DEVELOPMENT OF A NOVEL BIODEGRADABLE DRUG POLYMER FOR THE MODIFICATION OF INFLAMMATORY RESPONSE

by

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

MASTER OF APPLIED SCIENCE

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MASTER OF APPLIED SCIENCE, 2008

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ABSTRACT

The first objective of this thesis was to assess the feasibility of designing a “smart” degradable polymer that can release anti-inflammatory drugs in response to inflammatory-related enzymes. The drug polymer was synthesized using diisocyanates, poly(caprolactone) diols, and oxaceprol (OC) biomonomers. Biodegradation studies demonstrated that the trimethylhexamethylene diisocyanate-based drug polymer responded to an inflammatory enzyme to release more OC, while a 1, 12-diisocyanatododecane analog demonstrated minimal drug release. The drug delivery response was believed to be a direct function of the molecular structure and distribution of the hard segment.

The second objective of this thesis was to elucidate the anti-inflammatory mechanisms of OC by investigating its effects on cytokine-induced monocytic-cells adhesion in human umbilical vein endothelial cells (HUVECs) \textit{in vitro}. Results showed that OC had no direct effect on the monocyte-endothelium adhesion, suggesting that OC may mediate inflammation by mechanisms other than those suggested by the literature.
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Chapter 1 INTRODUCTION

In this study, polyurethane chemistry was used in conjunction with an atypical anti-inflammatory drug oxaceprol as a model anti-inflammatory polymer system.

This novel drug polymer has the following advantages:

- It allows local therapeutic delivery, minimizing adverse side effects (as oppose to administering systematically).
- It can maintain drug release over a long period of time; slow release (as oppose to diffusion-controlled release and/or polymer erosion or burst release)
- It is responsive to inflammatory enzymes, allowing the regulation of drug delivery rates (the inflammatory response causes the activation of macrophages, which causes the up-regulation of inflammatory enzymatic reactions, which in turn degrades the PUs to release the anti-inflammatory drug to control inflammation)
- It can be used as a drug carrier and as a biomaterial itself. It possesses PU’s mechanical properties, biocompatibility and degradability. It can be used in degradable scaffold systems for tissue regeneration applications or manage inflammatory processes in diseased tissues.

1.1 Background

The biodegradable behavior of polyurethanes has been of interest for the design of polymeric carriers for biologically active agents. Previous studies have demonstrated that cholesterol esterase (CE), an enzyme that is present in increasing quantities as
monocytes differentiate into macrophages during chronic inflammation, elicits the most hydrolytic degradation towards polyurethanes.\textsuperscript{5,6} Recently, a polyurethane that had antimicrobial agents incorporated into its backbone has been shown to release active free drug in the presence of CE, but very little work has yet been carried out to directly examine the use of polyurethanes as pro-drug macromolecules with anti-inflammatory activity.\textsuperscript{3,7}

1.2 Hypothesis

In the current thesis, an anti-inflammatory drug monomer was synthesized into the backbone of biodegradable polyurethane elastomers. It was hypothesized that:

1) the drug polymer would release oxaceprol in its original form, therefore potentially enabling the drug to effectively demonstrate an anti-inflammatory effect on the target cells.

2) the drug release profile of this material would be dependent on its degradation by inflammatory enzymes and the drug would be released proportionally to the magnitude of the esterase activity from these enzymes.

3) the drug release profile of this material would be dependent on the structure of the polymer hard segment.

4) oxaceprol would act by directly reducing the adhesion of monocytes to cytokine-stimulated endothelial cells.

The specific objectives of this project were:

1. To assess the feasibility of incorporating an anti-inflammatory drug, Oxaceprol, as a monomer unit into the backbone of a biodegradable polyurethane.
2. To examine and evaluate the effect of CE and diisocyanate structures on the degradation of the drug polymer and release of free drugs.

3. To establish an in vitro cell line model and determine if the drug activity directly affects the adhesion of monocytic cells to endothelial cells.

1.3 Thesis Outline

This document consists of six chapters. The outline of this document is as follows:

Chapter 2 – provides background and literature review on polyurethane chemistry, polymer drug delivery, inflammatory diseases and treatments.

Chapter 3 – presents results and discussions on the synthesis and characterization of the anti-inflammatory drug polymer using oxaceprol. The effect of enzyme and diisocyanate structures on degradation and drug release will also be discussed. (Appendices that are related to this chapter include: Appendix A)

Chapter 4 – examines the effect of Oxaceprol on TNFα-induced U937 Monocyte Adhesion on Human Umbilical Vascular Endothelial Cells. (Appendices that are related to this chapter include: Appendix B)

Chapter 5 – summarizes the significant findings of this work

Chapter 6 – provides recommendation for future work.
REFERENCE


Chapter 2 LITERATURE REVIEW

2.1 Polyurethane Chemistry, Synthesis and Characteristics

Polyurethane elastomers (PU) are regarded as some of the most versatile biomaterials currently available due to their superior mechanical properties, particularly tensile strength and fatigue resistance, and blood and tissue compatibility.\textsuperscript{1} They are widely used in medical applications, especially as long-term implants. However, during the last decade, a number of devices made from PUs have been withdrawn from long term implantation due to \textit{in vivo} degradation. Much research has been done since then in an effort to understand and modify the materials to increase their \textit{in vivo} biocompatibility as well as take advantage of their properties for the design of more complex systems such as scaffolds and polymeric carriers for biologically active agents\textsuperscript{2-5}. In this section, the materials chemistry and the synthesis of PU will be reviewed, which will be followed by an overview on PU degradation mechanisms in order to aid with the understanding and design of more biodurable materials, and to provide strategies for utilizing PUs’ degradative properties in the development of controlled-release applications.

2.1.1 Polyurethane Chemistry

Polyurethanes are a family of block copolymers which are composed of alternating blocks of soft and hard segment units linked together by a urethane or urea group.\textsuperscript{1,2,6} The soft segments are typically long chain polyols with low glass transition temperature (Tg), such as polyester, polyether or polyalkyldiol. The hard segments have high glass transition temperatures, and are typically diisocyanates with low molecular chain extenders consisting
of short chain diols, water or diamines.

There are two main characteristics of PUs that contribute to their wide range of properties. The first one is the two phase microstructure that they exhibit.\textsuperscript{1,6} There exists a degree of immiscibility between the hard polar urethane segments and the soft polyol segments. Phase separation of two segments occurs which produces a structure that can be considered as hard segment domains dispersed in soft segment matrix. The second important quality of PUs is their ability to incorporate other functional groups into the polymer network. In fact, urethane groups do not constitute the majority of the functional groups within a PU.\textsuperscript{1} Other functional groups include ether, allophanate, urea, biuret, carbodiimide, isocyanurate, acylurea as well as aliphatic chains and aromatic rings.\textsuperscript{7}

The two-phase micro-domain structures of PU and their scope for structural diversity allow many possible combinations of physiochemical and mechanical characteristics, making PU unique when compared to other polymeric materials. The properties of PU range from rigid hard thermosetting materials to those of much softer elastomers.\textsuperscript{1} PUs combine flexibility with high strength, wear resistance and a degree of hardness, and have relatively good blood compatibility and various degrees of hydrolytic stability, which explain why they have been used extensively over the past few decades as biomaterials, particularly as components of implanted devices.\textsuperscript{1,8}

PU’s dual-microphase structure with regions of hard or soft segments also contributes to one of its very important properties, which is also part of the focus in this thesis: \textit{in vivo}
degradation. In order to maintain the minimal interfacial free energy, the surface composition of PUs varies with the environment it is exposed to. This ability to change surface composition is largely due to the mobility of its soft segments. For instance, when the PUs are exposed to a polar environment, there will be a higher proportion of polar hard segments at the interface.

### 2.1.2 The synthesis of polyurethane

The synthesis of polyurethanes is based on the highly reactive nature of the isocyanate functional group. The electronic structure of the isocyanate group consists of several resonance states:

\[
\overset{\delta^-}{O} \overset{\delta^+}{C} \overset{\delta^-}{N}
\]

The resonance structures indicate that the highest net positive charge is on the carbon atom, and hence, this centre becomes susceptible to nucleophilic attack. Electrophilic groups adjacent to the isocyanate will draw electrons from the carbon, making it more positive and more susceptible to nucleophilic attack. Therefore, aromatic diisocyanates are more reactive than aliphatics because of the electron withdrawing nature of the benzene ring, which makes the isocyanate carbon even more susceptible to nucleophilic attack. In addition,
stereochemical factors also play an important role. Ortho substituents in aromatic compounds, branched or bulky substituents on aliphatic molecules will sterically hinder the approach of electron donors and retard the reaction rates.¹

The primary reaction for PU synthesis is the reaction of the isocyanate group with a hydroxyl terminated molecule. The mechanism is as follows: 1) the free electrons from the hydroxyl’s oxygen attack the nucleophilic carbon. In a subsequent step, 2) there is an electron rearrangement to establish balance on the oxygen and nitrogen, followed by 3) an immediate covalent bonding of the released hydrogen from the hydroxyl group to the nitrogen of the isocyanate.

The reactivity of primary alcohol is three times that of secondary alcohol and 200 times that of tertiary.¹ The rate of reaction can also be affected by the size of side groups on the alcohols, which sterically hinders the approach of isocyanates.

Isocyanate groups are very reactive and promote reactions with other functional groups such as amine, carboxylic acid and water. The isocyanate group is also able to react with the newly formed functional groups in the polymer, if the isocyanate is present in excess concentration. In the latter case, interactions between the polymer chains result in the
formation of crosslinks between the polymers, consolidating polymer networks.\textsuperscript{1,6} The crosslinks are primarily covalent bonds, which are extremely strong and stable and cannot be broken once formed. Other weaker intermolecular bonds such as hydrogen bonds also exist between urethane groups and ether or ester links, and serve to influence the stability of the separated microdomains within the material, as mentioned earlier.

The most common laboratory method used for the synthesis of biomedical polyurethanes is the two-step or prepolymer method.\textsuperscript{1,2,6} The prepolymer is first synthesized from an oligomeric diol in excess diisocyanate to produce an isocyanate terminated oligomer of low molecular weight. This prepolymer is then reacted with a diol or diamine chain extender to produce high molecular weight multi-block copolymer. This synthesis method offers greater control of reaction chemistry and produce narrower molecular weight distributions than single-step polymerization.\textsuperscript{1,6} Most of the syntheses are carried out in solution. The rate of the uncatalyzed reaction and the effectiveness of the catalyst may be influenced by the choice of solvent.\textsuperscript{6} Careful choice of solvents is also crucial because solvents which will complex with the catalyst or the active hydrogen compound can result in a slower reaction. Commercially produced polymers typically use solvent-free, bulk polymerization techniques.

\textbf{2.1.3 Polyurethane Degradation}

Although PUs have excellent mechanical properties, their \textit{in vivo} biostability has been found to be an issue because they are very susceptible to hydrolytic and oxidative
processes in biological environment. The inflammatory cells, specifically monocyte-derived macrophages (MDMs) and foreign body giant cells (FBGCs), have been shown to elicit both of these two degradation processes by providing a significant source of oxidative species and hydrolytic enzymes.\textsuperscript{2,9,10}

Polyester urethanes have been found to be especially susceptible to hydrolytic degradation\textsuperscript{8,11} while polyether urethanes are more resistant to hydrolysis but are readily susceptible to oxidation due to the soft polyether segments.\textsuperscript{12-14} The presence of enzymes, the hard segment structure, steric hindrance and hydrophilicity of the polymer strongly affect the rate of hydrolysis.\textsuperscript{1}

Many biological enzymes have been shown to have the capability of recognizing and acting upon synthetic substrates such as PU polymers, and a number of investigators have shown that many enzymes can in fact degrade polyurethanes \textit{in vitro}.\textsuperscript{15,16} Smith et al. synthesized \textsuperscript{14}C-labelled polyether urethanes and exposed them to solutions of esterase, papain and lysosomal liver enzymes and found that PUs incubated with esterase, cathepsin C and liver homogenate released higher concentrations of \textsuperscript{14}C labeled species than buffer controls.\textsuperscript{16} In addition, polyether urethanes with soft segment molecular weights of 1000 were more susceptible to papain and cathepsin C degradation than polymers with soft segment molecular weights of 650 and 2000. Santerre et al.\textsuperscript{17,18} specifically used enzymes known to be associated with MDM activity to study the cleavage of PU. They found that PU degradation was enzyme and material specific.\textsuperscript{17,19} They were able to demonstrate that enzymes that are associated with inflammatory cells, such as cholesterol esterase (CE),
degraded polyester urethanes over a 30 day period while other enzymes such as collagenase, cathepsin B and xanthine oxidase do not. Moreover, the urethane and urea linkages are found to be less susceptible to CE hydrolysis than the ester linkage.\textsuperscript{6,18,20}

When CE was used in the degradation of polyether urethane, they found that the degree of degradation was significantly less.\textsuperscript{17} In another study with polyether-based polyurea-urethanes, Santerre et al.\textsuperscript{21} showed that CE would hydrolyze the urethane to release hard segment components. However, their studies showed that the polymer that contained the most hydrolytically labile urea and urethane bonds (most hard segments) exhibited the least degradation. They suggested that the hard segment micro-domains have stable hydrogen-bonds that shield the urethane and urea bonds from hydrolytic attack. This finding agrees with previous \textit{in vivo} observations which indicated that the elevated hardness of hard-segment-containing Pellethanes have better \textit{in vivo} biostability.\textsuperscript{2,22} In a study with polycarbonate PU, Tang et al.\textsuperscript{23} demonstrated that hard-segment chemistry and density also influenced the degradation of polycarbonate PU by esterase. Polymer synthesized with methylene-diphenyl-diisocyanate (MDI) showed higher stability than the polymers synthesized with methylene-dicyclohexane-diisocyanate (HMDI) and hexane diisocyanate (HDI). The stability is believed to be due to the fact that MDI undergoes less molecular reorganizations on the surface and it retains a more hydrophobic nature when exposed to water. The results also suggested that the increase in density of the hard segment size causes more carbonyl groups to be integrated into secondary hard segment structures through hydrogen bonding, resulting in less degradation induced by CE. They ranked the different chemical groups’ susceptibility to hydrolysis as follows: non-hydrogen-bonded carbonate >

Many studies have been carried out in an effort to modify PUs to increase their \textit{in vivo} biocompatibility. Santerre et al. \textsuperscript{3,24-27} reduced the materials’ susceptibility to hydrolysis by adding surface-modifying macromolecules (SMM) containing fluorinated end groups to the base polyether urethane. They showed that different formulations of SMM provided varying degrees of enzyme resistance and also pro-actively reduce oxidation. Baumgartner et al. \textsuperscript{28} incorporated phosphorylcholine head groups into the PU backbones by using glycerophosphorylcholine (GPC) as the chain extender. They were able to increase biocompatibility and reduce bacterial adhesion with increased GPC content.

\section*{2.2 Polymeric Drug Delivery}

\textbf{2.2.1 Controlled Drug Delivery}

A major challenge with conventional oral or intravenous drug delivery methods is that many medications that are being used require high quantities of the drug to be administered in order to provide local relief and achieve a therapeutic effect. While distributing the drug to the target tissue, some drugs often accumulate in non-target organs, which can result in adverse side effects and gastrointestinal injuries. \textsuperscript{29,30} These modes of administration also rely heavily on patient compliance, which has been shown to have increased hospital admissions. \textsuperscript{31} Very often, there is no need for systemic administration of a drug that is meant to treat a localized malady. Therefore, a lot of attention has been put into the development of drug delivery systems that can administer drugs locally and with precise
drug release rates for prolonged times.

The potential advantages of controlled drug delivery include: 1) continuous maintenance of plasma drug levels in a desirable range, thereby eliminating the problems of drug under- or over-dosage; 2) local delivery to the target site increases effectiveness of drug and minimizes side effects, which is particularly important in the case of highly toxic drugs and sensitive bio-derived molecules such as peptides and proteins; 3) improvement in patient compliance; 4) lower drug waste; 5) possibility of delivering drugs to sites that are inaccessible under normal conditions such as the brain.  

Controlled drug delivery is especially useful in chronic conditions, in which long term treatment and multi-day dosing schedule is needed. Local drug-delivery implants will provide precise delivery with a single application for an extended period of time. A biodegradable polymer-drug system thus becomes a favorable approach because it has the advantage of dissolving away after its delivery mission.

2.3 Polymer-based drug delivery systems

Controlled drug delivery systems can be broadly classified into liposome-based, electromechanical and polymer-based delivery systems. The focus of this thesis is on the polymer-based systems and will hereby be discussed in detail. Chemical and physical means have been explored for the delivery of bioactive agents from the polymers. Diffusion and solvent activation, either through osmosis or swelling of the system are classified as physically-controlled release mechanisms. Systems that rely on chemical or enzymatic
reaction to cause degradation of the polymer or cleavage of the drug from the polymer are classified as chemically-controlled release systems. These methods include grafting or coating drug agents on the surface of polymers, as the backbone of polymers, or through drug encapsulation within a polymer matrix, microspheres, microcapsules, micelle or hydrogels. 32

Figure 2.1 shows some of the common approaches to the design of degradable devices. The most common drug delivery approach is to disperse the drug physically within the biodegradable matrix (Figure 2-1). Another approach is by polymeric-drug conjugation (Figure 2-2), which is a chemically-controlled release system.

\[ \text{D} = \text{drug} \quad \text{X} = \text{degradable bond} \]

**Figure 2-1:** Biodegradable drug delivery systems - drugs are physically entrapped within the biodegradable matrix. (Figure modified from 34)
Figure 2-2: Polymeric drug conjugates - drugs are chemically bonded to the polymer. (Figure modified from 34)

2.3.1 Diffusion-based systems

Figure 2-3a shows a polymeric drug reservoir device in which the drug is physically entrapped inside a solid polymer that can be injected or implanted. The classic example of this type of drug delivery is the implantable contraceptive Norplant™, which comprises a polydimethylsiloxane capsule containing steroids that are slowly released by diffusion through the polymer for 5 years.35

Another very similar system is the monolithic device in which the drugs are evenly embedded in polymers at high concentration (Figure 2-3b). The drug molecules in the matrix form a network of interconnected particles.36 The drug on the surface of the device becomes solubilized first and diffuses out of the matrix, leaving pores for the drugs deeper in the matrix to diffuse out slowly. For this type of system, the diffusion path length changes as the drug leaves the device, making constant release rates difficult to achieve. The earlier devices
were fabricated from non-degradable model polymers such as ethylene vinyl acetate copolymer and poly(dimethylsiloxane). However, due to the non-degradable nature of these devices, a second surgical procedure is often required to remove them after the implantation, resulting in very limited \textit{in vivo} applications of these systems. Therefore, many groups have later explored the concept of using biodegradable polymers, such as polylactic acid (PLA), polyglycolic acid (PGA) and poly lactic/glycolic acid copolymers (PLAGA), as carrier matrices. The combined mechanisms for the diffusion of medication through pores and the degradation of the polymer matrix provided better control of the drug release profile. By manipulating the polymer property, the therapeutic delivery profiles become more controllable.

Microsphere (monolithic) or microcapsule (reservoir)-based delivery systems (10-2000μm) were later developed. These dosage forms of the biodegradable matrices have very high surface areas, which increase the release rate of the drugs and also facilitate the administration of the drug carriers via parenteral routes. More complex systems such as core-shell systems and pH-sensitive spheres were also developed to add control over the released profiles. These type of delivery systems have been investigated for the delivery of many different drugs, such as anesthetics, antibiotics, anti-inflammatory agents, anticancer agents, hormones, steroids, and vaccines. As an example, Freitas et al. developed polylactic acid microspheres that contain nimesulide, a non-steroidal anti-inflammatory drug (NSAID). The system was prepared using the classical emulsion solvent-evaporation method, and \textit{in vitro} studies showed that 29% of the drug was released over 108 hours.
Other than PLAGA, poly(caprolactone) (PCL) has also been extensively explored as a matrix for macromolecular release and long term drug delivery due to its slow degradation rate and high permeability of drugs.\textsuperscript{41} However the biodegradable polyesters mentioned to date often undergo bulk erosion, in which the water influx becomes greater than the polymer erosion, hence resulting in a multiphase release characteristic rather than the favorable constant drug delivery.\textsuperscript{37} To tackle this problem, surface eroding polymers such as poly(ortho esters)\textsuperscript{42} and polyanhydrides\textsuperscript{43,44} were later employed as carrier candidates for monolithic implants and microspheres. Many other biodegradable polymer systems were investigated as drug delivery matrices, such as polyhydroxy alkanoates, poly(glutamic acid), poly(amino acid), polyphosphoesters and polyphosphazenes.\textsuperscript{32}
2.3.2 Water penetration-based systems

Hydrophilic hydrogels are excellent candidates as matrix devices for the delivery of bioactive agents because of their ability to absorb large quantities of water or body fluids and swell, and yet remain insoluble in aqueous solutions because of their chemical or physical cross-linking network of the polymer chains. The physical cross-links are usually formed by entanglement and crystallites while chemical cross-links are usually formed by difunctional cross-linking agents or high-energy radiation. Hydrogels can be prepared from natural or synthetic homo or copolymers. Depending on the extent of cross linking of the polymer, the chemical structure of the monomer components, the amount of drug loading, nature of the drug molecule, method of drug loading, and external stimuli such as pH, temperature and ionic strength, the release profile of the bioactive agents could be very different. (See Figure 2-4) There is usually more than one mechanism that governs the delivery of bioactive agents from the hydrogel systems. Many models have been developed in an attempt to predict the release of the bioactive agents from a hydrogel device. The mechanisms that control the release profile include diffusion, swelling and chemical reactions that occur within the hydrogel matrix.

Acrylic acid (AA) and methacrylic acid (MAA) are very commonly-used monomers for the fabrication of anionic pH-sensitive hydrogels. 2-(dimethylamino)ethyl methacrylate (DMAEMA) is used for cationic hydrogel fabrication. Other polymers that are used include N-isopropylacrylamide (NIPAAM) and polypropylene oxide–polyethylene oxide–polypropyleneoxide (PPO–PEO–PPO) block copolymers. Controlled-drug release systems with biodegradable thermosensitive polyesters and polyphosphazenes have also been
developed in recent years. Polymers such as polycaprolactone (which can be degraded by lipase) were copolymerized with poly (ethylene glycol) (PEG) to make enzymatically degradable gels.

Figure 2-4: Swelling of hydrogel as water penetrates the system. Mesh size changes in response to the degree of crosslinking, chemical structure and external stimuli to release the drug.

More examples of cross-linked polymers that have been explored as candidates for the polymeric carriers of swelling systems to deliver anti-inflammatory agents are copolymers of guar gum with acrylamide cross-linked with glutaraldehyde, sodium alginate and methylcellulose cross-linked with glutaraldehyde, chitosan and methylcellulose cross-linked with glutaraldehyde. One group used a supercritical fluid method to impregnate ethylcellulose/methylcellulose micropheres with naproxen, another NSAID. They managed to obtain 40% release over 8-10 hours. Saravanan et al. synthesized magnetic gelatin micropheres that were loaded with diclofenac sodium in an attempt to improve targeting efficiency of the micropheres. They were able to generate a slow release of drug over more than 18 days via ultrasonically controlled-release mechanism. After injecting the micropheres intravenously, however, they found that the majority of the injected dose was trapped in non-target organs.
2.3.3 Polymeric drug conjugates

Another approach to polymeric drug delivery is the design of polymeric prodrugs or polymer conjugates \(^{59-62}\) (Figure 2-2). One design of polymeric prodrugs was proposed by Prof. Ringsdorf in 1975 \(^{60}\) (Figure 2-5). This delivery system consists of a polymeric backbone, a drug, a biodegradable spacer and optionally a homing device. The idea is to have drugs fixed directly or via the spacer arm onto the polymer backbone and the bonds connecting the two are cleavable by hydrolysis and enzymes.\(^{59,62}\) (See Figure 2-2a)

![Figure 2-5: Ringsdorf-proposed model for polymeric prodrugs. \(^{60,61}\) Bonds connecting drug and polymer are cleavable by hydrolysis or enzymes.]

Two different synthetic methods have been reported for the preparation of these polymers that contain pendant drug substituents. The first method requires the conversion of the drug into a polymerizable monomer by consecutive aminolysis or transesterification and then polymerized or copolymerized with other monomers to produce the final drug polymer. Davaran et al. \(^{62}\) converted an anti-inflammatory drug (5-amino salicylic acid (5-ASA)) into polymerizable derivatives (see Figure 2-6) and then free radically copolymerizing them with methacrylic acid (MA) or hydroxyethyl methacrylate (HEMA). The resulting polymer bears
the drug units as side substituents of the acrylic backbone, and the drug is connected to the
carrier via degradable ester or amide bonds. Drug release studies showed that the hydrolytic
behaviour of this system strongly depends on pH, with up to 50% of the drug being delivered
at pH 8.5 over 24 hours. Degree of swelling, type of comonomer and the nature of the
hydrolysable bond are believed to have a strong effect on the release profiles. Davaran also
prepared acrylic formulation for NSAIDs, in which the drug was covalently linked to a
polymer backbone via hydrolysable bonds.\textsuperscript{63-65} They successfully synthesized polyurethanes
and polyetherurethanes that have Ibuprofen pendant groups linked to the polymer directly or
with a methylene spacer group via ester bonds.\textsuperscript{63} They demonstrated that the release of
Ibuprofen by hydrolysis (pH 8, 37$^\circ$C) can be increased by the introduction of long
hydrophilic units along the polymer chain. They believed that these soft segments increased
the hydrophilicity and flexibility of the polymer, which promoted the susceptibility of the
ester bond to hydrolysis. The spacer group also increased the mobility of the pendant group
to cause more hydrolysis of the ester bonds to release more drugs. pH also plays an important
role in the hydrolytic behavior of the polymer.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{5-ASA.png}
\caption{The polymerizable derivatives of the anti-inflammatory agent 5-ASA, which
was then copolymerized by free radical technique to form copolymers with 8-20
wt\% drug.\textsuperscript{62}}
\end{figure}

The second method to synthesize drug conjugate is to attach the drug directly to
preformed polymer backbones via degradable chemical bonds. Zovko et al.\textsuperscript{66} synthesized
polymer-fenoprofen conjugates by modifying NSAID fenoprofen into benzotriazolide and amino acid amide derivatives, and then reacting them with polyhydroxy aspartamide-type polymers. Babazadeh et al.\textsuperscript{61,67} attached diclofenac and ibuprofen directly to a HEMA – acrylamide copolymers. They showed that the drug release is linked to selective hydrolysis (37° C) of the ester or amide bonds from side chain of the drug moieties. Giammona et al.\textsuperscript{68} used a the biodegradable polymer, α,β-poly(N-hydroxyethyl)-dl-aspartamide (PHEA), as a drug carrier for the attachment of naproxen and 4-Biphenylacetic acid. The macromolecular conjugates retained both \textit{in vivo} analgesic and anti-inflammatory activities and showed maximum activity at 4 hours subsequent to administration as oppose to 1 hour for the free drugs.

Many different combinations of polymer and bioactive agents have been explored to develop pendant drug conjugate. Many of them have frequently been synthesized to deliver anticancer agents. One of the carriers that have been widely used to carry anticancer agent is N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers.\textsuperscript{69} Some groups have tried to synthesize drug conjugates that carry anti-inflammatory agents. Boudreaux et al.\textsuperscript{70} synthesized acrylic acid and alkylacrylamide copolymers with pendent beta-naphthol esters moieties. Chang et al.\textsuperscript{71} made NSAIDs macromolecular prodrugs by covalently linking ibuprofen, ketoprofen and naproxen with HEMA and then copolymerizing with MA. Recently, many other polymer-drug conjugates of NSAIDs were synthesized with this method. Some of the therapeutic agents that were conjugated are ibuprofen\textsuperscript{63-65,71-73}, ketoprofen\textsuperscript{71,73}, naproxen\textsuperscript{71,73} and indomethacin\textsuperscript{65,74}. In one study, Udipi et al.\textsuperscript{75} conjugated a low molecular weight anti-inflammatory superoxide dismutase mimic (SODm) to small
disks of poly(etherurethane urea). This surface-bound drug exhibited anti-inflammatory effects which included a dramatic reduction in both acute and chronic inflammation associated with implant materials. It also has a potent inhibitory effect on matrix production and fibrosis at the implant interface and has a protective effect on poly(etherurethane urea) biodegradation in vivo.

Other than the pendant drug-polymer conjugate design proposed by Ringsdorf, other groups have attempted to incorporate bioactive agents directly as part of the biodegradable polymer backbone (Figure 2-2b).76,77 These agents are inserted into the polymer as monomers. Mahkam et al.76 synthesized morphine-containing polyetherurethanes by polymerizing morphine with a prepolymer that consisted of PEG and 1, 4-diiodocyanatocubane. Degradation (pH 8, 37°C) results showed that 1,4-diaminocubane and morphine were slowly released from the polymer by hydrolysis. The drug release was much slower when a similar polyurethane drug polymer that does not contain PEG was degraded. The introduction of PEG is believed to have increased the hydrophilicity of flexibility of the polymer which promoted the hydrolysis of the urethane bond that connects the drug and the rest of the polymer. Similar results were shown in another study that aims at producing drug-polymer for colon-specific delivery.77 In this study, 5-[4-(hydroxyl phenyl)azo] salicylic acid (HPAS) and 4,4'-dihydroxyazobenzene (DHAB) were incorporated into the backbone of a polyetherurethane that consist of 1,6-hexamethyleneisocyanate (HDI) and PEG. HPAS and DHAB act as low molecular weigh diols in the polymer synthesis.

2.3.4 Responsive delivery systems
With the development in synthetic biodegradable polymers, a wide range of bioactive agents, from therapeutic agents to macromolecules such as proteins, can be effectively delivered. Most of the systems mentioned earlier on in this section manage to retain a constant drug concentration over a period of time, but the release tends to be passive, that is, the drug will be continuously administered regardless of the need until all of the drug is exhausted.

More recent studies have tried to develop “smarter” delivery systems where the drug delivery is controlled by an external or internal stimulus, other than just hydrolysis. As mentioned earlier, there are some hydrogel-based systems which respond to external stimuli such as pH, ionic strength, and temperature and exhibit variable swelling patterns. 32,45,47,48,51-53

Pulsed drug delivery systems have also been developed and the release is either governed by an inner mechanism of the device or by the changes in the physiological environment or external stimuli. 78 An example of such delivery systems is the hyaluronic acid-based system developed by Yui et al. 79,80 The hyaluronic acid responds to the increase in concentration of hydroxyl radicals and hyaluronidase during inflammation to release drug. 79-81

An alternate approach is to incorporate drugs directly into the backbone of a biodegradable polymer which contain moieties that are sensitive to specific hydrolytic processes within the biological environment. Santerre et al. 4,82 incorporated drugs into the
backbone of a polyurethane as main chain monomers. A fluoroquinolone antimicrobial drug (Ciprofloxacin) was synthesized into the backbone of the polyester urethane. Their concept was to take advantage of the enzymes released during the inflammatory process, namely CE, to trigger the release of the antimicrobial drug. This design is based on the knowledge that the inflammatory response is activated in the presence of bacteria or injury, leading to infections. The activation of macrophages then up-regulates the inflammatory enzymatic reactions which then results in the degradation of the PUs to release the antibiotics. Their in vitro studies showed that CE was able to cleave the polymer (pH 7, 37°C) to produce multiple antibiotic-containing degradation products. However, despite of the increase in degradation products, the amount of free drug released was not significantly more than when the polymer was incubated in buffer solution. Nevertheless, the antibiotics released from the polymer were enough to inhibit the growth of bacteria. In another study, ciprofloxacin was converted into a biomonomer which consisted of two drug molecules connected together via a spacer arm triethyl glycol (TEG). The drug polymers were synthesized by reacting the diisocyanates with polycaprolactone (PCL) and then chain extending the polymer with the biomonomer. In a study done by Tangpasuthadol et al., they synthesized a polycarbonate polymer that has pendant chains connected to it via ester bonds. Their degradation results demonstrated that the backbone carbonate bond is hydrolyzed faster than the pendant chain ester bond. Increased alkyl pendant chain length further decreased hydrolysis, due to harder accessibility of water to the hydrolysable linkages.

2.4 Medical Device Related Inflammation and Inflammatory Diseases

The efficacy of a biomedical device that is placed in vivo is inherently linked to the
ability of the device to become incorporated into and accepted by the body. Upon the placement of the implant, a response of injury is usually initiated, activating a sequence of healing mechanisms. Specifically, the reaction of the vascularized connective tissue to cell injury is called inflammation.\textsuperscript{9,85,86} The vascularized connective tissue includes the plasma, circulating cells, blood vessels, and cellular and extracellular constituents of connective tissue.

Inflammation serves to contain, destroy, dilute or wall off the injurious agent, while it sets into motion the process of repair – a series of events that may heal and reconstitute the implant site.\textsuperscript{85} Repair involves the replacement of the injured tissue by regeneration of native parenchymal cells, or the formation of fibroblastic scar tissue or, most commonly, a combination of these two processes.\textsuperscript{9,85,87} Although inflammation plays a very crucial role in the body’s defense and wound healing, it can also be potentially harmful because it can lead to fibrous capsule formation and also affect the structural integrity of implanted devices, which will ultimately have an effect on the biocompatibility of the implant.\textsuperscript{9} Consequently, an ideal biocompatible implant would then be one that can enhance the beneficial effects of inflammation yet control its harmful sequelae.

2.4.1 Inflammation Responses and Repair

When the tissue suffers an injury from implantation, the macrophages present in the affected tissues quickly detect for foreign matter, bind to them and try to engulf them by phagocytosis.\textsuperscript{9} Cytokines such as IL-1β and TNF-α are secreted by the activated macrophages. Mast cells at the injury site also secrete histamine to cause vasodilation and
Cytokines induce nearby blood vessels to produce adhesion molecules called selectins, which protrude from the vessel’s interior endothelial cells and attach loosely to the leukocytes that are in the bloodstream. Integrins on the leukocytes are also activated, which leads to a firm attachment of the leukocytes to the endothelial cells. Adhesion is then followed by diapedesis. The leukocytes then migrate to the site of injury by chemotaxis, depending on the specific receptors for chemotactic agents on their cell membranes. The predominant cell type present in the inflammatory response varies with the age of the injury. During acute inflammation, which is the immediate and early response to an injury and is usually short (hours and days), the leukocytes that play the major role are neutrophils. After a few days, monocytes become the predominant cell type, and they differentiate into macrophages after diapedesis, which are very long-lived (months). Inflammation is regarded as chronic when the inflammation is of prolonged duration (weeks and months) and is associated with the infiltration of lymphocytes and macrophages.

Neutrophils and macrophages at the implant site will secrete cytokines that help prolong and coordinate the body’s response to injury, and engage in phagocytotic activities. During normal phagocytosis, the neutrophils or macrophages will engulf its prey, kill and degrade it. However, in the case of an implanted biomaterial, the size disparity between the biomaterial surface and the attached cell (the biomaterials are often too large to be engulfed) will cause “frustrated phagocytosis”, resulting in the extracellular release of a wide variety of biologically active products in an attempt to degrade the non-phagocytosable biomaterial,
including inflammatory cytokines, lysosomal enzymes, and reactive oxygen and acid species which are potentially harmful to the biomaterial’s integrity. In addition, activated macrophages can fuse to form inflammatory giant cells or foreign body giant cells (FBGCs), which will cause more for secretion. Macrophages and FBGCs may persist at the tissue-implant interface for the lifetime of the implant.

### 2.4.2 Biomaterials, Tissue Engineering and Regeneration

The nature of the foreign body reaction to an implant depends on the extent of injury created by the implantation, the chemical properties, charge, free energy and topography of the surface of the biomaterial. The extent and duration of inflammation and wound healing determines the biocompatibility of the material. Prolonged inflammation can lead to fibrous encapsulation of the implant, which can cause cell death around the biomaterial and eventually lead to the failure of the implant. Extensive secretion of enzymes from macrophages or FBGC can lead to the decomposition of the biomaterial in vivo, which can result in cell toxicity and device failure.

For biodegradable materials, such as biodegradable polymers, the intensity of the inflammatory responses may be modulated by the biodegradation process which may lead to topographic changes of the materials and the release of polymeric oligomer and monomer as degradation products. Internal fracture fixation devices made with biodegradable synthetic polymers such as Poly(glycolic acid) (PGA) and Poly(lactic acid) (PLA) have been demonstrated clinically to invoke non-specific foreign body reactions that comprised of polymorphonuclear leukocytes and PBGCs phagocytosing degradation products.
Phagocytosis of Poly(L-lactic acid) fragments by peritoneal macrophages have also been shown to result in cell damage and necrosis.\textsuperscript{91}

Over the last few years, interest in tissue engineering and regeneration has burgeoned. Tissue engineered devices that are composed of transplanted cells and biomaterials have been investigated as devices for the restoration or modification of tissue or organ function. In order for these devices to achieve their intended function, appropriate host responses are desired.\textsuperscript{86} An apparent short term favourable response must have minimal issues of inflammatory response towards a biomaterial and immunologic response towards transplanted cells. The interconnections between the inflammatory response and the immune response increase the complexity of host response in tissue engineering devices. In Mikos et al.’s \textsuperscript{86} thorough review, they compiled experimental evidence on inflammatory response towards biodegradable polymer scaffold materials for various applications including bone, nerve, and skin regeneration, therefore highlighting the need for biomaterials that can control these inflammatory processes as they undergo resorption.

### 2.4.3 Inflammatory Diseases

Sometimes, the inflammation process can also be triggered even when there are no foreign invaders, as in the cases of autoimmune diseases. In such cases, the body’s normally protective immune system mistakenly recognizes its own tissue as alien, and as a result initiates a series of events to destroy its own normal tissue and progressively aggravate inflammation. Rheumatoid arthritis, heart disease and atherosclerosis are examples of severe
pathologies closely associated with uncontrolled inflammation.\textsuperscript{92,93} These diseases have caused disability, and claimed the lives of many.

2.4.4 Anti-inflammatory Medications

Most anti-inflammatory agents that are available usually fall under the following categories: Non-steroidal anti-inflammatory drugs (NSAIDs), and disease-modifying antirheumatic drugs (DMARDs) such as methotrexate, and corticosteroids. The different groups of drugs differ not only by their mechanisms of action, but also by their side-effects.\textsuperscript{94-97} The NSAIDs, such as salicylates and ibuprofen, are important in the pharmaceutical treatment of pain (at low doses) and inflammation (at higher doses). They generally act through the inhibition of cyclo-oxygenases (COX), which in turn inhibit the synthesis of prostaglandin (PG). Research suggested that most of the analgesic effects of NSAIDs are mediated by blocking the COX-2 inducible enzyme and the adverse effects of NSAIDs, especially the gastrointestinal injuries, are caused by blocking the constitutive COX-1.\textsuperscript{95,97} Inhibition of the synthesis of PG, specifically PGE\textsubscript{2}, prevents vasodilation, and hence reduces plasma exudation, explaining NSAIDs’ ability to relieve inflammatory pain, erythema and swelling. However, a big body of evidence suggests that NSAIDs have additional prostaglandin-independent anti-inflammatory mechanisms of action.\textsuperscript{97} Methotrexate, one of the most widely used DMARDs, inhibits methylation reactions, which may account for inhibitory effects on cytokine production and promotes adenosine release, which as a result inhibit the adhesion of neutrophils.\textsuperscript{94} Corticosteroids possess both anti-inflammatory and immunosuppressive abilities. They act by increasing or decreasing transcription of genes, such as cytokine genes. Although they inhibit inflammatory disease
progression, they are generally restricted due to long-term side effects.

Despite their difference in mode of action, all the anti-inflammatory drugs that are currently used share the property of inhibiting the recruitment of neutrophilic leukocytes to the site of inflammation. Accumulation of polymorphonuclear leukocytes is a characteristic of acute inflammatory responses. Active joint inflammation is consistently accompanied by a histopathological infiltration of activated neutrophils into synovial fluid.

The major challenge with many anti-inflammatory medications that are being used is that high quantities of the drug must be systematically administered in order to provide local pain relief and achieve therapeutic effect. While distributing the drug to the target tissue, some drugs often accumulate in non-target organs, which can result in minor unwanted side effects, such as nausea, vomiting, dizziness and fatigue or more serious complications such as gastrointestinal injury with NSAIDs or COX inhibitors. Very often, there is no need for systemic administration of a drug that is meant to treat a localized malady. Therefore, a lot of attention has been put into the development of drug delivery systems that can administer anti-inflammation drugs locally, while minimizing systemic side effects.

Oxaceprol is a synthetic N-acetylated derivative of hydroxyproline, which is known as an atypical inhibitor of inflammation. It was introduced for the treatment of degenerative joint diseases over 20 years ago and was widely used in France and Germany. Oxaceprol is used to treat osteoarthritis and rheumatoid arthritis and its clinical efficacy is similar to the classical non-steroidal anti-inflammatory drugs (NSAID) ibuprofen and diclofenac for
pain relief.\textsuperscript{98} However, Oxaceprol has been shown to have no effect on PGE\textsubscript{2} or leukotriene C\textsubscript{4} production by ionophore-stimulated mouse peritoneal macrophages \textit{in vitro}, showing that it does not have COX inhibitory activity.\textsuperscript{101} Its low incidence of gastrointestinal side-effects is most likely due to the fact that it does not inhibit prostaglandin synthesis, unlike the traditional NSAIDs. Ionac et al.\textsuperscript{101} demonstrated that Oxaceprol administered daily for 15 days to arthritic rats markedly inhibited secondary tail and ear lesions. Inhibition of synovial membrane neutrophil infiltration was also observed at day 15, which was comparable to that achieved with indomethacin. However, in contrast to some NSAIDs, Oxaceprol had very little effect on the primary paw swelling and cartilage damage. In fact, high concentrations of Oxaceprol have been demonstrated to have stimulatory effects on cartilage proteoglycan metabolism \textit{in vitro}.\textsuperscript{94,100} Veihelmann at al.\textsuperscript{99} investigated the leukocyte-endothelial cell interactions in oxaceprol-treated mice with antigen-induced arthritis (AiA) using intravital microscopy and found that the leukocyte adherence to the endothelium and swelling were significantly reduced. Literature suggests that Oxaceprol’s anti-inflammatory activity is closely related to its ability to inhibit neutrophil infiltration into the inflamed site and to inhibit the extravasation of neutrophilic leukocytes. Although the precise mechanism of action remains to be established, it has been proposed that Oxaceprol acts predominantly by inhibiting leukocyte adhesion and migration.\textsuperscript{94}
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Chapter 3 Synthesis and Characterization of a novel Anti-inflammatory Drug Polymer Using Oxaceprol

Abstract
This study assessed the feasibility of incorporating an anti-inflammatory drug, Oxaceprol (OC), as a monomer unit into the backbone of a biodegradable poly(ester)urethane (PEU) to form a polymer that can release anti-inflammatory drugs in response to inflammatory-related enzymes. The model drug polymer was successfully synthesized using diisocyanates, poly(caprolactone) diol, and oxaceprol biomonomers as chain extenders. The latter consisted of a symmetrical unit that contained two anti-inflammatory oxaceprol drug molecules linked together by a tri(ethylene glycol). Biomonomers were characterized by mass spectrometry, infrared spectroscopy (FT-IR) and nuclear magnetic resonance spectroscopy (NMR) while the polymer was characterized by size-exclusion chromatography (SEC) and differential scanning calorimetry (DSC). In vitro biodegradation studies for 4 weeks at 37°C, pH 7.0, demonstrated that a trimethylhexamethylene diisocyanate (THDI)-based drug polymer was sensitive to increases in the concentration of an inflammatory enzyme, cholesterol esterase, and released more free drug as compared to the buffer-incubated samples. A 1, 12-diisocyanatododecane (DDI) analog of this drug polymer was synthesized as a comparison and demonstrated minimal drug release even when incubated with enzyme. The drug delivery response of the two polymers was believed to be a direct function of the molecular structure and distribution of the PEU hard segment, and the degree of interaction between the polymer chains.
Introduction

Polymeric drug delivery technology has evolved into a very active area of research in recent years. With the advances in synthetic biodegradable polymer development, a wide range of bioactive agents, from therapeutic agents to macromolecules such as proteins, can be effectively delivered. Controlled drug delivery has the advantage of regulating drug delivery rates and localizing the therapeutic agents,¹,² and hence minimizing adverse side effects. Chemical and physical approaches have been explored for the incorporation of bioactive agents into polymers. These methods include grafting or coating drug agents onto the surface of polymers, or drug encapsulation within a polymer matrix, microspheres, microcapsules, micelle or hydrogels.²-⁴ In these systems, drug molecules are released by diffusion and/or polymer erosion. Although these systems manage to retain a constant drug concentration over a period of time, the release tends to be passive, that is, the drug will be continuously administered regardless of the need until all of the drug is exhausted.

More recent studies have tried to develop “smarter” delivery systems where the drug delivery is controlled by an external or internal stimulus. Hydrogel-based systems respond to external stimuli such as pH, ionic strength, and temperature ²,⁵-¹⁰ and exhibit variable swelling patterns. Pulsed drug delivery systems have also been developed and the release is either governed by a secondary internal mechanism of the device or by the changes in the physiological environment or external stimuli.¹¹ An example of such delivery systems is a hyaluronic acid-based appliance developed by Yui et al.¹²,¹³ The hyaluronic acid responds to the increase in concentration of hydroxyl radicals and hyaluronidase during inflammation to release drug.¹²-¹⁴

In other work, Udipi et al.¹⁵ covalently conjugated a low molecular weight
superoxide dismutase mimic (SODm) to small disks of poly(etherurethane urea), which imparted anti-inflammatory character to the material. SODm belongs to a new class of anti-inflammatory drugs consisting of a Mn(II) complex of a macrocyclic polyamine ring that catalyzes the dismutation of superoxide to less reactive hydrogen peroxide and oxygen at rates equivalent to that of native enzyme. This surface bound anti-inflammatory drug down-regulates superoxide anion and the associated reactive oxygen species. Udipi et al. demonstrated that active SODm has a dose-dependent anti-inflammatory effect including a dramatic reduction in both acute and chronic inflammation on materials. It also has a potent inhibitory effect on matrix production and fibrosis at the implant interface and has a protective effect on poly(etherurethane urea) biodegradation \textit{in vivo}.

An alternate approach is to incorporate drugs directly into the backbone of a biodegradable polymer which contain entities that are sensitive to specific hydrolytic processes within the biological environment. Woo et al.\textsuperscript{16,17} incorporated drugs into the backbone of a polyurethane (PU) as the main chain monomers. A fluoroquinolone antimicrobial drug (Ciprofloxacin) was synthesized into the backbone of the polyester urethane. The concept was to take advantage of the enzymes released during the inflammatory process, namely cholesterol esterase (CE) and carboxyl esterase, to trigger the release of the antimicrobial drug. This design is based on the knowledge that the inflammatory response is activated at sites of injury or bacterial infections. The activation of macrophages then up-regulates the inflammatory enzymatic reactions which then results in the degradation of the PUs to release the antibiotics. \textit{In vitro} studies showed that CE was able to cleave the polymer (pH 7, 37°C) to produce multiple antibiotic-containing degradation products. While polyurethanes synthesized with hexamethylene diisocyanate
(HDI) did not show an increased amount of drug release when comparing CE to buffer,\textsuperscript{16} subsequent materials synthesized with 1,12-diisocyanatododecane (DDI) showed a significant increase in drug release with enzyme vs buffer alone.\textsuperscript{17} In the latter work, the amount of antibiotics released from the polymer, in the presence of either CE or inflammatory cells, was enough to inhibit the growth of bacteria. In a more recent study, ciprofloxacin was converted into a biomonomer which consisted of two drug molecules covalently bound together via triethylene glycol (TEG). The drug polymer was synthesized by reacting the diisocyanates with polycapro lactone (PCL) and then chain extending the polymer with the biomonomer.\textsuperscript{18} The latter studies showed that drug and degradation product release profiles were highly dependent on the type of diisocyanate used.\textsuperscript{16-18}

Polyurethanes (PUs) are widely used in medical applications, including long-term implants, and more recently biodegradable scaffold systems for tissue engineering and drug delivery concepts.\textsuperscript{19} Although PUs have excellent mechanical properties, their \textit{in vivo} biostability has been found to be an issue that has limited their traditional applications because they are very susceptible to biodegradation. The two dominant chemical reactions at work in the breakdown of PUs are believed to be oxidation and hydrolytic enzyme processes. Inflammatory cells, and specifically monocyte-derived macrophages (MDMs) and foreign body giant cells (FBGCs), are involved in these two degradation processes by providing a significant source of oxidative species and hydrolytic enzymes.\textsuperscript{19-21} Much research has been done in an effort to understand and modify PU materials to increase their \textit{in vivo} biocompatibility as well as to conceive the design of more complex systems such as tissue engineering scaffolds and polymeric carriers for biologically active agents.\textsuperscript{16,19,22,23}

The goal of the current study was to synthesize anti-inflammatory biodegradable PUs
which could be used to control the inflammatory processes that are triggered at the time of implant placement. This would be particularly useful in tissue regeneration applications or to manage inflammatory processes in diseased tissues. Ideally, the drug would be released in a proportional magnitude to the initial inflammatory response.\textsuperscript{24-26}

The model anti-inflammatory drug that was used to synthesize the biomonomers for the polymer work was oxaceprol, a synthetic N-acetylated derivative of hydroxyproline that is known as an atypical inhibitor of inflammation.\textsuperscript{27-31} It was introduced for the treatment of degenerative joint diseases over 20 years ago and was widely used in France and Germany.\textsuperscript{27} It is used to treat osteoarthritis and rheumatoid arthritis and its clinical efficacy is reported to be similar to that of the classical non-steroidal anti-inflammatory drugs (NSAID) ibuprofen and diclofenac for pain relief.\textsuperscript{28} However, oxaceprol has been shown to have no effect on PGE\textsubscript{2} or leukotriene C\textsubscript{4} production by ionophore-stimulated mouse peritoneal macrophages \textit{in vitro}, showing that it does not have COX inhibitory activity.\textsuperscript{31} Its low incidence of gastrointestinal side-effects is most likely due to the fact that it does not inhibit prostaglandin synthesis, unlike the traditional NSAIDs. Oxaceprol administered daily for 15 days to arthritic rats markedly inhibited secondary tail and ear lesions. Inhibition of synovial membrane neutrophil infiltration was also observed at day 15, which was comparable to that achieved with indomethacin.\textsuperscript{27,30}

In this work, the polymers will be characterized for their biodegradation potential in order to determine the quantity of drug that could be released and to assess their degradation sensitivity to the inflammatory enzyme, CE.
3.2 Materials and Methods

3.2.1 Drug Biomonomer Synthesis

The materials used for the synthesis of the biomonomer were all purchased from Sigma-Aldrich Co., Milwaukee, WI, unless otherwise specified. Trans-1-acetyl-4-hydroxy-L-proline (Oxaceprol (OC), purity 99%), t-butyldimethylsilyl chloride (TBDMS-Cl), acetonitrile (anhydrous), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), pentane (>99%), methanol (EMD Chemicals Inc., HPLC grade), tetrahydrofuran (THF, ACS grade), tri(ethylene glycol) (TEG, anhydrous), dichloromethane (DCM, 99.8%, EMD Chemicals Inc., Gibbstown, NJ), dimethylaminopyridine (nucleophilic catalyst; DMAP), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), tetra n-butyl ammonium fluoride (TBAF), chloroform (ACS grade, EMD Chemicals Inc., Gibbstown, NJ), ethyl ether (ACS grade, EMD Chemicals Inc., Gibbstown, NJ) were used directly as received.

The anti-inflammatory biomonomer was synthesized by reacting the carboxylic acid group on OC with the hydroxyl groups of TEG, while leaving the hydroxyl group available for later polymerization. Synthesis was carried out under nitrogen purge. In a first step, 10 g of OC (57.75 mmol) and TBDMS-Cl (144.36 mmol) were added into 25 mL of acetonitrile in a 0°C ice bath. DBU (150.15 mmol) was then added and the reaction solution was left stirring overnight at ambient temperature and under a nitrogen purge. A white precipitate developed in this step and was filtered out. The filtrate was then concentrated by removing the solvent under reduced pressure. It was then treated with a 2:1 ratio of water to pentane (80 mL: 40 mL). A white precipitation layer developed in between the solvents and was filtered out. This residue was then dissolved in methanol (20 mL), THF (10 mL), water
(10mL) and 2N aqueous sodium hydroxide (16mL) and the mixture was stirred for 1.5 hours at room temperature. 1N hydrochloric acid was used to adjust the pH to 3 at which point the product began to recrystallize. The white precipitate was filtered out and dried. (Figure 3-1: Product A, 10.1g, 61% yield, MW: 287) (For mass spec analysis and NMR of Product A, see Appendix A - 1)

**Figure 3-1**: Reaction scheme for the synthesis of the drug biomonomer

Following step 1, 28.19 mmol of product A were allowed to react with TEG, DMAP and EDAC (respectively in the mole ratio of 1: 0.454: 0.046: 1.135) in 40 mL of DCM,
under nitrogen purge. This reaction took place at 0°C in an ice bath for 1 hour, after which the cooling was removed and the mixture was stirred for 9 days in ambient temperature. The solvent was then removed under vacuum pressure with a rotor evaporator and the residue was treated with a saturated NaCl solution (20mL) and extracted with chloroform (10 mL x 3). The chloroform extract was then dried using sodium sulphate and filtered, and the solvent was removed under reduced pressure. (Mass spectrometry analysis at this point showed that there were side products which included one that had a very similar structure as Product B, but only one end of the TEG was connected to a drug molecule. See Product pre-B in Appendix A-2 for details.) To further remove the side products generated during the reaction, the residue was passed through a small silica column (liquid chromatography column: I.D. × L 1.0 cm × 60 cm, Sigma-Aldrich Co., Milwaukee, WI, packed with silica gel, 200 mesh and finer, EMD Chemicals Inc., Gibbstown, NJ, collected in 10 mL fractions), with 2.5% methanol and 97.5% chloroform. Samples were collected in glass tubes and characterized by layer chromatography (TLC) (data not shown). Three different spots were observed at different locations on the TLC plate. The top spot was isolated, and the solvent was removed at low pressure using the rotorvap. The dominant product in this isolate was confirmed to be the protected biomonomer product. (Figure 3-1: Product B, 8.99g, 93% yield, MW: 688) (For mass spec analysis and NMR, see Appendix A -2)

For the hydroxyl group deprotection steps, 13 mmol of Product B were dissolved in THF (75mL) and reacted with TBAF (26 mmol) for 5 mins in an ice bath, after which the ice bath was removed and the reaction continued for another 2 hours at room temperature, under a nitrogen purge. The solvent was then removed under low pressure with a rotor evaporator. Multiple purification procedures were then established to remove the unreacted agents and
by-products from the pure product. Two different purification procedures were implemented in an attempt to isolate the pure product. An earlier attempt included the use of small silica gel chromatography separation (liquid chromatography column: I.D. \( \times \) L 1.0 cm \( \times \) 60 cm, Sigma-Aldrich Co., Milwaukee, WI, packed with silica gel, 200 mesh and finer, EMD Chemicals Inc., Gibbstown, NJ, collected in 10 mL fractions). The sample was passed through the column with a solvent mixture of 2.5% methanol and 97.5% chloroform. Samples were then collected using small glass tubes and spotted using capillary tubes on a silica thin layer chromatography (TLC) plate. Three different products were observed in the TLC (Figure A-15). The different products were isolated using the column and the solvent was removed from these samples by using a rotor evaporator. The residues were analysed by mass spectrometry separately (Figure A-16, 17 and 18). The dominant product in the middle isolate was confirmed to be the biomonomer product. While this approach was successful in recovering the desired product, the yield of this separation method was quite low (Figure 3-1: Product C, 36% yield, MW: 460.5). An alternate approach used solvent extraction and resulted in a higher yield. The residue was first treated with saturated NaCl solution (20mL) and then extracted with ethyl ether (10 mL x 3) to remove the cleaved protecting group (TBDMS) group. The water phase, which mainly consisted of both the desired product and residual tetra n- butyl ammonium ion, was then isolated. The aqueous phase was directly treated with chloroform (10 mL x 3), with the aqueous phase being isolated again and the solvent removed under reduced pressure. The final product contained the drug monomer (Figure 3-1: Product C, 83% yield, MW: 460.2) and some tetra n-butyl ammonium ion at a mole ratio 3:1. (For the purification results and procedures, including mass spec analysis and NMR, see Appendix A-3.)
3.2.2 Drug Polymer Synthesis

The reagents used for the polymer synthesis were all purchased from Sigma-Aldrich Co., Milwaukee, WI, unless otherwise specified. 1,12-Diisocyanatododecane (DDI) and trimethylhexamethylene diisocyanate (THDI; mixture of 2, 2, 4- and 2, 4, 4-isomers) were vacuum distilled before use and used within 72 hours. Polycaprolactone diol (PCL, average molecular weight from titration 2067g/mol) was degassed under nitrogen for 2 hours just prior to use. Dimethylsulphoxide (DMSO, purity 99.9%, anhydrous), dibutyltin dilaurate (DBTDL, purity 95%), methanol (HLPC grade, EMD Chemicals Inc., Gibbstown, NJ), ethyl ether (ACS grade, EMD Chemicals Inc., Gibbstown, NJ) and acetone (ACS grade, EMD Chemicals Inc., Gibbstown, NJ) were directly used as received. Hydroxyl groups were titrated prior to use of the diols.32 (See Appendix A -4 for details on distillation and titration)

The synthesis of the drug polymer was carried out under a dry nitrogen atmosphere (Figure 3-2). The stoichiometry of the polymerization was 3:2:1 of THDI (or DDI): PCL: drug biomonomer. In the first step, the prepolymer was synthesized by reacting 2.50 mmol of PCL with 3.75 mmol of THDI or DDI in 20mL DMSO at 65℃. 0.25mmol of DBTDL was added into the reaction mixture and was stirred for 1 hour. In the second step, 1.23 mmol of the anti-inflammatory drug biomonomers were dissolved in 10 mL of DMSO and mixed in with the prepolymer and allowed to react for 4 hours, after which the heat was removed and the reaction was allowed to proceed for another 16 hours at ambient temperature. The polymerization was finally terminated with 2mL of methanol, and precipitated in 400mL of diethyl ether. The precipitate was left to cool down, removed from the solvent and redissolved in 20mL of acetone. The precipitation and redissolving steps were repeated for 2 more times and the polymer was finally precipitated in distilled water and dried in a vacuum
oven at 30mmHg, 30°C for 48 hours.

This synthesis was repeated with 1.25mmol of TEG as a substitute for the drug biomonomer chain extender to make a non-drug control for the biodegradation studies.

Figure 3-2: Reaction scheme for the synthesis of the THDI drug polymer

3.2.3 Characterization of Biomonomer and Polymer

Nuclear magnetic resonance spectroscopy (NMR). $^1$H and $^{13}$C Nuclear magnetic resonance spectra were recorded on a Varian Mercury 300 NMR (Varian Inc., Palo Alto, CA) at room temperature. All NMR samples were run in the Department of Chemistry at the University of Toronto (Toronto, ON). The spectrometer was operated at 300 MHz for $^1$H and 75.5 MHz
for $^{13}$C measurements. All samples were prepared in 15% wt deuterated chloroform (Sigma-Aldrich Co., Milwaukee, WI), which has a proton chemical shift at $\delta=7.26$ relative to tetramethylsilane (TMS) from the residual protons and a carbon chemical shift of $\delta=77.0$ from the carbon resonance of the methyl group.

Infrared spectroscopy (FT-IR). A Perkin Elmer Spectrum 1000 FT-IR System (PerkinElmer Life and Analytical Sciences, Inc., Woodbridge, ON) located at the ANALEST facilities (Department of Chemistry) at the University of Toronto was used to carry out functional group analysis. The samples were prepared by solvent casting from dichloromethane solutions directly onto disposable PTFE IR cards (Sigma-Aldrich Co., Milwaukee, WI). 128 scans were made and averaged into one final spectrum.

Mass Spectroscopy. The AB/Sciex QStar electrospray ionization mass spectrometer (AME Bioscience A/S, Norway) that was used for the analysis work was located at the Advanced Instrumentation for Molecular Structure Laboratory (Department of Chemistry) at the University of Toronto. During the ionization process, the molecules were protonated into ions (MH$^+$). Sodium (MNa$^+$) or potassium (MK$^+$) analog molecules were also formed, because they were present in the liquid chromatography mobile phase. Tandem Mass Spectrometry (MS/MS) was also performed on selected parent ions to confirm molecular structures. Mass spectrum results were plotted as relative ion intensity vs. mass-to-charge (m/z) ratio.

For the mass analysis of the biomonomer, samples were purified and all the solvent was removed under low pressure using a rotor evaporator. For the detection of impurities in the polymer, 1 mg of polymer sample was dissolved in 0.5 mL of DMAC and then filtered.
using Microcon centrifugal filter devices (Millipore Corp., Bedford, MA) with a molecular weight cutoff of 3000. Samples were filtered for 90 minutes at 14000rpm (CentrifugeMPW65R, MPW Med. Instruments, Warsaw, Poland). This step ensured the separation of the large molecular weight polymers from the small molecules. The filtrate was collected and solvent was removed under vacuum pressure using a Vacufuge Concentrator (Eppendorf Inc., Hamburg, Germany). The residue was then analyzed using the mass spectrometer for signs of free drug, monomers or other possible impurities.

**Size-exclusion chromatography (SEC).** Molecular weights of the polymers were determined using SEC. The analysis was carried out using an Agilent 1100 Series Binary Pump (G1312A) for solvent delivery, an auto sampler (Agilent 1100 Series G1367A), a diode array detector (Agilent 1100 Series G1315A) set at wavelength of 210 nm, a refractive index detector (Agilent 1100 Series G1352A) set at 35°C, and three columns (Phenogel 100 Å, 104 Å and 106 Å) assembled in series, which were housed in an Agilent column compartment, with temperature set at 40°C. The mobile phase solvent was THF (Caledon Laboratory Ltd., Ontario, Canada) and the flow rate was 1 mL/min. The injection volume was 30 µL and the polymer concentration was approximately 20 g/l. Polystyrene external standards (Fluka, Buchs, Switzerland) were used as a reference material. Styrene internal standards (Sigma-Aldrich Co., Milwaukee, WI) were also injected alongside with each polymer sample to adjust for the baseline shifts and variations in each injection. All molecular weight (MW) data were reported as polystyrene equivalents.

**Differential Scanning Calorimetry (DSC).** DSC was carried out using a DSC2910 (TA
Instruments, Newcastle, Delaware) with the standard DSC cell. Temperature and cell constant calibrations were done using Indium as the standard, and samples were run at 10 °C/min under nitrogen flow of 10 cc/min. The baseline calibration using empty standard aluminum pans was performed using the same heating rate and nitrogen atmosphere over the temperature range of interest. Polymer samples of approximately 3 mg were cooled to -120 °C using liquid nitrogen and then heated past the melting temperature to approximately 80 °C, and the 1st heating curve was recorded. The sample was then removed and quenched to room temperature on an aluminum cooling plate. The run was then repeated under the same conditions as the first one under a nitrogen atmosphere, and the 2nd heating curve was recorded. No cooling curves were recorded. This work was done at the Brockhouse Institute for Material Research in the Thermal Analysis Lab, McMaster University, Hamilton, ON, Canada.

### 3.2.4 Biodegradation studies

Cholesterol esterase (EC 3.1.1.13) was used for the degradation studies because it has been shown to be an enzyme that is present in increasing quantities as monocytes differentiate into macrophages during chronic inflammation, and elicits the most degradation towards polyurethanes.33-35 Bovine pancreatic CE (Sigma-Aldrich Co., Milwaukee, WI) was dissolved in Dulbecco’s phosphate buffered saline 1X, 0.05mM, pH 7.0 (DPBS, Gibco, Invitrogen, Grand Island, NY). It was then stored in 3mL aliquots at -70°C until time of use.

The activity of CE was determined using a p-nitrophenylbutyrate (p-NPB) assay. One CE unit was defined as the amount of enzyme required for the generation of 1 nmol of p-nitrophenol from p-NPB per minute.26 The generation of p-nitrophenol can be detected by UV spectrophotometer (Ultrospec II, LKB Biochrom Ltd., Cambridge UK) measurements at
room temperature at pH 7.0, 25°C and wavelength of 401nm. P-nitrophenol has a molar extinction coefficient of 16000 L/(mol.cm) under these conditions.

4mM p-NPB substrate solutions were prepared by mixing 71μL of p-NPB (Sigma-Aldrich Co., Milwaukee, WI) into roughly 15 mL of acetylnitrile (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ) and 78 mL of DPBS. The substrate solutions were then divided into 3.2 mL aliquots and stored frozen in -70°C until they were needed. The enzyme activity was determined by adding 20 μL of enzyme solution into a 3 mL cuvette containing 980 μL of DPBS and 500 μL of p-NPB substrate. The mixture was then inverted a few times to mix and immediately analyzed for UV absorbance change rate over 200 seconds.

The purified drug polymers were dissolved in dimethylacetamide (DMAC, Sigma-Aldrich Co., Milwaukee, WI) at a 10% (w/v) concentration. The polymer solutions were then centrifuged at 4000rpm for 10 minutes and decanted onto sterilized 5cm x 5cm x 0.8cm Teflon plates to make thin films. The films were dried in a 40°C oven for 20 hours and then transferred to a vacuum oven for 3 days at 45°C. Under a sterile laminar flow hood, these thin polymer films were then cut into 0.5cm x 5cm strips, curled with a pair of tweezers and placed into autoclaved glass screw cap vials (4 mL), in a configuration such that the polymer strips had minimal contact area with the vial walls. The total surface area of each polymer strip that was exposed to buffer or enzyme solution was roughly 5 cm².

Each type of drug polymer was subjected to three different incubation solutions: DPBS alone at pH 7.0, low concentration CE solution (1 unit/mL) and high concentration CE solution (10 unit/mL). All the incubation solutions were supplemented with 1X antibiotic-antimycotic (Gibco Inc., Grand Island, NY) which contained penicillin G sodium, streptomycin sulfate and amphotericin β as fungizone. The degradation was performed at
37°C with constant shaking (150rpm) over four weeks. On the first day, 1 mL of incubation solution was added to each vial. On subsequent days, an equal volume of aliquot (~70µL) of concentrated CE solution (approx.15 unit/mL or 150 unit/mL) was added to each vial in order to maintain enzyme activity of approximately 1 unit/mL and 10 unit/mL, respectively. Buffer controls were also replenished with DPBS to keep all volumes the same. All the solutions were withdrawn at the end of each week (day 7, day 14, day 21 and day 28) and stored at -70°C until required for high performance liquid chromatography and mass spectrometry analysis. After the withdrawal of all the liquid in the vials at the end of each week, the polymer strips were then resubmerged in fresh CE or buffer solution with the same conditions as the first day. Degradation studies were performed in triplicates.

3.2.5 High Performance Liquid Chromatography

Reverse phase HPLC analysis was carried out using an HPLC system consisting of a Waters 600E pump and controller, Waters 2996 photodiode array detector and a PC with Waters Millenium Software for data processing and peak analysis. A Synergi Fusion-RP C18 polar embedded HPLC column (250 x 4.6mm, particle size 4µm) was used, which was protected with a Fusion RP SecurityGuard cartridge system (4 x 3.0mm) (Phenomenex Co., Torrance, CA). The column was maintained at ambient temperature (25±1°C).

A gradient method (Table 3-1) was established to analyze the degradation products. The gradient method used a mobile phase consisting of acetonitrile (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ), and 40mM potassium phosphate (monobasic, Sigma-Aldrich Co., Milwaukee, WI) adjusted to pH 2.5 with phosphoric acid (Caledon Laboratory Ltd., Ontario, Canada). Water (filtered using Millipore Milli-Q Gradient system with 0.22µm
filter) was used to flush the column of the buffer salts. The mobile phase was degassed by using a helium sparge. The flow rate was 1mL/min with an initial system pressure of approximately 1500 psi. A sample injection volume of 50 μL was used. A full UV spectrum was recorded and the data was later plotted at 215nm for free drug analysis. Column maintenance was performed by flushing with 10 column volumes of 95% water and 5% acetonitrile, followed by 10 column volumes of THF, followed by 10 column volumes of 95% acetonitrile and 5% water.

### Table 3-1: HPLC gradient program

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL/min)</th>
<th>% Acetonitrile</th>
<th>% 40mM Potassium Phosphate, pH 2.5</th>
<th>% Water</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>Linear</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>Linear</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>Linear</td>
</tr>
<tr>
<td>60</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>95</td>
<td>Linear</td>
</tr>
<tr>
<td>65</td>
<td>2</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>Linear</td>
</tr>
<tr>
<td>70</td>
<td>1</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>Linear</td>
</tr>
</tbody>
</table>

Prior to injection, the polymer degradation solutions were thawed to room temperature and filtered using Microcon centrifugal filter devices (Millipore Corp., Bedford, MA) with a molecular weight cutoff of 3000. Samples were filtered for 90 minutes at 14000rpm (CentrifugeMPW65R, MPW Med Instruments). This step was done to remove large molecular weight soluble products, particulate, and residual enzyme in the degradation solutions which would affect the performance of the column. Drug standards were prepared by dissolving free OC in DPBS, with concentrations ranging from 0.4 to 20 mg/L. (See Appendix A-6 for the HPLC drug calibration curve)
3.2.6 Scanning electron microscopy (SEM)

The polymer samples before and after degradation were imaged using the SEM. The polymer samples were removed from the buffer solution and submerged in distilled water in small glass vials. They were sonicated for 30 minutes to remove salt that might have been left behind from the buffer solutions, after which the water was removed and the samples were left in a vacuum oven overnight at room temperature. The dried samples were then affixed on steel stubs and coated with 5-7 nm of platinum in a Polaron SEM coating unit (SC515). They were viewed and photographed using a Hitachi 2500 Scanning Electron Microscope.

3.2.7 Statistical Analysis

All data represent the average of at least triplicate measurements. When only 2 groups were compared, student’s t-test was used. ANOVA was used when 3 or more groups were compared for their significant difference with each other (i.e. the difference in free drug release in the degradation studies). Where values compared are referred to as different, statistically significant differences at a $p$ value < 0.05 are implied, unless otherwise stated.

3.3 Results

3.3.1 Biomonomer synthesis and characterization

The yields for the various steps of the biomonomer synthesis were as follows: protection step (product A): 61%; coupling step (product B): 93%; deprotection and purification step (product C, final biomonomer): 36% (with column purification) and 83% (with solvent extraction). For the purpose of this study, the pure product that was isolated
using column purification was reported here. Mass spectrometry, $^1$H and $^{13}$C NMR and IR analysis indicated the successful synthesis of the biomonomer with the desired structure. Mass spectrometry data revealed that there were only 2 peaks in Product C (Figure 3-3). These two peaks corresponded to the sodium analog and the protonated form of the pure biomonomer, which had a calculated mass of 460.5 amu. The $^1$H NMR spectrometry of the biomonomer is shown in Figure 3-4 and the key defining peaks in the spectrum are listed in Table 3-2. The key defining peaks in the $^{13}$C NMR spectrometry are shown in Table 3-3 and Figure 3-5. FTIR spectra for the biomonomer is shown in Figure 3-6 and confirms the presence of multiple functional groups. The peak located at the wavelength of approximately 3400 cm$^{-1}$ is associated with the free hydroxyl groups that were available for later polymerization.

![Mass Spectrometry of biomonomer](image)

**Figure 3-3**: Mass Spectrometry of biomonomer (positive mode), demonstrating two peaks that are associated to the drug biomonomer (calculated mass of 460.2 amu).
Table 3-2: H-NMR key defining peaks for the biomonomer. Italicized protons indicate the protons associated with the respective shift.

<table>
<thead>
<tr>
<th>Chemical Formula</th>
<th>$CHCO_2$</th>
<th>$CHOH$</th>
<th>$CH_2$</th>
<th>$CHHN$</th>
<th>$CH_3$</th>
<th>$CHHN$</th>
<th>$CHOH$</th>
<th>$CH_3$</th>
<th>$COCH_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shift (ppm rel to TMS)</td>
<td>4.65</td>
<td>4.5</td>
<td>4.29</td>
<td>3.73</td>
<td>3.7</td>
<td>3.65</td>
<td>3.53</td>
<td>3.33</td>
<td>2.3, 2.1</td>
</tr>
<tr>
<td>Relative number of protons</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Area under peak</td>
<td>1.9</td>
<td>2</td>
<td>4</td>
<td>6.1</td>
<td>4.1</td>
<td>2.0</td>
<td>2.3</td>
<td>2.1</td>
<td>+0.8</td>
</tr>
</tbody>
</table>

Figure 3-4: H-NMR (400MHz, CDCl$_3$) confirms the biomonomer’s structure
Table 3-3: $^{13}$C-NMR key defining peaks for the biomonomer

<table>
<thead>
<tr>
<th>Chemical Formula</th>
<th>$C^n$</th>
<th>CHCO$_2^m$</th>
<th>$CH_2^b$</th>
<th>$CH_2^g$</th>
<th>$CH_2^f$</th>
<th>$CHOH^c$</th>
<th>CHCO$_2^d$</th>
<th>$CH_2^c$</th>
<th>$CH_2^b$</th>
<th>$CH_1^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shift (ppm rel to TMS)</td>
<td>172.6</td>
<td>170.5</td>
<td>70.9</td>
<td>70.4</td>
<td>69.3</td>
<td>64.5</td>
<td>57.8</td>
<td>56.4</td>
<td>38.4</td>
<td>22.4</td>
</tr>
</tbody>
</table>

Figure 3-5: C-NMR (400MHz, CDCl$_3$) confirms the biomonomer’s structure
3.3.2 Polymer synthesis and characterization

The weight average molecular weights (polystyrene equivalent weight) of the polymers were as follows: THDI-PCL-OC had a Mw of $5.1 \times 10^4$, with a polydispersity (PD) of 1.5; THDI-PCL-TEG (control) had a Mw of $4.2 \times 10^4$, with a PD of 1.5; DDI-PCL-OC had a Mw of $4.5 \times 10^4$, with a PD of 1.4; and DDI-PCL-TEG (control) had a Mw of $3.2 \times 10^4$, with a PD of 1.4. All the polymers have similar molecular weights and polydispersities. In theory, the final drug polymers consisted of 10 wt% drug biomonomers, which could be translated into 7.5 wt% of pure drug. Mass spectrometry analysis of the dissolved polymer solutions showed that there was no residual free drug, monomers or other low molecular
impurities associated with the drug polymers (See Appendix A-5 for the results).

DSC thermograms for the polymers after the 1\textsuperscript{st} and 2\textsuperscript{nd} heating are shown in Figure 3-7. Key values from the 2\textsuperscript{nd} heating are summarized in Table 3-4. The transition temperatures reported are the midpoint values. The change of heat for the melt transitions (Tm) is also reported in each thermogram while the change in heat capacity is reported for the Tg values. DSC scans show that all three types of polymers have a weak inflexion point (Tg) near -42 to -55°C, for both the 1\textsuperscript{st} and 2\textsuperscript{nd} heating. The initial DSC curves also show an endotherm (Tm1) related to the melting of the soft PCL segment of the polymers, and is recorded between 34°C to 48°C, which is similar to the typical values for polyurethanes synthesized with PCL as a soft segment.\textsuperscript{36,37} The Tg for pure PCL is around -60°C and the melt temperature ranges between 59 and 64°C, depending upon its crystalline nature.\textsuperscript{38} The degree of crystallinity, $\alpha_c$, of the PCL phase was calculated using a value of the enthalpy of melting for completely crystalline PCL, which is 142 J/g.\textsuperscript{39}

DDI-PCL-OC has the lowest Tg (1\textsuperscript{st} heating), the lowest melting temperature (Tm1) and also the lowest change of heat at this transition (Table 3-4). This implies phase separation and lower degree of crystallinity of the PCL segment. After quenching and reheating, the Tm1 peaks shift to lower temperatures for all three polymers, with the shift in DDI-PCL-OC yielding the greatest change (Figure 3-7c). The use of the drug biomonomer as the chain extender in THDI-PCL-OC resulted in a polymer with higher degree of crystallinity than its no-drug analog THDI-PCL-TEG (Table 3-4). It was also noted that only DDI-PCL-OC had higher order melt transitions (Tm2 and Tm3), indicating the presence of distinct higher temperature crystalline zones in the polymer. These latter endotherms were believed to be related to the migration of hard segment into domains. The small values of Hm2 and
Hm3 (Table 3-4) are evidence that the degree of crystallinity of the rigid segment is small.

There are no distinct melt transitions identified for the hard segments of the THDI polymers.

When cast into films, the THDI polymers had a white appearance while the DDI polymers appeared semi-transparent and more flexible (Table 3-5).

**Table 3-4**: Thermal properties and degree of crystallinity of polymers after 2nd heating

<table>
<thead>
<tr>
<th>Polymer</th>
<th>T_g (°C)</th>
<th>ΔHm1 (J/g)</th>
<th>Tm1 (°C)</th>
<th>α_c * (%)</th>
<th>ΔHm2 (J/g)</th>
<th>Tm2 (°C)</th>
<th>ΔHm3 (J/g)</th>
<th>Tm3 (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>After 1st heating</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THDI-PCL-TEG</td>
<td>-48.4</td>
<td>48.3</td>
<td>47.5</td>
<td>41</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>THDI-PCL-OC</td>
<td>-41.9</td>
<td>60.8</td>
<td>46.2</td>
<td>55</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DDI-PCL-OC</td>
<td>-55.8</td>
<td>28.5</td>
<td>44.2</td>
<td>26</td>
<td>0.61</td>
<td>65.8</td>
<td>1.84</td>
<td>107.2</td>
</tr>
<tr>
<td><strong>After 2nd heating</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THDI-PCL-TEG</td>
<td>-54.8</td>
<td>50.6</td>
<td>42.8</td>
<td>43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>THDI-PCL-OC</td>
<td>-53.3</td>
<td>53.3</td>
<td>42.7</td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DDI-PCL-OC</td>
<td>-54.3</td>
<td>26.8</td>
<td>34.1</td>
<td>25</td>
<td>1.3</td>
<td>63.5</td>
<td>0.67</td>
<td>113.6</td>
</tr>
</tbody>
</table>

* With respect to wt% PCL in PU.

**Table 3-5**: Polyurethane Chemistry & Appearance

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Reaction Stoichiometry</th>
<th>PCL wt %</th>
<th>Diisocyanate wt %</th>
<th>Hard Segment Characteristics</th>
<th>Film Appearance before incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>THDI-PCL-TEG</td>
<td>3:2:1</td>
<td>84</td>
<td>13</td>
<td>Short, branched, low polarity</td>
<td>White</td>
</tr>
<tr>
<td>THDI-PCL-OC</td>
<td>3:2:1</td>
<td>79</td>
<td>12</td>
<td>Short, branched, low polarity</td>
<td>White</td>
</tr>
<tr>
<td>DDI-PCL-OC</td>
<td>3:2:1</td>
<td>77</td>
<td>15</td>
<td>Long, linear chain, low polarity</td>
<td>Semi-transparent</td>
</tr>
</tbody>
</table>
Figure 3-7: DSC thermograms of polymers after 1st heat and 2nd heat: (a) THDI-PCL-TEG, (b) THDI-PCL-OC, (c) DDI-PCL-OC.
### 3.3.3 Degradation Profile and Degradation Products

Figure 3-8 shows the HPLC chromatograms for the THDI-PCL-OC polymers incubated at pH 7.0, 37°C for 7 days. Many peaks on these chromatograms are associated with the protein components in the CE preparation and the antibiotics that were added to the buffer during incubation (Figure 3-8, chromatogram d). Unique peaks at 11-13 min, 31 min and 33.2 min (next to the 33.4 enzyme fragment peak) were detected in the high concentration CE incubated polymer sample (Figure 3-8, chromatogram a). These peaks are also present but in significantly smaller quantity for the 1 unit/mL CE incubated polymer (Figure 3-8, chromatogram b) and appear in very low magnitude for the buffer incubated polymer (Figure 3-8, chromatogram c). These peaks may be associated with either solely polymer-derived products or protein/polymer product complexes. The peak at 7.6 min corresponds to free oxaceprol, as shown by the injection of the standard (Figure 3-8, chromatogram e). This drug peak is observed in all of the other chromatograms for the degraded drug polymers (Figure 3-8, chromatogram a-c).

Figure 3-9 shows the HPLC chromatograms for the DDI-PCL-OC polymers. In contrast to the THDI-PCL-OC polymers, the peaks at 11-13 min and 31 min were relatively small even for the high concentration CE incubated DDI polymer (Figure 3-9, chromatogram a). The peak at 33.2 min which was observed in the THDI samples was not found in the DDI chromatograms. The drug peak at 7.6 min was also relatively smaller in all the DDI polymer samples. Figure 3-10 shows the HPLC chromatograms for the drug-free THDI-PCL-TEG polymers. Similar to its drug analog, the peaks at 11-13 min and 31 min were observed for the high concentration CE incubated THDI polymer (Figure 3-10, chromatogram a).
However, the peak at 33.2 min which was observed in the drug analog was not found in the non-drug chromatograms. An additional product peak, however, was observed at 37 min for the high CE-incubated sample. No drug peaks were observed, as expected. For all the high CE concentration samples (chromatograms 3-8a, 3-9a and 3-10a), there is a small peak at 7.1 min located near the OC retention time. The areas under this peak for three polymers are very similar. This is an enzyme related peak that is seen for the CE +buffer solution (Figure 3-8, chromatogram d).

![Typical HPLC Chromatograms for the THDI-PCL-OC polymer incubated at 37°C for 7 days. *OC had a retention time of approximately 7.6 minutes.](image-url)

**Figure 3-8:** Typical HPLC Chromatograms for the THDI-PCL-OC polymer incubated at 37°C for 7 days. *OC had a retention time of approximately 7.6 minutes.
Figure 3-9: Typical HPLC Chromatograms for the DDI-PCL-OC polymer incubated at 37°C for 7 days. *OC had a retention time of approximately 7.6 minutes.
Figure 3-10: Typical HPLC Chromatograms for the THDI-PCL-TEG polymer incubated at 37°C for 7 days. No OC peaks were observed in the chromatograms for this polymer. *OC had a retention time of approximately 7.6 minutes.

The OC release profile was determined by calculating the area under the OC peak on the HPLC chromatograms (retention time: 7.6 minutes, see Figure 3-8). It should be noted that the OC peak overlaps partially with one of the smaller CE fraction peaks when CE is used. In the latter case, the amount of OC is then determined by subtracting the area associated with the CE fraction from the total area. This particular CE peak has been found to be always proportional to another CE peak at 6.4 minutes, making the approximation of the peak area possible. (See Appendix A-7 and A-8 for detailed calculations and UV spectra)
The cumulative OC release profiles from THDI-PCL-OC and DDI-PCL-OC are shown in Figure 3-11. The cumulative OC profile demonstrates an increasing trend over the 28 days for the THDI-PCL-OC polymer exposed to enzyme or the buffer alone solutions. There is a very significant increase in cumulative OC release when 10 unit/mL CE was added, when compared to the buffer controls (P<0.01) and when 1 unit/mL CE was added (p<0.05). However, no significant differences (P>0.05) in cumulative OC release between 1 unit/mL CE and buffer controls was observed.

**Figure 3-11:** Cumulative oxaceprol release from THDI-PCL-OC and DDI-PCL-OC polymers. Data represent mean ± S.D., n=3. *Significantly greater than buffer and 1 unit/mL CE, P <0.01. ** Significantly greater than buffer, P <0.05.

Comparatively, DDI-PCL-OC had a lower level of drug release. The effect between buffer and enzyme treatments on the release of drug was not statistically significant, except for past 30 days at elevated concentration. Figure 3-12 showed the weekly incremental
release of drug from the THDI-PCL-OC polymer. Week 1 had the highest free drug release for the polymers that were exposed to 10 unit/mL CE. The free drug release rate was pretty constant for polymers that were exposed to 1 unit/mL and buffer alone. Figure 3-13 showed that the DDI-PCL-OC only had a differentially large release in the first week.

![Graph showing drug release over weeks for different concentrations of CE.](image)

**Figure 3-12:** Free drug release from THDI-PCL-OC polymer at week 1, 2, 3 and 4. Data represent mean ± S.D., n=3. *Significantly greater than buffer, P <0.05. **Significantly greater than buffer, P <0.01.

![Graph showing drug release over weeks for different concentrations of CE.](image)

**Figure 3-13:** Free drug release from DDI-PCL-OC polymer at week 1, 2, 3 and 4. Data represent mean ± S.D., n=3.
3.3.5 SEM

Scanning electron microscope images of all surfaces were taken before and after incubation. The surfaces showed no sign of cracking or fissuring. Figure 3-14 shows the picture of the three polymers before and after 4 weeks of incubation with buffer and enzyme solutions. The surface morphology for the three polymers before incubation is nonporous and smooth (Figure 3-14 a, e, i). Figure 3-14c and d show pitting of the THDI-PCL-TEG surfaces upon co-incubation with enzyme, with the pitting more severe when the high concentration CE was used (d). There is a significant difference in surface morphology for the enzyme-incubated material (c, d) and buffer-incubated material (b). The buffer-incubated polymer showed very little evidence of polymer degradation and has similar surface morphology as the control (a). Similar effects were observed with the THDI-PCL-OC polymer. The pitting on the surface of the enzyme incubated THDI-PCL-OC samples (g, h), however, was relatively less severe than those observed on the THDI-PCL-TEG polymers (c, d). There was no difference between the appearance of the polymer incubated with enzyme and that of the control for the DDI-PCL-OC polymer, and there was no evidence of surface pitting, as shown in Figure 3-14(i) –(l). See Appendix A-9 for more SEM images.
<table>
<thead>
<tr>
<th>Before Degradation</th>
<th>Incubated with Buffer</th>
<th>Incubated with Enzyme (1 unit/mL)</th>
<th>Incubated with Enzyme (10 unit/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THDI-PCL-TEG Polymer (Control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>(b)</td>
<td>(c)</td>
<td>(d)</td>
</tr>
<tr>
<td>THDI-PCL-OC Polymer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(e)</td>
<td>(f)</td>
<td>(g)</td>
<td>(h)</td>
</tr>
<tr>
<td>DDI-PCL-OC Polymer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i)</td>
<td>(j)</td>
<td>(k)</td>
<td>(l)</td>
</tr>
</tbody>
</table>

**Figure 3-14:** SEM for drug polymers degraded by cholesterol esterase, incubated for 4 weeks: (a, e, i) prior to incubation, (b, f, j) incubated with buffer, (c, g, k) incubated with 1 unit/mL enzyme and (d, h, l) incubated with 10 unit/mL enzyme.
3.4 Discussion

The two diisocyanates used in the synthesis of the drug polymers differed in molecular weight and chemical structure. DDI is a long aliphatic linear chain while THDI has a shorter branched structure. Their individual character ultimately defines the size and shape of the hard segment within the polymer chains, which determines in part the degree of phase separation for the polymer chains and the formation of hydrogen bonding, as well as hard-segment crystallization. These characteristics will have the potential to influence degradation rate and profile of these polymers, and consequently will affect their drug delivery properties. In this study, polyurethane chemistry was used in conjunction with an atypical anti-inflammatory drug oxaceprol as a model anti-inflammatory polymer system.

The drug polymers were synthesized by first forming a diisocyanate-PCL prepolymer. The traditional chain extender was replaced by the oxaceprol biomonomer, a symmetrical unit that contained two oxaceprol drug units linked together by tri(ethylene glycol). The use of the biomonomer form for the drug eliminates the carboxylic acid moieties which typically have more complex reactions with the diisocyanates than do hydroxyl groups. Further, a symmetric drug biomonomer will provide non-competing reactions on each end of the molecule. This would provide better control over the polymerization, and would result in a more uniform molecular weight since both terminal ends have the same reactive groups. The TEG segment in the biomonomer segment improves the polymer hydrophilicity and hence increases the potential for hydrolysis and release of the drug. Furthermore, TEG is an ether and therefore enhances chain movement during polymerization of the larger molecular weights biomonomers, and thereby yielding enhanced potential for reaction with the
prepolymer chains.

The weight average molecular weight of the control polymer (chain-extended with TEG) in this study was similar to that of the drug polymer and demonstrated that the reactivity of the biomonomer is on par with lower molecular molecules.

The anti-inflammatory drug polymers synthesized in this study, and in particular the material with THDI as the hard segment, released more drug in the presence of enzymes associated with inflammatory cells. Since inflammation occurs during the initial surgical implantation, enzymes will be present near polymer drug implants to release the drug. This will in turn serve to control the level of inflammation at wound-healing sites. The findings with the THDI polymer (Figure 3-8) indicate that there are more degradation products in the presence of enzymes, agreeing with the SEM images of the drug polymer in Figure 3-14(e) to (h), which show an increase in the number of pits on the surface of the enzyme-incubated samples. These findings also agree with earlier studies which showed that CE increased the degradation of $^{14}$C-radiolabelled-polycaprolactone-based polyurethanes. Cholesterol esterase (CE) was used as the model enzyme to hydrolytically degrade the latter polymers. Santerre et al. had shown that CE could cause a greater amount of radiolabel release than buffer for a polyesterurethane consisting of radiolabeled 2, 4-toluene diisocyanate, polycaprolactone and ethylene diamine. Similar findings were recorded for a drug polymer that was synthesized with radiolabeled 1,6 - hexane diisocynate, polycaprolactone and fluoroquinolone antibiotics.16

The results of the HPLC analysis of the DDI-PCL-OC polymer in the current studies did not show significant differences between the chromatograms of the enzyme and buffer incubated samples (Figure 3-9). Longer retention time peaks, such as the 31 min peak
(usually associated with high molecular-weight/hydrophobic compounds) were relatively small as compared to those found in the enzyme-incubated THDI samples. The 33.2 min peak was not seen at all and the drug peak at 7.6 min was very small, especially for the buffer-incubated sample (Figure 3-9c), and was almost masked by the base-line peaks. Another observation that was in line with the HPLC findings was the SEM images for this material, which showed that there was no significant degradation of the DDI material surface (Figure 3-14 (i)-(l)). However, the HPLC peak at 11-13 min did indicate the presence of degradation products in the DDI incubation solution. It is believed that the 31 min and 11-13 products do not contain oxaceprol drug since these two peaks were also observed in the HPLC chromatogram of the drug-free THDI-PCL-TEG incubation solutions (Figure 3-10). The differences between the degradation of the THDI and DDI drug polymers could be explained by the relative length and steric effect of the diisocyanate molecules used in their respective syntheses. Although THDI and DDI are both hydrophobic in nature, the THDI monomers have side methyl groups. These side groups may cause the polymer chains of the THDI polymer to be less closely packed, allowing the buffer and enzyme to access the hydrolysis sites in the polymer within the time frame of this experiment. The long linear DDI monomers on the other hand are believed to have facilitated the close packing of its polymer chains and specifically forming dense hard segment domains, thereby limiting the available hydrolysis sites around the drug. Evidence for enhanced structure appears in the DSC data (Figure 3-7) where the DDI polymer showed the presence of higher order structures at 64 and 114°C, where these were not present for the THDI polymers. In addition, there was evidence that hard/soft separation was taking place in the DDI polymer. These results are consistent with other studies where investigators showed that polyurethanes
synthesized from side chain-containing diisocyanates inhibited chain packing.\textsuperscript{43,44}

Another possibility for the differences observed between the two drug polymers may relate to the fact that DDI-containing drug polymers have a low solubility in polar solvent (i.e., tetrahydrofuran and dimethylformamide) in comparison to THDI-containing polymers. Therefore, it may require a long time to take up water into its backbone in order to initiate noticeable degradation. This observation agrees with previous studies that show that hydrophobicity of polymer film surfaces is dependent on the structure of the diisocyanate used.\textsuperscript{44} The long induction period of the hydrolysis of polymers have been shown to be related to the water molecules not being able to structurally associate with the polymer chains during those periods.\textsuperscript{45} Previous studies have shown that a slow degradation rate is not uncommon for DDI-PCL polymers. A degradation study has shown that a DDI-PCL-antibiotic drug polymer that had radiolabels on its DDI segments released dramatically less radiolabelled products and free drug than its shorter hexane-diisocyanate analog with a higher ratio of polar urethane to hydrocarbon units, even when exposed to 40 units/mL of CE, over a 30-day period.\textsuperscript{17} When only buffer was used, there was almost zero cumulative radiolabel release over the same time length.

In the current study, it is demonstrated that the high concentration CE (10 unit/mL) was able to cause a significant increase of free drug release from the THDI-PCL-OC polymer when compared to the sample with no CE (Figure 3-11). The difference in the cumulative drug release was mainly contributed by the initial burst release of drug from the 10 unit/mL CE-incubated polymers during week 1 (see Figure 3-12), while in weeks 2 to 4 a slower release pattern was observed for the different incubation solutions. The initial burst release may be due to the quick exhaustion of readily available hydrolysable bonds on the surface of
the material and the inability for the CE to penetrate into the bulk of the polymer.\textsuperscript{36,46} This observation suggests that surface area is the key limiting step and not enzyme activity.

Another consideration to explain the burst effect is that there could be a high concentration of enzyme or degradation products accumulated at the surface of the material due to adsorption of enzyme. This may slowly limit polymer degradation at the surface by blocking available hydrolysis sites.\textsuperscript{46} It has been previously shown that the removal of this CE layer and reestablishing the “burst” effect could be achieved through ultrasonicating the samples and re-incubating them in fresh media.\textsuperscript{46,47} In other work with polyurethanes, it has been reported that there was an exchange between the active and adsorbed enzyme on the polymer surface, and it was observed that a continuous radiolabel release from the polyurethane occurred, even after 10 weeks.\textsuperscript{42} When incubating the THDI polymer with buffer alone, drug release was at a steady rate throughout the 4 weeks, except for a slight increase during week 2. The results also indicated that 1 unit/mL CE was not enough to demonstrate any significant increase in free drug release when compared to using buffer alone, taking into account the error bars (Figure 3-11 and 3-12). The absence of a differentiation effect between CE (1 unit/mL) and buffer was in agreement with a previous study whereby a similar antimicrobial polymer containing THDI (THDI-PCL-CiproTEG) did not demonstrate significant differences in drug release (see Table 3-6).\textsuperscript{18}

The DDI-PCL-OC polymer yielded a very low concentration of drug release, with or without the presence of enzyme (Figure 3-11 and 3-13), which is in agreement with the HPLC chromatograms which did not show any unique degradation product peaks (see Figure 3-9). Taking the standard deviations into account, it is not possible to conclude that there was drug release for the buffer-incubated samples over the four weeks. As well, the total
drug release in 4 weeks for the 10 unit/mL enzyme-incubated samples was only 0.9±0.3
µg/cm² (see Table 3-6), which was almost 7 fold lower than that of the THDI-containing
polymer. Although there is a significant difference in cumulative drug release between the
enzyme (10 unit/mL) and buffer incubated polymer (Figure 3-11), the difference was mainly
observed after the first week, which was the only time point that had drug release
significantly above zero (see Figure 3-13). Possible explanations for the initial burst of
release was discussed above for THDI samples.

Table 3-6: Cumulative free drug release after 28 days and structure of the chain extenders.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>CE used (units/mL)</th>
<th>With CE (µg/cm²)</th>
<th>Structure of chain extender</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) THDI-PCL-OC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.6±0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.2±0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>6.2±0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) DDI-PCL-OC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.2±0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.5±0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.9±0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c) HDI-PCL-Cipro</td>
<td></td>
<td>0.60±0.02</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.65±0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d) DDI-PCL-Cipro</td>
<td></td>
<td>0.22±0.01</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.38±0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e) THDI-PCL-CiproTEG</td>
<td></td>
<td>3.5±0.1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.9±0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f) DDI-PCL-CiproTEG</td>
<td></td>
<td>5.5±0.2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.5±0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g) THDI-PCL-NorfTEG</td>
<td></td>
<td>0.078±0.002</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.086±0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>h) DDI-PCL-NorfTEG</td>
<td></td>
<td>4.0±0.2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.5±0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Estimated from data in reference16.  b Estimated from data in reference17.  Cipro is a fluoroquinolone
antimicrobial drug (Ciprofloxacin). It was incorporated directly onto the backbone of the
polyurethane.  c Estimated from reference18.  CiproTEG and NorfTEG are fluoroquinolone
antimicrobial drug biomonomers that consist of two drug molecules linked together via a TEG soft
segment, in an analogous manner to OC.
In previously reported work, the drug release rate of a DDI-PCL-Cipro polymer was very low (Table 3-6(d)) when compared to its HDI analog (Table 3-6(c)), which had a shorter and less hydrophobic diisocyanate component.\textsuperscript{17} It is interesting to note that the drug release rates recorded in that study with the DDI polymer was very similar to the ones in this study (Table 3-6 (b)), despite of the difference in the structure of the chain extender used for the two polymer systems. In another study with DDI-PCL-ciproTEG polymer and DDI-PCL-NorfTEG polymers, although the cumulative drug release rate was much higher than the DDI polymer in this study (Table 3-6 (f) and (h)), there was a 1-week delay in the onset of drug release from these polymers,\textsuperscript{18} indicating that the polymer needed quite some time to take up enough water into its backbone to initiate degradation. All of this implied that the molecular and material structures around the drug were critical in terms of defining release rates.

As was highlighted in earlier discussions, the DSC data provide insight into the differences of material structure and hence a possible explanation for drug release rates of the DDI and THDI polymers (Figure 3-7 (b) and (c)). The elevated soft segment melt transition temperature in the THDI polymers in the current study is a good indication of increased phase mixing,\textsuperscript{48} in which the hard segments containing the drug are relatively less excluded from the soft segment phase. The significantly lower crystallinity of the PCL soft segment observed in the DDI polymer implied that the aliphatic diisocyanate hard segment had an impact on the ability of the soft segment to organize into crystals. One possible explanation is the increase of conformational mobility of the polymer chains.\textsuperscript{49} The higher order melt transitions observed (Tm2 and Tm3) in the DSC thermograms are assigned to the hard segments, indicating that the structure of the DDI-chain extender (containing the drug)
formed detectable domains, presumably a result of the phase separation process. Lower crystallinity within the soft segment phase of the DDI drug polymer most likely accounts for its semi-transparent appearance (Table 3-5), in contrast to the milky appearance of the THDI polymers, which is an indication of the presence of crystalline ordering.

Tang et al. 26,41 have shown that the biostability of polyurethane surfaces are highly correlated to the H-bonded state of the polyurethane. Specifically, they found that H-bonding among urethanes within the subsurface layers plays an important role in increasing the resistance to hydrolysis at the early stages of degradation.41 In DDI-PCL-OC, the H-bonding would be facilitated by the linear and repeated methyl structure of DDI which provides an uninhibited ability for hard segments to interact. Such H-bonding around the drug molecule would provide a protective structure around the soft-hard segment interface, creating a hindrance effect on the cleavage of linkages by the enzyme.25 The side groups in the hard segments of the THDI based-polymers are believed to have filled the free volume between the domains of rigid segments50 and prevented the development of strong hydrogen bonds of the urethane-urethane type in these domains.51

It was interesting to observe the difference in the cumulative drug release amounts between polymers synthesized with different drug structures (Table 3-6). The oxaceprol units in this study were small (MW= 173.2) and were very hydrophilic in nature, containing a hydroxyl, an amide and an acid group on each unit. The drug was bonded to the diisocyanate via a urethane bond and to the TEG via an ester bond. In order to release free drugs, both bonds had to be cleaved. One would expect that the hydrophilic nature of the drug would render the oxaceprol drug polymer more susceptible to CE degradation than previous drug-polymers containing the fluoroquinolone biomonomers, which were less
hydrophilic and had a higher molecular weight (MW = 331.3 for ciprofloxacin and 319.3 for norfloxacin). (Table 3-6 (a) and (b) vs (e), (f), (g) and (h)). While this analysis was true for the THDI polymers synthesized with OC and norfloxacin, it did not hold true for the Ciprofloxacin analog. The latter implies that small changes in drug structure may have a dramatic effect on the structural arrangement of the polymer chains and in turn on the immediate environment of the drug in the polymeric material. The introduction of DDI into the different drug polymers (Table 3-6 (b), (f) and (h)), yielded the complete opposite effect. The oxaceprol polymer delivered minimal free drugs while the fluoroquinolone polymers reported on by Cai et al. 18, although having a late onset, managed to deliver a significant amount of free drugs. It is believed that since the oxaceprol drug was a lot less bulky than the fluoroquinolones, the polymer chains were able to be more closely-packed in the DDI-PCL-OC than in the fluoroquinolone polymers, and thereby delaying the onset of the drug release to a time point beyond the scope of this study.

Cholesterol esterase is known to preferentially catalyze the hydrolysis of long-chain fatty acid esters or cholesterol. 52,53 Although it exhibits little or no substrate specificity, 54 studies have indicated that the active sites of CE substrates often contain a hydrophobic ring and a hydrocarbon chain. 52,55,56 CE activity has been demonstrated on nitrophenyl esters 55,57 as well as natural substrates such as cholesterol oleate 56, all of which have the features aforementioned. In addition, the length of the side-chain of the esters has been shown to have a significant impact on CE activity. 55,56 Substrates that have longer side-chain esters demonstrated a higher hydrolysis rate. The THDI-biomonomer and DDI-biomonomer polymer segments have structures that resemble p-nitrophenylbutyrate, a water soluble carboxylic ester that is commonly used as a substrate for CE (Figure 3-15). 55 Therefore a
possible explanation for the increase in free oxaceprol product observed for the enzyme versus buffer solution in the THDI-PCL-OC polymer is provided by the similarity in structures. From the structure of the individual polymer chain alone, one would expect that the long DDI chain would further facilitate the binding of CE to the drug polymer. However, as noted earlier, the latter effect was not shown in the degradation studies for the DDI-PCL-OC polymer and was masked by the closely-packed linear aliphatic DDI that is believed to have inhibited the access of water molecules and CE altogether. It is thus hypothesized that the addition of side groups to the long DDI backbone might facilitate the access of CE to the ester-carbonyl hydrolysis site, and could potentially demonstrate an even higher difference in drug release for the enzyme versus buffer solution than the THDI drug polymer in this study.

Figure 3-15: Structures of (a) THDI-PCL-OC polymer segment, (b) DDI-PCL-OC polymer segment and (c) p-nitrophenylbutyrate.
3.5 Conclusion

In summary, this study demonstrated the feasibility of incorporating an anti-inflammatory drug as a biomonomer into the backbone of a polyurethane, to generate a biodegradable anti-inflammatory drug delivery system. It was shown that the polymer could be degraded by an inflammatory cell-derived enzyme, cholesterol esterase. Analysis of the solutions showed that there was an enhanced release of free oxaceprol from the THDI drug polymer in the presence of the enzyme at a physiologically relevant concentration.34

The enzyme dose response of the polyester-drug-urethanes was different for materials synthesized with DDI vs THDI in the CE-catalyzed biodegradation studies. The polymeric material with the branched THDI as the diisocyanate group was shown to be more responsive to the hydrolytic degradation induced by CE, whereas the polymer synthesized with long linear DDI units showed very high stability and had very minimal drug release over the 4 week incubation period.

The drug delivery response itself was shown to be a direct function of the surface chemistry and molecular arrangement of the hard segment domains made up of drug and diisocyanate components. Future work will focus on investigating the biological activity of the materials in an implant environment, and a further analysis of high molecular weight degradation products. The use of other long chain diisocyanates that contain side groups could be explored as a potential candidate for CE-controlled drug-release polymers.
3.6 Acknowledgements

This study was supported by a University of Toronto Fellowship and Interface Biologics Inc. The assistance of Dr. Frank LaRonde on polymer synthesis and Dr. Peng Chen on molecular weight analysis were greatly appreciated.


Abstract

Oxaceprol (OC) has been demonstrated to exert atypical anti-inflammatory properties \textit{in vivo} and has been widely used as a treatment for joint diseases and osteoarthritis and rheumatoid arthritis. Previous \textit{in vivo} studies in animals demonstrated that oxaceprol-treatment reduced leukocyte adhesion to endothelium and inhibited neutrophil infiltration to arthritic joints. With a view to elucidating the anti-inflammatory mechanisms of OC, the present study aimed to investigate the direct effects of OC on cytokine-induced monocytic-cells adhesion in human endothelial cells \textit{in vitro}. Human umbilical vein endothelial cells (HUVECs) were treated with OC and subsequently with 10ng/mL of TNF-\(\alpha\). U937 monocytes were then added to the HUVECs for 30 min. The adhesion of U937s was measured qualitatively and quantitatively. Results showed that treatment with TNF-\(\alpha\) markedly increased U937 monocytes adhesion. However, treatment of HUVECs with OC did not modulate the cell adhesion. In conclusion, OC showed no effect on cytokine-induced monocyte-endothelial cell interactions in HUVECs. This study suggests that the reduction of leukocyte adherence to the endothelium by oxaceprol observed in \textit{in vivo} experiments are not explained by direct drug interactions with the adhesion molecules expressed on the endothelium and may alter arthritic processes by other mechanisms.
4.1 Introduction

Chronic inflammation is the major cause of various severe pathologies such as rheumatoid arthritis and atherosclerosis. The adhesive interactions between circulating leukocytes and endothelial cells lining the vascular wall, and the transendothelial migration of these cells are important steps of inflammation. Various stimuli, such as the proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-γ (IFN-γ), can induce chemotactic factors and other cytokines to be released, and can activate endothelial cells by causing an increase in expression of adhesion molecules on the surface of these cells. All of these actions contribute to the recruitment and migration of the leukocytes from the blood to the inflamed tissues.

Most anti-inflammatory agents that are available to control the above inflammatory events usually fall under the following categories: Non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs) such as methotrexate, and corticosteroids. The different groups of drugs differ not only by their mechanisms of action, but also by their side-effects. The NSAIDs, such as salicylates and ibuprofen, are important in the pharmaceutical treatment of pain (at low doses) and inflammation (at higher doses). They generally act through the inhibition of cyclo-oxygenases (COX), which in turn inhibit the synthesis of prostaglandin (PG). Research suggested that most of the analgesic effects of NSAIDs are mediated by blocking the COX-2 inducible enzyme, while its adverse effects, especially the gastrointestinal injuries, are caused by blocking the constitutive COX-1. Inhibition of the synthesis of PG, specifically PGE₂, prevents vasodilation, and hence reduces plasma exudation, explaining NSAIDs’ ability to relieve inflammatory pain, erythema and swelling. However, a big body of evidence suggests that NSAIDs have
additional prostaglandin-independent anti-inflammatory mechanisms of action.\textsuperscript{6} Methotrexate, one of the most widely used DMARDs, inhibits methylation reactions, which may account for inhibitory effects on cytokine production and promotes adenosine release, which as a result inhibit the adhesion of neutrophil.\textsuperscript{3} Corticosteroids are both anti-inflammatory and immunosuppressive.\textsuperscript{7} They act by increasing or decreasing the transcription of genes, such as cytokine genes. Although they inhibit inflammatory disease progression, they are generally restricted due to long term side effects. Despite their difference in mode of action, all the anti-inflammatory drugs that are currently used share the property of inhibiting the recruitment of neutrophil leukocytes to the site of inflammation.\textsuperscript{3} Accumulation of polymorphonuclear leukocytes is a characteristic of acute inflammatory response.\textsuperscript{8,9} Diseased joints in rheumatoid arthritis are consistently accompanied by a histopathological infiltration of activated neutrophils into the synovial fluid.\textsuperscript{9}

Oxaceprol is a synthetic N-acetylated derivative of hydroxyproline, which is known as an atypical inhibitor of inflammation.\textsuperscript{3,10-13} It was introduced for the treatment of degenerative joint diseases over 20 years ago and was widely used in France and Germany.\textsuperscript{3} It is used to treat osteoarthritis and rheumatoid arthritis and its clinical efficacy is similar to the classical NSAIDs ibuprofen and diclofenac for pain relief.\textsuperscript{10} However, oxaceprol has been shown to have no effect on PGE\textsubscript{2} or leukotriene C\textsubscript{4} production by ionophore-stimulated mouse peritoneal macrophages \textit{in vitro}, showing that it does not have COX inhibitory activity.\textsuperscript{13} Its low incidence of gastrointestinal side-effects is most likely due to the fact that it does not inhibit prostaglandin synthesis, unlike the traditional NSAIDs. Ionac et al.\textsuperscript{13} demonstrated that oxaceprol administered daily for 15 days to arthritic rats markedly inhibited secondary tail and ear lesions. Inhibition of synovial membrane neutrophil
infiltration was also observed at day 15, which was comparable to that achieved with indomethacin. However, in contrast to some NSAIDs, oxaceprol had very little effect on the primary paw swelling and cartilage damage. In fact, high concentrations of oxaceprol have been demonstrated to have stimulatory effects on cartilage proteoglycan metabolism in vitro.\textsuperscript{3,12} Veihelmann at al.\textsuperscript{11} investigated the leukocyte-endothelial cell interactions in oxaceprol-treated mice with antigen-induced arthritis (AiA) using intravital microscopy and found that the leukocyte adherence to the endothelium and swelling were significantly reduced. Literature suggests that oxaceprol’s anti-inflammatory activity is closely related to its ability to inhibit neutrophil infiltration into the inflamed site and to inhibit the extravasation of neutrophilic leukocytes. Although the precise mechanism of action remains to be established, it has been proposed that oxaceprol acts predominantly by inhibiting leukocyte adhesion and migration.\textsuperscript{3} No studies exist that examine the direct effect of oxaceprol on monocytic cell-endothelial adhesion in vitro.

4.2 Objectives and Approach
The objective of this study, therefore, was to investigate the effects of oxaceprol on U937 monocyte adhesion onto human umbilical vein endothelial cells (HUVECs). Both cell types are widely used for investigating monocyte-endothelial cell interactions.\textsuperscript{14} The U937 cell line was originally derived from a histiocytic lymphoma patient.\textsuperscript{15} The U937s display many monocytic characteristics, are homogenous and simple to culture, rendering them a useful model as monocytes in adhesion studies.\textsuperscript{14-16} HUVEC is one of the most explored endothelial cell models and is commonly used for in vitro studies of inflammation.\textsuperscript{17} The potential of oxaceprol to modulate the TNF-\(\alpha\)-stimulated enhancement in monocyte adhesion was evaluated. TNF-\(\alpha\)-stimulated cells treated without drug were used as controls. The
effects were also compared with those of aspirin as a reference substance, which was shown to modulate monocyte-endothelial cell interactions.18,19

4.3 Materials and Methods

4.3.1 Materials
Trans-1-acetyl-4-hydroxy-L-proline (oxaceprol (OC), purity 99%), tumour necrosis factor - α (TNF-α), 3-[4,5-Dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide (MTT cell proliferation assay), and trypan blue stain were all purchased from Sigma-Aldrich Co., Milwaukee, WI.

4.3.2 Cell Culture
Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex Bio Science, Walkersville, MD, and cultured in endothelial growth media (EBM-2 basal medium, supplemented with EGM-s SingleQuots) according to Cambrex Clonetics’s recommended protocol. These cells were cultured at St. Michael’s Hospital, Toronto, ON. The cells were grown in T-75 flasks (Corning, Fisher Scientific, St. Louis, MO), at 37°C in a humidified atmosphere of 5% CO₂ and the growth medium was changed every other day until cells reached 80% confluence. Only cells from passages 3-5 were used for this study. Twenty-four hours before the experiments, the medium was removed and replaced with growth-supplement-free medium. (See Appendix B-1 for a detailed description of the HUVEC culture protocol.)

U937 monocytic cells (American Type Culture Collection, ATCC, Manassas, VA) were grown in suspension culture in RPMI 1640 medium (containing 0.3g/L L-glutamine, 2.0g/L sodium bicarbonate) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 1% sodium pyruvate and 100 units/ml penicillin streptomycin. All medium and
supplements were from Gibco, Grand Island, NY, unless otherwise mentioned. Cells were grown in T-75 flasks and the medium was replaced every 2 days. Cell density was maintained between $10^5$ and $10^6$ viable cells/mL. (See Appendix B-2 for a detailed description of the U937 cell culture protocol.)

4.3.3 Assessment of Cell Viability

Oxaceprol has been traditionally administered systematically.\textsuperscript{10,20} Osteoarthritis patients that were treated with oxaceprol (3x400 mg/day or 3x2.3 mmol/day for 6 weeks) experienced reduction in pain (at rest and under load) and the Ritchie articular inflammation index.\textsuperscript{20} In a study with hamsters fitted with a dorsal skinfold chamber, a bolus intravenous dose of 50 mg/kg (or 290 μmol/kg) of oxaceprol markedly inhibited ischemia/reperfusion-induced leukocyte adhesion.\textsuperscript{21} In vitro studies with ionophore-stimulated mouse peritoneal macrophages showed that Oxaceprol of up to 10 μmol/L had no effect on PGE\textsubscript{2} or leukotriene C4 production.\textsuperscript{13} Since there were no previous studies that examined the direct effect of oxaceprol on HUVEC and U937 cell viability and also adhesion, a wide drug window was chosen to be investigated in this study, using a low drug concentration (5 μmol/L) to extremely high concentration (10,000 μmol/L).

The effect of oxaceprol on HUVEC viability was determined using the MTT assay.\textsuperscript{22} HUVECs were cultured in a 96-well culture plate (Costar polystyrene flat-bottom micro-plate, Corning, Corning, NY) at $10^6$ cells/ml for 48 h. Oxaceprol of various concentrations (0, 5, 50, 500, 5000 and 10,000 μmol/L) were added into the wells in serum-free medium for 12 h. MTT dye was then added to each well and the plate was further incubated at 37°C for 4 h. Afterwards, detergent reagent (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added to the wells and the plate was put in the dark for 4 hours. The spectrometric
absorbance at 570 nm was read using a microplate reader (BioTek Instruments, Inc., Winooski, VT). Cells incubated in control media served as negative controls.

Passaged U937 cells were labeled with fluorescent dye, 2, 7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester (BCECF-AM, Sigma-Aldrich Co., Milwaukee, WI), at 10μM final concentration in RPMI-1640 medium at 37°C and 5%CO₂ for 30 mins. The acetoxymethyl esters of BCECF are initially non-fluorescent. Once they diffuse across the cell membranes, they can be enzymatically hydrolyzed by nonspecific intra-cellular esterases to the fluorescent BCECF, resulting in a charged form that is trapped inside the cell membranes. The labeled cells were harvested by centrifugation and washed three times with 1% FBS in phosphate buffered saline (PBS, Cambrex Bio Science, Walkersville, MD) to remove excess dye. The cells were finally resuspended in serum-free RPMI-1640 medium and added into polystyrene 96-well plates (Costar micro-plate, Corning, Corning, NY) at 2 x 10⁵ cells/well. Oxaceprol of various concentrations (0, 4, 40, 400, 4000, 10000 μmol/L) were added into the wells for 0.5h and 24h. The 0.5 h time point was chosen to match the time duration for the adhesion experiment with HUVEC described in the next section. The 24 h time point was chosen to demonstrate a longer term effect of the drug on these cells. The fluorescence intensity of each well was measured with a fluorescence microplate reader set at excitation and emission wavelengths of 485nm and 535nm, respectively. Cells incubated in control media served as negative controls. Trypan blue exclusion test was performed according to standard protocol to confirm the cell viability before and after drug incubation. (See Appendix B-3 for a detailed description of the BCECF-AM marker optimization studies and the investigation of BCECF-AM as an effective marker for the U937. See Appendix B-4 for the trypan blue assay & standard curves.)
4.3.4 Endothelial-Monocyte Adhesion Assay

HUVEC were plated into polystyrene 96-well flat-bottom plates at 3 x 10^3 cells/cm² for 3 days before the assay. The cells were grown to confluence and incubated as indicated above. HUVECs were exposed to oxaceprol of various concentrations (2.5 to 7000 µmol/L) for 12 h in serum-free medium and subsequently treated with human TNF-α (100ng/ml) for 6h, in the presence of the drug, at 37°C and 5%CO₂.

U937 cells were labeled with BCECF-AM as described before. The cells were resuspended (2 x 10^5/ well) in endothelial cell EBM-2 medium and then added to the HUVECs (treated with and without oxaceprol as described above). The two cells were co-incubated for 30 min at 37°C, after which the non-adhering U937s were removed by gently washing each well three times with 1% FBS-PBS. The fluorescence intensity of each well was measured with a fluorescence microplate reader. To determine the standard curves of fluorescence units per cell, a separate plate containing known numbers of U937 cells labeled with BCECF-AM was prepared with each set of experiments. Cell concentrations for the standards used to generate the calibration curves were determined by staining with trypan-blue and counting with a hemocytometer following a standard protocol.24 (See Appendix B-4 for the trypan blue assay & standard curves)

For all the experiments, EC cells treated without oxaceprol for 12 h and subsequently treated with TNF-α for 6h were used as “stimulated controls” while cells treated without drugs for 18 h and without addition of TNF-α were used as “unstimulated controls”. Cells treated for 12 h with aspirin (2000 and 7000 µmol/L) in place of oxaceprol were used as “relative drug controls”. Aspirin has been shown to modulate U937-endothelial cell interactions at these concentrations.18,19
4.3.5 Confocal microscopy observation and quantification of adhesion

Adhesion experiments were repeated in 24-well plates, where HUVECs were cultured on coverslips and placed on the bottom of each well before the addition of drug. The rest of the experiments were carried out following the exact procedures described for the 96-well plates, with the cells, drugs, and medium amount scaled up to match the size of the 24 well-plates. After the co-incubation and the gentle wash with FBS-PBS solution to remove the non-adhering U937s, the cells were fixed with 10% formalin for 1 h, and then rinsed with PBS. All samples were imaged within 1 hour of the last wash. Observations were carried out by confocal microscopy (Zeiss LSM510, Advanced Optical Microscopy Facility, Princess Margaret Hospital, Toronto, ON).

The BCECF-labeled U937 appeared bright under the confocal microscope. The image contrast and brightness was manipulated to maximize the contrast between U937 cells and the HUVEC monolayer. In order to determine the number of U937s adhered on the HUVEC, the total number of pixels that were fluorescent (U937 cells) was measured using Image J (Java-based image processing program, U. S. National Institutes of Health, Bethesda, Maryland, USA). The total pixel area covered by the cells was then divided by the average total fluorescent area of the stimulated controls to calculate an estimated percentage of cells adhered relative to the stimulated control. Three regions of interest (ROI) were investigated per sample and their relative size was 1.2 mm x 1.2 mm.
4.3.6 Scanning Electron Microscopy

The cells were imaged using scanning electron microscopy (SEM). After the final wash of the adhesion experiments, the cells were first fixated in 10% formalin solution overnight. They were then dehydrated through immersion in increasing ethanol concentrations (50%, 70%, 90%, 95%, and 100%). Dehydrated samples were then processed using a Polaron CPD7501 Critical Point Drier (Fisons Instruments, Uckfield, England) to ensure cell morphology was not compromised. The samples were then affixed on steel stubs and coated with 3 nm of platinum in a Polaron SEM coating unit (SC515). Finally, the samples were viewed and photographed using a Hitachi 2500 Scanning Electron Microscope.

4.3.7 Statistical Analysis

All data represent the average of at least triplicate measurements. When only 2 groups were compared (mean from 1 treatment condition compared with that of control), student’s t-test was used. ANOVA was used when 3 or more groups were compared for their significant difference with each other (i.e. means from different concentrations of drug addition). When values are referred to as being significantly different, it implies that the statistical difference is for a \( p \) value < 0.05, unless otherwise stated.
4.4 Results

4.4.1 Cell Viability

The viability of cells in the confluent monolayer of HUVECs was not affected by the different oxaceprol treatments (5 to 10,000 $\mu$mol/L) for the 12 h test period when compared to negative controls with no drug treatment, as shown by the MTT data in Fig 4-1.

![Graph showing cell viability](image)

**Figure 4-1:** Effects of oxaceprol on HUVEC cell survival and proliferation. The % viable cells after incubation for 12 h with oxaceprol at indicated concentrations were determined by MTT assay. Data represent mean ± S.D., n=3, and are expressed as percentage of HUVEC survival compared to negative controls (=100% ± 0%). None of the values are significantly different from controls ($p<0.05$).

With the BCECF-labeled U937s, the fluorescent intensity of the cells treated with 4 to 10,000 $\mu$mol/L of oxaceprol for up to 24 h did not vary significantly from the negative control, showing that the drug did not have an effect on the number of living cells under these concentrations. Data is shown in Fig 4-2. Trypan blue exclusion test showed that >95% of the oxaceprol-incubated cells were excluding the dye even after 24 h of co-
incubation. The cells still appeared round and healthy under the light microscope observation.

![Graph showing effects of oxaceprol on U937 cell survival.](image)

**Figure 4-2**: Effects of oxaceprol on U937 cell survival. The % viable cells after incubation for 0.5 h and 24 h with oxaceprol at indicated concentrations were determined by measuring the fluorescence intensity of the live BCECF-labeled U937. Data represent mean ± S.D., n=3 and are expressed as a percentage of U937 viable cells compared to negative controls (=100% ± 0%). ANOVA analysis shows that values are not significantly different from controls (p < 0.05).

### 4.4.2 Effect of Oxaceprol on U937 Monocyte Adhesion to HUVEC

Figure 4-3 shows that the stimulation of HUVEC with 10ng/ml of TNF-α for 6 h caused a significant increase in U937 monocyte adhesion to HUVEC when compared with unstimulated controls (p<0.01). The TNF-α-induced U937 monocyte adhesion, however, did not differ between HUVECs cultured with 2.5 to 7000 μmol/L oxaceprol or without drug. When aspirin (NSAID) was added, a dose-dependent inhibition of TNF-α adhesion was
observed, which agrees with previous studies.\textsuperscript{18,19} Adhesion, in the presence of either 2000 or 7000 \(\mu\text{mol/L}\) of aspirin, was reduced to 45±8\% and 27±8\% respectively, which are significantly different from the stimulated control (\(p<0.01\)) and significantly different from each other (\(p<0.05\)). The trypan blue exclusion test of the U937 before and after aspirin incubation showed that >95\% of the cells are viable at the concentrations used in this experiment.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4-3.png}
\caption{Effects of oxaceprol on U937 cell adhesion onto HUVECs. HUVECs were exposed to drugs of various concentrations for 12 h, followed by 6 h of exposure to TNF-\(\alpha\) prior co-incubation with BCECF-labeled U937s. U937s were allowed to adhere to HUVECs for 30 mins, after which the non-adherent monocytes were removed and fluorescence signal from the U937s was measured. Cells treated without drugs for 18 h (no addition of TNF-\(\alpha\)) are “unstimulated control” (\(\square\)), while cells treated without drugs for 12 h and subsequently treated with TNF-\(\alpha\) are “stimulated control” (\(\square\)). Cells treated with 12 h of aspirin and then subsequently with TNF-\(\alpha\) were the “relative drug controls” (\(\bigotimes\)) while samples treated with oxaceprol are the test sample (\(\blacksquare\)). Data represent mean ± S.D., \(n=3\), and are expressed as a percentage of U937 adhesion for TNF-\(\alpha\) stimulated controls (\(=100\% ± 0\)). *Significantly different from “stimulated controls”, \(P <0.01\).}
\end{figure}
4.4.3 Confocal microscopy observation and quantification of adhesion

When observed under the confocal microscope, the stimulation of HUVEC with 10ng/ml of TNF-α for 6 h caused a significant increase in U937 monocyte adhesion to HUVEC (Figure 4-5) in comparison to unstimulated controls (p<0.01), agreeing with the results from the microplate fluorescent readings (Figure 4-3). The confocal images (Figure 4-5) clearly show the reduction of U937 adhesion on aspirin-treated HUVECs (2000 μmol/L) when compared with the TNF-α stimulated control. However, a significant reduction was not observed with oxaceprol-treated HUVECs (2000 μmol/L).

To quantitatively determine the percentage of U937s adhered on the HUVEC, the total number of pixels that were fluorescent (cells) was measured and then compared with controls. Results are shown in Figure 4-4.

![Graph](image_url)

**Figure 4-4:** Quantitative evaluation of the fluorescence signal from the labelled U937s using the confocal image analysis (Figure 4-5). Data represent mean ± S.D., n=9 and are expressed as percentage of U937 adhesion for TNF-α stimulated controls (=100±0%).*Significantly different from “stimulated controls”, P <0.01.
Figure 4-5: Confocal microscopy images showing the adhered U937s (white) on HUVEC. HUVECs were exposed to drugs for 12 h, followed by 6 h of exposure to TNF-α and drug prior to co-incubation with BCECF-labeled U937s. U937s were allowed to adhere to HUVECs for 30 mins, after which the non-adherent monocytes were removed and imaged. a) Cells treated without drugs for 18 h and with no addition of TNF-α (“unstimulated control”); b) Cells treated without drugs for 12 h and subsequently treated with TNF-α (“stimulated control”); c) Cells treated with 12 h of 2000 μmol/L aspirin and then subsequently with TNF-α and drug. (“relative drug control”); d) Cells treated with 2000 μmol/L oxaceprol for 12 h, followed by TNF-α and drug.
4.4.4 Effect of TNF-α on cell morphology

The effect of TNF-α (100ng/mL) on the adhesion of U937s to HUVECs was observed under the SEM and micrographs are shown in Figure 4-6. The significant increase of U937s adhering on the surface of HUVEC can be seen under low magnification (x200) (Figures 4-6 a and b). When TNF-α was added, the higher magnification micrographs show adhered U937s that had deviated from their unactivated spherical shapes and demonstrated numerous protrusions of the cell surface, and small pseudopodia spreading out (Figures 4-6 c and d).
**Figure 4-6:** SEM micrographs of U937s (spherical cells) interacting with HUVECs (monolayer). There is a marked increase in adhesion of U937s upon exposure of HUVECs to 100ng/mL TNF-α prior to the addition of U937s (b) as compared to the control without TNF-α exposure (a). Images (c) and (d) show the activated U937s, with microvillar structures and small pseudopodia spreading.
4.5 Discussion

One of the earliest events in inflammation is the adhesion of monocytes to the endothelium, followed by migration and differentiation into macrophages. Inflammatory cytokines such as TNF-α which is secreted during acute inflammation, can strongly induce the expression of adhesion molecules on cultured HUVECs, such as ICAM-1, VCAM-1 and E-selectin. The upregulation of these surface molecules contribute to an increase in monocyte adhesion and chemokine release in endothelial cells.

In the present study, the effect of TNF-α on monocyte adhesion was clearly demonstrated by the adhesion tests. By measuring the UV absorbance from the fluorescently-labeled monocytes that remained on the HUVEC surface after rinsing loosely-adhered U937 cells, it was found that upon 6h of exposure to TNF-α, the percentage of U937 that was adhering to HUVEC increased significantly (Figure 4-3). This finding agrees with previous findings in literature. Similar induced-adhesion patterns were observed when the adhesion was investigated using confocal microscopy (Figure 4-4) and SEM (Figure 4-6). From the SEM images, one can clearly see that the adhered monocytes on the surface of the HUVECs underwent morphological changes, from the unstimulated smooth, round structures in suspension, to ones that had activated microvillar structures and even pseudopodia extending from their surfaces. These changes suggest activation and functional changes of the U937 cells, as one would expect during acute inflammation.

Oxaceprol has been shown to exert anti-inflammatory actions and has been widely used as a treatment for joint diseases and osteoarthritis and rheumatoid arthritis. Its action has been found to be atypical of most NSAIDs, since it does not inhibit COX activity. Previous in vivo studies with mice that had antigen-induced arthritis revealed that

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13 Previous in vivo studies with mice that had antigen-induced arthritis revealed that
oxaceprol-treatment had resulted in a significantly lower number of adhered leukocytes to the endothelium of these mice.\textsuperscript{11} Other studies have shown that oxaceprol markedly inhibits neutrophil infiltration into the joints of rats with adjuvant arthritis\textsuperscript{13}. Studies on surgical ischemia reperfusion in hamsters \textit{in vivo} also showed that oxaceprol inhibited leukocyte adhesion and extravasation.\textsuperscript{21} These findings triggered the investigation in this paper which sought to determine if oxaceprol would act directly to reduce the adhesion of monocytic-cells to human endothelial cells.

The unaltered cell viability in the present study showed that even with high concentrations of oxaceprol (of up to 10,000 μmol/L), the survival of both monocytes and HUVECs was not altered, as shown in Figures 4-1 and 4-2 and trypan blue results. This opens up the possibility of studying the effects of oxaceprol on monocytic cells and endothelial cell interactions over a very large range of concentrations. In the present adhesion studies, cells were treated with oxaceprol of concentrations from 2.5 to 7000 μmol/L. The adhesion test results clearly demonstrated that oxaceprol did not modulate the cytokine-stimulated monocytic-cell adhesion to HUVECs over the studied concentration range (Figures 4-3 and 4-4). In contrast, aspirin, a drug with well-documented NSAID character\textsuperscript{18,19}, showed a dramatic reduction in monocytic-cell adhesion towards the stimulated endothelial cells (Figures 4-3 to 4-5). Therefore, it is concluded that the model successfully demonstrated the direct effect of NSAIDs on the reduction on cell adhesion and showed that oxaceprol must have a more subtle mechanism of anti-inflammatory action that differs from directly affecting adhesion molecules on endothelial cell surfaces. Since TNF-α acts primarily by inducing the expression of ICAM-1, VCAM-1 and E-selectin on endothelial cell surface\textsuperscript{14,25-27}, it is inferred by these findings that oxaceprol most likely did not directly
inhibit the expression of these molecules. Further studies using direct antibodies to these molecules would be able to confirm this.

Although *in vivo* animal studies demonstrated a reduction of leukocyte adherence to the endothelium and extravasation of these cells by oxaceprol, the effects with human cells could not be observed in this *in vitro* study, demonstrating that the precise mechanism of action of this drug is most likely not the direct inhibition of cytokine-induced endothelial cell adhesion molecule expression nor monocyte adhesion, as the literature had suggested. This study has not been repeated on animal cell lines or on other types of leukocytes that are activated during inflammation, but it clearly demonstrated that the drug effect was not observed on the human endothelial cell lines and the U937 monocytic cells used in this study.

It is important to note that the model used in this study provides only a restrictive view of the inflammatory reaction. Differences in the origin in the cell culture, type of inflammatory cells and treatment regimes (concentration and time of exposure) could also affect the results concerning the effects of this drug. For example, inflammation involves a complex cytokine network and TNF-α is just one of the numerous cytokines participating in this network. There exists other cytokines, such as interleukin-1, interleukin-4 or interferon-γ, which possess very specific profiles of cellular adhesion molecule expression of their own. Moreover, the recruitment of inflammatory cells can also be modulated by other mechanisms such as the expression of chemotactic factors. Nevertheless, the model described here provided an expanded insight on the mechanistic action by which oxaceprol affects cytokine-induced monocytic adhesion on HUVECs, and has raised new hypotheses that need to be further tested.
4.6 Conclusion

In summary, the data suggest that oxaceprol, even at non-physiologically relevant concentrations, has no effect on cytokine-induced monocyte adhesion in HUVECs. This suggests that the reduction of leukocyte adherence to the endothelium by oxaceprol observed in in vivo animal experiments are not explained by direct drug interactions with the adhesion molecules expressed on the endothelium. This raises the possibility that oxaceprol may alter the process of osteoarthritis and rheumatoid arthritis during earlier stages of inflammation or by mechanisms other than those addressed in this study.

4.7 Acknowledgements

This study was supported by a University of Toronto Fellowship and Interface Biologics Inc. The assistance of Kuihua Cai (Santerre Lab) and Effat Rezaei (Dr. D. Courtman’s laboratory, St. Michael’s Hospital, Toronto, ON) on cell culture was greatly appreciated.
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Chapter 5 CONCLUSIONS

In the current thesis, an anti-inflammatory drug monomer is successfully synthesized into the backbone of biodegradable polyurethane elastomers. The hypothesis that this drug polymer would release oxaceprol to potentially demonstrate an anti-inflammatory effect on the target cells was demonstrated to be true. The 2\textsuperscript{nd} hypothesis that the drug release profile of this material would be dependent on its degradation by inflammatory enzymes and the drug would be released proportionally to the magnitude of the esterase activity from these enzymes was also proven to be true. The drug release profile of this material was also shown to be dependent on the structure of the polymer hard segment, which agreed with the 3\textsuperscript{rd} hypothesis. However, the 4\textsuperscript{th} hypothesis that oxaceprol will act by directly reducing the adhesion of monocytes to cytokine-stimulated endothelial cells, was disproved in this thesis.

The major findings are listed out as follows:

**On the Synthesis and Characterization of a novel Anti-inflammatory Drug Polymer Using Oxaceprol:**

1. Mass spectrometry, NMR and UV spectroscopy confirmed the structure of the oxaceprol biomonomer. The biomonomer had two hydroxyl groups available for polymerization. It was very hydrophilic and behaved very similarly to tetra n-butyl ammonium ion (TBA\textsuperscript{+}), the deprotection agent used in the last step of the synthesis. Separating the residual ammonium ion and the product monomer without comprising yield was difficult. Chain-extending PCL-diisocyanate prepolymer with the biomonomers resulted in final drug polymers that had a MW similar to the control.
non-drug polymers, which had tri-ethylene glycol as the chain extender. The final drug polymer was reprecipitated in water during the last purification step to ensure no residue free drug, monomers or other water-soluble impurities (such as TBA\(^+\)) remained on the polymer mixture. Mass spectrum of the dissolved drug polymer solution, indicated no low molecular weight impurities were associated with the drug polymers.

2. HPLC chromatograms and SEM results demonstrated that THDI-PCL-OC drug polymer experienced the most degradation, released more degradation products and showed the highest cumulative free drug release in the presence of cholesterol esterase, an inflammatory cell enzyme. 10 unit/mL enzyme catalyzed the hydrolysis of the drug polymer, as shown by the significantly higher free drug release in enzyme compared to the buffer solution. A lower enzyme concentration (1 unit/ml) did not cause the drug polymer to release a significantly different amount of oxaceprol compared to buffer.

3. There is an initial burst release of free drug from the 10 unit/ml CE-incubated samples during week 1 and a steady release in following weeks. The temporal difference is believed to be related to the exhaustion of available hydrolysable bonds on the polymer surface, the slow penetration of CE into the bulk, and also the accumulation of enzyme products on the material’s surface that blocks hydrolysis sites. The same polymer incubated in buffer showed relatively steady release of free drug throughout the 4 weeks. Samples incubated with 1 unit/ml CE demonstrated
similar free drug release amounts as the buffer-incubated samples. The hypothesis that the enzyme had a dose-dependent effect on the hydrolysis of the drug polymer was validated.

4. SEM and HPLC results demonstrated that the analog DDI-PCL-OC polymer showed very low overall degradation rates and minimal drug release with or without the presence of enzyme. Total drug release over 4 weeks for the 10 unit/ml enzyme-incubated samples was almost 7 fold lower than that of the THDI samples. HPLC chromatograms also did not show any unique degradation product peaks. Only when 10 unit/ml of CE was used, and only in week 1, did the drug polymer release an oxaceprol amount that was significantly above zero. The differences in degradation and drug release rates between the THDI and DDI drug polymers are believed to be related to:

a. Diisocyanate structure: DDI’s long aliphatic linear structure resulted in densely-packed hard segment domains (based on DSC data) and hence limited the accessibility of water to the hydrolysis sites, while THDI’s side methyl groups permitted the THDI polymer to be less densely-packed in the region of drug segment.

b. Polymer hydrophobicity: The DDI polymer has longer hydrophobic chains which has low solubility. Taking up water into the backbone could take a long time and could delay the onset of degradation. It is hypothesized that a longer incubation period might demonstrate a burst release of drug and degradation products in the DDI polymer.
c. Polymer crystallinity: The DSC demonstrated that the DDI drug polymer was more phase separated, had a lower soft-segment crystallinity and had hard segment crystalline domains. Possible H-bonds around the soft-hard segment interface and crystalline layer on the material surface might explain why the degradation of the DDI drug polymer proceeded at a very slow rate.

d. Nature of drug molecule: There is a significant difference in the cumulative drug release amounts for polymers that carry different drugs. In the case of DDI polymers, the oxaceprol polymer delivered minimal free drugs while a norfloxacin analogy polymer\(^1\), although with a late onset, managed to deliver a significant amount of free drug. It is believed that, aside from factors such as the hydrophilicity and the nature of the hydrolysable bonds around the drug, the size of the drug can also be a significant factor that affects the accessibility of water and enzymes to the hydrolysable bonds, especially in a system where the polymer chains are very closely-packed (i.e. DDI-PCL-OC polymer).

5. As a result of conclusions 2 to 4, it is believed that by changing the chemistry of the diisocyanate and the drug, the specificity of enzymatic cleavage and subsequently the release pattern of drug could be altered.

**On the Interaction of Oxaceprol on TNFα-induced U937 Monocyte Adhesion on Human Umbilical Vascular Endothelial Cells:**

1. Upon 6 h of TNF-α exposure, the percentage of monocytic cell adhering to HUVEC was significantly increased *in vitro*, as demonstrated by SEM, confocal micrograms, as well as UV quantification of the fluorescently-labeled monocytic cells. SEM
images demonstrated morphological changes on the cell surface of adhered monocytic cells, demonstrating signs of activation.

2. The cell viability data in the present study showed that even with high concentrations of oxaceprol (of up to 10,000 μmol/L), the survival of both monocytes and HUVECs was not altered. It is concluded that any adhesion effects of this drug, if any, could not be due to the killing of the cells within the concentration range studied.

3. The cell model successfully demonstrated the dramatic dose-response effect of Aspirin (a well-documented NSAID) on the reduction on cell adhesion, agreeing with results reported in the literature.²,₃ This result demonstrated that the cell model is valid for the demonstration of the effects of drugs on direct cell-adhesion.

4. This cell model successfully demonstrated that oxaceprol, even at very high concentrations (up to 7000 μmol/L), has no effect on TNF-α -induced monocytic cells-adhesion onto HUVECs. Since TNF-α acts primarily by inducing the expression of ICAM-1, VCAM-1 and E-selectin on endothelial cell surfaces,⁴,⁷ it is inferred by these findings that oxaceprol must have a more subtle mechanism of anti-inflammatory action that differs from directly affecting adhesion molecules on endothelial cell surfaces. Hence, the reduction of leukocyte adherence to the endothelium by oxaceprol observed in in vivo animal experiments could not be explained by direct drug interactions with the adhesion molecules expressed on the endothelium. It is hypothesized that oxaceprol may alter the process of osteoarthritis
and rheumatoid arthritis during earlier stages of inflammation or by mechanisms other than those addressed in this study.

5. The model used in this study provides only a restrictive view of the inflammatory reaction. It is believed that the differences in the origin in the cell culture, type of inflammatory cells, cytokines, and treatment regimes (concentration and time of exposure) could possibly affect the results concerning the adhesion effects of this drug. To establish a simple *in vitro* cell model to demonstrate the direct therapeutic effect of oxaceprol is believed to be a difficult task.
REFERENCE


Chapter 6 RECOMMENDATIONS

On the Synthesis and Characterization of a novel Anti-inflammatory Drug Polymer Using Oxaceprol:

1. Modifications of the biomonomer purification procedures should be made in order to simplify and increase the yield and purity of the biomonomer. Other types of columns (i.e. ion-exchange columns) could be explored to separate TBA\(^+\) with the biomonomer. Precipitating the TBA\(^+\) with ionic salts such as potassium iodide could also be examined as a mean to extract TBA\(^+\) from the aqueous phase.\(^1\)

2. The effect of the diisocyanate chemistry should be further investigated, by using diisocyanates with varying hydrocarbon segment lengths, as well as varying locations, sizes and numbers of side groups. Since substrates that have longer side-chain esters has been shown to increase CE activity,\(^2,3\) it is hypothesized that an addition of side groups to the long DDI backbone might cause less polymer packing,\(^4,5\) and prevent hard segment crystallization and facilitate the access of CE to the ester-carbonyl hydrolysis site, and could potentially demonstrate a higher difference in drug release for the enzyme versus buffer solution, than the THDI drug polymer in this study.

3. If the degradation studies are to be repeated, antibiotics should not be added to the degradation buffer because these antibiotics fragments had many HPLC peaks at the detection wavelength of 215nm, which complicated the process of finding the optimized method for the HPLC and also isolating the drug product peaks. By
making sure that all steps were performed in a very sterile environment, one could possibly avoid the use of antibiotics altogether.

4. HPLC methods could be further optimized to isolate the free drug peak from any enzyme fragment peaks. Further HPLC method development should be done with a different solvent system that does not contain potassium salt so that the degradation product fragments could be collected and analyzed using mass spectrometry for their possible structures.

5. Further polymer characterization could be completed in order to determine: a) the total amount of drug that was incorporated in the drug polymer, so that it could be compared to the theoretical value that was based on stoichiometry; b) the degree of H-bonding on the different polymer surfaces, i.e. using infrared spectroscopy; c) the hydrophobicity by measuring the contact angle; and d) film density by floatation techniques.  

6. It was found in this study that DDI/PCL/OC had very little degradation with or without CE in the solution over the 30 day incubation period. Further biodegradation tests should be performed over a longer period of time to examine the hypothesized slow onset of the hydrolysis due to the slow uptake of water into the hydrophobic DDI backbone.

7. Since CE had a dose response effect on the drug release in the THDI/PCL/OC
polymers and had minimal effect on the DDI/PCL/OC polymer, it would be interesting to perform a degradation experiment on a blend polymer that consists of a mixture of both the DDI and THDI polymer. It is hypothesized that the methyl groups on the THDIs will result in a less densely-packed structure in this mixed polymer than pure DDI drug polymer, and will result in a higher drug release rate, while showing a more distinct effect of CE on the hydrolysis rate than the pure THDI drug polymer. (See recommendation 2)

8. Alternate components could be used to replace the water absorbing tri-ethylene glycol component in the biomonomer in order to introduce a higher specificity of CE on the cleavage of the bonds to release the free drug. One alternative could be the use of JEFFAMINE® polyetheramines. The amines will form amides upon reaction with the carboxylic acids on the OC drug molecules. Since it was demonstrated that the urethane and urea linkages are found to be susceptible to CE hydrolysis at a much slower rate than ester linkages, it is hypothesized that the amides will also undergo slower hydrolysis, and possibly resulting in a higher specificity of CE activity on drug release.

9. Alternate monomers that contain other degradable bond types and other inflammatory cell-derived enzymes should be examined and compared for their effects on the release of oxaceprol.

10. Other anti-inflammatory drugs could be incorporated into a polyurethane backbone
using the same technique. When choosing another drug for the synthesis of a drug polymer, in conjunction with making sure that the drug has the necessary chemistry for the polymerization, it is strongly recommended (although not absolutely necessary) that the drug should also have:

a. established and well-documented *in vitro* and *in vivo* models for easy testing of the drug’s effect after it is released;

b. an established HPLC method for easy drug peak isolation

c. a distinct feature for easy detection, such as a very unique UV peak that is much higher than the base polymer or a unique element that could be detected and quantified by performing elemental analysis.

11. Further testing should be done to confirm that the drug structure was not altered during the synthesis and that the free drugs released from the drug polymer still reserve their anti-inflammatory effects.

12. Biocompatibility testing should be done to examine the toxicity of the degradation products.

**On the Interaction of Oxaceprol on TNF-α-induced U937 Monocyte Adhesion on Human Umbilical Vascular Endothelial Cells:**

1. Adhesion molecule expressions (e.g ICAM-1, VCAM-1 and E-selectin) on endothelial cells are strongly induced in response to TNF-α.\(^8\)-\(^11\) The ability of OC to modulate the TNF-α stimulated enhancement in these adhesion molecule-expressions could be evaluated to support the findings in this study that indicated OC had no
effect on leukocyte adherence to the endothelium.

2. The drug effect of OC could be specific to the origin in the cell culture, type of inflammatory cells, cytokines, and treatment regimes (concentration and time of exposure). More adhesion tests could be done to examine the effects of the different combinations of the above factors on direct leukocyte-endothelial cells interactions.
REFERENCE


APPENDIX A

Appendix A-1: Biomonomer Product A, Mass Spectrometry and NMR results

Figure A-1: Structures of molecules related to the synthesis of Product A
Figure A-2: Mass Spectrometry of Product A (negative mode), demonstrating the deprotonated product peak at 286 amu (calculated mass of Product A: 287 amu).

Figure A-3: MS/MS of peak 286 (from Figure A-2), demonstrating that the peaks at 244 and 131 amu are associated to the breakdown of the 286-peak during analysis.
Figure A-4: H-NMR (400MHz, CDCl3) shifts confirms the structure of Product A: a) the spectrum b) H-NMR shifts and relative ratio of areas under the peaks; and c) H-NMR shift estimations of Product A, correlating to the shifts in the spectrum.
Appendix A-2: Biomonomer Product B, Mass Spectrometry and NMR results

Figure A-5: Structure of Product B

MW: 688.4

Figure A-6: Structure of suspected side product

MW: 419.2
Figure A-7: Mass Spectrometry of Product pre-B (positive mode), before passing through a silica column demonstrating the protonated product peak at 689 amu, its sodium-analog peak at 711 amu (calculated mass of Product B: 688 amu), and several impurity peaks. One possible structure of the impurity is shown in the figure above. The protonated peaks associated with this side product are at 420 amu and its sodium analog at 443 amu. Only a very small peak is found at 288 amu, demonstrating that almost all of the Product A was exhausted.

Figure A-8: MS/MS of peak 689 (from figure above), demonstrating that the impurity peaks at 443.3, 364.2, 174.2, 129.1, 123.1 found in the spectrum above are not due to
breakdown of the 689-peak during analysis, since their m/z positions are not found in the MS/MS.

Figure A-9: Mass Spectrometry of Product B (positive mode) after passing through a silica column, demonstrating the product peak at 689 amu, its sodium-analog peak at 711 amu (calculated mass of Product B: 688 amu), and reduced impurity peaks, showing that the purification was successful.

Figure A-10: MS/MS of peak 689 (from Figure A-9 above), which is similar to Figure A-8, demonstrating that the mass spectrometer broke down the 689-peak into several minor components during analysis, none of which were present in significant amount in the actual MS (Figure A-9).
Figure A-11: H-NMR (400MHz, CDCl₃) confirms the structure of the purified Product B:
a) the spectrum b) H-NMR shifts and relative ratio of areas under the peaks; and c) H-NMR shift estimations of Product B, correlating to the shifts in the spectrum.
Appendix A-3: Biomonomer Product C, Purification, Mass Spectrometry and NMR results

After the deprotection of the hydroxyl group on the biomonomer, there were multiple impurities and side-products in the residue. The main suspected impurities are the derivatives of the deprotecting agent: tetrabutyl ammonium ions and tributyl-amine, and the derivative of the deprotected group: tert-butyldimethylsilyl products. The structures of these molecules are shown in Figure A-13. Mass spectrometry analysis of the residue before purification showed multiple peaks that could be associated with these impurities (Figure A-14).

![Structure of Product C (Biomonomer)](image)

**Figure A-12:** Structure of Product C (Biomonomer)
Figure A-13: Structures of suspected impurities: TBA$^+$ (a), and tributylamine (b), which are derivatives of the deprotecting agent used in the deprotection step; and tert-butyl-dimethyl silyl products (c), which are products of the deprotecting groups that were cleaved off during the deprotection step.
Figure A-14: Mass Spectrometry of Product C (positive mode), before any purification. The protonated product peak is demonstrated at 483 and its sodium analog at 461 amu (Figure A-12, calculated mass of Product C: 460 amu). There are also many impurity peaks. The strongest impurity is the TBA\(^+\) peak at 242 amu (Figure A-13a, calculated mass: 242). The peak at 186 is assigned to tributylamine (Figure A-13b, calculated MW: 185) and the peak at 142 is assigned to tert-butyl-dimethyl silyl products. (Figure A-13c, calculated MW: 115\(^+\)). No peak is observed at 689 amu, demonstrating that all of the Product B was deprotected and the reaction had reached completion.

**Purification Procedure A: Silica Column**

One of the purification procedures attempted included the usage of a small silica column. 100% chloroform was used to pack the column. The solvent that was used to load the sample and to run the sample through the column consisted of 2.5% methanol and 97.5% chloroform. Samples were collected in small glass tubes and then spotted on a silica thin layer chromatography (TLC) plate (Figure A-15).
Figure A-15: TLC plates results (SK-1-89), ran with 10% methanol and 90% chloroform. Three main spots were observed. Fractions 3-22 were collected in order to identify the “top spot”. Fractions 35-43 were collected to identify the “middle spot”, while fractions 55-65 were collected to identify the “bottom spot” (not shown). All fractions were collected using a silica column (liquid chromatography column: I.D. × L 1.0 cm × 60 cm, Sigma-Aldrich Co., Milwaukee, WI, packed with silica gel, 200 mesh and finer, EMD Chemicals Inc., Gibbstown, NJ, collected in 10 mL fractions) run with 2.5% methanol and 97.5% chloroform. Solvent was removed from these samples by using a rotor evaporator and the residues were analysed by mass spectrometry and NMR separately.
Figure A-16: Mass Spectrometry of the “top spot” (positive mode) collected from the column. Peak at 186 amu is believed to be associated with tributyl amine (Figure A-14b, calculated MW: 185).

![Mass Spectrometry Image](image.png)

Figure A-17: Mass Spectrometry of the “middle spot” (positive mode) collected from the column. The protonated product C peak is demonstrated at 483 and its sodium analog at 461 amu (Figure A-13, calculated mass of Product C: 460 amu). There are no other impurity peaks.

![Mass Spectrometry Image](image.png)
**Figure A-18:** Mass Spectrometry of the “bottom spot” (positive mode) collected from the column. The TBA$^+$ peak is clearly observed at 242 amu (Figure A-14a, calculated MW: 242).

Although successful, the yield of this separation method was quite low (36% yield), mainly due to the close association between the TBA$^+$ and the desired product C. Further attempts were made with different concentrations and compositions of solvent for the silica column but none of them produced a higher yield. The biomonomer (Figure A-13) was highly hydrophilic, and appeared to have very similar solubility and affinity towards water as the TBA$^+$.

**Purification Procedure B: Solvent Extraction**

Since the column method produced low yield, solvent extraction was used to remove some of the impurities after the deprotection step. The objective was to remove the protecting group (Figure A-13c) and most of the TBA-related products (Figure A-13a and b)
while conserving most of the desired product C (Figure A-12). After treating the impure residue (Figure A-14) with NaCl solution (conc.) and extracting it with diethyl ether (ACS grade, EMD Chemicals Inc., Gibbstown, NJ) three times, the protecting group (Figure A-13c) was removed into the diethyl ether phase. NMR showed peaks that correlate with the shifts in tert-butyl dimethylsilyl products (See Figure A-19) in the ether phase, and no product C peaks at all, showing that product C was pretty hydrophilic and preferred to remain in the aqueous salt phase. By mass loss calculations, approximately 79 wt% of the tert-butyl dimethylsilyl groups (TBDMS) was removed by this ether extraction step.

**Figure A-19:** H-NMR (400MHz, CDCl₃) showing that the main product from the ether extraction was indeed tert butyl dimethylsilyl-related products.
Figure A-20: H-NMR shift estimation of tert-butyl dimethylsilyl-related product. The ratio of area under methyl group shift 0.1ppm: shift 0.9ppm is expected to be 6:9. The ratio based on Figure A-19 was 5.6:9.

The NaCl solution (conc.) phase, which mainly consisted of the product C and residual tetra n- butyl ammonium ion, was then directly treated with dichloromethane, in an attempt to extract some of the product out of the aqueous phase and directed into the hydrophobic layer. However, to one’s surprise, more TBA+ (Figure A-13a) and the tributyl amines (Figure A-13b) were found in the organic layer than product C, indicating that product C had a strong affinity to water and behaved very similarly to tetra n-butyl ammonium ions. This extraction procedure was repeated with chloroform for two more times, and more of the TBA+ and tributyl amines were pulled into the organic phase. It is important to note that some product C did diffuse out of the aqueous phase and into the organic phase as well, although in a lesser amount. The 3rd extraction with chloroform showed that the majority of the product in the organic phase was TBA+, with some of product C, at a mole ratio of 5.4 (See H-NMR results on Figure A-21).

The later aqueous phase was dried under reduced pressure using a rotor evaporator, and the sodium chloride (that was in the NaCl solution) was removed from the residue by dissolving the residue in dichloromethane and passing the solution through a filter funnel (Whatman qualitative filter papers, Grade 3, pore size: 6µm, Whatman Inc., NJ). The
sodium chloride crystals were filtered out, and then the dichloromethane was removed again under reduced pressure with a rotor evaporator. The final product contained the drug biomonomer (product C) and some tetra n-butyl ammonium ion at a mole ratio 3:1 (see NMR spectrum on Figure A-24).

**Figure A-21:** H-NMR (400MHz, CDCl3) of the chloroform phase, showing that there are two main products in this phase, the main product being TBA+ (peaks (i)-(iv)), and the secondary being the biomonomer (peaks a-j). Under the area under the peak ratios, it can be derived that the mole ratio of TBA+ to biomonomer was 5.4:1.
Figure A- 22: H-NMR shift estimation of TBA⁺, the ratio of peak i:ii:iii:iv are expected to be 12:8:8:8.

Figure A- 23: H-NMR shift estimations of drug biomonomer, correlating to the ones find in Figure A-21.
**Figure A-24:** H-NMR (400MHz, CDCl3) of the residue in the aqueous phase confirms the structure of the biomonomer (peaks a-j) and some TBA⁺ (peaks (i)-(iv)). Under the area under the peak ratios, it can be derived that the mole ratio of TBA⁺ to biomonomer was 1:3.
Appendix A-4: Distillation of diisocyanates and the titration of hydroxyls

The DDI and THDI were distilled prior to syntheses, using the standard distillation apparatus shown in the figure above. Under a vacuum of 0.05 mmHg, the distillate was...
collected at 110°C. The diisocyanates were stored in a sealed flask and used within 72 hours. The PCL was titrated to determine the number of hydroxyl groups available for polymer reaction. The method used was the acetic anhydride/pyridine titration method, 1 in which the acetic anhydride converted the R-OH groups into R-COOH groups, which were then titrated with sodium hydroxide. The moles of acid correspond to the moles of hydroxyl in each diol sample.

PCL samples were transferred into 3 dried 125mL titration bottles, with approximately 1g in each bottle. The bottles were then degassed in a vacuum oven at 70°C overnight, and then purged with nitrogen. The weighed samples were gently dissolved in 5mL of acetic anhydride solution, which consisted of a 1:3 mixture of acetic anhydride (98% A.C.S Reagent) and 75% pyridine (distilled), capped, under nitrogen purge. Each flask in turn was then connected to a condenser and heated on an electric hotplate until small bubbles were observed. The solution was then gently mixed and let boil for 5 minutes. After the heat was removed, 20 mL of distilled pyridine was added through the septum at the top of the condenser to rinse the residues into the flask. The condenser was removed and the resultant mixture was quenched with 20mL of distilled water and 3 drops of phenolphthalein were added to each flask. The samples were then titrated with standard 1N sodium hydroxide solution. Parallel empty assay (control) was carried out simultaneously and the net cost of sodium hydroxide solution was used to calculate the amount of hydroxyl group of the sample. Table A-1 contains the results for the PCL titration. The calculation to determine total hydroxyl content and molecular weight are as follows:
total acid groups = total hydroxyl number = \( \Delta (mL) \times \text{molarity of titrant (mol / mL)} \times \frac{1}{\text{mol NaOH}} \times \frac{1}{\text{hydroxyl group per mol diol}} \)

\[
M_W = \frac{\text{weight of diol sample (g)}}{\text{Total hydroxyl number}} \times \frac{1}{2 \text{ hydroxyl groups per mol diol}}
\]

where \( \Delta = \) NaOH solution volume required for control – NaOH solution volume required for sample.

**Table A-1: Determination of PCL hydroxyl groups by acetic anhydride/pyridine titration**

<table>
<thead>
<tr>
<th>PCL Titration</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample weight (g)</td>
<td>0.96</td>
<td>1.13</td>
<td>0.99</td>
<td>-</td>
</tr>
<tr>
<td>NaOH volume (mL), 1.0204N*</td>
<td>23.35</td>
<td>23.2</td>
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<tr>
<td>Total hydroxyl number</td>
<td>0.97</td>
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</tr>
<tr>
<td>Moles PCL (mmol)</td>
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<td>0.56</td>
<td>0.46</td>
<td>-</td>
</tr>
<tr>
<td>MW PCL (g/mol)</td>
<td>1981</td>
<td>2013</td>
<td>2156</td>
<td>-</td>
</tr>
</tbody>
</table>

* Average MW = 2050±93 g/mol

* NaOH was titrated with 0.25N HCl to determine its actual concentration prior to PCL titration. Phenolphthalein was used as indicator. The concentration calculated was 1.0204N, which was slightly higher than what the bottle indicated (1.000N).

**REFERENCE**

Appendix A-5: Polymer impurity investigation

To detect if there were any small molecular weights molecules, including free drug, monomers or other possible impurities such as TBA$^+$ that remained with the final polymer after the multiple washing steps, the polymer was dissolved in DMAC and filtered (See Chapter 3 Methods and Materials). The filtrate was collected and the solvent removed. Mass spectrometry of the residue (Figure A-26) showed no traces of biomonomer (MW460; expected protonated form at 461 amu, sodium analog at 483 amu), TBA$^+$ (MW 242), or free drug (MW 173).

![Mass spectrum of the DDI-PCL-OC polymer that was dissolved in DMAC. The high molecular weight products were filtered out and the solvent was removed prior to analysis. The spectrum shows no TBA$^+$ nor monomer nor free drug.](image-url)
Appendix A-6: Oxaceprol Calibration Curve for HPLC

**Figure A-27:** HPLC chromatograms of oxaceprol of different concentrations. Retention time of drug is approximately 7.6 minutes (at wavelength 215 nm).

**Figure A-28:** Oxaceprol HPLC calibration curve
Appendix A-7: HPLC separation of polymer drug products

When enzyme was used, the HPLC peaks from the enzyme (peak 2 and 3) overlapped with that of the drug peak in integration zone B (see Figure A-29).

**Figure A-29:** HPLC separation of enzyme products and free drug. Integration zones are defined as A (6.1 to 6.8 minutes) and B (6.8 to 7.9 minutes).

In order to determine the area under the HPLC curve that belonged to the drug, the following calculation was used:

Two integration zones were defined: Zone A from 6.1 to 6.8 minutes and Zone B from 6.8 to 7.9 minutes (Figure A-29). Zone A contains peak 1 of the enzyme products. Zone B contains peaks 2 and 3 of the enzyme products, as well as the oxaceprol peak.

To determine if peaks 1, 2 and 3 are specific to cholesterol esterase products, the area under peaks 1, 2 and 3 are investigated for their relationship with each other. The hypothesis
is that peak 1 is always proportional to peak 2 +3. To test this hypothesis, enzymes of different amounts (20, 30 and 40 units/ml) were incubated at 37°C for 14 days and 28 days respectively. Peaks 1, 2 and 3 were isolated in the HPLC chromatograms of all the samples (Figure A-30). Integrations of the areas under zone A (peak 1) and B (peak 2 + 3) were done and it was found that the area under zone A was consistently proportional to the area under zone B, regardless of the amounts of CE and the incubation time. The relationship was found to be:

\[
\frac{\text{Peak 1}}{\text{Peak 1} + 2 + 3} = 0.4 \pm 0.02; \ n = 6
\]

**Figure A-30**: HPLC chromatograms of cholesterol esterase solutions incubated for 14 or 28 days, at different concentrations

The oxaceprol drug peak overlaps with the enzyme peaks 2 and 3 in zone B. Figure A-31 shows a representative chromatogram of polymer THDI/PCL/OC, which was exposed to 10 unit/ml of CE for 28 days. It can be clearly observed that the peaks observed in zone B
(peak 4 and 5) of this sample are a combination of both the drug peak OC and the enzyme peaks 2 and 3.

**Figure A-31:** HPLC chromatograms of cholesterol esterase (a), pure drug (b) and THDI/PCL/OC polymer (c) at 28 days after degradation with 10 units/ml CE.

Pure buffer has no peaks in zones A and B (Figure A-29). UV absorbance spectra further proved that the buffer (which contains antibiotics) did not interfere with the UV absorbance, or shape of peaks 1, 2 and 3 of the enzyme (Figure A-32). In fact, the proportion of the peaks of the enzyme + buffer samples obey the same formula: \( \frac{A}{A+B} = 0.4 \).
Figure A-32: UV absorbance spectra of peak 1, 2 and 3 of pure enzyme sample (a, c, e) and enzyme sample with buffer that contains antibiotics (b, d, f). Both samples were incubated at 37°C for 14 days.

UV absorbance spectrum of oxaceprol is compared to that of the pure enzyme product (peak 3) (Figure A-33 a and b). The two are distinctly different, and traces of both can be seen in the UV absorbance spectra of the degradation products for the THDI/PCL/OC polymer that was exposed to 1 or 10 units/ml of CE and buffer (peak 5, Figure A-33 d and e). As CE concentration increased from 1 to 10 units/ml, the 277 nm UV peak became more
evident in the drug polymer UV spectra. However, the dominating shapes of these spectra still resemble that of the pure oxaceprol, showing that peak 5 of the drug polymers that were exposed to CE is related to both the drug and the enzyme. In the case where the polymer was not exposed to any CE, the spectrum of the polymer peak 5 (c) looks identical to that of the pure drug (a), confirming that peak 5 in the buffer is only related to pure drug.

(a) Peak OC – Pure oxaceprol

![Graph (a)](image)

(b) Peak 3 – Pure enzyme

![Graph (b)](image)

(c) Peak 5 – polymer in buffer

![Graph (c)](image)

(d) Peak 5 – polymer in 1unit/ml CE

![Graph (d)](image)

(e) Peak 5 – polymer in 10unit/ml CE

![Graph (e)](image)

**Figure A-33:** UV absorbance spectra of pure oxaceprol standard (peak OC) (a), pure enzyme standard (Peak 3) (b) and polymer THDI/PCL/OC(9) (peak 5) that was exposed to buffer (c) or enzyme (d,e), for 28 days.
The DDI/PCL/OC polymer that was exposed to 1 or 10 units/ml of CE or buffer also showed similar UV absorbance chromatograms under the peak 5 (Figure 34 d and e). Since this polymer released a significantly lower amount of drug than the THDI/PCL/OC polymer (Figure 33 c,d,e), the UV absorbance from the CE products are relatively stronger. The 277 nm UV peak that is associated with the CE is definitely more evident in the drug polymer that was exposed to 1 unit/ml CE, and even more so in the one that was exposed to 10 unit/ml CE. Again, the shapes of these spectra clearly showed that peak 5 of the drug polymers that were exposed to CE (d and e) is related to both the drug and the enzyme. In the case where the polymer was not exposed to any CE, the spectrum of the polymer peak 5 (c) again looks identical to that of the pure drug (a), confirming that peak 5 in the buffer is only related to pure drug.
Figure A-34: UV absorbance spectra of pure oxaceprol standard (peak OC) (a), pure enzyme standard (Peak 3) (b) and polymer DDI/PCL/OC(1) (peak 5) that was exposed to buffer (c) or enzyme (d,e), for 7 days.
Appendix A-8: Drug Peak Isolation and Calculation

Figure A-35: HPLC chromatograms of cholesterol esterase (a), pure drug (b) and THDI/PCL/OC polymer (c) at 7 days after degradation with 10 units/ml CE.

In order to accurately estimate the area under a HPLC spectrum that is associated with free drug, the following enzyme peak relation was used:

\[
\frac{\text{peak } 1}{\text{peak } (1+2+3)} = 0.4 \pm 0.02
\]

By simple rearrangements, one gets:

\[
\text{peak } (2+3) = \frac{\text{peak } 1}{0.4} - \text{peak } 1
\]

When this formula is used for polymer peak calculations, peak 6 is peak 1, and it is assumed that the area for peak (4+5) – OC = peak (2+3). Therefore,

\[
OC = \frac{\text{peak } (4+5) - \text{peak } 6}{0.4} + \text{peak } 6
\]
## Appendix A-9: SEM images of polymer surfaces

<table>
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<tr>
<th></th>
<th>100X</th>
<th>5000X</th>
<th>10,000 X</th>
</tr>
</thead>
<tbody>
<tr>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
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<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>CE 10 unit/ml</td>
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<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure A-36:** SEM images of the THDI/PCL/OC polymer
Before Degradation

In Buffer

CE 1 unit/ml

CE 10 unit/ml

Figure A-37: SEM images of DDI/PCL/OC polymer
<table>
<thead>
<tr>
<th></th>
<th>100X</th>
<th>5000X</th>
<th>10,000 X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Degrada-tion</td>
<td><img src="100X.png" alt="Image" /></td>
<td><img src="5000X.png" alt="Image" /></td>
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<tr>
<td>CE 1 unit/ml</td>
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<td><img src="5000X.png" alt="Image" /></td>
<td><img src="10000X.png" alt="Image" /></td>
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<tr>
<td>CE 10 unit/ml</td>
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<td><img src="5000X.png" alt="Image" /></td>
<td><img src="10000X.png" alt="Image" /></td>
</tr>
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</table>

**Figure A-38**: SEM images of THDI/PCL/TEG polymer
APPENDIX B

Appendix B-1: U937 cell culture protocol

B-1-1: Complete Media Preparation (1L)
1) Combine:
   - 10 mL sodium pyruvate (100 mM, Gibco, Grand Island, NY)
   - 10 mL penicillin streptomycin (10,000 units/mL, Gibco, Grand Island, NY)
   - 100 mL FBS (Hyclone, Logan, UT)
   - 880 mL RPMI 1640 medium (containing 0.3g/L L-glutamine, 2.0g/L sodium bicarbonate)
2) Aliquot media into 50mL falcon tubes and refrigerate.

B-1-2: Thawing U937
1) Warm a tube of complete media in a 37°C water bath for 20 minutes.
2) To set up cultures, add the complete media to a T75 flask and allow the flask to equilibrate in a 37°C, 5% CO₂ humidified incubator for at least 30 minutes.
3) Thaw the cryovial of U937 cells by gentle agitation in a 37°C water bath, for 2 minutes
4) Remove vial from water bath and spray with 70% ethanol.
5) Transfer the cells in the cryovial into the culture flask set up earlier, using a micropipette.
6) Gently rock the culture flask to evenly distribute the cells and return to the incubator.
B-1-3: Subculturing

1) Subculture the cells when they are 70%-80% confluent. Remove RPMI complete medium from 4°C storage and allow it to warm up in a 37°C water bath.

2) Prepare new culture T-75 flasks with 50 mL of warm medium in each of them.

3) In a sterile flow hood, transfer the cell suspension to a 50 mL centrifuge tube and spin down at 1500 rpm for 5 minutes, at room temperature.

4) Aspirate the medium from the culture vessel.

5) Resuspend the cell pellet with 10 mL warmed complete media (~1 million cells/mL).

6) Transfer 1 mL of the suspension to each of the T-75 flasks setup earlier.

7) Gently rock the culture flask to evenly distribute the cells. Incubate at 37°C, 5% CO₂, humidity.

B-1-4: Cryopreserving U937

1) When cells reach 80% confluent, they can be harvested and frozen (~10 million cells per T-75 flask). Prepare 5 mL cryopreservation medium: 250 μL DMSO and 4.75 mL FBS.

2) Centrifuge to collect cells into a pellet: 1500rpm for 5 min, at room temperature.

3) Resuspend cells in 5 mL of cold cryopreservation medium. The cell concentration should be around 2 million cells per mL.

4) Pipet aliquots (1 mL each) into sterile cryovials, keeping them chilled in the refrigerator for 2 hours.

5) Transfer the vials to a -70°C freezer or place in liquid Nitrogen for long-term storage (-200°C).
Figure B - 1: Light optical microscopy of U937 cells after 5 days of culturing
Appendix B-2: HUVEC culture protocol
(Protocol adapted from Cambrex Clonetics Endothelial Cell Systems Instructions: AA-1001)

B-2-1: Complete Media Preparation (500mL)
1) Decontaminate the external surfaces of all supplement vials and the medium bottle
   (EBM-2 BulletKit, Cambrex Bio Science Walkersville, Inc., Walkersville, MD)
   with 70% ethanol.
2) In a flow hood, add from the supplement vials to 500mL EBM-2 basal medium
   (Cambrex Bio Science Walkersville, Inc., Walkersville, MD):
   - rhEGF
   - Ascorbic Acid
   - GA-1000
   - R³-IGF-1
   - rhFGF-B
   - VEGF
   - FBS
   - Heparin
   - Hydrocortisone
3) Aliquot 10mL complete media into each 15mL falcon tube and refrigerate. Avoid
   repeated warming and cooling of the medium. Only warm up the required volume.

B-2-2: Thawing HUVECs
1) To set up cultures, calculate the number of T-75 flasks needed based on the
   recommended seeding density of approximately 2500-5000 cells/cm². Each cryovial
   usually contains ~2 million cells/mL.
2) Warm up the needed amount of complete media in a 37°C water bath for 20 minutes.

3) Add 10mL complete media to each T75 flask and allow the flasks to equilibrate in a 37°C, 5% CO₂ humidified incubator for at least 30 minutes.

4) Thaw the cryovial of HUVECs by gentle agitation in a 37°C water bath, for 2 minutes. Remove vial from water bath and spray with 70% ethanol.

5) Resuspend the cells and transfer the appropriate amount of cells in the cryovial into each culture flask set up earlier, using a micropipette.

6) Gently rock the culture flask to evenly distribute the cells and return to the incubator.

7) *** DO NOT centrifuge the cells to remove cryoprotectant cocktail. This will cause damage to the cells.

**B-2-3: Subculturing**

Reagents needed: Reagent Pack (Cambrex Bio Science Walkersville, Inc., Walkersville, MD), which contains trypsin-neutralising solution (TNS), trypsin-EDTA and HEPES buffered saline solution.

1) Subculture the cells when they are 70%-80% confluent (~ 2 million cells for each confluent T-75 flask). Remove EBM-2 complete medium from 4°C storage and allow it to warm up in a 37°C water bath. Allow TNS, trypsin-EDTA and HEPES solutions to warm up to room temperature.

2) Aspirate medium from one culture flask with a pipette.

3) Rinse cells with 10 mL of HEPES solution.

4) Aspirate the HEPES solution from flask.

5) Repeat steps 3 and 4.
6) Add 10 mL trypsin-EDTA solution to flask. Rock the flask to make sure cells have contact with the trypsin solution.

7) Examine cells under microscope and set timer for 5 minutes. Continue to rock the flask during the 5 minutes and tap the bottom of the flask to facilitate cell detachment.

8) After 5 minutes, check under the microscope to make sure that all the cells are detached.

9) Add 20 mL of TNS to the flask to neutralize the Trypsin solution.

10) Using a pipette, transfer the cell solution to a centrifuge tube and rinse the flask with 5 mL EGM-2 full medium and add the rinse to the centrifuge tube. (The number of cells left in flask should be <5%)

11) Centrifuge cells at 1200rpm for 5 minutes.

12) Aspirate the supernatant with a 25 mL pipette, except for the last 1-2 mL.

13) Aspirate the late 1-2 mL of supernatant with a 1 mL pipette, gently and carefully so as to keep the cell pellet undisturbed.

14) Resuspend the cells in 1 mL of EGM-2 medium.

15) Prepare new culture T-75 flasks with 10 mL of warm medium in each of them.

16) Transfer needed amount of the cell suspension to each of the T-75 flasks.

17) Gently rock each culture flask to evenly distribute the cells. Incubate at 37°C, 5% CO₂, humidity.

**B-2-4: Maintenance**

1) After seeding for 24 hours, examine the cells under the microscope. Most cells should have attached to the culture flask, in single or in small colonies.

2) Change the culture medium to remove residual DMSO, trypsin and non-attached cells.
3) Change the culture medium every other day thereafter.

**B-2-5: Cryopreserving HUVECs**

1) When cells reach 80% confluent, they can be harvested and froze (~2 million cells per T-75 flask). Prepare 5 mL cryopreservation medium: 4mL growth media, 0.5 mL DMSO and 0.5 mL FBS.

2) Harvest cells following steps 2-10 under “B-2-3: Subculturing” and centrifuge to collect cells into a pellet: 1200 rpm for 5 min, at room temperature.

3) Resuspend cells in 1 mL of cold cryopreservation medium. The cell concentration should be around 2 million cells per mL.

4) Pipet 1 mL of cell solution into chilled sterile cryovials, keeping them on ice.

5) Transfer the vials to a -70°C freezer or place in liquid Nitrogen for long-term storage (-200°C).
B-2-6: Images of HUVEC under the light optical microscope

Figure B - 2: Light optical microscopy of HUVEC cells after 2 days of culturing (Passage 4)

Figure B - 3: Light optical microscopy of HUVEC cells after 5 days of culturing (Passage 4)
Appendix B-3: BCECF-AM marker optimization and the investigation of BCECF-AM as an effective marker for the U937

In order to quantitatively determine the number of U937s in this study (for cytotoxic testing of OC and the adhesion studies), the U937s were labelled with a 2, 7, bis (2 carboxyethyl) 5 (6) carboxyfluorescein acethoxymethyl ester (BCECF/AM) fluorescent marker. BCECF-AM is a fluorescent marker that is commonly used for cell viability and proliferation studies \(^1,2\). It is fast, accurate, not labour-intensive and does not require use of radioisotopes \(^3\) or toxic compounds \(^1,4\). These markers come in a non-polar form which is not fluorescent. They can passively cross the membranes of the living cells, and are converted by non-specific cytoplasmic esterases to a membrane impermeable polar form (with 4-6 negative charges/molecule), which will get trapped inside the cells [from Analytix 2.2000 (analytical newsletter published by Fluka, Sigma-Aldrich Co., Milwaukee, WI)]. The markers that have been cleaved give a fluorescent signal, making the quantification of living cells with a fluorescence well-plate reader or a confocal microscope possible.

The objective of the following tests was to find the conditions under which BCECF-AM will perform as an efficient marker for U937 cells. The two parameters that were examined were the marker concentration and incubation time. The ranges tested for these parameters were selected to cover the ranges that have been shown to work in the literature. \(^1,4-6\)

**Experimental procedures**

1) Warm RPMI-1640 incomplete medium.

2) Prepare culture ware, 6- well culture plate and pipettes.
3) Centrifuge one flask of U937 cells (70%-80% confluent) at 1500 rpm, for 5 minutes. Resuspend the pellet in ~6 mL fresh RPMI medium (or approximately 1.5 million cells/mL). Add 1 mL of cells into 4 of the wells of the 6-well culture plate.

4) Thaw BCECF-AM (2, 7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyleneester, 1 mg/mL, Sigma-Aldrich Co., Milwaukee, WI), in a 37°C water bath. (BCECF-AM is light sensitive. Minimize light exposure!)

5) Dilute BCECF-AM into different concentrations using warmed RPMI medium and add the marker solutions into the wells that have cells, with the final BCECF-AM concentration in each well being 0, 5, 10, 19 μM respectively, for the 4 wells.

6) Put the plate in a 37°C, 5% CO₂ environment for 30 minutes. Set up 3 other identical plates beside this plate and incubate them for 45, 55 and 65 minutes respectively.

7) Once the incubation time period is up, remove each well’s content into a centrifuge tube and centrifuge the labelled cells.

8) After aspirating the supernatant, wash the cells three times with 1% FBS in phosphate buffered saline (PBS, Cambrex Bio Science, Walkersville, MD) to remove excess dye.

9) Resuspend the cells in 1 mL RPMI-1640 medium and use the trypan blue method (see Appendix B-4) to determine the cell concentration. Add the labelled cells of different BCECF and cell concentrations into different wells in a 96-well plate, in triplicates.

10) Measure the fluorescence intensity of the marker with a fluorescence microplate reader set at excitation and emission wavelengths of 485nm and 535nm, respectively.

**Experimental results**

Trypan blue stains showed that cell survival was > 95% for all concentrations of
BCECF-AM. The 0 µM BCECF-AM samples acted as controls, and showed that there was no background fluorescence from the U937 and the media (Figure B-4). Fluorescent marker tests demonstrated that BCECF/AM was an efficient marker for U937s at concentration of 5 to 19µM at 30 minutes. The fluorescence count is directly proportional to live cell number at all these concentrations. The BCECF-AM concentration that was later chosen for oxaceprol cytotoxicity and U937-HUVEC adhesion studies was 10 µM. The effect of BCECF-AM incubation time is shown in Figure B-5. 30 minutes of incubation was sufficient to show a linear relationship between fluorescent count and cell number ($R^2 > 0.99$), and was therefore chosen as the incubation time for all the experiments.

Figure B-4: Effect of marker concentration. U937s were incubated with BCECF-AM for 30 minutes. Fluorescence count was plotted against the number of U937s in each well, n = 3. Data are plotted with standard deviation.
Figure B-5: Effect of incubation time. U937s were incubated with 10 μM BCECF-AM for 30 to 65 minutes. Fluorescence count was plotted against the number of U937s in each well, n = 3. Data are plotted with standard deviation.
Appendix B-4: Trypan blue assay & standard curves

Trypan Blue is a dye that is used to access the viability of cells. Trypan blue dye stains the cells that are damaged and dead, leaving the viable cells unstained. The reactivity of this dye is based on the negatively charged chromophore that does not interact with cells unless the cell membrane is damaged.7,8

Procedure:
1) Cells are diluted to a desired concentration
2) Take 0.1ml of the cell solution and mix thoroughly with 0.1ml of trypan blue dye (0.4%).
3) Pipet 10 μL of this mixture under a glass cover slip on a hemocytometer for cell counting
4) Under a microscope, live (unstained) and dead (stained) cells in each of the quadrants were counted, and averaged.
5) Cell suspension concentration is determined by the following equation:
   \[\text{Cell Concentration (cells/mL)} = \text{average # of cells in each quadrant} \times 2 \times 10^4,\]
   where 2 is the dilution factor (cell suspension : Trypan blue stain = 1:1) and 10^4 is the conversion factor for the hemocytometer.
**Figure B-6**: A hemocytometer

**Figure B-7**: Under an optical microscope, live ( unstained) and dead (stained) cells in the grids can be counted.
REFERENCE


