drugs....... 77

3.3.6 Compression Testing ......................................................................................................... 79

3.3.7 In vitro release of CXB from acetyl and methacryl capped PCLA-PEG-PCLA hydrogels ............................................................................................................................................................................. 82

3.3.8 In vivo CXB release from acetyl and methacrylate hydrogels in horse joints...... 83

3.4 Conclusions ......................................................................................................................... 86

3.5 References ............................................................................................................................ 87

4 Conclusions and Future Work .............................................................................................. 90

5 Appendices ............................................................................................................................. 92
5.1 Appendix 1: Permission to Reuse Copyrighted Material ........................................... 92
5.3 Appendix 2 - Supporting Information for Chapter 2 ............................................ 93
5.4 Appendix 3 – Supporting Information for Chapter 3.......................................... 100
List of Tables

Table 2.1. Molecular masses and chemical analyses of the polymers ........................................... 38

Table 2.2. Results of dissolution and vial inversion tests to determine polymer solubility at 4 °C and gelation at 37 °C ........................................................................................................................................ 40

Table 2.3. Rheology results. G’ and G” of drug-free and CXB-loaded hydrogels measured at 1 Hz and 1% strain amplitude at 4 and 37 °C ........................................................................................................ 43

Table 2.4. Rheology results. G’, G”, and compressive moduli of drug-free and drug-loaded hydrogels measured at 1 Hz and 1% strain amplitude at 4 and 37 °C .................................................................................. 51

Table 3.1. Molar mass and chemical analysis data for the polymers ............................................. 71

Table 3.2. Results of tests to determine polymer solubility at 4 °C and initial assessment of gelation at 37 °C based on the vial-tilt test ........................................................................................................................................ 73

Table 3.3. Time to gelation in the presence of KPS/TEMED as well as G’ and G” for 1.5k3150-MA hydrogels after 80 min at different temperatures ................................................................................................. 82

Table 3.4. Mechanical testing. G’, G”, and compressive moduli of drug-free and drug-loaded (5%) \textbf{1.5k3150-MA} hydrogels ........................................................................................................................................ 78
List of Figures

Figure 1.1. A. Triblock copolymers below their critical micelle concentration (CMC) ........ 6
Figure 1.2. Depiction of drug release models................................................................. 10
Figure 1.3. Schematic of micelles and their self-assembly into micelle networks to form hydrogels. .................................................................................................................. 11
Figure 1.4. Schematic representation of IA delivery of different therapeutics................. 17
Figure 1.5. Schematic of types of mechanical testing. ....................................................... 19

Figure 2.1. G’ and G” of acetyl-capped PCLA-PEG-PCLA hydrogels during temperature sweep and temperature hold (37 ºC) experiments............................................................... 42
Figure 2.2. Depiction of the possible effects of drug loading on the hydrogel structure....... 45
Figure 2.3. In vitro CXB release ..................................................................................... 46
Figure 2.4. Chemical structures of drugs studied............................................................ 47
Figure 2.5. Syneresis (water loss) measured for 15% 2k hydrogels loaded with 5 wt% of different drugs. ......................................................................................................................... 48
Figure 2.6. G’ and G” of 15% 2k acetyl-capped PCLA-PEG-PCLA hydrogels during temperature sweep and temperature hold (37 ºC). .............................................................................. 50
Figure 2.7. Compressive stress versus strain for 15% 2k hydrogels with and without drugs incorporated .......................................................................................................................... 52

Figure 3.1. G’ and G” of 20% 1.5k3150-MA hydrogels without KPS/TEMED during Temperature sweep and Temperature hold (37 ºC) .................................................................................. 75
Figure 3.2. G’ and G” of 1.5k3150-MA hydrogels as a function of time in the presence of KPS/TEMED ............................................................................................................................... 81
Figure 3.3. Chemical structures of NSAIDs tested................................................................ 76
Figure 3.4. Syneresis measurements for 20% 1.5k-MA hydrogels loaded with 5 wt% of different NSAIDs……………………………………………………………………………… 77

Figure 3.5. G’ of 20 wt% 1.5k-MA hydrogels loaded with 5% of different NSAIDs .. 78

Figure 3.6. Compressive stress versus strain for 20 wt% 1.5k-MA hydrogels with and without drugs incorporated. ........................................................................................................ 80

Figure 3.7. In vitro CXB release into 20 mg/mL polysorbate 80 sink solution............. 83

Figure 3.8. CXB concentrations measured in synovial fluid after 1 ml injection of 15% 2k + 10% CXB and 20% 1.5k-MA + 10% CXB .................................................................................. 85

Figure A2.1. 1H NMR spectrum of 1.5k polymer (400 MHz, CDCl3).......................... 93

Figure A2.2. 1H NMR spectrum of 2k polymer (400 MHz, CDCl3)............................ 94

Figure A2.3. 1H NMR spectrum of 3k polymer (400 MHz, CDCl3)........................... 95

Figure A2.4. Overlay of SEC traces of polymers 1.5k, 2k, and 3k.............................. 96

Figure A2.5. Vial inversion test that was used to differentiate between mobile and immobile gels at different temperatures. ........................................................................................................ 96

Figure A2.6. Amplitude sweeps and frequency sweeps of 20% 1.5k hydrogels........... 97

Figure A2.7. Amplitude sweeps and frequency sweeps of 15% 2k hydrogel............ 98

Figure A2.8. Amplitude sweeps and frequency sweeps of 10% 3k hydrogel............... 99

Figure A3.1. 1H NMR spectrum of 1k2080 polymer (400 MHz, CDCl3). ................. 1020

Figure A3.2. 1H NMR spectrum of 1k2840 polymer (400 MHz, CDCl3). ............... 10301

Figure A3.3. 1H NMR spectrum of 1.5k3150 polymer (400 MHz, CDCl3). .............. 1042

Figure A3.4. 1H NMR spectrum of 1.5k4190 polymer (400 MHz, CDCl3). ............. 1053

Figure A3.5. 1H NMR spectrum of 2k4600 polymer (400 MHz, CDCl3). ............... 104
Figure A3.6. Overlay of SEC traces of polymers………………………………………….105

Figure A3.7. Temperature sweep experiments of $1.5k_{3150}$-MA loaded with either 5% or 10% CXB.
………………………………………………………………………………………………105
List of Schemes

Scheme 2.1. Synthesis of PCLA-PEG-PCLA triblock copolymers. .......................... 37

Scheme 3.1. Synthesis of methacrylate capped PCLA-PEG-PCLA triblock copolymers. .... 70
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>Hydrophobic-hydrophilic-hydrophobic</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>BAB</td>
<td>Hydrophilic-hydrophobic-hydrophilic</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSC</td>
<td>Biological safety cabinet</td>
</tr>
<tr>
<td>CCS</td>
<td>Corticosteroids</td>
</tr>
<tr>
<td>CL</td>
<td>ε-Caprolactone</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CXB</td>
<td>Celecoxib</td>
</tr>
<tr>
<td>d</td>
<td>days</td>
</tr>
<tr>
<td>D</td>
<td>Dispersity</td>
</tr>
<tr>
<td>DA</td>
<td>Diels-Alder</td>
</tr>
<tr>
<td>DCLO</td>
<td>Diclofenac</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug delivery system</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>ETO</td>
<td>Etodolac</td>
</tr>
<tr>
<td>G’</td>
<td>Storage modulus</td>
</tr>
<tr>
<td>G’’</td>
<td>Loss modulus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>GLD</td>
<td>Glutualdehyde</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>IA</td>
<td>intra-articular</td>
</tr>
<tr>
<td>IBU</td>
<td>Ibuprofen</td>
</tr>
<tr>
<td>KPS</td>
<td>potassium persulfate</td>
</tr>
<tr>
<td>LA</td>
<td>Lactide</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower critical solution temperature</td>
</tr>
<tr>
<td>MAA</td>
<td>Dimethacrylate methacrylic acid</td>
</tr>
<tr>
<td>Mal</td>
<td>Maleimide</td>
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<tr>
<td>MEL</td>
<td>Meloxicam</td>
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<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OHIP</td>
<td>Ontario health insurance plan</td>
</tr>
<tr>
<td>PBCL</td>
<td>Poly(α-benzyl carboxylate ε-caprolactone)</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(caprolactone)</td>
</tr>
<tr>
<td>PCLA</td>
<td>poly(caprolactone-co-lactide)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PHE</td>
<td>Phenylbutazone</td>
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PLA  Poly(lactide)
PLGA Poly(D,L-lactide-co-glycolide)
PMMA Poly(methyl methacrylic acid)
PNIPAAM Poly(N-isopropylacrylamide)
PPO Poly(propylene glycol)
PVA Poly(vinyl alcohol)
PVE Poly(vinyl ether)
PXL Paclitaxel
S  Seconds
SEC Size exclusion chromatography
SF Synovial fluid
T Temperature
TEMED $N,N,N',N'$-Tetramethylethylenediamine
THF Tetrahydrofuran
TMC Trimethyl chitosan
UTM Universal testing machine
UV Ultraviolet
$\Delta G$ Change in free energy
$\Delta H$ Change in enthalpy
$\Delta S$ Change in entropy
Chapter 1

1 Introduction

Many drugs, including both those that are approved and those still under development, are intended for oral administration and have many shortcomings. For example, many drugs suffer from poor aqueous solubility.\textsuperscript{1} In addition, high throughput screening and combinatorial chemistry have lead to the rapid discovery and development of many drugs that have poor biopharmaceutical properties.\textsuperscript{2,3} Poor solubility in water limits drug absorption and leads to a poor bioavailability profile. It may lead to the need for high doses with undesirable side effects. Several approaches are available to improve the aqueous solubility of hydrophobic drugs for oral administration, including a reduction in particle size, surfactants, nanosuspension, cryogenic techniques, supercritical fluid technology, floating granules, and more.\textsuperscript{4} Oral drug administration aims to deliver a desired drug concentration systematically throughout the body to achieve a pharmacological response. Many other administration methods exist with their own specific purposes, advantages and disadvantages. Alternatives to oral administration are considered when a patient cannot take the drug by mouth, rapid response is required, or when the digestive tract absorbs the drug poorly. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly prescribed group of drug globally.\textsuperscript{5} As an example, aspirin can damage the stomach and small intestine, while others can have more serious side-effects, like Celecoxib being linked to cardiovascular toxicity. In addition to bioavailability issues, many therapeutics suffer from short half-lives and chemical instabilities. Harsh physiological environments further contribute to drug metabolism and consequently degradation by causing redox reactions, hydrolysis and racemisation.\textsuperscript{6}
1.1 Classes of drug delivery systems

Drug delivery systems (DDSs) can improve the pharmacological properties of many drugs by altering the pharmacokinetics, biodistribution or by acting as reservoirs to provide a sustained release. The simplest ways to improve oral administration of hydrophobic drugs are by using low molecular weight surfactants to increase solubilization or by adding polymeric coatings to tablets to provide a timed release. These simple DDSs have made great contributions to disease treatment, however, a strong need exists for advanced delivery systems capable of targeted tissue delivery, and the ability to respond to biological stimuli in vivo. Targeted DDSs are developed not only to increase the solubility, but also to protect the drug from degradation, reduce side effects and increase the drug concentration at the target site. Several drug carriers, such as microparticles, nanoparticles, micelles, and hydrogels have been used to achieve these benefits.

1.1.1 Polymer microparticles

Microparticles are spherical in shape, are between 1 and 1000 µm in diameter and are commonly employed as DDSs. There is a diverse range of uses and administration techniques available for microparticles and they can be prepared from a wide range of different materials. For instance, insulin has been delivered orally to patients suffering from diabetes mellitus, a chronic metabolic disease diagnosed by pancreatic islet cell destruction or lack of sensitivity to endogenous insulin. Insulin-loaded chitosan-based microparticles have been prepared by Kondiah et al. The authors used copolymeric trimethyl chitosan-poly(ethylene glycol) and dimethacrylate methacrylic acid (TMC-PEGDMA-MAA) to encapsulate insulin. After oral administration, the insulin encapsulated in the microparticles was protected from the harsh environment of the stomach and a therapeutic 24-hour plasma insulin concentration was exhibited in rabbits. The versatility of microparticles
was also shown in the study by Kondiah et al.,\textsuperscript{11} through the incorporation of pH-responsive (TMC) and mucoadhesive (MAA) moieties.

Microparticles are often administered directly into target tissues by intramuscular, intraperitoneal or subcutaneous injection.\textsuperscript{12} Poly(D,L-lactide-co-glycolide) (PLGA) is a hydrophobic material that has been proven to be biodegradable and to exhibit a tolerable host response in a number of applications. Since hydrophilic therapeutics, such as proteins, are structurally sensitive, they are incompatible with the gastrointestinal tract, and therefore significant research has been dedicated to their delivery in microparticles. PLGA microparticles are the most widely studied DDS for proteins and peptides.\textsuperscript{13} PLGA was approved by the US Food and Drug Administration for human use and is commercially available from several suppliers that follow good manufacturing practice. For these reasons, it is easy to imagine that there are many research and development activities using PLGA and its derivatives. The most successful applications include commercially available hydrophobic therapeutic-loaded microparticles such as, Lupron Depot\textsuperscript{®} and Suprecur\textsuperscript{®} for the treatment of prostate cancer.\textsuperscript{13}

1.1.2 Polymer nanoparticles

Nanoparticles are similar to microparticles but smaller in size. Nanoparticles range from 1 to 250 nm and differ in their synthesis, formulation and processing techniques. They can be hollow or solid and can be composed of lipids, polymers or surfactants.\textsuperscript{14} The small sizes of nanoparticles allows them to be easily taken up by cells. Their cellular uptake provided motivation for early nanoparticle research to target macrophages for the treatment of AIDS. Macrophages act as reservoirs for the human immunodeficiency virus.\textsuperscript{15} Nanoparticles are also good candidates to be used as drug carriers because they can reduce drug toxicity, increase therapeutic efficacy and distribute well through the body.\textsuperscript{9} These traits are important for managing chronic pain when the
patient requires frequent and high doses of medication. Pain management is a common problem all over the world, affecting 37% of people in developed countries and 41% in developing countries. Several nanocarriers have been developed for the delivery of local anesthetics, such as bupivacaine, ropivacaine or benzocaine in PLGA nanoparticles, lidocaine in poly(caprolactone) (PCL) nanoparticles and benzocaine in poly(lactide) (PLA) nanoparticles. There are also numerous reports of PLA, PCL and PLGA materials being used to prepare NSAID-load nanoparticles for oral administration to alleviate gastrointestinal side effects. For instance, Ibrahim et al. prepared celecoxib-loaded nanoparticles by an emulsification solvent diffusion method using PLC, PLA and PLGA for topical optical delivery. The authors achieved high CXB encapsulation efficiency (>79%) in the nanoparticles and prepared an eyedrop formulation that provided drug release over 24 hours.

1.1.3 Polymer micelles
Micelles can be considered nanoparticles based on their size (10-100 nm). However, micelles have a specific structure that is made up of a core-shell morphology that self-assembles from amphiphilic molecules (Figure 1). Micelles can be prepared by diblock or triblock copolymers or less commonly from more elaborate copolymers. NSAIDs generally have poor water solubility and a short plasma half-life, which makes them good candidates for encapsulation into micelles. When hydrophobic drugs are encapsulated, micelles can protect then from degradation and increase their water dispersibility, as shown by Bhat et al. for naproxen. The authors used several amphiphiles, differing by charge (nonionic, cationic, and anionic), poly(ethylene glycol) (PEG) chain length, in different surfactant combinations to study the solubilization of naproxen. Mixed surfactant systems were superior to single surfactants in general for maximizing the drug loading in the system. Poloxamers are amphiphilic triblock copolymers prepared from PEG and
poly(propylene glycol) (PPO). Different poloxamers have different ratios of PEG:PPO, block sequences (PEG-PPO-PEG or PPO-PEG-PPO) and polymer chain molar masses, which give different physical properties for use in various applications including drug delivery.\textsuperscript{23} Amaral \textit{et al.}\textsuperscript{24} prepared meloxicam (MEL)-loaded micelles by a thin-film method using poloxamer P123 and F123 to improve oral administration. They tested the ability of poloxamer micelles to encapsulate MEL by varying the concentration during preparation. All formulations tested had high encapsulation efficiency (>75%), but higher micelle loading of MEL was achieved by increasing the concentration during preparation. The level of drug encapsulation is determined by the degree of interaction of the micelle core and the drug. Drug-loaded polymeric micelles can also be administered intravenously, and because they typically have lower toxicity than the free drug, higher doses can be delivered. For instance, in chemotherapy, the toxicity of the doxorubicin (DOX) is often the limiting factor for the dose administered per chemotherapy session. Higher doses can be achieved by the DOX-loaded PEG-poly(\(\alpha\)-benzyl carboxylate \(\varepsilon\)-caprolactone) (PBCL) micelles.\textsuperscript{25} DOX has an estimated toxicity of 10 mg/Kg in mice and a dose of 5 mg/kg is not high enough to suppress C26 tumor growth making free drug dangerous to administer. The DOX-loaded PEG-PBCL micelles allowed for doses as high as 50 mg/kg making treatments far more effective.
Figure 1.1. A. Triblock copolymers below their critical micelle concentration (CMC). B. Micelles form by self-assembly above the CMC. The blue segments represent hydrophobic blocks and the red segments represent the hydrophilic blocks.

1.1.4 Hydrogels

1.1.4.1 Background

Hydrogels are three dimensional polymer networks that are capable of absorbing large amounts of water, giving them properties that mimic some biological tissues, and making them suitable for biomedical applications. Hydrogels can be prepared from a large variety of natural or synthetic materials and are chemically stable or biodegrade and dissolve. In general, hydrogels have broad uses. One of the most common commercial uses of hydrogels is in contact lenses. In 1960, poly(2-hydroxyethylmethacrylate) was used to synthesize the first hydrogel for contact lens applications. The properties of contact lenses can be modulated by changing the composition of the polymer. For instance, the less common “hard” contact lenses are prepared from poly(methyl methacrylate). The market has trended toward shorter lasting materials prepared from hydrogels because they have shorter adaptation periods due to their higher elasticity and porosity. Unlike other polymer DDSs discussed above, which can be used for systemic drug delivery (oral and
intravenous), hydrogels are generally used for local drug delivery amongst other biomedical applications. Their porous structure makes them well suited to provide high local drug concentrations for extended periods. Generally, a distinction can be made for hydrogels used as micro or nanoparticles and they are referred to as microgels, which can form building blocks of hydrogels. For local drug delivery, hydrogels can mainly be used in two ways: prepared outside of the body and surgically implanted or they can be injected and form a gel \textit{in situ}. Das \textit{et al.} used preformed amyloid hydrogels loaded with stem cells to promote the differentiation of neurons by implantation into the brains of mice. The hydrogel in this case provided a 3-fold increase in the area and viable cells compared to free cells. However, preformed gels require invasive implantation due to their high elasticity and cannot take the form of the cavity within the body. For these reasons, \textit{in situ} forming hydrogels have attracted significant attention.

\subsection{1.1.4.2 Physically crosslinked injectable hydrogel}

\textit{In situ} forming hydrogels are injectable fluids that form a gel within the body that can be used for cell encapsulation, tissue repair or as DDSs. These gels have significant advantages over preformed hydrogels, including that surgical implantation is not required, and also that a broad range of therapeutics can be easily incorporated. \textit{In situ} forming hydrogels can be formed either by chemical or physical crosslinking. Physical crosslinking is typically achieved by an increase in temperature. At the lower critical solution temperature (LSCT), which is characteristic to the polymer system, a phase separation ensues and is governed by the hydrophobic-hydrophilic balance of the polymer chains. According to the equation, $\Delta G = \Delta H - T \Delta S$, enthalpy and entropy changes contribute to the overall free energy change associating with polymer chain dissolution. Dissolution of the polymer chains is typically favoured enthalpically due to the formation of
hydrogen bonds between the polymer chain and water. However, it is entropically unfavourable due to the organization of water molecules associated with the polymer chains. As temperature increases above the LCST of the polymers, the $T\Delta S$ term becomes larger, making $\Delta G$ for dissolution positive (unfavourable) and polymer chain association (aggregation) is favored over polymer-water association.\textsuperscript{32}

Poly($N$-isopropylacrylamide (PNIPAAM) has an LCST of 32 $^\circ$C and is one of the earliest polymers to be studied for its thermo-responsive properties.\textsuperscript{33} As the temperature increases, the linear PNIPAAM rapidly transforms into a coil and subsequently a globule above its LCST. Unfortunately, PNIPAAM experiences significant syneresis (water loss) and shrinking upon gelation that has made its practical use as an \textit{in situ} forming gel impractical.\textsuperscript{34} The degree of syneresis can be improved by synthesizing diblock, triblock and star copolymers with PEG. Lin \textit{et al.}\textsuperscript{35} reported that the gelation mechanism changes in relation to linear PNIPAAM when the PEG blocks were added. PEG-PNIPAAM formed micelles and the gelation mechanism was assumed to be one of micellar packing and chain entanglement. This is one example of how micelles have an extended range of use beyond the solubilization of drugs for systemic delivery.

As discussed earlier, poloxamers can have different properties depending on their PEO:PPO ratio and molecular weights.\textsuperscript{36} poloxamer F127 (PEG$_{101}$-PPO$_{56}$-PEG$_{101}$) and poloxamer F68 (PEG$_{80}$-PPO$_{27}$-PEG$_{80}$) are known to form gels \textit{in situ} by a micelle aggregation mechanism. Khateb \textit{et al.}\textsuperscript{37} investigated their use for ocular delivery of the antibiotic ofloxacin. In this study, 20 wt% individual and mixtures of the polymers were prepared with drug-free and ofloxacin-loaded (0.3 v/w\%) formulations and the authors studied the gelation properties to establish feasibility and conditions for \textit{in situ} gelation. The properties of the hydrogels, measured by differential scanning calorimetry and rheometry were not greatly influenced by ofloxacin, although the drug loading
concentration was low (0.3 w/v%). Dynamic light scattering (DLS) showed that the polymer mixtures formed hybrid micelles, as indicated by a monodisperse diameter profile. The formulations of 20 wt% F127 and F68 were tested on rabbits. The F127 formulation gelled upon contact with the corneal surface providing even coverage. The gel residence time on the eye was 10 - 15 minutes, which was shorter than expected based on results from the in vitro experiments because blinking cleared material away. The performance of F68 in vivo was relatively poor as the polymer was washed away by the first blinks.

Poly(vinyl ether)s (PVEs) also exhibit thermo-responsive gelation properties. PVEs can be modified by adding a variety of pendant oxyethylene units to modulate the hydrophobic-hydrophobic interaction and therefore gelation. Recently, Moreno et al.\textsuperscript{38} used bovine serum albumin (BSA), rKPM-11 (a protein) and dextran to evaluate the release behaviour of poly(methyl vinyl ether-co-maleic anhydride) (PVE-mal) modified poloxamer F127 hydrogels. PVE-mal has anhydride groups that were crosslinked with F127 by ring opening polymerization to form chemical bonds along the polymer chains. Introducing PVE-mal to F127 resulted in an increase in viscosity with temperature and a 5-fold increase in the drug release time in vivo (rats) of model drugs over poloxamer F127 alone.

Poloxamers have been proven to be useful for drug delivery, but they also have many disadvantages, which have created a need for the development of different materials. Specifically, poloxamers for in situ forming gels have very low mechanical strength, short residence times at their injection site, and are not biodegradable. PEG-polyester copolymers can provide some advantages and have been investigated in several different block configurations. PEG-PLGA-PEG copolymers were prepared by Jeong et al.\textsuperscript{39} and loaded with ketoprofen (moderately hydrophobic drug) and spironolactone (hydrophobic drug). They observed a 90% release of ketoprofen within
3 d and a 90% release of spironolactone in 55 d. The polymer structure (block lengths), polymer concentration, drug hydrophobicity and drug concentration were shown to influence release rates and profile. Early drug release was diffusion dominant and the secondary release was caused by a combination of diffusion and polymer degradation. Drug release from hydrogels can be explained by two models. Model I, proposed by Higuchi,\textsuperscript{40} assumes there is a homogeneous mixture of drug within the dissolved copolymer and represents the release by diffusion and degradation equally (Figure 2A). Model II assumes the hydrophobic drug is partitioned within the hydrogel in a domain structure. In Model II, the drug can be released from the hydrophilic domain by diffusion, while release from the hydrophobic core is a function of drug concentration, diffusion coefficient, permeability, thickness and degradation rate of the polymer (Figure 2B).

**Figure 1.2.** Depiction of drug release models. A Model I shows a homogeneous dispersion of drug throughout the hydrogel. B shows partitioning of the hydrophobic drug in a core-shell orientation.

Block configuration of PEG/polyester polymer chains can have a significant impact on the gelation temperature, ease of polymer synthesis and gelation mechanism.\textsuperscript{41} For copolymers prepared from PEG and PLGA, BAB-type (hydrophilic-hydrophobic-hydrophilic) PEG-PLGA-PEG copolymers
can be rearranged to ABA-type PLGA-PEG-PLGA. The relative gelation temperatures are different for BAB and ABA versions of PEG/PLGA triblock copolymer hydrogels.\cite{42} When the hydrophobic blocks flank the hydrophilic block, they can connect one micelle to another, which is not possible with only one hydrophobic block per polymer chain (Figure 3). Zentener \textit{et al.}\cite{43} prepared the PLGA\textsubscript{1500}-PEG\textsubscript{1000}-PLGA\textsubscript{1500} and studied thermal gelation \textit{in vitro} as well as the \textit{in vivo} release of proteins, paclitaxel (PXL), and porcine growth hormone. The delivery times had a broad range of time scales from one to six weeks. PXL was fully released \textit{in vitro} after 50 d from the hydrogel, compared to 5 d from poloxamer F127. The PLGA-PEG-PLGA PTX delivery system afforded an additional 45 d survival time in tumor-bearing rats over the control.

\begin{figure}[h]
\centering
\includegraphics{figure13.png}
\caption{Schematic of micelles and their self-assembly into micelle networks to form hydrogels. A. Representation of a micelle formed from a BAB-type triblock copolymer B. Micelle aggregation mechanism proposed by Yu \textit{et al.}\cite{44} As temperature increases, micelle aggregation begins to form extended networks. At a critical temperature micelle networks collapse due to maximum hydrophobicity and this results in phase separation. Figure 3B reproduced from reference.\cite{44} C. Representation of a micelle formed from an ABA-type triblock copolymer D. Micelle bridging mechanism proposed where micelles connect one-another as temperature increases.}
\end{figure}
increases to form a micelle network. Blue segments represent hydrophobic blocks and red segments represent hydrophilic blocks.

Different hydrophobic blocks such as poly(lactide)(PLA), poly(caprolactone)(PCL), and poly(caprolactone-co-lactide) (PCLA can be substituted for PLGA. Cho et al.45,46 prepared PCLA-PEG-PCLA hydrogels for parental drug delivery. 5-Fluorouracil was used as a model drug and its release was measured *in vitro*. Polymer chain compositions were varied and their effects on drug release were measured. PEG chains lengths (220 – 1,500), PCLA block lengths (3,800 – 7,200) and total copolymer molar masses varied from 8,800 – 15,900 g/mol. 5-Fluourourcil loading ranged from 0.2 to 3.3 wt% and full release occurred within 4 d for all formulations. Later, Petit et al.47 prepared acetyl capped PCLA\textsubscript{1600}-PEG\textsubscript{1500}-PCLA\textsubscript{1600} (4,700 g/mol) loaded with 0.125 wt% celecoxib (CXB) and observed that its release *in vitro* in 0.2% polysorbate 80 occurred for 100 d. This shows the effect that end-capping of polymer chains can have on the drug release rate.

1.1.4.3 Chemically crosslinked injectable hydrogel

Physically crosslinked hydrogels, as discussed previously, have contributed significantly to the field of drug delivery, as indicated by numerous formulations commercially available and their exploration in clinical trials. Despite their importance, their application is limited due to their weak mechanical strength, which often leads to short tissue residence time. Chemically crosslinked hydrogels contain permanent junctions that provide superior mechanical strength and better control the dissolution of polymer chains.48 Hydrogels with chemical crosslinks are used for drug delivery applications and form *in situ*. A polymer backbone that contains appropriate functional groups is required to chemically crosslink to form hydrogels. Functional groups can react to form chemical crosslinks by small molecules, click reactions, and radical polymerization.
Small molecules that are bifunctional have the potential to be used as chemical crosslinkers. Zan et al. used glutaraldehyde (GLD) as a chemical crosslinker for a hybrid polymer hydrogel network composed of chitosan and poly(vinyl alcohol) (PVA). Chitosan contains many accessible amine groups that can be used for chemical crosslinking. GLD can form two Schiff bases with amino groups of the chitosan chains. A mechanically resilient material was formed at body temperature (37 °C) with 10 minutes by the reaction between chitosan and GLD. The gelation time and mechanical strength could be modulated by changing the GLD concentration and subsequently the degree of crosslinking. Drug release was demonstrated using lysozyme and drug release times could be extended from 75% in 10 d (33 µM) to 50% in 30 d by increasing the crosslinker concentration to 100 µM. Small molecule crosslinkers can form hydrogels with desirable mechanical and drug release properties but GLD and other agents have been shown to be toxic. The choice of small molecule crosslinking agents that have been proven safe are limited. However, genipin is an extract from the gardenia fruit and has been shown to be a safer alternative estimated to be 5,000 – 10,000 times less cytotoxic than GLD. Injectable hydrogels using genipin as the crosslinking agent have been prepared with chitosan grafted with PEG by Bhattarai et al. PEG-graft-chitosan was prepared by converting PEG to PEG-aldehyde by oxidation with dimethyl sulfoxide (DMSO)/ acetic anhydride and subsequently grafting the PEG to chitosan by Schiff base formation. BSA (1 wt%) was used a model protein to measure in vitro release with 1 – 3% polymer hydrogels and 0.5 mM genipin. Comparison of the genipin-added and genipin-free formulations showed that the presence of genipin increased the release time from 5 h to 40 d.

Click reactions are rapid, versatile, highly specific, regiospecific, and occur with mild conditions that usually yield a single product. The Diels-Alder (DA) reaction between an alkene and a diene is one example that meets these criteria and has been used extensively for hydrogel
formation. Yu et al. used a DA reaction to crosslink hyaluronic acid (HA) and PEG for cell encapsulation and delivery for repair to articular cartilage.\textsuperscript{54} A furyl group was grafted to HA polymer chains and the degree of substitution was used to control the degree of crosslinking with PEG flanked by maleimide groups (mal-PEG-mal). The gelation time was modulated between 50 – 410 minutes depending on the ratios of materials used. Hydrogels with high elasticity and fatigue resistant were achieved (loading up to 200 shear cycles). DA reactions for hydrogel formation may be useful for some cell and drug delivery applications and do not require a catalyst. However, long reaction times limit their use because drug may diffuse from the formulation before crosslinking reaches a level to effectively encapsulate the payload.

A Michael addition is a nucleophilic addition reaction of a nucleophile to an $\alpha,\beta$-unsaturated carbonyl compound. Reactions between thiols and maleimides or methacrylates have been extensively employed in bioconjugate and hydrogel chemistry.\textsuperscript{48} For example, Elbert et al. used the reactions between PEG-thiols and PEG-acrylates to form hydrogels.\textsuperscript{55} The reaction time was less than one minute to reach a gel at 37 °C for their initial formulation but could be increased to about six minutes by changing the PEG-acrylate concentration, making them suitable for in situ forming gels. The authors investigated the release of protein cargo from the hydrogels and found that it occurred within 12 d. The authors claimed that a hydrophilic hydrogel should reduce the amount of protein denaturation compared to hydrogels prepared from PLGA due to the limited hydrophobic protein adsorption sites.

Hydrogel formation by radical polymerization involves the decomposition of an initiator to form free radicals by either redox reactions, light or temperature.\textsuperscript{48} Functionalized polymer chains react with free radicals and lead to hydrogel network formation. Methacrylate and acrylate
moieties can be used to crosslink polymers by exposure to UV light or redox initiators. Dai et al.\textsuperscript{56} studied both photo and redox initiated crosslinking between acrylate-capped PLA-PEG-PLA copolymers where the PEG block ranged from 10 to 20 Kg/mol. The photoinitiator used was α,α-dimethoxy-a-phenylacetophenone. For photopolymerization to proceed, exposure to UV-light (365 nm, 50mW/cm\textsuperscript{2}) for 40 s in a mold was required. Ferrous gluconate/t-butyl hydroperoxide was used as the redox initiator. The redox polymerization occurred within seconds of adding the initiator. The compressive moduli were used as indicators of the mechanical strengths of the hydrogels prepared by two different initiating systems and were found to be the same. For both initiation methods, a critical initiation concentration initiator was determined, and for those samples prepared above this concentration, no increase in mechanical strength was measured (6.20 and 1.94 mM for redox and photo initiators, respectively). Photoinitiated hydrogels have practical complications for \textit{in situ} forming gels since the UV light must penetrate the tissue depth. However, Ono et al.\textsuperscript{57} showed that this type of hydrogel system is well suited for use in biological adhesive applications.

Using more efficient redox initiator systems help mitigate the associated toxicity of conventional options. Macromers can be crosslinked \textit{in situ} in the presence of ammonium or potassium persulfate (APS, KPS) and \textit{N,N,N',N'-Tetramethylethlenediamine} (TEMED) as initiator and catalyst respectively. The polymerization is initiated by the free radicals generated on TEMED molecules and can produce hydrogels with relatively high homogeneity with varying crosslink densities. The tertiary amine reacts immediately with peroxysulfate to generate a free radical that initiates vinyl polymerization.\textsuperscript{58} Ching et al. used dextran functionalized with 2-hydroxyethyl methacrylate to prepare \textit{in situ} forming gels.\textsuperscript{59} They showed that the mechanical properties could be modulated by controlling the amount of KPS added. Further, Hennink et al.
used this technique for the release of model proteins (lysozyme, IgG, and albumin) from a similar glycidyl methacrylate derivatized dextran. They found that the protein release time could be manipulated by the hydrogel polymer concentration, and crosslink density up to 40 d. With the addition of dextranase, release rates were further manipulated.

1.2 Intra-articular drug delivery systems

Hydrogels are particularly attractive for the potential localized treatment of joint conditions. Osteoarthritis (OA) is caused by progressive deterioration of articular cartilage. The biochemical mechanisms of OA are not yet fully understood, and no curative treatment is available. All currently available treatments are focused on the treatment of OA symptoms such as pain and inflammation. NSAIDs are commonly used to alleviate symptoms but have low bioavailability. As a result, they must be administered at high doses to reach therapeutic levels in the joint when taken orally, which leads to cardiovascular and gastrointestinal problems. Intra-articular (IA) injection, meaning direct injection into the joint cavity is a promising alternative to circumvent the systemic toxicity of oral administration and the low bioavailability of common NSAIDS. Limitations exist for IA injections of free drugs, which most notably include the rapid efflux of drug from the joint cavity in addition to the abrasive nature of poorly water-soluble NSAIDS (Figure 4). For these reasons, DDSs are required for IA injection. Significant challenges exist in designing DDSs for IA delivery due to the anatomy of the joint, where the joint cavity is covered by a fibrous capsule filled with synovial fluid (SF). The SF permeates joint cartilage to shuttle nutrients and oxygen. SF permeation contributes to a leaky vasculature that gives delivered drugs a short residence time and a rapid breakdown of DDS formulation.
Hyaluronic acid (HA) is a viscoelastic glycosaminoglycan and is widely used to treat OA of the knee by articular injection. This treatment is not covered by Ontario Health Insurance Plan (OHIP) and costs approximately $130 per injection. There are several formulations commercially available as viscosupplementation agents. The evidence for the efficacy of HA is conflicting. However, Arrich et al. have reported that there is no demonstrated clinical effectiveness of HA injections alone and they should not be used for OA treatment due to risks of adverse events. However, Park et al. used HA as a DDS for the delivery of piroxicam and showed the formulation to reduce OA experimentally induced swelling up to 40%. There was a synergistic effect of the formulation, showing a combination of HA:piroxicam ratio of 1:1 had greater effect than either one individually. IA injections of corticosteroids (CCSs) are also commonly used for pain associated with OA. Unlike HA, CCS injections have been proven effective and shown to reduce pain by 22% over placebo within the first week. Unfortunately, after week one, CCSs do not appear
to have a long term effect.\textsuperscript{67} \textit{In situ} forming hydrogels have recently been investigated for OA treatment. Brucine is a toxic, yet potentially useful compound for the treatment of joint diseases through its ability to act as a pain killer and anti-inflammatory. Chen \textit{et al.}\textsuperscript{68} prepared brucine-loaded microspheres and suspended them in a 20wt\% chitosan thermally responsive hydrogels. A 7 d sustained release was observed in rats. PCLA-PEG-PCLA triblock copolymer hydrogel DDS have been investigated for IA delivery \textit{in vivo} in horses.\textsuperscript{69} Cokelaere \textit{et al.} reported the use of formulations containing 4 and 12 wt\% CXB to achieve elevated levels in synovial fluid for up to 30 d. Unfortunately, they observed no anti-inflammatory effects from lipopolysaccharide induced synovitis.

1.3 Mechanical properties for IA drug delivery

Hydrogels are viscoelastic materials that have both solid and liquid characteristics. An important physical property of viscoelastic materials is the dynamic modulus, which is the ratio of stress to strain under a set of testing parameters. Stress is the force exerted and strain is the displacement measured. Materials can experience physical stress in several ways including shear, compression, indentation, tension, and bending. Mechanical properties can influence the drug release behaviour, specifically in the environment of the joint cavity, which experiences large and repetitive stress. Hydrogels that are used for drug delivery have physical properties that should be tuned according to the tissue to which they will be delivered, so the high stresses that will be subjected to a material in the joint cavity need to be considered in the design of the material.
1.3.1 Syneresis

Syneresis is the spontaneous release of water or solvent from a gel. This phenomenon is an undesirable trait that is a practical disadvantage to implementation because it is accompanied by hydrogel shrinking. DDSs designed for IA delivery should experience limited syneresis in order to fill the joint cavity and maintain their size and shape. For hydrogels composed of a micelle network, syneresis can lead to loss of mechanical strength. This can lead to more rapid hydrogel erosion and consequently drug release. It is understood that thermo-responsive hydrogels are dynamic systems. However, shrinking and syneresis lead to further practical complications when performing mechanical testing. For instance, phase separation during rheological testing produces an upper film of water on top of the sample that causes the plates to slip giving erroneous measurements.

1.3.2 Rheology

Rheology is the study of the flow of matter and is useful in characterizing materials for DDSs. Rheology is appropriate for characterizing hydrogels because it is fast, sensitive and requires small sample sizes. The information acquired can be used to understand differences in hydrogel
network architecture as well as specific gelation behaviours in the case of in situ forming gels. For rheological measurements, a sample is placed on a lower flat plate and sandwiched by an upper plate, which can have different geometries, such as a parallel plate or cone. Shear stress is applied to the sample when torsional oscillation is applied (Figure 5B).

In the linear viscoelastic regime, where the data collected is independent of the amplitude and the frequency of the stress, the data can be used to understand important aspects of gelation behaviour and structure. Biomaterials like hydrogels are rarely purely solid or purely fluid. Rather, they possess both solid and fluid properties and are consequently viscoelastic. The dynamic moduli collected are the measure of the energy stored (storage modulus, $G’$) and the energy dissipated (loss modulus, $G”$). More energy is stored when the material is solid, which allows it to recover to its original form when the force is removed and has a high $G’$ value. More energy is dissipated when the material is fluid, which gives a high $G”$. Small amplitude oscillatory shear (SAOS) experiments are useful to collect information about changing systems. Unlike experiments with continuous rotational shear, SAOS can collect information quickly as a function of time. Using frequency and amplitude parameters in the linear viscoelastic regime, gelation behaviour can then be studied by varying the temperature. For most thermo-responsive hydrogels, the material is fluid dominant at low temperatures (e.g., 4 °C), and $G’’ > G’$. At higher temperatures (e.g., 37 °C), gelation results in the material becoming solid-dominant, and $G’ > G”$. The cross-over temperature, where $G’ = G”$, is considered the gelation temperature under a given set of conditions (e.g., polymer concentration) and rheology parameters (i.e., frequency and amplitude).
1.3.3 Compression testing

Hydrogels injected into the joint experience high compressive forces. Therefore, the measurement of compressive strength is an important consideration in the design and testing of IA DDSs. A universal testing machine (UTM) is the most common instrument for compressive testing but can perform a variety of tests. Most often, compressive tests are performed uniaxially, in that the stress and strain are generated along a single axis (Figure 5A). UTMs can range significantly in size and capabilities from those used for civil engineering applications (mega-Newton) to those relevant here for testing biomaterials (milli-Newton). During compressive testing, the normal force ($F_n$) and gap height are measured. The compressive stress is calculated as from $F_n$/cross-sectional area of the sample. Compressive strain is calculated as vertical displacement/sample height.
1.4 Thesis Scope

The goal of this thesis was the development of an IA delivery system capable of encapsulating drugs and releasing them over a period of at least 30 d, in order to minimize the need for frequent injections. While the target system could potentially be applied to new OA therapeutics, the initial goal was to deliver NSAIDs, as they are commonly used to treat OA symptoms and could benefit from a localized delivery strategy. Based on prior work, a thermo-responsive PCLA-PEG-PCLA triblock copolymer system was selected and the incorporation of drugs into the gels was investigated. Early in this research, we observed that the loading of different drugs had a significant effect on gelation, prompting further exploration of this phenomenon, and attempts to minimize drug incorporation effects through tuning of the length of the PEG blocks. Thus, Chapter 2 explores the effect of PEG molar mass and the incorporation of different drugs on the properties of thermo-responsive PCLA-PEG-PCLA hydrogels. Chapter 3 will describe the use of chemical crosslinking to improve the mechanical properties of in situ forming hydrogels with incorporated drugs. The release of CXB from the hydrogels both in vitro and in vivo in horses is also described. Finally, Chapter 4 describes the conclusions of the thesis work and suggestions for future research.
1.5 References


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2  Effect of Drug Loading on the Properties of Temperature-responsive Polyester-poly(ethylene glycol)-polyester Hydrogels

2.1  Introduction

Drug delivery systems combine engineered technology with traditional methods of drug administration. These systems can improve drug solubility, tissue targeting, patient compliance, provide sustained release, and reduce side effects.\textsuperscript{1, 2} Strategies implementing biodegradable polymers have attracted attention due to the ease with which polymer properties can be tuned.\textsuperscript{3} Using polymer-based delivery systems in various forms, researchers have sought to provide sustained release of drugs to local tissues while at the same time minimizing plasma drug concentrations.\textsuperscript{4} Hydrogels, nanoparticles, microparticles, liposomes, polymer assemblies, and micelles have been investigated and in many cases can be directly injected to the targeted tissues.\textsuperscript{5-8}

Hydrogels are three-dimensional polymer networks capable of absorbing and retaining large amounts of water.\textsuperscript{9} Their high water content gives them mechanical properties that can mimic biological tissues, making them excellent candidates for drug delivery applications.\textsuperscript{10} Temperature-responsive hydrogels are viscoelastic liquids at one temperature, and exist as hydrogels at another temperature.\textsuperscript{11-13} In many cases, temperature-responsive gels are formed from amphiphilic block copolymers that self-assemble into micellar morphologies.\textsuperscript{14, 15} At increased temperatures, self-assembly of the micelles results in the formation of non-covalent networks and consequently gelation.
The poly(ethylene glycol)(PEG)-block-poly(propylene glycol)-block-PEG triblock copolymer poloxamer 407 is an example of a polymer that gels at a particular temperature. At 20 wt% in aqueous solution and below room temperature (37 °C), it is a transparent gel. Formulations composed of poloxamer 407 and the anticancer drug paclitaxel were investigated for drug delivery via direct injection to esophageal carcinoma tumors. The drug release period was less than 4 d, limiting this system to short-duration therapy applications. Poloxamers also suffer from undesirable rapid surface dissolution and poor biodegradability, meaning they dissolve in vivo but do not break down. Biodegradable thermo-sensitive polymers have also been investigated. For example, systems based on PEG-block-poly(L-lactic acid)(PLA)-block-PEG triblock copolymers were shown to exist as viscoelastic liquids at 45 °C and as gels at 37 °C. When glycolic acid was incorporated into the copolymer as PEG-(D,L-lactic acid-co-glycolic acid)-PEG, the formulations were viscoelastic liquids at room temperature and transparent gels at 37 °C. These hydrogels showed superior release kinetics compared to poloxamers when tested in vitro using ketoprofen as a hydrophilic drug and spironolactone as a hydrophobic drug. Structurally related acyl-capped poly(ε-caprolactone-co-lactide)(PCLA)-PEG-PCLA triblock copolymers have since been shown to have improved drug release and degradation kinetics.

Temperature-responsive hydrogels are particularly attractive for the localized treatment of joint conditions such as osteoarthritis, as they can be injected into the joint and undergo gelation without any additional chemical additives or steps. While no curative treatment for the underlying molecular mechanism of osteoarthritis exists, anti-inflammatory drugs such as celecoxib (CXB) have been used to alleviate symptoms. Unfortunately, these drugs can have adverse cardiovascular and other side effects when taken orally in high doses or for extended periods.
into the joint is a promising alternative to circumvent the systemic toxicity of oral administration, but a delivery system is needed to address challenges such as the abrasive nature of poorly soluble drugs and their rapid efflux from the joint cavity.\textsuperscript{28}

Many different thermo-responsive gels have been investigated for use in biomedical applications including cellulose derivatives,\textsuperscript{29} chitosan and glycerophosphate,\textsuperscript{30} and poly($N$-isopropylacrylamide) copolymers\textsuperscript{31, 32} as well as those mentioned above. Only a small number have been investigated for joint conditions, however, including chitosan\textsuperscript{33} and PCLA-PEG-PCLA.\textsuperscript{34} One consideration is that network formation occurs entirely through non-covalent interactions and their disruption can lead to early disintegration of the gel in the body and/or compromised mechanical properties.\textsuperscript{35} The mechanical properties of thermo-responsive hydrogels have been reported extensively, but in most cases, the effect of drug incorporation on the hydrogel properties has not been investigated. The few studies that have looked into the effects of drug loading on hydrogel properties have revealed mixed results.\textsuperscript{36, 37} Given the importance of maintaining the gel state to control drug release, particularly in the load-bearing and shear intensive joint environment, it is critical to understand and optimize the effects of drug incorporation on thermo-responsive hydrogels.

Here we report the preparation and characterization of three different acetyl-capped PCLA-PEG-PCLA triblock copolymers containing PEG molar masses of 1500, 2000 and 3000 g mol\textsuperscript{-1}. We describe the effect of PEG chain length and CXB loading on the temperature-responsive gelation, rheology and \textit{in vitro} drug release rate. CXB was selected as the initial drug for study as it has previously been incorporated into PCLA-PEG-PCLA hydrogels.\textsuperscript{38} Subsequently, using the triblock copolymer prepared with 2000 g mol\textsuperscript{-1} PEG, we investigate the effects of a range of incorporated drugs, including CXB, phenylbutazone (PHE), methotrexate (MTX), ibuprofen
(IBU), diclofenac (DCLO), and etodolac (ETO), on the syneresis, rheology, and compressive moduli of the gels.

2.2 Experimental

2.2.1 General materials and procedures

ε-Caprolactone (CL), L-lactide (LA), and sodium chloride were obtained from Alfa Aesar (USA). Pentane, ethyl ether, CH₂Cl₂, sodium phosphate monobasic and sodium phosphate dibasic were obtained from Caledon (Canada). Acetonitrile was from VWR Analytical (USA). Toluene was obtained from Caledon Laboratories, dried using an Innovative Technologies Inc. solvent purification system, collected under vacuum, and stored under a N₂ atmosphere over 4 Å molecular sieves. Water used to prepare buffer solution was obtained from a Barnstead Easypure II system and had a measured resistivity of 15 MΩcm or greater. All other chemicals were obtained from Sigma Aldrich (USA) and were used as received. ¹H NMR spectroscopy was performed using a 400 MHz Bruker AvIII HD NMR instrument. Spectra were obtained in CDCl₃. Chemical shifts (δ) were referenced to the residual solvent signal of CDCl₃ (δ 7.26 ppm) and are expressed as part per million (ppm). Size exclusion chromatography (SEC) experiments were performed on an instrument equipped with Viscotek GPC Max VE2001 solvent module, Viscotek VE3580 RI detector operating at 30 °C, and a Malvern 270 Dual detector. Samples were dissolved in tetrahydrofuran (THF) at 5 mg mL⁻¹, passed through 0.22 µm syringe filters, and injected with a 100 µL loop. The THF eluent was run at 1 mL min⁻¹. Number average molar mass (Mₙ) and dispersity (Đ) values were determined relative to poly(methyl methacrylate) (PMMA) standards.
2.2.2 Synthesis of PCLA-PEG\textsubscript{1500}-PCLA (1.5k)

The synthesis was performed as previously reported.\textsuperscript{34} In a 100 mL three-neck round bottom flask fitted with a Dean-Stark trap and condenser, PEG\textsubscript{1500} (8.0 g, 5.3 mmol, 1.0 equiv.), LA (3.1 g, 21.5 mmol, 4.0 equiv.), CL (10 g, 88 mmol, 16 equiv.) and anhydrous toluene (40 mL) were combined, heated to reflux (130 °C) under an Ar atmosphere, and stirred. 15 mL of toluene/water mixture was removed azeotropically. The reaction flask was then cooled to 114 °C and tin(II) ethyl hexanoate (30 µL, 90 µmol, 0.02 equiv.) was added into the reaction mixture. Ring-opening polymerization proceeded at 114 °C for 16 h. Next, the triblock copolymer was capped by adding NEt\textsubscript{3} (9.0 mL, 89 mmol, 22 equiv.) and acetic anhydride (5.2 mL, 68 mmol, 13 equiv.) by syringe and acylation proceeded for 4 h. Upon completion, the reaction mixture was cooled to 70 °C and precipitated in pentane while stirring rapidly. After storage at −20 °C for 30 min, a waxy solid was isolated by decanting the solvent. The resulting polymer was dried by heating at 50 °C for 18 h under ambient atmosphere. Yield = 21 g, 95%; \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): δ 5.19–5.03 (m, 8.5 H), 4.35–4.24 (m, 2.9 H) 4.23–4.19 (m, 4.0 H), 4.16–4.09 (m, 8.0 H), 4.05 (t, \textsuperscript{3}J\textsubscript{HH} = 6.63 Hz, 26.9 H), 3.63 (s, 132 H), 2.46–2.32 (m, 14.6 H), 2.31 (t, \textsuperscript{3}J\textsubscript{HH} = 7.46 Hz, 29.4 H), 2.13 (s, 2.7 H), 2.04 (s, 3.0 H), 1.72–1.58 (m, 71.2 H), 1.58–1.54 (m, 27.9 H), 145- 1.32 (m, 37.8). SEC: M\textsubscript{n} = 5900 g mol\textsuperscript{-1}, \textit{D} = 1.30.

2.2.3 Synthesis of PLCA-PEG\textsubscript{2000}-PCLA (2k)

The polymer was synthesized by the same procedure as for 1.5k except that 2000 g mol\textsuperscript{-1} PEG was used as the macroinitiator. The amounts were: PEG\textsubscript{2000} (8.0 g, 4.0 mmol, 1.0 equiv.), LA (3.1 g, 22 mmol, 5.4 equiv.), CL (7.1 g, 62 mmol, 16 equiv.) and anhydrous toluene (40 mL). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): δ 5.19–5.03 (m, 11.4 H), 4.35–4.24 (m, 2.2 H), 4.23–4.19 (m, 2.3 H), 4.16–4.09 (m, 10.5 H), 4.06 (t, \textsuperscript{3}J\textsubscript{HH} = 6.63 Hz, 21.5 H), 3.62 (s, 176.0 H), 2.46–2.32 (m, 11.2 H), 2.31
(t, 3\text{J}_{HH} = 7.67 \text{ Hz}, 20.8 \text{ H}), 2.13 (s, 4.0 H), 2.04 (s, 1.7 H), 1.72–1.58 (m, 60.0 H), 1.58–1.54 (m, 40.7 H), 1.44–1.32 (m, 32.4 H). SEC: $M_n = 7900 \text{ g mol}^{-1}$, $D = 1.22$.

2.2.4 Synthesis of PLCA-PEG$_{3000}$-PCLA (3k)

The polymer was synthesized by the same procedure as for 1.5k except that 3000 g/mol PEG was used as the macroinitiator. The amounts were: PEG$_{3000}$ (12 g, 4.0 mmol, 1.0 equiv.), LA (2.8 g, 19 mmol, 4.9 equiv.), CL (10 g, 88 mmol, 22 equiv.) and anhydrous toluene (40 mL). $^1$H NMR (400 MHz, CDCl$_3$): δ 5.20–5.03 (m, 9.1 H), 4.35–4.24 (m, 2.4 H) 4.23–4.19 (m, 2.1 H), 4.16–4.09 (m, 6.8 H), 4.06 (t, 3\text{J}_{HH} = 6.67 \text{ Hz}, 24.0 H), 3.63 (s, 264.9 H), 2.46–2.32 (m, 8.4 H), 2.29 (t, 3\text{J}_{HH} = 7.67 \text{ Hz}, 23.0 H), 2.12 (s, 3.5 H), 2.04 (s, 2.5 H), 1.72–1.58 (m, 59.3 H), 1.58–1.54 (m, 32.1 H), 1.44–1.32 (m, 31.2 H). SEC: $M_n = 8800 \text{ g mol}^{-1}$, $D = 1.10$.

2.2.5 Hydrogel Preparation

Phosphate-buffered saline (PBS) was prepared from ultra-pure water, 20 mM Na$_2$HPO$_4$, 5 mM NaH$_2$PO$_4$ and 120 mM NaCl. The pH was adjusted to 7.4 with concentrated NaOH. Hydrogels were prepared by weighing molten polymer into glass vials, and adding 4 °C PBS to provide polymer concentrations of 5, 10, 15, 20, or 25 wt%. Immediately, before allowing the molten polymer to cool upon contact with cold PBS, the materials were vortexed vigorously for 20 s and then stored at 4 °C for 4 d. After 1 d of incubation, vials were placed in a 37 °C oven for 30 min to facilitate further dissolution and then returned to 4 °C. Drug-loaded gels were prepared by adding powdered drug to previously prepared solutions at 4 °C to yield 5 or 10 wt% drug and then vortexing. For example, 1.0 g of CXB was added to 9.0 g of hydrogel formulation to provide 10 wt% CXB.
2.2.6 Rheometry

Rheometry was performed on an Anton Paar MCR 302 rotational shear rheometer with a 50 mm diameter parallel plate geometry. The instrument was equipped with a Peltier plate to control temperature to ± 0.01 °C and a hood with solvent trap to assist in temperature control and to limit evaporation and dehydration of samples. 280 grit waterproof emery paper was glued to the upper and lower plates of the measurement geometry to limit wall slip. 2.2 mL liquid samples were pipetted onto the rheometer stage, which was precooled to 4 °C, and the gap between the plates was set to 1 mm. The viscous and elastic moduli, $G''$ and $G'$ respectively, were determined by small-amplitude oscillatory shear measurements at a fixed strain amplitude of 1%, and a fixed frequency of 1 Hz, as a function of temperature. We confirmed that, at this amplitude and frequency, the oscillations were in the linear viscoelastic regime (Figures S6-S8). The temperature was increased at a constant rate of 1 °C min$^{-1}$ from 4 to 37 °C, after which the temperature was held at 37 °C for 30 min to allow for relaxation of the polymer chains and to investigate post-gelation changes in the materials. The normal force on the rheometer tool was fixed at 10 mN to ensure that contact with the sample was maintained as the temperature varied; as a result, the tool gap varied slightly with temperature. This was accounted for in our data analysis. In this work, the gel point was taken to be the temperature at which $G'$ (1 Hz) = $G''$ (1 Hz). The experiments were performed in triplicate.

2.2.7 In Vitro CXB Release

CXB-loaded hydrogels were prepared as described above. 1.0 g of CXB-loaded hydrogel was weighed into a 1 cm diameter x 1 cm height cylindrical cup and placed in a 37 °C oven for 30 min to form a gel. The gel contained in the cup was lowered into 100 mL of pre-heated (37 °C) 2 mg mL$^{-1}$ polysorbate 80 in pH 7.4 PBS (solvent release medium). The free drug samples were weighed
and contained in nylon mesh bags (2.5 cm x 4 cm, 400 mesh) and sealed shut with dialysis tubing clips. 100 mL samples of the solvent release medium were taken at regular time intervals and were replaced with fresh solvent release medium until 22 d. The CXB concentration in the solvent release medium was measured on a Varian Cary 300 Bio UV-Vis Spectrophotometer at 253 nm based on an extinction coefficient of $1.65 \times 10^4$ L·mol$^{-1}$·cm$^{-1}$ for CXB in the same medium. The experiments were performed in triplicate.

2.2.8 Syneresis Measurements

Syneresis (water loss) was measured gravimetrically as previously described.\textsuperscript{39} Approximately 1 g of drug-free or drug-loaded 15% 2k hydrogel was placed into a 3 mL screwcap vial and the vial with gel was accurately weighed. The sealed vials were placed in a 37 °C oven. The vials were periodically removed from the oven, unsealed, inverted onto a paper towel and let sit for 1 min before being weighed, resealed and placed back in the oven. The mass loss was calculated. The experiments were performed in triplicate.

2.2.9 Compression Testing

The compression moduli of drug-free 15% 2k hydrogels and hydrogels loaded with 5 wt% CXB, PHE, and MTX were measured by compression testing. Formulations were gelled in 6 mL, 1.92 cm inside diameter syringes at 37 °C for 30 min and then cut into 6.5 mm thick cylindrical slices. Unconfined stress-strain measurements were performed on these samples using a Univert mechanical tester (CellScale, Guelph, ON, Canada), equipped with a 0.5 N load cell. Samples were immersed in a 37 °C water bath and preloaded with 0.01 N. Uniaxial compression was applied at a constant rate of 4% s$^{-1}$ (relative to the initial height) to a total strain of 40%. The nominal stress was calculated by dividing the applied force by the original cross-sectional area of the sample. The
secant modulus, reported as compressive modulus, was calculated as the slope of the stress-strain curve between 5 and 20% strain. Triplicate samples were measured.

2.3 Results and Discussion

2.3.1 Synthesis and Characterization of PCLA-PEG-PCLA Triblock Copolymers

The properties of thermo-responsive hydrogels based on PCLA-PEG-PCLA can be tuned according to the chemical structures of the copolymers.\textsuperscript{40,41} For example, LA/CL monomer ratios and the molar masses of the blocks can be tuned. In the current work, we synthesized PCLA-PEG-PCLA triblock copolymers containing 1500 (PEG\textsubscript{1500}), 2000 (PEG\textsubscript{2000}), and 3000 (PEG\textsubscript{3000}) g mol\textsuperscript{-1} PEG. PCLA-PEG\textsubscript{1500}-PCLA (labeled as 1.5k) was synthesized as previously described.\textsuperscript{34} Briefly, ring-opening copolymerization of LA and CL in toluene was performed using PEG\textsubscript{1500}-diol as the macroinitiator and tin(II) 2-ethylhexanoate [Sn(Oct)\textsubscript{2}] as the catalyst (Scheme 1). The resulting polymers were end-capped with acetyl end groups using an excess of acetic anhydride. The new PCLA-PEG\textsubscript{2000}-PCLA (2k) and PCLA-PEG\textsubscript{3000}-PCLA (3k) copolymers were synthesized under similar conditions, starting from PEG\textsubscript{2000}-diol and PEG\textsubscript{3000}-diol respectively. Yields for all polymerizations were >94%.

\textbf{Scheme 2.1.} Synthesis of PCLA-PEG-PCLA triblock copolymers.
The polymers were characterized by $^1$H NMR spectroscopy (Figures S1-S3). The PEG block gave rise to peaks at 3.6 ppm in the spectra. The incorporation of the LA into the PCLA block was confirmed by the observation of resonances corresponding to the backbone methine protons at 5.0 - 5.2 ppm. The CL content was confirmed by the presence of peaks corresponding to the methylene groups adjacent to the ester oxygen at ~4.0 and 4.1 ppm, depending on the neighbouring monomer. The monomer ratios in 1.5k, 2k, and 3k were calculated by comparing the integrals of the peaks of the PEG initiator (3.6 ppm) with those of the CL (4.1 and 4.0 ppm) and LA (5.10 ppm) blocks. The CL/LA mass ratios ranged from 2.22 to 3.25 while the masses of the PCLA blocks for all three polymers were very similar, ranging from 1205 - 1320 g mol$^{-1}$ (2410 - 2640 g mol$^{-1}$ total for the two polyester blocks of each triblock copolymer). The degree of acetylation was calculated using the integrals of terminal methyl protons ($\text{CH}_3$-CO-LA at δ2.13, $\text{CH}_3$-CO-CL at 2.04 ppm, and $\text{CH}_3$-CO-PEG at 2.04–2.06 ppm) relative to that of the PEG peak at 3.6 ppm. The extent of acetylation was ≥ 95% for all three polymers.

**Table 2.1.** Molecular masses and chemical analyses of the polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>PEG (g mol$^{-1}$)</th>
<th>PCLA $^\dagger$ (g mol$^{-1}$)</th>
<th>$M_n^\dagger$ full polymer (g mol$^{-1}$)</th>
<th>CL/LA$^\dagger$ mass ratio</th>
<th>PCLA/PEG$^\dagger$ mass ratio</th>
<th>$M_n^\ddagger$ (g mol$^{-1}$)</th>
<th>$\mathcal{D}^\ddagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5k</td>
<td>1500</td>
<td>2600</td>
<td>4100</td>
<td>3.25</td>
<td>1.73</td>
<td>5900</td>
<td>1.30</td>
</tr>
<tr>
<td>2k</td>
<td>2000</td>
<td>2640</td>
<td>4640</td>
<td>2.22</td>
<td>1.33</td>
<td>7900</td>
<td>1.22</td>
</tr>
<tr>
<td>3k</td>
<td>3000</td>
<td>2410</td>
<td>5410</td>
<td>2.68</td>
<td>0.80</td>
<td>8800</td>
<td>1.10</td>
</tr>
</tbody>
</table>

$^\dagger$Calculated using $^1$H NMR spectroscopy. $^\ddagger$Calculated using SEC.
The polymers were also characterized by SEC relative to PMMA standards (Table 1, Figure S4). The $M_n$ values determined by SEC were higher than those of calculated using the $^1$H NMR spectra, which can likely be attributed to the differences in hydrodynamic volumes of the polymers relative to those of the PMMA standards. Nevertheless, $M_n$ increased with PEG molar mass, as expected. The $D$ values decreased with increasing PEG length as the higher dispersity PCLA blocks represented a decreasing mass fraction of the polymer.

2.3.2 Thermo-responsive Gelation

A suitable thermo-responsive hydrogel formulation for injection into joints is one for which the polymer forms a low-viscosity solution that can easily be drawn into an 18-gauge needle at 4 °C (fridge temperature) and that forms a gel at 37 °C (body temperature). Polymers were added to PBS at 5, 10, 15, 20 and 25 wt% and were first evaluated qualitatively for their ability to dissolve at 4 °C and then to gel at 37 °C. It should be noted that while we refer to the process as dissolution, it is known that the amphiphilic block copolymers are not technically dissolved at the molecular level but actually form micelles. The micelles can be detected by dynamic light scattering but due to their sub-100 nm diameter do not scatter much light. Therefore, the suspensions appear transparent to the naked eye. The vial inversion method was used to differentiate gels from viscous liquids at 37 °C. Vials containing the formulations were brought to 37 °C for 30 min, then inverted and examined after 10 min (Figure S5). The absence of flow was taken to indicate gelation.

At 5 wt% polymer, all the polymers dissolved in PBS, but none gelled at 37 °C (Table 2). At 10 wt%, only the 3k polymer gelled at 37 °C. At 15 wt%, the 2k system exhibited the desired dissolution and gelation behavior, but the 3k polymer no longer dissolved. At 20 wt%, the 1.5k
polymer exhibited the desired behavior, while the 2k polymer no longer fully dissolved. At 25 wt%, even the 1.5k polymer only partially dissolved. Based on these results, polymer concentrations of 20, 15 and 10 wt% for the 1.5k, 2k, and 3k polymers respectively were selected for further study.

Table 2.2. Results of dissolution and vial inversion tests to determine polymer solubility at 4 °C and gelation at 37 °C. “Yes” indicates dissolution or the ability to form gels that did not flow over 10 min

<table>
<thead>
<tr>
<th>Polymer</th>
<th>5 wt% Polymer</th>
<th>10 wt% Polymer</th>
<th>15 wt% Polymer</th>
<th>20 wt% Polymer</th>
<th>25 wt% Polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dissolve at 4 °C</td>
<td>Gel at 37 °C</td>
<td>Dissolve at 4 °C</td>
<td>Gel at 37 °C</td>
<td>Dissolve at 4 °C</td>
</tr>
<tr>
<td>1.5k</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2k</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>3k</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Partially</td>
</tr>
</tbody>
</table>

2.3.3 Rheological Properties of Drug-free and CXB-loaded Hydrogels

Figure 1 shows the elastic and viscous moduli, G’ and G”, of the unloaded and CXB-loaded (10 wt% CXB) hydrogels as a function of temperature T while heating, and as a function of time while T was held at 37 C. Measurements were performed at a frequency of 1 Hz and a strain amplitude of 1%. Table 3 summarizes G’ and G” for these systems at 4 and 37 °C. For the 20% 1.5k
hydrogels at 4 °C, $G''$ was greater than $G'$ as the formulation was a liquid (Figure 1A, Table 3). Both $G'$ and $G''$ increased as the temperature increased and at 37 °C, $G'$ was greater than $G''$, indicative of a gel. The crossover of $G'$ and $G''$ was at 28 °C, indicating gelation. Our results are in reasonable agreement with results of Petit et al.\textsuperscript{24}, who found drug-free acetyl capped PCLA\textsubscript{1700-PEG\textsubscript{1500}-PCLA\textsubscript{1700}} hydrogels to have $G' \sim 360$ Pa at 36 °C in a temperature sweep from 4 – 50 °C.\textsuperscript{24} The gradual increase in $G'$ when $T$ was held constant at 37 °C can be attributed to the time required for micelle aggregation and structural reorganization of the gel (Figure 1B). $G'$ for the drug-loaded 20% 1.5k + 10% CXB hydrogel behaved similarly to that of the 20% 1.5k, but its value at 37 °C was a factor of 10 lower than for the drug-free hydrogel. The $G''$ at 37 °C was also lower for the CXB-loaded hydrogel than in the CXB-free case. It is possible that the CXB incorporation impeded the aggregation of the polymer micelles or polymer chain entanglements, thereby weakening the gels. This would be undesirable for an injectable drug delivery system.
**Figure 2.1.** $G'$ and $G''$ of acetyl-capped PCLA-PEG-PCLA hydrogels during temperature sweep (A, C, E) and temperature hold (37 °C) (B, D, F) experiments performed at 1 Hz and 1% strain amplitude. A-B) 20% 1.5k and 20% 1.5k + 10% CXB; C-D) 15% 2k and 15% 2k + 10% CXB; E-F) 10% 3k and 10% 3k + 10% CXB. Open markers represent drug-free hydrogels and filled markers represent CXB-loaded hydrogels. Error bars represent standard deviations of triplicate samples.
Table 2.3. G’ and G’’ of drug-free and CXB-loaded hydrogels measured at 1 Hz and 1% strain amplitude at 4 and 37 °C. Errors on the measurements correspond to the standard deviations of triplicate samples.

<table>
<thead>
<tr>
<th></th>
<th>4 °C</th>
<th>37 °C (after 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G’ (Pa)</td>
<td>G’’ (Pa)</td>
</tr>
<tr>
<td>20% 1.5k</td>
<td>0.05 ± 0.02</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>20% 1.5k + 10% CXB</td>
<td>0.08 ± 0.04</td>
<td>0.70 ± 0.09</td>
</tr>
<tr>
<td>15% 2k</td>
<td>0.06 ± 0.01</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>15% 2k + 10% CXB</td>
<td>0.53 ± 0.12</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>10% 3k</td>
<td>0.18 ± 0.04</td>
<td>0.80 ± 0.08</td>
</tr>
<tr>
<td>10% 3k + 10% CXB</td>
<td>0.52 ± 0.05</td>
<td>2.27 ± 0.18</td>
</tr>
</tbody>
</table>

Like the 20% 1.5k hydrogels, the 15% 2k hydrogels also had G’’ greater than G’ at 4 °C as they were liquid at this temperature. Both G’ and G’’ began increasing at the onset of the temperature ramp, with a gelation temperature of 27 °C (Figure 1C). G’ reached ~600 Pa after 30 min at 37 °C (Figure 1D), significantly higher than that of the 20% 1.5k hydrogel. Notably, the incorporation of drug in 15% 2k + 10% CXB did not significantly change G’ at 37 °C (Figure 1D), although G’’ increased ~2-fold on drug loading at 37 °C. The material remained dominantly elastic, although weakly so.

Both the 10% 3k and 10% 3k + 10% CXB hydrogels were liquids at 4 °C with G’’ greater than G’ (Figure 1E). G’ and G’’ increased with increasing temperature for both systems with crossover points of 28 °C and 29 °C for the 10% 3k and 10% 3k + 10% CXB hydrogels respectively. 10%
3k + 10% CXB had ~5-fold higher G’ than 10% 3k for the duration of the temperature sweep and after 30 min at 37 °C (Figure 1F). G” was also higher for 10% 3k + 10% CXB than for 10% 3k. Thus, drug loading had a large impact on gelation and viscoelastic properties of the 10% 3k system. In contrast to the 1.5k hydrogels, for which drug loading impeded gelation and resulted in lower moduli, both the elastic and viscous moduli of the 3k system increased with drug loading.

Overall, the differences between the 1.5k, 2k, and 3k systems show that even a modest change in the PEG block length can lead to substantial differences in the effects of drug loading on gel properties. We propose that the gelation in these involves both micelle aggregation and bridging of micelles by polymer chains (Figure 2).43 One would expect less micelle bridging for shorter PEG chains, such as in the 1.5k hydrogel, and in this case drug incorporation may disrupt micelle aggregation, resulting in lower viscous and elastic moduli (Figure 2E). In contrast, micelle bridging should be more important for the 3k hydrogel. The results suggest that CXB incorporation does not disrupt micelle bridging in this system and may even enhance it. It may be that aggregates of hydrophobic drug that are incorporated into the network provide sites for additional bridging that results in increases to both G’ and G” (Figure 2C). The 2k hydrogel may represent an intermediate case in which competing influences of the drug result in modest overall changes to the properties of the hydrogel.
2.3.4 In Vitro Release of CXB From the Hydrogels

Several parameters can affect in vitro drug release rates in hydrogel systems, including degradability of the hydrogel, pore size, drug solubility, solvent release medium, micellar-drug interactions and drug concentration. In the current study, the release of CXB into a solution containing 2 mg/mL polysorbate 80 (non-ionic surfactant) in PBS was studied for 20% 1.5k + 10% CXB, 15% 2k + 10% CXB, and 10% 3k + 10% CXB hydrogels and compared with the dissolution of unencapsulated solid CXB contained in nylon mesh bags. The polysorbate 80 was
used to increase the solubility of CXB in the solution.\textsuperscript{4, 44} Drug release was detected on day one for all systems (Figure 3), in contrast to the ~10-d lag period observed by Petit \emph{et al.} \textsuperscript{4} However, the authors of that study admitted that the lag period was difficult to explain. The 15\% 2k + 10\% CXB formulation had the slowest release, with 30\% of the CXB released over 22 d. The 20\% 1.5k + 10\% CXB and 10\% 3k + 10\% CXB formulations had similar release rates up to day 10 and at day 22 the total release was 42 and 55\% for 1.5k and 3k respectively. The faster release from the 10\% 3k + 10\% CXB formulation was initially surprising but might be explained by the fact that it is relatively more viscous and less elastic than the other hydrogels. Overall, considering the slow CXB release and modest effect of drug incorporation on the rheological properties, the 15\% 2k hydrogel was deemed most suitable for further studies with different drugs.

\textbf{Figure 2.3.} \emph{In vitro} CXB release into 2 mg/mL polysorbate 80 in PBS solvent release solution. Error bars correspond to the standard deviations on triplicate samples.
2.3.5 Syneresis of 15% 2k Hydrogels Loaded with Different Drugs

While the above work provided an indication of the behavior of the 15% 2k hydrogel upon loading of CXB, we were also interested in its behavior upon the loading of different drugs. Based on interest in applying thermo-responsive hydrogels for the delivery of drugs to joints, several additional molecules, mainly anti-inflammatory drugs including phenylbutazone (PHE), ibuprofen (IBU), diclofenac (DCLO), etodolac (ETO), as well as the immunosuppressant methotrexate (MTX) were selected (Figure 4). First, the hydrogel phase behaviour was investigated. Syneresis is well documented for poly(N-isopropylacrylamide) (PNIPAM)\textsuperscript{45, 46} and poly(vinyl alcohol)\textsuperscript{45} hydrogels. Unfortunately, syneresis is associated with a collapse of the hydrogel, weakening its physical properties. Syneresis was measured by placing known volumes of drug-loaded 15% 2k hydrogel into vials at 37 °C and the released water was removed at time points over 4 h. Five wt% of drug, relative to the total formulation, was selected because this loading would provide a suitable dosage of drug in an appropriate injection volume for the drugs studied.

![Chemical structures of drugs studied.](image)

**Figure 2.4.** Chemical structures of drugs studied.
A wide range of water loss, ranging from ~1 to 39 wt% was observed from the different drug-loaded hydrogels (Figure 5). Etodolac had the highest syneresis, and methotrexate the lowest. MTX, PHE, CXB, and DCLO loaded hydrogels had reduced syneresis relative to drug-free 15% 2k. There were no obvious relationships between the chemical functional groups on the drugs and the degree of syneresis. For example, drugs containing carboxylic acid groups led to both the highest (ETO, IBU) and lowest (MTX) degrees of syneresis. However, there did appear to be a relationship between drug aqueous solubility and syneresis, as MTX and PHE have the highest solubilities (0.45 and 0.70 mg mL\(^{-1}\), respectively)\(^47\) and had the lowest syneresis. ETO and IBU have the lowest aqueous solubilities (0.01 and 0.038 mg mL\(^{-1}\) respectively)\(^47\) and had the highest degrees of syneresis. The addition of highly hydrophobic drugs may result in large drug aggregates that disrupt the hydrogel network structure and lead to macroscopic precipitation.\(^11\) CXB appears to be an exception in this case, since it has low solubility (0.005 mg mL\(^{-1}\)),\(^47\) yet has an intermediate degree of syneresis after 4 h.

**Figure 2.5.** Syneresis (water loss) measured for 15% 2k hydrogels loaded with 5 wt% of different drugs. Error bars correspond to the standard deviations on triplicate samples.
2.3.6 Rheology of 15% 2k Hydrogels Loaded with Different Drugs

CXB, PHE, and MTX-loaded 15% 2k hydrogels were selected for further rheological studies because they showed the lowest degree of syneresis, suggesting that they maintained the most integral hydrogel networks.\textsuperscript{48} For the 15\% 2k + 5\% PHE and 15\% 2k + 5\% CXB hydrogels, $G''$ was higher than $G'$ at 4 °C (Figure 6, Table 4). Both $G'$ and $G''$ increased with temperature and the cross-over temperatures were 25 °C, and 29 °C for the CXB and PHE-loaded hydrogels respectively. The 15\% 2k + 5\% MTX hydrogel did not have a measurable cross-over temperature because $G'$ was higher than $G''$ for the entire temperature range, even at 4 °C (Figure 6C). The $G'$ value at 37 °C was highest for the 15\% 2k + 5\% MTX hydrogel, followed by 15\% 2k + 5\% PHE, and 15\% 2k + 5\% CXB. $G''$ values followed the same trend. Thus, the hydrogels with lower syneresis had higher moduli, consistent with the abilities of some drugs to more effectively enhance network formation (Figure 2). Overall, the 15\% 2k hydrogel networks were strengthened by addition of certain drugs (CXB, PHE, and MTX) that likely acted as nodes for additional linkages but differ in the way energy is transferred; polymer-polymer interactions increase $G'$ (energy is stored) and polymer-drug interactions increase $G''$ (energy is dissipated).
Figure 2.6. G’ and G” of 15% 2k acetyl-capped PCLA-PEG-PCLA hydrogels during temperature sweep (A, C, E) and temperature hold (37 °C) (B, D, F) experiments performed at 1 Hz and 1% strain amplitude. A-B) 15% 2k + 5% CXB; C-D) 15% 2k + 5% PHE; E-F) 15% 2k + 5% MTX. Open circles represent drug-free hydrogels and filled circles represent CXB-loaded hydrogels. Error bars represent standard deviations of triplicate samples.
Table 2.4. G’, G”, and compressive moduli of drug-free and drug-loaded hydrogels measured at 1 Hz and 1% strain amplitude at 4 and 37 °C. Errors on the measurements correspond to the standard deviations of triplicate samples.

<table>
<thead>
<tr>
<th></th>
<th>4 °C</th>
<th>37 °C (after 30 min)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G' (Pa)</td>
<td>G” (Pa)</td>
<td>G’ (Pa)</td>
</tr>
<tr>
<td>15% 2k</td>
<td>0.4 ± 0.3</td>
<td>0.8 ± 0.4</td>
<td>640 ± 110</td>
</tr>
<tr>
<td>15% 2k + 5% CXB</td>
<td>0.5 ± 0.1</td>
<td>1.9 ± 0.4</td>
<td>640 ± 200</td>
</tr>
<tr>
<td>15% 2k + 5% PHE</td>
<td>0.4 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>830 ± 100</td>
</tr>
<tr>
<td>15% 2k + 5% MTX</td>
<td>7 ± 5</td>
<td>5 ± 2</td>
<td>1300 ± 10</td>
</tr>
</tbody>
</table>

2.3.7 Compression Testing of Drug-loaded 15% 2k Hydrogels

Uniaxial unconfined compression testing was performed on drug-free as well as the CXB, PHE, and MTX-loaded 15% 2k hydrogels at 37 °C in water. This information is important when designing delivery systems for joint diseases because the material will experience both shear and compressive forces in situ. The results are plotted as stress-strain curves in Figure 7. The drug-loaded hydrogels had compressive moduli that were roughly a factor of two lower than those of the drug free formulation (Table 4). The compressive moduli for the hydrogels were substantially lower than the shear moduli. These results are consistent with those published by Knapp et al., who observed a reduction in compressive modulus compared to shear modulus in collagen gels.49
Figure 2.7. Compressive stress versus strain for 15% 2k hydrogels with and without drugs incorporated. Representative examples for each system are shown.

2.4 Conclusions

It is important to consider the intended drug delivery application when designing and testing in situ forming hydrogels and to consider the possible effects that drug loading can have on the physical properties of the gels. In this paper, we first investigated the effects of different PEG block lengths on the thermal gelation of acetyl-capped PCLA-PEG-PCLA triblock copolymers. We found that even within a relatively small range of 1500 – 3000 g mol⁻¹, the PEG block length can have a substantial effect on the thermal gelation, leading to more than a 20-fold difference in G’ and ~7-fold differences in G” at 37 °C with CXB incorporated. We attribute this to the differences in the relative extents of micelle aggregation and micelle bridging in these hydrogels formed from polymers with different PEG lengths and varying effects of drug incorporation on these networks. Overall, the 15% 2k hydrogel exhibited the smallest change in G’ upon loading of 10 wt% CXB, and also exhibited the slowest release of CXB in vitro, which are favorable for application in intra-articular drug delivery. Subsequently, the loading of different drugs into the 15% 2k system was investigated and it was found that different drugs led to large differences in
the extent of syneresis of the hydrogels at 37 °C. The different extents of syneresis appear to be related to aqueous drug solubility and therefore may reflect the influence of hydrophobic drug aggregates on the hydrogel network structure. Lower syneresis was associated with higher $G'$ and $G''$ in subsequent rheological studies. Furthermore, drug loading reduced the compressive modulus. Thus, different drugs can have very different effects on gelation and mechanical properties and these changes need to be studied for each drug-hydrogel system, particularly where the physical properties of the gel are important in their application, such as in load-bearing joints. Future work should investigate methods to increase the mechanical strength of drug-loaded hydrogels. Furthermore, additional research is needed to better understand and predict how different drugs will impact mechanical properties of hydrogels.
2.5 References

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3 Covalently crosslinkable thermo-responsive hydrogels for intra-articular drug delivery

3.1 Introduction

Inflammation from chronic diseases is often treated through the oral administration of drugs.\(^1\) For example, nonsteroidal anti-inflammatory drugs (NSAIDS) are commonly used to treat osteo and rheumatoid arthritis, diseases that affect up to 1.5 billion people worldwide and are increasing in prevalence with the aging population.\(^2\) Unfortunately, NSAIDS have been tied to adverse cardiovascular events, along with other side effects including gastrointestinal and kidney problems.\(^3,4\) A large phase IV study was conducted to investigate the cardiovascular toxicity of celecoxib (CXB), naproxen, and ibuprofen in over 24,000 patients.\(^5\) The authors found that 1 in 20 patients experienced major toxicity within 2 years. Consequently, there has been significant interest in using drug delivery systems to locally administer drugs, thereby increasing the drug efficacy at the desired site of action, while reducing the side effects that result from systemic exposure to high concentrations of drugs.\(^6\)

Hydrogels are a class of materials under investigation for localized drug delivery.\(^7\) Hydrogels are crosslinked three-dimensional structures that can be made from many different types of molecules and are characterized by their ability to absorb large amounts of water.\(^7\) Their high water content allows them to mimic tissues in the body, thereby affording an acceptable host response, while their porosity allows for high drug loading and controlled release.\(^1\) It is important for hydrogel drug delivery systems to be designed with specific criteria in mind, such as ease of administration, suitable mechanical properties for the application of interest, the ability to provide sustained drug release, and biodegradation into non-toxic products.\(^8\)
An *in-situ* forming hydrogel is a system that can be injected into the body as a viscoelastic liquid but that transitions into a gel post-injection. *In situ* hydrogels can be separated into two major classes depending on the types of crosslinks: covalent or physical. Covalently crosslinked hydrogels require the addition of chemical modifiers and/or for the polymer chains to be pre-functionalized for covalent linkages to be formed post-injection. Many different covalent crosslinking strategies have been explored to prepare hydrogels for different applications. Horse radish peroxidase and hydrogen peroxide were used to prepare *in situ* forming hydrogels composed of dextran-tyramine conjugates. The authors demonstrated the ability to form stable viscoelastic hydrogels under physiological conditions via enzyme-catalyzed crosslinking. Other approaches that have utilized small molecule crosslinking agents include the reaction of poly(N-isopropylacrylamide)-co-poly(L-lysine) with genipin, and alginate containing phenyl moieties with oxidative coupling reaction via peroxidase and calcium ions.

Although useful, small molecule crosslinkers have the potential to remain unreacted in small amounts and cause toxicity. Strategies that involve pre-functionalized polymers have been developed to eliminate the use of potentially toxic small molecule crosslinking agents. For example, chitosan hydrogels were prepared by Michael reactions between thiolated poly(ethylene glycol) (PEG) and acrylated chitosan. Polysaccharides functionalized with amino and aldehyde groups have been used to prepare *in situ* forming hydrogels by imine formation for the delivery of chondrocytes. Photopolymerization after ultraviolet (UV)-light exposure in the presence of a photoinitiator has been used with acrylate capped PEG and it was shown that the photoinitator (2,2 dimethoxy-2-phenyl acetophenone) used was cytocompatible. Photopolymerization eliminates the risk of early gelation due to high level of temporal control that is achievable using a UV-light source. However, the use of UV light for crosslinking has practical challenges for
applications where the hydrogel is administered into locations such as joints that are not easily accessible to light.

Many physically crosslinked hydrogels are capable of reversible thermally-induced gelation.\textsuperscript{16} The polymers composing thermo-responsive hydrogels are typically amphiphilic and are water soluble/dispersible at low temperatures, but form gels at higher temperatures. The gelation temperature depends on polymer concentration, polymer structure, and the lengths of the hydrophilic and hydrophobic blocks. The amphiphilic nature of the polymer chains, either triblock or diblock, enables the formation of micelles by self-assembly above their critical micelle concentration (CMC). For example, copolymers with the composition PEG-\textit{block}-poly(propylene oxide)-\textit{block}-PEG, commonly referred to as poloxamers have been extensively explored in drug delivery applications.\textsuperscript{17} Poloxomer 407 forms a thermo-reversible gel that has been used to deliver the anti-tumour drug paclitaxel.\textsuperscript{18,19} Over the duration of the study, a 67\% decrease in tumor size was achieved in mice. Although promising, poloxomer hydrogels have relatively short delivery periods as drugs are released quite rapidly from these systems. More sustained drug release can be achieved by increasing the hydrophobicity of the amphiphilic copolymer. For example, the commercially available ReGel\textsuperscript{®} is composed on poly(lactide-\textit{co}-glycolide)(PLGA)-PEG-PLGA. Preclinical trials with paclitaxel for esophageal cancer showed longer release times and high tolerability than the poloxomer system.\textsuperscript{20} However, further clinical investigations showed that survivor rate did not improve with the treatment.\textsuperscript{21} Further modifications have been made to the chemical structure of the ABA block copolymer structures to further increase hydrogel stability and degradation times. For example, poly(caprolactone-\textit{co}-lactide)(PCLA)-PEG-PCLA systems have been used to provide improved gelation, and slow the release of hydrophobic drugs relative to previous systems.\textsuperscript{22} While significant advancements have been made in improving the gelation
of physically crosslinked hydrogels, they were found to have large burst releases of drug in vivo and very weak mechanical properties, which is problematic,\textsuperscript{23} particularly for administration into load-bearing joints.\textsuperscript{23,24}

In the present study, we describe a hybrid gelation system involving both physical and chemical crosslinking, which is designed to provide hydrogels with mechanical properties and drug release profiles suitable for intra-articular drug delivery. The synthesis and characterization of methacrylate capped PCLA-PEG-PCLA triblock copolymers are reported, with the amphiphilicity inducing physical crosslinking at physiological temperatures and the methacrylate groups enabling chemical crosslinking between polymer chains in the presence of a thermally sensitive free radical initiation system [potassium persulfate (KPS)/ \(N,N,N',N'\)-tetramethylethlenediamine (TEMED)] that has previously been shown to be non-toxic and tolerable for drug delivery applications.\textsuperscript{25,26} Characterization of the resulting gels by rheology and compression testing was performed. Furthermore, in vitro drug release of celecoxib (CXB), along with in vivo trials using horses were used to investigate the clinical potential of these formulations. These trials also included the acetyl-capped hydrogel formulation described in Chapter 2 for comparison.\textsuperscript{27}

3.2 Experimental

3.2.1 General materials and procedures

\(\varepsilon\)-Caprolactone (CL), L-lactide (LA), and KPS were obtained from Alfa Aesar (USA). Pentane, ethyl ether, CH\(_2\)Cl\(_2\), PEG, tin (II) octanoate, TEMED and Phenylbutazone (PHE) were obtained from Sigma Aldrich (USA). Meloxicam (MEL) was purchased from Fisher Scientific. Celecoxib (CXB), methotrexate (MTX) and diclofenac (DCLO) were purchased from Ontario Chemicals Inc. Acetonitrile was from VWR Analytical (USA). Toluene was obtained from Caledon Laboratories,
dried using an Innovative Technologies Inc. solvent purification system based on aluminum oxide columns, collected under vacuum, and stored under a N₂ atmosphere over 4 Å molecular sieves. Phosphate buffered saline (PBS) powder packs were purchased from Sigma Life Science and were used to prepare the pH 7.4 PBS solutions from according to the manufacturer’s instructions. Water used to prepare the buffer solution was obtained from Barnstead Easypure II system with a measured resistivity of 15 MΩ or greater. All other chemicals were used as received. ¹H NMR spectroscopy was performed using a 400 MHz Bruker AvIII HD NMR instrument. Spectra were obtained in CDCl₃. Chemical shifts (δ) were referenced to the residual solvent signal of CHCl₃ in CDCl₃ (δ 7.26 ppm) and are expressed as part per million (ppm). SEC experiments were performed on an instrument equipped with Viscotek GPC Max VE2001 solvent module, Viscotek VE3580 RI detector operating at 30 °C, and a Malvern 270 Dual detector. Samples were dissolved in tetrahydrofuran (THF) at 5 mg/mL, passed through 0.22 µm syringe filters, and injected with a 100 µL loop. The eluent, filtered THF, was run at 1 mL/min. Number average molar mass (Mₙ) and dispersity (Đ) values were determined relative to poly(methyl methacrylate) (PMMA) standards. Sonication was performed using a Fisher Scientific FS20H immersion system.

3.2.2 Synthesis of MA-PCLA-PEG₁₅₀₀-PCLA-MA (1.5k₃₁₅₀-MA)

This polymer was synthesized by a modification of previously reported procedures.²⁷ All glassware was depyrogenized by placing in 265 °C oven for at least 16 h. In a 100 mL three-neck round bottom flask fitted with a Dean-Stark trap and condenser, PEG₁₅₀₀ (8.0 g, 8.0 mmol, 1.0 equiv.), LA (2.9 g, 20 mmol, 2.5 equiv.), CL (9.0 g, 79 mmol, 9.9 equiv.) and anhydrous toluene (40 mL) were combined, and heated to reflux (130 °C) under an Ar atmosphere with stirring. 15 mL of toluene/water mixture was removed azeotropically. The reaction flask was then cooled to 114 °C and tin(II) ethyl hexanoate (30 µL, 90 µmol, 0.02 equiv.) was added into the reaction
mixture. Ring-opening polymerization proceeded at 114 °C for 16 h. Next, the resulting triblock copolymer was capped by adding NEt3 (9.0 mL, 89 mmol, 11 equiv.) and methacrylic anhydride (5.2 mL, 34 mmol, 4.2 equiv.) by syringe and acylation proceeded for 4 h. The reaction mixture was then cooled to 70 °C and precipitated into pentane while stirring rapidly. After storage at -20 °C for 30 min, a waxy solid was isolated by decanting the solvent. The resulting polymer was dried by heating at 40 °C for at least 18 h under ambient atmosphere. Yield = 12.3 g, 77%.

\[ {^1}H \text{ NMR (400 MHz, CDCl}_3\text{): } \delta 6.29–6.27 (m, 0.3 H), 6.21–6.18 (m, 0.9 H), 6.13–6.12 (m, 0.1 H), 6.10–6.07 (m, 0.6 H), 5.71–5.69 (m, 0.3 H), 5.64–5.62 (m, 0.9 H), 5.58–5.56 (m, 0.1 H), 5.56–5.53 (m, 0.6 H), 5.21–5.01 (m, 4.9 H), 4.33–4.24 (m, 1.7 H), 4.22 (t, \( ^3J_{HH} = 4.98 \text{ Hz} \), 2.6 H), 4.18–4.09 (m, 7.4 H), 4.05 (t, \( ^3J_{HH} = 6.63 \text{ Hz} \), 15.4 H), 3.63 (s, 132 H), 2.45–2.33 (m, 7.0 H), 2.30 (t, \( ^3J_{HH} = 7.67 \text{ Hz} \), 16.1 H), 1.99–1.98 (m, 1.1 H), 1.97–1.95 (m, 2.8 H), 1.94–1.93 (m, 1.8 H), 1.73–1.59 (m, 46.7 H), 159–1.44 (m, 17.4), 1.44–1.32 (m, 23.1). SEC: \( M_n = 5630 \text{ g/mol, } D = 1.25. \]

3.2.3 Synthesis of MA-PCLA-PEG\textsubscript{1000}-PCLA-MA (1k\textsubscript{2080}-MA)

The polymer was synthesized by the same procedure as for 1.5k\textsubscript{3150}-MA except that 1000 g/mol PEG was used as the macroinitiator and the quantities of reagents were: PEG\textsubscript{1000} (8.0 g, 8.0 mmol, 1.0 equiv.), LA (1.9 g, 13 mmol, 1.6 equiv.), CL (6.0 g, 53 mmol, 6.6 equiv.) and anhydrous toluene (40 mL). Yield = 13.8 g, 87%.

\[ {^1}H \text{ NMR (400 MHz, CDCl}_3\text{): } \delta 6.18–6.15 (m, 0.1 H), 6.10–6.06 (m, 0.8 H), 6.02–6.00 (m, 0.1 H), 5.98–5.95 (m, 0.8 H), 5.62–5.58 (m, 0.1 H), 5.54–5.51 (m, 0.8 H), 5.48–5.45 (m, 0.1 H), 5.45–5.42 (m, 0.8 H), 5.09–4.89 (m, 3.1 H), 4.24–4.13 (m, 1.5 H), 4.11 (t, \( ^3J_{HH} = 4.98 \text{ Hz} \), 2.8 H), 4.07–3.98 (m, 5.6 H), 3.95 (t, \( ^3J_{HH} = 6.63 \text{ Hz} \), 9.5 H), 3.53 (s, 88 H), 2.36–2.23 (m, 5.0 H), 2.19 (t, \( ^3J_{HH} = 7.67 \text{ Hz} \), 10.6 H), 1.89–1.86 (m, 0.5 H), 1.86–1.84 (m, 2.5 H), 1.83–1.79 (m, 2.8 H), 1.63–1.48 (m, 31.2 H), 1.48–1.34 (m, 11.7), 1.34–1.20 (m, 15.0). SEC: \( M_n = 2735 \text{ g/mol, } D = 1.18. \]
3.2.4 Synthesis of MA-PCLA-PEG$_{1000}$-PCLA-MA (1k$_{2840}$-MA)

The polymer was synthesized by the same procedure as for 1.5k$_{3150}$-MA except that 1000 g/mol PEG was used as the macroinitiator and the quantities of reagents were: PEG$_{1000}$ (8.0 g, 8.0 mmol, 1.0 equiv.), LA (3.1 g, 22 mmol, 2.7 equiv.), CL (10 g, 88 mmol, 11 equiv.) and anhydrous toluene (40 mL). Yield = 18.4 g, 87%. $^1$H NMR (400 MHz, CDCl$_3$):  δ 6.12–6.10 (m, 0.1 H), 6.05–6.01 (m, 0.9 H), 5.97–5.95 (m, 0.1 H), 5.94–5.90 (m, 0.5 H), 5.57–5.54 (m, 0.1 H), 5.50–5.45 (m, 0.9 H), 5.43–5.40 (m, 0.1 H), 5.40–5.36 (m, 0.5 H), 5.06–4.83 (m, 5.2 H), 4.20–4.08 (m, 1.5 H), 4.06 (t, $^3$J$_{HH}$ = 4.98 Hz, 2.5 H), 4.02–3.93 (m, 8.0 H), 3.90 (t, $^3$J$_{HH}$ = 6.63 Hz, 17.8H), 3.48 (s, 88 H), 2.30–2.19 (m, 6.7 H), 2.15 (t, $^3$J$_{HH}$ = 19.6 Hz, 26.3 H), 1.84–1.81 (m, 0.5 H), 1.81–1.79 (m, 2.9 H), 1.78–1.76 (m, 1.9 H), 1.58–1.43 (m, 53.3 H), 1.43–1.29 (m, 19.2), 1.29–1.16 (m, 25.4). SEC: $M_n = 3035$ g/mol, $Đ = 1.38$.

3.2.5 Synthesis of MA-PCLA-PEG$_{1500}$-PCLA-MA (1.5k$_{4190}$-MA)

The polymer was synthesized by the same procedure as for 1.5k$_{3150}$-MA except that the quantities of reagents were: PEG$_{1500}$ (8.0 g, 5.3 mmol, 1.0 equiv.), LA (3.1 g, 22 mmol, 4.0 equiv.), CL (10 g, 88 mmol, 16 equiv.) and anhydrous toluene (40 mL). Yield = 17.8 g, 84%. $^1$H NMR (400 MHz, CDCl$_3$): δ 6.27–6.24 (m, 0.2 H), 6.19–6.15 (m, 0.8 H), 6.11–6.09 (m, 0.1 H), 6.07–6.05 (m, 0.3 H), 5.69–5.66 (m, 0.2 H), 5.63–5.58 (m, 0.8 H), 5.55–5.53 (m, 0.1 H), 5.53–5.50 (m, 0.3 H), 5.19–4.99 (m, 7.5 H), 4.34–4.21 (m, 1.8 H), 4.19 (t, $^3$J$_{HH}$ = 4.98 Hz, 2.3 H), 4.16–4.07 (m, 10.5 H), 4.03 (t, $^3$J$_{HH}$ = 6.63 Hz, 27.2 H), 3.61 (s, 132 H), 2.44–2.31 (m, 10.4 H), 2.28 (t, $^3$J$_{HH}$ = 7.67 Hz, 28.3 H), 1.97–1.95 (m, 0.7 H), 1.95–1.92 (m, 2.7 H), 1.92–1.89 (m, 1.3 H), 1.70–1.58 (m, 78.4 H), 1.59–1.42 (m, 26.0), 1.44–1.29 (m, 38.1). SEC: $M_n = 6125$ g/mol, $Đ = 1.28$. 
3.2.6 Synthesis of MA-PCLA-PEG\textsubscript{2000}-PCLA-MA (2k\textsubscript{4600}-MA)

The polymer was synthesized by the same procedure as for 1.5k\textsubscript{3150}-MA except that 1000 g/mol PEG was used as the macroinitiator and the quantities of reagents were: PEG\textsubscript{2000} (8.0 g, 4.0 mmol, 1.0 equiv.), LA (3.1 g, 22 mmol, 5.4 equiv.), CL (7.1 g, 62 mmol, 16 equiv.) and anhydrous toluene (40 mL). Yield = 14.4 g, 79% \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 6.21–6.18 (m, 0.1 H), 6.13–6.09 (m, 0.9 H), 6.01–5.98 (m, 0.6 H), 5.64–5.61 (m, 0.1 H), 5.57–5.53 (m, 0.9 H), 5.48–5.44 (m, 0.6 H), 5.14–4.93 (m, 10.9 H), 4.28–4.16 (m, 2.7 H), 4.14 (t, \(J_{HH} = 4.98\) Hz, 1.8 H), 4.10–4.00 (m, 9.9 H), 4.05 (t, \(J_{HH} = 6.63\) Hz, 21.8 H), 3.56 (s, 176 H), 2.41–2.27 (m, 8.8 H), 2.22 (t, \(J_{HH} = 7.67\) Hz, 23.3 H), 1.91-1.89 (m, 0.4 H), 1.88 (s, 2.9 H), 1.86–1.83 (m, 2.1 H), 1.65–1.53 (m, 60.4 H), 1.53–1.37 (m, 39.7), 1.37-1.23 (m, 32.2). SEC: \(M_n = 5500\) g/mol, \(Đ = 1.13\).

3.2.7 Hydrogel Preparation

Hydrogels were prepared by weighing molten polymer into glass vials and adding 4 °C PBS to provide polymer concentrations of 10, 15, 20, or 25 wt%. Immediately, before allowing the molten polymer to cool upon contact with cold PBS, the hydrogels were vortexed vigorously for 20 s and then stored at 4 °C. After 24 h of incubation at 4 °C, vials were placed in 37 °C oven for 30 min to facilitate further dissolution and then returned to 4 °C for 24 h. Gelation was achieved by adding 60 µL KPS (50 mg/mL) and 20 µL TEMED (10 wt%) per gram of polymer solution to produce a final concentration of 10 mM KPS and 20 mM TEMED. Drug-loaded gels were prepared by adding powdered drug to previously prepared polymer solutions at 4 °C to yield 5 or 10 wt% drug and then vortexing.
3.2.8 Rheometry

Rheometry was performed on an Anton Paar MCR 302 rotational shear rheometer with a 50 mm diameter parallel plate geometry. The instrument was equipped with a Peltier plate to control temperature to ± 0.01 °C and a hood to limit evaporation and dehydration of samples as well as to help regulate the temperature. Waterproof emery paper (280 grit) was fastened to the upper and lower plates of the measurement geometry to limit sample slip. Samples (2.2 mL) were pipetted onto the precooled stage (4 °C) and the gap between the plates was set to 1 mm. The viscous and elastic moduli, G" and G’ respectively, were determined by small-amplitude oscillatory shear (SAOS) measurements at a fixed strain amplitude of 1%, and a fixed frequency of 1 Hz for all experiments. In all experiments, the normal force on the rheometer tool was fixed at 10 mN to ensure that contact with the sample was maintained; as a result, the tool gap varied slightly with temperature and this was taken into account in the analyses. Temperature sweeps were run at a constant rate of 1 °C min\(^{-1}\) from 4 to 37 °C, after which the temperature was held at 37 °C for 30 min to allow for relaxation of the polymer chains and to investigate post-gelation changes in the material. Measurements over time were performed at constant temperatures (4, 21, or 37 °C). Simulated injection conditions involved a rapid temperature (approximately 20 s) increase to 37 °C at the onset of measurements to mimic the temperature change occurring upon injection in vivo. The gel point was taken as the temperature at which G’ (1 Hz) = G” (1 Hz). The experiments were performed in triplicate.

3.2.9 Syneresis Measurements

Gravimetric analysis was used to measure water loss. Approximately 1 g of drug-free or drug-loaded hydrogel was added into a 3 mL screwcap vial and the vial with gel was accurately weighed. The sealed vials were placed in a 37 °C oven. At specified time points, the vials were unsealed,
inverted onto a paper towel and let sit for 1 min before being weighed, sealed and placed back in the oven. The loss in mass corresponded to water loss and thus the degree of syneresis.

### 3.2.10 Compression Testing

The moduli of CXB, PHE, MTX, DLO, and MEL (5 wt% of drug) loaded and drug-free 20% 1.5k/3150-MA hydrogels were measured by compression testing. Formulations were gelled in 6 mL syringes (internal diameter of 19.2 mm) at 37 °C for 30 min and then cut into approximately 65 mm thick cylinders prior to testing. Unconfined stress-strain measurements were performed on a Univert universal testing instrument (CellScale, Guelph, ON, Canada), equipped with a 10 N load cell. Samples were preloaded with 0.01 N prior to each test. Uniaxial compression was applied at a constant rate of 4% s\(^{-1}\) to a total strain of 30%. The nominal stress was calculated by dividing the applied force by the cross-sectional area of the sample.\(^{26}\) The secant modulus was calculated from the slope between 5 and 20% strain.

### 3.2.11 In Vitro CXB Release from 20% 1k/3150-MA and 15% 2k

CXB-loaded hydrogels were prepared as described above. CXB-loaded hydrogel (1.0 g of 10 wt%) was weighed into a 1 cm diameter x 1 cm height cylindrical cup and placed in a 37 °C oven for 30 min to form a gel. The cup, containing the gel, was lowered into 100 mL of pre-heated (37 °C) 20 mg/mL polysorbate 80 in pH 7.4 PBS (solvent release medium). The free CXB samples were weighed and contained in nylon mesh bags (2.5 cm x 4 cm, 400 mesh) and sealed shut with dialysis tubing clips. Solvent release medium samples (100 mL) were taken at regular time intervals and replaced with fresh release medium until 22 d. The CXB concentration in the solvent release medium was measured on a Varian Cary 300 Bio UV-Vis Spectrophotometer at 253 nm based on an extinction coefficient of 1.65 × 10\(^4\) L·mol\(^{-1}\)·cm\(^{-1}\) for CXB in the same medium.
3.2.12 Intra-articular injection of the CXB-loaded hydrogels and synovial fluid sampling in horses

All procedures were done in compliance with the guidelines of The Canadian Council on Animal Care guidelines (University of Guelph Protocol 3974). Two in vivo trials were performed to compare an optimized acetyl capped formulation (15% 2k + 10% CXB) and a methacylated formulation (20% 1.5k3150-MA + 10% CXB). Three horses were used for the 15% 2k + 10% CXB trial and 6 horses used for the 1.5k3150-MA + 10% CXB trial. The 2k + 10% CXB formulation was prepared as previously described (Chapter 2) except drug was loaded with CXB and drawn into syringes in a biological safety cabinet (BSC). The 1.5k3150-MA + 10% CXB formulation was prepared sterile into 2 mL aliquots as described above and KPS /TEMED additions were made on site in a portable BSC. From the 2 mL aliquots, 1 mL was drawn into each 1 mL syringe that were pre-chilled to limit early crosslinking. An immune response test was performed first by injecting 1 mL of formulation subcutaneously into 3 horses. Swelling was observed at 8 h and 24 h. For subsequent intra-articular injections, 1 mL of formulation was administered to a carpal joint.

Horses underwent a general physical exam and more specific musculoskeletal exam for lameness and joint abnormalities. All horses were normal adults between 425 and 550 kg and group housed in a large pasture, fed hay with a known nutritional analysis and potable water. For assessments and collection, they were admitted into a small pen and taken into an indoor facility at ambient temperature. No restraint of the horses was required other than a halter and lead rope. Blood was aseptically collected into 10 mL heparinized Vacutainer tubes using a 20-gauge needle from one of the jugular veins, the blood was mixed by turning the tube over end to end several times and then placed on ice until centrifugation to collect plasma. Synovial fluid was collected from the metacarpophalangeal (fetlock) joint of one of the front legs by the approach through the
sesamoidian collateral ligament. This avoided much of the synovial vasculature and reduced the chances of blood contamination during synovial fluid collection. With the fetlock joint flexed and held by the operator or an assistant, a 20-gauge 1.5” needle was advanced through the sesamoidian collateral ligament into the palmar compartment of the fetlock joint and 1 mL of synovial fluid was withdrawn into a 3 mL syringe. Synovial fluid was expressed into a 3 mL eppendorf tube and held on ice until aliquots were made.

3.2.13 Determination of celecoxib concentration in synovial fluid
Analyses were performed based on a modification of a previously reported procedure.\textsuperscript{28} CXB was extracted from synovial fluid (SF) by taking 100 µL aliquots and pipetting into 15 mL falcon tubes. 10 µL of 1 mg/mL ibuprofen internal standard was added and thoroughly mixed with the aliquot. 3 mL of HPLC grade acetonitrile was added, then the sample was vortexed for 20 s vigorously and placed on shaker for 18 h. Next, each sample was centrifuged at 6000 rpm for 20 min and placed in -20 freezer for at least 1 h. Acetonitrile was decanted from the pellet into a 15 mL centrifuge tube and allowed to evaporate completely. 1 mL of mobile phase was added, vortexed for 30 s, sonicated for 30 s, and then vortexed again. Next, each sample was filtered through a 0.2 µm Supor filter into HPLC glass vials.

The HPLC was equipped with a Waters Separations Module 2695, a Photodiode Array Detector (Waters 2998) and a Kinetex C18 5 µm (4.6x100 mm) column connected to a C18 guard column. The PDA detector was used to monitor CXB and ibuprofen at 254 nm. Analyte separation was obtained using an isocratic run method with Acetonitrile:0.1M aqueous KH\textsubscript{2}PO\textsubscript{4} pH 2.4 (48:52). The retention time of ibuprofen was 4.5 min and CXB was 6 min.
The calibration curve was obtained from the CXB spiked and extracted standards using ibuprofen as the internal standard. The standard solutions of 10, 5, 2, 1, 0.5, and 0.2 µg/mL CXB were added to 100 µl of blank SF fluid samples along with 10 µg/mL ibuprofen as the internal standard. Each sample was extracted using the standard extraction method described above and re-dissolved in 1 mL of mobile phase. All samples were filtered through 0.2 µm membrane filters and 100 µl was injected using the instrument method described above.

3.3 Results and Discussion

3.3.1 Synthesis and Characterization of Methacrylate Capped PCLA-PEG-PCLA Triblock Copolymers

Ring-opening polymerizations of LA and CL in toluene were performed using 1000, 1500 and 2000 g/mol PEG-diols as the macroinitiators and tin(II) 2-ethylhexanoate [Sn(Oct)2] as the catalyst (Scheme 1). Different ratios of LA and CL relative to PEG were used to achieve varying ratios of CL/LA. A higher CL/LA ratio has been reported to provide higher crystallinity, which results in longer degradation times. The resulting triblock copolymers were capped with methacrylate end groups using an excess of methacrylic anhydride. Yields for all reactions were at least 77%.

Scheme 3.1. Synthesis of methacrylate capped PCLA-PEG-PCLA triblock copolymers.
The polymers were characterized by $^1$H NMR spectroscopy (Figures S1-S5). In the spectra, the PEG blocks gave rise to peaks at 3.6 ppm. The incorporation of the LA into the PCLA block was confirmed by the observation of resonances at 4.8 - 5.2 ppm corresponding to the methine protons on the polymer backbone. The presence of peaks at ~4.0 and 4.1 ppm confirmed the CL content corresponding to the presence of methylene groups adjacent to the ester oxygen, depending on the neighboring monomer. The monomer ratios in the polymers were calculated by comparison of the integrations of the peaks of the PEG initiator (3.6 ppm) with those of the CL (4.1 and 4.0 ppm) and LA (5.10 ppm) blocks. Most of the CL/LA mass ratios ranged from 3.68 - 3.93, except for 2k4600-MA which had a ratio of 2.30. The molar masses of the PCLA blocks for all five polymers were varied significantly, ranging from 540 - 1350 g/mol (single block) and the PCLA/PEG ratios varied from 1.08 to 1.79. The degree of methacrylation was calculated using the integrations of terminal methyl protons (3 peaks between 1.8 and 1.9) relative to that of the PEG peak at 3.6 ppm. The extent of methacrylation was between 73 and 98% for all polymers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>PEG (g/mol)</th>
<th>PCLA $^a$ (g/mol)</th>
<th>$M_a^a$ full co-polymer (g/mol)</th>
<th>CL/LA$^a$ mass ratio</th>
<th>PCLA/PEG$^a$ mass ratio</th>
<th>$M_n^b$ (g/mol)</th>
<th>$\mathcal{D}^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1k2080-MA</td>
<td>1000</td>
<td>540</td>
<td>2,080</td>
<td>3.86</td>
<td>1.08</td>
<td>2,735</td>
<td>1.18</td>
</tr>
<tr>
<td>1k2840-MA</td>
<td>1000</td>
<td>920</td>
<td>2,840</td>
<td>3.93</td>
<td>1.85</td>
<td>3,035</td>
<td>1.38</td>
</tr>
<tr>
<td>1.5k3150-MA</td>
<td>1500</td>
<td>825</td>
<td>3,150</td>
<td>3.68</td>
<td>1.10</td>
<td>5,630</td>
<td>1.25</td>
</tr>
<tr>
<td>1.5k4190-MA</td>
<td>1500</td>
<td>1,345</td>
<td>4,190</td>
<td>3.98</td>
<td>1.79</td>
<td>6,125</td>
<td>1.28</td>
</tr>
<tr>
<td>2k4600-MA</td>
<td>2000</td>
<td>1,300</td>
<td>4,600</td>
<td>2.30</td>
<td>1.30</td>
<td>5,500</td>
<td>1.13</td>
</tr>
</tbody>
</table>

Table 3.1. Molar mass and chemical analysis data for the polymers
The polymers were also characterized by SEC relative to PMMA standards (Table 1, Figure S6). As reported in Chapter 2, the $M_n$ values determined by SEC were higher than those of calculated from $^1$H NMR spectroscopy. The discrepancy was attributed to the use of PMMA standards, which have different hydrodynamic volumes than PCLA-PEG-PCLA. The $Đ$ values ranged from 1.13-1.38, which was also in agreement with those of the analogous polymers with acetyl end-caps (Chapter 2).

### 3.3.2 Feasibility Screening of Methacrylated Hydrogels

It has previously been established that changing the end-cap can have substantial effects on polymer solubilities and gelation because the end-group influences the hydrophobic-hydrophilic balance of the block copolymers.$^{29,30}$ Therefore, initial feasibility screening was performed on the five different polymers described above to establish the appropriate PEG length and corresponding PCLA block length. Solubility was tested in PBS at 4 °C at different concentrations (10, 15, 20 and 25 wt%) and was determined by whether the solution was transparent or translucent (Table 2) with sufficiently low viscosity to enable injection using an 18-gauge needle. The primary goal was to exploit covalent gelation. Initial screening focused on the abilities of the formulations to form gels at 37 °C in the presence of KPS/TEMED using the well-established vial-tilt test.$^{31}$ The vial-tilt test involved incubating the formulation at 37 °C for 30 min, inverting the vial, and examining it after 10 min.$^{31}$ The absence of flow indicated gelation. The secondary goal was to produce a hydrogel that would possess thermo-reversible gelation through physical crosslinking as it was hypothesized that this would enhance the properties of the resulting gel.
We found that among the polymers evaluated, the examples with the highest PCLA/PEG mass ratios (1.30 and above) afforded suspensions with low viscosity but with high opacity/turbidity, suggesting that the polymers were dispersed as large particles or aggregates. These turbid suspensions had some thermo-reversible activity at 37 °C in the absence of KPS/TMED. Interestingly, they formed solid gels at 37 °C when KPS and TEMED were added, but these gels had high syneresis and course textures (1k\textsubscript{2840} and 1.5k\textsubscript{4190}) or were very weak and fell apart when probed (2k\textsubscript{4600}). When attempts were made to dissolve the 1k\textsubscript{2080} sample at 4 °C, it resulted with large polymer globules that minimally absorbed water (1k\textsubscript{2080}). Finally, the 1.5k\textsubscript{3150} sample, formed an ideal translucent, free-flowing liquid at concentrations up to 20 wt%. It also underwent gelation at 37 °C both with and without the addition of KPS/TEMED. The 20 wt% 1.5k\textsubscript{3150} formulation was selected for further study, as lower concentrations would be expected to afford weaker hydrogels.

Table 3.2. Results of tests to determine polymer solubility at 4 °C and initial assessment of gelation at 37 °C based on the vial-tilt test. “Yes” indicates full dissolution into a free-flowing
translucent solution capable of being drawn into an 18-gauge needle or the ability to form gels at 37 °C that did not flow over 10 min with the addition of KPS/TEMED.

<table>
<thead>
<tr>
<th>MA hydrogels</th>
<th>10% Dissolve at 4 °C</th>
<th>Gel at 37 °C</th>
<th>15% Dissolve at 4 °C</th>
<th>Gel at 37 °C</th>
<th>20% Dissolve at 4 °C</th>
<th>Gel at 37 °C</th>
<th>25% Dissolve at 4 °C</th>
<th>Gel at 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1k2080</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>1k2840</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>1.5k3150</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Partially</td>
<td>Yes</td>
</tr>
<tr>
<td>1.5k4190</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>2k4600</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
</tr>
</tbody>
</table>

a significant syneresis

3.3.3 Thermal Gelling Rheological Properties of 1.5k3150-MA

The rheological properties of the gels during thermal and the combination of thermal and covalent crosslinking were investigated. First, thermo-reversible crosslinking of 20 wt% 1.5k3150-MA was investigated by omitting the addition of KPS/TEMED. Temperature sweep followed by temperature hold experiments were performed at a heating rate of 1 °C/min and a constant frequency (1 Hz) and amplitude (1%) (Figure 1). At 4 °C, G’’ was greater than G’, indicating the material was a liquid. At 25 °C, G’ became greater than G’’ indicating that gelation had occurred. At 37 °C, G’ was 1.5 kPa and G’’ was 640 Pa. Overall, the rheological properties of 1.5k3150-MA were quite similar to those of a acetyl capped PCLA-PEG-PCLA hydrogels reported by Petit et al.29 and described in Chapter 2 of this thesis.27
3.3.4 Syneresis of 20 wt% 1.5k\textsubscript{3150}-MA Hydrogels Loaded with Different Drugs

Syneresis occurs when the hydrogel network displaces water from its pores causing dehydration and collapse of the network structure, resulting in two phases.\textsuperscript{32} During gelation in the presence of a diluent (water), the network reaches its maximum swelling and then collapses.\textsuperscript{33} It was shown in Chapter 2 that the degree of syneresis was related to the moduli of thermo-reversible acetyl capped PCLA-PEG-PCLA hydrogels; the greater the syneresis, the less stiff the gel was. Syneresis was measured gravimetrically for formulations prepared from 20 wt\% 1.5k\textsubscript{3150}-MA and loaded with five different NSAIDS, each loaded at 5 wt\% (Figure 3). Different drugs led to different degrees of syneresis, as previously observed for the acetyl capped PCLA-PEG-PCLA hydrogels. Some drugs tested increased water loss (CXB, MTX, and DCLO), while others caused a reduction in water loss (MEL and PHE) compared to the control (drug-free 1.5k\textsubscript{3150}-MA). DCLO-loaded hydrogels exhibited the highest degree of syneresis of 13.6\% ± 0.7\% and PHE-loaded hydrogels
exhibited the lowest of 4.7% ± 1.3%. Overall, the extent of water loss was much lower for the covalently crosslinked 1.5kMA hydrogels described here, compared to the physically crosslinking acetyl capped PCLA-PEG-PCLA hydrogels reported in Chapter 2. The results indicate that covalently crosslinking fixed the network structure in place, preventing its reorganization and collapse.

![Chemical structures of NSAIDs tested](image)

**Figure 3.2.** Chemical structures of NSAIDs tested
Figure 3.3. Syneresis measurements for 20 wt% 1.5k$_{3150}$-MA hydrogels loaded with 5 wt% of different NSAIDs. The error bars represent the standard deviations of triplicate experiments.

3.3.5 Rheology of 20 wt% 1.5k$_{3150}$-MA Hydrogels Loaded with Different Drugs

The five different NSAIDS were loaded into the 20 wt% 1.5k$_{3150}$-MA hydrogel formulation at 5 wt% and dynamic rheometry was performed under the simulated injection conditions where the liquid formulations at 4 °C were brought to 37 °C over a period of 20 s. G' and G” were measured over time at 1 Hz and a amplitude of 1% of 60 min. Initially, all formulations were liquid dominant, with G” greater than G’. Gelation, as indicated by the crossover of G’ and G”, occurred after ~2 min for each system. G’ had plateaued after ~10 min for all formulations except DCLO, which took ~20 min (Figure 5). The addition of PHE, CXB, MTX and DCLO increased G’ for the hydrogels, compared to the drug-free hydrogel, while only MEL showed a reduction in G’ (Table 4). The incorporation of PHE caused the largest (4-fold) increase in G’. Modest increases in G” were also observed for the incorporation of most drugs except MEL and DCLO. Overall, it is
apparent that different chemical properties (molecular mass, hydrophobicity, ionic charge, functional groups) of the drug can have a significant effect on rheological properties of the gel, even for the covalently crosslinked gels.

Figure 3.4. G' of 20 wt% 1.5k3150-MA hydrogels loaded with 5 wt% of different NSAIDs over time following a rapid increase in temperature to 37 °C. Experiments were performed at 1 Hz and 1% strain amplitude. Error bars represent standard deviations of triplicate samples.

Table 3.3. G', G", and compressive moduli of drug-free and drug-loaded (5 wt%) 1.5k3150-MA hydrogels measured at 1 Hz and 1% strain amplitude after 60 min at 37 °C. Errors on the measurements correspond to the standard deviations of triplicate samples.

<table>
<thead>
<tr>
<th></th>
<th>G' (kPa)</th>
<th>G&quot; (Pa)</th>
<th>Compressive modulus (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5k3150-MA</td>
<td>7.7 ± 1.2</td>
<td>1.2 ± 0.7</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>1.5k3150-MA + 5% MEL</td>
<td>5.2 ± 0.3</td>
<td>0.8 ± 0.4</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Drug Loading</td>
<td>Compressive Modulus</td>
<td>Strain</td>
<td>Modulus</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>1.5k3150-MA + 5% DLO</td>
<td>8.4 ± 0.6</td>
<td>1.1 ± 0.7</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>1.5k3150-MA + 5% MTX</td>
<td>13.7 ± 0.3</td>
<td>2.0 ± 0.4</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>1.5k3150-MA + 5% CXB</td>
<td>17 ± 8</td>
<td>2.8 ± 1.5</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>1.5k3150-MA + 5% PHE</td>
<td>31 ± 10</td>
<td>1.8 ± 0.9</td>
<td>29 ± 2</td>
</tr>
</tbody>
</table>

### 3.3.6 Compression Testing

Unconfined compression testing was used to determine the compressive moduli of drug-free and drug-loaded 1.5k3150-MA hydrogels. Stress-strain curves are shown in Figure 6. The PHE-loaded hydrogel had a higher compressive modulus when compared to drug-free and the other drug-loaded hydrogels (Table 4). Conversely, MTX, DLO, and MEL-loading reduced the compressive moduli and CXB-loading had no significant effect. The compressive moduli for the covalently crosslinked hydrogels are ~1000-fold higher than those of the previously studied acetyl-capped PCLA-PEG-PCLA hydrogels (Chapter 2). Furthermore, all of the acetyl-capped PCLA-PEG-PCLA hydrogels had reduced compressive moduli when loaded with drugs. The stiffer properties of the hybrid covalent-physically crosslinked systems should enable these hydrogels to better support the mechanical loading in the joint. In addition, they are less susceptible to degradation of their properties upon loading with drugs.
Figure 3.5. Compressive stress versus strain for 20 wt% $1.5k_{3150}$-MA hydrogels with and without drugs incorporated. Error bars represent standard deviations of triplicate experiments.

3.3.7 Temperature Kinetics of Phenylbutazone-loaded $1.5k_{3150}$-MA

Gelation of 20 wt% $1.5k_{3150}$-MA + 5% PHE in the presence of the KPS/TEMED system was investigated by dynamic rheometry. Measurements at 4 °C were performed to determine the stability of the formulation containing the initiator and catalyst at this temperature. PHE was selected because it had the highest moduli from shear and compression tests. Gelation, indicated by a crossover of $G’$ and $G’’$, required 23 min at 4 °C (Figure 2). To simulate an injection or removal of the system from a refrigerator, tests were used where the sample was placed on the rheometer at 4 °C and measurements began along with a rapid increase in temperature to 21 °C or 37 °C over a period of 20 s. Gelation occurred within 2 min at 37 °C and within 4 min at 21 °C. $G’$ values plateaued at 27 – 31 kPa after 80 min for all temperatures investigated (Table 3). Compared to the system described above without KPS/TEMED, the covalent crosslinking
increased $G'$ by a factor of $\sim$30-fold. $G''$ values also increased for all systems with temperature, indicating an increase in viscosity. The increase was highest for the system at 37 °C, suggesting that the thermal gelation via physical crosslinking plays a significant role in increasing $G''$.

**Figure 3.6.** $G'$ and $G''$ of 1.5k$_{3150}$-MA + 5% PHE hydrogels as a function of time in the presence of KPS/TEMED performed at 1 Hz and 1% strain amplitude. Closed symbols represent $G'$ and open symbols represent $G''$. Squares represent gelation at 37 °C, triangles represent gelation at 21 °C and circles represent gelation at 4 °C.
Table 3.4. Time to gelation in the presence of KPS/TEMED as well as $G'$ and $G''$ for $1.5k_{3150}$-MA + 5% PHE hydrogels after 80 min at different temperatures at 1 Hz and 1% strain amplitude for gelation kinetics of covalent crosslinking. Errors on the measurements correspond to the standard deviations of triplicate samples.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time to gelation (min)</th>
<th>$G'$ (kPa)</th>
<th>$G''$ (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>2</td>
<td>27 ± 5</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>21</td>
<td>4</td>
<td>29 ± 6</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>31 ± 6</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

3.3.8 In vitro release of CXB from acetyl and methacryl capped PCLA-PEG-PCLA hydrogels

*In vitro* release of CXB from the acetyl capped 15% 2k PCLA-PEG-PCLA hydrogel containing 10 wt% CXB (Chapter 2) and from 20 wt% $1.5k_{3150}$-MA containing 10 wt% CXB were measured and compared over 22 d at 37 °C. Rheological behavior was tested to confirm the physical properties were not significantly affected by the increase in drug concentration (Figure A3.7). While the loading of PHE enhanced the mechanical properties the most, CXB was selected for release studies to enable comparison with previously reported studies and because the release protocol and synovial fluid analysis for CXB had already been developed in our lab. Polysorbate 80 (2 w/v %) was used to increase the solubility of CXB to provide appropriate solvent sink conditions. In contrast to Chapter 2, where only 0.2 w/v% polysorbate 80 was used, here the concentration of polysorbate was increased to provide a measurable release profile within 22 d. We suspected the release profile would not be high enough to provide a good comparison if 0.2% polysorbate 80 was used. The release of CXB was much slower for the $1.5k_{3150}$-MA hydrogel. After 22 d, ~20% CXB was released into the solvent release medium. The drug release profile
follows the Higuchi equation,\textsuperscript{35} that assumes a homogeneous distribution of drug throughout the hydrogel matrix that release is governed by diffusion. Acetyl capped 15\% 2k was reported in Chapter 2 to release 30\% of CXB over 22 d in a 0.2\% polysorbate 80 solution.\textsuperscript{27} With a 10-fold increase in polysorbate 80 concentration to 2\%, the CXB release increased to 93\%. The release profile followed a first-order kinetic model that assumes the drug is partitioned by a core-shell domain. The rapid early stage CXB release up to day 5 occurred when the gel had high water content and the slower rate in the later stage was due to decreased hydration.\textsuperscript{36} Overall, the slower release of from the covalently crosslinked gel \textit{in vitro} suggested that it could also provide a more sustained release \textit{in vivo}.

![Figure 3.7](image)

**Figure 3.7.** \textit{In vitro} CXB release into 20 mg/mL polysorbate 80 solvent sink solution. Error bars represent standard deviation of triplicate experiments.

3.3.9 \textit{In vivo} CXB release from acetyl and methacrylate hydrogels in horse joints

Subcutaneous injections into horses were performed first to test the immune response to the material. These subcutaneous tests showed only mild swelling after 8 h and no response after 24 h. Next, 20 wt\% 1.5k\textsubscript{3150}-MA hydrogel was injected into the carpels of 3 horses to further test its
safety. Again, no adverse reactions were observed after 24 h. In addition, Petit et al. have previously studied the \textit{in vivo} release of acetyl capped PCLA-PEG-PCLA hydrogels in detail and determined that these materials were well tolerated.\textsuperscript{23}

The acetyl capped hydrogel formulation \textbf{15\% 2k + 10\% CXB} (1 mL) was injected into the metacarpophalangeal (fetlock) of 3 healthy horses. CXB concentrations in SF samples were measured over time (Figure 7). A burst release was observed and $C_{\text{max}}$ was measured on day 1 for all animals (270 ± 17 µg/mL). This concentration is about 10-fold higher that measured by Petit \textit{et al.} \textsuperscript{23} who observed a $C_{\text{max}}$ of 24 ± 7 µg/mL and 36 ± 4µg/mL for 5 and 26 wt\% CXB loaded hydrogels composed of a similar 1.5k PEG version of the hydrogel. Following the $C_{\text{max}}$ at day 1, a rapid decrease in CXB concentration was measured in the SF and the concentration reached 0.03 µg/mL on day 16. These results are in general agreement with the trials performed by Cokelaere \textit{et al.}\textsuperscript{24} and Petit \textit{et al.},\textsuperscript{23} who both reported a burst release followed by decrease in drug concentrations until 14 d.
Figure 3.8. CXB concentrations measured in synovial fluid after 1 ml injection of 15\% 2k + 10\% CXB (Circles) and 20\% 1.5k-MA + 10\% CXB (Triangles). Error bars correspond to the standard deviations on 3 samples.

The methacrylated formulation (20\% 1.5k-MA + 10\% CXB) were injected into metacarpophalangeal (fetlock) joints of 6 horses. They provided a burst release and a $C_{\text{max}}$ of 45 ± 24 µg/mL on day 1. After day 7, the drug concentration plateaued at ~0.44 µg/mL until day 32, after which point the drug concentration began to decrease. The early stage burst release, which was not seen in the $\textit{in vitro}$ studies might be explained by the forced diffusion of drug out of the hydrogel, along with fluid, when the hydrogel underwent mechanical compression in the joint. Following this initial compression, the hydrogel or its fragments may have been forced to the edges of the joint, where they experienced less stress, consequently slowing the release. The middle stage release (day 29 and 32) might be attributed to further chemical degradation of the polymer network, exposing a high surface area for rapid diffusion. Overall, the results indicated that covalent crosslinking increased the time frame of drug release, which would reduce the need for repeated
injections. Gika et al. measured the concentration of CXB and etoricoxib in the SF of patients suffering from OA.\textsuperscript{37} Patients received oral doses of 100 mg of CXB twice per day for 5 d (1000 mg total per treatment period) before having their SF sampled and analyzed. The authors measured CXB at concentrations between 0.344 – 0.789 µg/mL. Further, Gika et al. report that CXB in SF samples could not be detected for 65% of the patients. Here, we report CXB was detectable in all animals who were administered with 100 mg per injection. The results reported here appear promising from a clinical point of view.

3.4 Conclusions

It is important to consider the mechanical properties for the intended use when designing \textit{in situ} forming hydrogels. This chapter described the synthesis, characterization and screening of several triblock copolymers of varying PEG and PCLA block lengths for their ability to dissolve and form hydrogels that were crosslinking through both chemical and physical mechanisms. We found that an appropriate balance between hydrophilic and hydrophobic domains were important for both dissolution and gelation. The \textbf{1.5}_{3150}-MA polymer composed of PEG\textsubscript{1500} flanked by PCLA\textsubscript{825} blocks provided the best properties during initial screening and was selected for further investigations. Subsequently, several commonly prescribed NSAIDs were loaded and used for mechanical testing by SOAS rheometry and compressive analysis. Chemical crosslinking greatly increased both the shear and compressive moduli, compared to the acetyl capped PCLA-PEG-PCLA hydrogels studied in Chapter 2. In addition, chemical crosslinking was well-suited to maintaining or exhibiting improved mechanical properties upon loading of several different NSAIDS. We found that release of CXB from the chemically crosslinked hydrogel \textbf{20\% 1.5}_{3150}-MA + 10\% CXB} was much slower \textit{in vitro} than from the physically crosslinked hydrogel \textbf{15\%}
This also translated to a more sustained release of CXB from the 20% 1.5\textsubscript{3150}-MA system \textit{in vivo} and is believed to result from the improved mechanical properties of the covalently crosslinked material. Overall, we conclude that covalent crosslinking is an effective approach to “fix” the network structure of physically crosslinked thermo-responsive hydrogels, and to make the properties resistant to degradation upon drug loading. Future work should include strategies to reduce the initial burst release of drug \textit{in vivo}.

### 3.5 References


4 Conclusions and Future Work

This thesis described the development of polymer systems for the delivery of hydrophobic drugs for the treatment of joint diseases, such as osteoarthritis (OA). There is no cure for OA and currently, all treatments are to alleviate symptoms. Oral administration of non-steroidal anti-inflammatory drugs (NSAIDs) results in poor bioavailability and can cause serious side effects. Therefore, intra-articular injection is a possible strategy to circumvent the risks associated with oral NSAID delivery. A strong need exists for the development of drug delivery systems capable of providing a sustained release, because of the rapid efflux of free drug from the joint. This thesis described the synthesis of PCLA-PEG-PCLA triblock copolymers, the investigation and comparison of two different end-caps that caused gelation by different mechanisms. Hydrogels that gelled by physical crosslinking could be tuned by modulating the middle PEG block and it was found that small changes in molar mass significantly affected the ability to maintain gelation when loaded with various NSAIDs. The optimized formulation was composed of PEG 2k and had more robust gelation when drug-loaded than its 1.5k predecessor. However, due to the poor mechanical properties associated with all physically crosslinked gels, covalently crosslinked gels were investigated. Adding methacrylate end-caps to the polymers significantly improved their mechanical properties upon gelation in the presence of a free radical initiation system. Furthermore, covalently crosslinked hydrogels showed improved mechanical properties when loaded with drugs. Phenylbutazone significantly enhanced gelation properties when compared to the drug-free hydrogel.
To further test the applicability for the treatment of OA, animal trials with horses were performed using the acetyl capped \textbf{2k} and the methacrylate capped \textbf{1.5k3150-MA} hydrogels loaded with CXB. The latter formulation was superior in providing a sustained release of CXB, as measured in the synovial fluid (SF) for up to 32 d compared to 16 d for the former. These results are promising from a clinical perspective and warrant further development.

Future work should include investigations to reduce the burst drug release. This release profile might be mitigated by incorporating other DDSs, such as microparticles within the \textit{in situ} forming gels. This might add another layer of protection against the compressive and shear forces experienced in the joint cavity. Also, the relatively larger size of microparticles will prevent them from being cleared out by the leaky vasculature of the synovial membrane, that otherwise smaller compounds will pass through.

Phenylbutazone had a positive effect on gelation. Future work might include the investigation of why it has gelation enhancing properties. Perhaps, combinatorial experiments and computer modelling could play an important role in this. In understanding what causes gelation-inhibition or gelation enhancement from drug compounds, better pairing can be achieved for different applications. Although this research focuses on treating symptoms of OA, future work should include formulations that have chondroprotective or enhancing properties.
5 Appendices

5.1 Appendix 1: Permission to Reuse Copyrighted Material

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Figure A2.1. $^1$H NMR spectrum of 1.5k polymer (400 MHz, CDCl$_3$). Assignments of key peaks used in the calculation of the polymer composition are provided. In the structure above, “a” denotes the CH$_2$ adjacent to a lactide unit, while “b” denotes the CH$_2$ adjacent to another caprolactone unit. “e” denotes an acetylated lactide unit while “f” denotes an acetylated caprolactone unit. The integration of the PEG peak at 3.6 ppm was set to 132 corresponding to 1500 g/mol. The number of LA units was determined from the integration of the peak at 5.1 ppm (8.5 units of lactic acid in this case) while the number of CL units was calculated as the sum of the integrations of the peaks at 4.14 and 4.06 ppm divided by 2 protons per repeat unit (17.4 units in this case).
Figure A2.2. $^1$H NMR spectrum of 2k polymer (400 MHz, CDCl$_3$). Assignments and calculations are as described in the caption of Figure A2.1.
Figure A2.3. $^1$H NMR spectrum of 3k polymer (400 MHz, CDCl$_3$). Assignments and calculations are as described in the caption of Figure A2.1.
Figure A2.4. Overlay of SEC traces of polymers 1.5k, 2k, and 3k.

Figure A2.5. Vial inversion test that was used to differentiate between mobile and immobile gels at different temperatures.
Figure A2.6. A) Amplitude sweep of 20% 1.5k hydrogel. B) Amplitude sweep of 20% 1.5k + 10% CXB formulation. C) Frequency sweep of 20% 1.5k hydrogel. D) Frequency sweep of 20% 1.5k + 10% CXB formulation.
Figure A2.7. A) Amplitude sweep of 15% 2k hydrogel. B) Amplitude sweep of 15% 2k + 10% CXB formulation. C) Frequency sweep of 15% 2k + 10% CXB hydrogel. D) Frequency sweep of 15% 2k + 10% CXB formulation.
Figure A2.8. A) Amplitude sweep of 10% 3k hydrogel. B) Amplitude sweep of 10% 3k + 10% CXB formulation. C) Frequency sweep of 10% 3k + 10% CXB hydrogel. D) Frequency sweep of 10% 3k + 10% CXB formulation.
5.4 Appendix 3 – Supporting Information for Chapter 3

Figure A3.2. $^1$H NMR spectrum of 1k$_{2080}$ polymer (400 MHz, CDCl$_3$). Assignments of key peaks used in the calculation of the polymer composition are provided. In the structure above, “a” denotes the CH$_2$ adjacent to a lactide unit, while “b” denotes the CH$_2$ adjacent to another caprolactone unit. “e” denotes a methacryl methyl unit, while “f” and “g” denotes methacryl methylene units. The integration of the PEG peak at 3.6 ppm was set to 88 corresponding to 1000 g/mol. The number of LA units was determined from the integration of the peak at 5.1 ppm (3.1 units of LA in this case) while the number of CL units was calculated as the sum of the integrations of the peaks at 4.03 and 3.98 ppm divided by 2 protons per repeat unit (17.4 units in this case).
Figure A3.2. $^1$H NMR spectrum of 1k$_{2840}$ polymer (400 MHz, CDCl$_3$). Assignments and calculations are as described in the caption of Figure A3.3.
Figure A3.3. $^1$H NMR spectrum of 1.5k $^{3150}$ polymer (400 MHz, CDCl$_3$). Assignments and calculations are as described in the caption of Figure A3.4.
Figure A3.4. 1.5k \textit{4190} $^1$H NMR spectrum of 1.5k$_{4190}$ polymer (400 MHz, CDCl$_3$). Assignments and calculations are as described in the caption of Figure A3.5.
Figure A3.5. $^1$H NMR spectrum of 2k$_{4600}$ polymer (400 MHz, CDCl$_3$). Assignments and calculations are as described in the caption of Figure A3.6.
Figure A3.6. Overlay of SEC traces of polymers.

Figure A3.7. Temperature sweep experiments of 1.5k3150-MA loaded with either 5% or 10% CXB.
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Publications/ Patents:
