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DEVELOPMENT OF NOVEL BIODEGRADABLE ANTIMICROBIAL POLYMERS FOR BIOMATERIALS APPLICATIONS

by

Gregory Lu-Yuen Woo

A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Graduate Department of Chemical Engineering and Applied Chemistry
University of Toronto

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ABSTRACT

Bacterial infection is a frequent complication associated with the use of medical devices. Antimicrobial agents have been incorporated into or applied directly onto the surfaces of devices to combat infection. This thesis will assess the feasibility of using a novel biodegradable polymer to release antibiotic drugs in response to inflammatory related enzymes. A series of biodegradable drug polymers were synthesized using diisocyanates, oligomeric compounds, and an antibiotic, ciprofloxacin. The drug polymers were characterized by gel permeation chromatography (GPC), and elemental analysis. Biodegradation studies were carried out by incubating the polymers with solutions of cholesterol esterase (CE). Degradation was assessed by high performance liquid chromatography (HPLC), mass spectrometry (MS) and radiolabel release. Subsequently, the activity of the released antibiotic was assessed against a clinical isolate of Pseudomonas aeruginosa. For one of the polymer formulations, the release of ciprofloxacin was approximately two-fold higher in the presence of enzyme as compared to buffer alone. Another formulation using a different diisocyanate showed the release of multiple degradation products. The results of this study suggest that these novel antimicrobial polymers or similar analogs show good potential for use in the control of medical device associated infections.
ACKNOWLEDGEMENTS

A number of people contributed to the completion of this thesis, and I would like to express my gratitude for their assistance. Firstly, I would like thank my research supervisor, Dr. Paul Santerre, for his guidance and support throughout the research and preparation of this thesis. I would also like to thank Dr. Marc Mittelman at the Toronto Hospital for lending his knowledge and suggestions to my research, and I also acknowledge Selva Sinnadurai and the other members of the lab at the Toronto Hospital for their assistance in introducing me to microbiology. I thank Doreen Wen and the members of the Carbohydrate Research Laboratory at the Faculty of Medicine for the mass spectrometry analysis. I would also like to thank Meilin Yang for providing synthesizing additional polymers and Fayaaz Jaffer for the enzyme stability tests.

I would like to thank all the students and residents of the Biomaterials Lab at the Faculty of Dentistry for providing a relaxing and fun work environment. In particular, I would like to thank Leylanaz Shajii, Ali Jahangir, Vijendra Sahi, Vivek Patel, Jeannette Ho, Dr. Frank Wang, and Fayaaz Jaffer.

I would also like to thank my family for their support and assistance throughout the years.

This work was financially supported by Bayer Healthcare, the Medical Research Council PMAC program, and the University of Toronto Open Fellowship.
LIST OF ABBREVIATIONS

CE  cholesterol esterase
CFU  colony forming units
DDI  1,12 diisocyanatododecane
DMSO dimethylsulphoxide
dimethylacetamide
DMAC  dimethylformamide
DMF  Guelph Chemical Laboratories
GPC  gel permeation chromatography
HDI  1,6 hexane diisocyanate
HPLC  high performance liquid chromatography
LiBr  Lithium bromide
\( M_n \)  number average molecular weight
\( M_w \)  weight average molecular weight
MBC  minimum bactericidal concentration
MIC  minimum inhibitory concentration
MS/MS mass spectrometry/mass spectrometry
m/z mass-to-charge ratio
PCL  polycaprolactone diol
PDA  photodiode array
PBS  phosphate buffered saline
PPE  poly(oxyethylene) diamine
TEA  triethylamine
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1.0 INTRODUCTION

1.1 Medical Device Related Infection

The use of biomaterial implants has become of great value by both saving and improving the quality of lives. Progress in materials engineering and the aging population have led to annual increases of 7 to 15% in device implantations in North America alone (Mittleman, 1996). Biomaterial device applications range from contact lenses and dental fillings, to more invasive devices such as artificial hips, catheters, vascular grafts, and heart valves. Table 1.1 provides estimates of the number of biomaterial devices implanted each year. Each of these devices were engineered to be biocompatible, i.e., to produce an appropriate host response. For instance, vascular grafts must be non-thrombogenic, contact lenses must allow oxygen diffusion, orthopaedic implants must be able to withstand repeated loading. In the majority of cases, biomaterial implants have been successful in achieving the performance for which they were designed.

Table 1.1 Frequency of Medical Device Implantation in North America (Mittleman 1996)

<table>
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<tr>
<th>Device</th>
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<tr>
<td>Urinary catheters and stents</td>
<td>100 000 000</td>
</tr>
<tr>
<td>Breast prosthesis</td>
<td>100 000</td>
</tr>
<tr>
<td>Hip and knee implants</td>
<td>150 000</td>
</tr>
<tr>
<td>Dental implants</td>
<td>20 000</td>
</tr>
<tr>
<td>Intraocular lenses</td>
<td>1 500 000</td>
</tr>
<tr>
<td>Contact lenses</td>
<td>18 000 000</td>
</tr>
<tr>
<td>Vascular grafts</td>
<td>350 000</td>
</tr>
<tr>
<td>Pacemakers</td>
<td>130 000</td>
</tr>
<tr>
<td>Peritoneal dialysis catheters</td>
<td>40 000</td>
</tr>
<tr>
<td>Heart valves</td>
<td>75 000</td>
</tr>
<tr>
<td>Vascular catheters and stents</td>
<td>150 000</td>
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</table>
Despite this success, one of the major barriers to the extended use of medical devices is biomaterial associated infection (Dankert et al. 1986; Dougherty et al., 1982; Gristina and Naylor, 1996; Mittelman, 1996). The rates of infection vary according to the type of implant and clinical study. Some rates that have been quoted in the literature include 1%-4% for orthopaedic implants (Gristina et al., 1993; Chang and Merritt, 1991), 1 to 6% for vascular grafts (O’Brien et al. 1992), and up to 100% for total artificial hearts (Gristina et al., 1988; DeVries, 1988) and certain urinary catheters (Nickel, 1993; Stamm, 1991). Although some of these rates may seem acceptable, the consequences of biomaterial associated infections are severe. For instance, vascular grafts which become infected have been reported to result in death or amputation in 50% of the cases (Elliot and Faroqui, 1993; Gristina et al., 1985). The sheer number of devices implanted annually in North America (Table 1.1) and the rest of the world make the cost of this problem very high.

1.2 Bacterial Adhesion and Biofilms

When an inert substratum (biomaterial or damaged tissue) is introduced into the biological environment, it may become a preferred site for bacterial adhesion (Gristina, 1987). Studies have shown that the presence of a biomaterial increases the risk of infection by at least a factor of four (Christensen et al., 1989). The natural ability of bacteria to adhere to surfaces, which is seen in other environments (such as marine and industrial ecosystems) is likely a principal factor (Mittelman, 1996). The process of bacterial adhesion is complex, and is influenced by many factors, including properties of the bacteria, the surface properties of the biomaterial, and the surrounding environment (Gristina et al., 1991). For instance, a biomaterial which has been implanted rapidly becomes coated with a conditioning film, which is composed of protein and polysaccharide components of blood, urine, saliva, or other secretions (Gristina 1987; Mittelman 1996). These coatings may provide specific receptor sites which bacterial cells can recognize and adhere to. However, the adherence of microorganisms is merely the first step in a sequence of events which leads to the development of an infection. Once the bacteria adhere, they may excrete extracellular polymeric substances (EPS), which, together with the bacteria, form what is known as a
The biofilm has been implicated as a major factor responsible for the difficulty in treating biomaterial associated infections. The EPS assists in bacterial adhesion, provides nutrients, and acts as a protective barrier. Biofilm encased bacteria are several times more resistant to antimicrobial agents than planktonic (free-floating) bacteria (Reid et al., 1993; Khoury et al., 1993). In many cases, biomaterial infections cannot be eradicated unless the implant is removed (Gristina et al., 1991).

The integration of tissue with the surfaces of biomaterials is a requirement for the biocompatibility of certain medical devices, and problems with the tissue-material interaction may ultimately be responsible for the failure of the biomaterial (Anderson, 1988). The tissue-biomaterial interaction has been recognized to be a similar phenomenon as the bacteria-biomaterial interaction. Gristina (1987) suggested that the fate of an implanted biomaterial is part of a "race for the surface" between macromolecules, tissue cells, and bacteria (Figure 1.1). The introduction of a biomaterial causes tissue injury and disrupts the local host defence mechanism. Since the surfaces of synthetic biomaterials are relatively acellular and inanimate, microorganisms can easily adhere and form biofilms, which can damage tissue while resisting antibodies and host defence mechanisms. However, if tissue cells arrive first and integrate with the surface, they provide a living, cellular barrier which can resist bacterial colonization through the usual host defense mechanisms. Since tissue cells have not been programmed by nature to adhere to synthetic materials, the enhancement of the wound healing process may provide a key to reducing the incidence of biomaterial associated infection.

Figure 1.1 The colonization of a biomaterial surface by tissue cells, bacteria, and macromolecules
Currently, a number of strategies are used to treat biomaterial associated infections, which include the use of topical or systemic antibiotics. Systemic antibiotics administered prior to implantations are effective since they act on bacteria in solution, before the bacteria have a chance to form resistant, adhesive colonies (Gristina et al., 1991). However, the more widespread use of antibiotics may lead to increased prevalence of antimicrobial resistance (Dankert et al., 1986). Additionally, post operative use of antibiotics is complicated by the incidence of infections caused by hematogenous (through the bloodstream) spread of bacteria from a secondary site, such as a dental procedure (An et al., 1996).

The use of direct antimicrobial or antibiotic coatings on medical devices such as catheters has been investigated, and in some cases, already being tested in clinical trials (Raad et al., 1993). This approach has the benefit of a localized delivery, thus minimizing systemic effects. Higher concentrations are also possible which may help treat biofilm laden bacteria. However, problems with these devices include the rapid release of antibiotic (Trooskin et al., 1985, Sherertz et al., 1989, Jansen and Peters, 1991) and concerns of antibiotic resistance (Bach et al., 1994; Raad et al., 1995). Some clinical trials have also failed to show effectiveness in reducing the incidence of infections (Riley et al., 1995; Ciresi et al., 1996).

A significant advancement to the ability of providing antimicrobial coatings onto biomaterials was the application of polymer drug delivery systems. Currently, a number of these systems are under investigation, including liposomes (Hope and Wong, 1995), bioerodible polymers (Heller, 1988), chemical and physical stimuli responsive polymers (Heller, 1996), and polymer drugs (Ottenbrite, 1991). The use of a drug delivery system would allow for a more extended, controlled release of antibiotic, and also the potential for designing a control system which could modulate the rate of release.

Recently, the modification of biomaterial surfaces to elicit a biological response has been suggested as a means of the design of new biomaterials (Ratner, 1993). With respect to infection, the ideal surface would be nonadhesive toward bacteria, while being adhesive toward tissue cells. Some proposed ideas to reduce adhesion include the use of heparin,
(Paulsson et al., 1994), polyethylene oxide (Desai et al., 1992), or plasma modified surfaces (Jansen and Kohnen, 1995; Mittelman, 1996b). However, since bacterial adhesion is such a complex process, it may not be possible to completely prevent bacteria from adhering to the surface of the biomaterial.

1.3 Research Objectives

From the above discussion, a need exists for an effective method to reduce the incidence of biomaterial associated infections. A proposed solution to this problem is to develop a novel biodegradable, polymer drug which can sustain delivery of effective concentrations of antibiotics to target tissues. This polymer, with an antibiotic synthesized into the backbone of the macromolecule, could be used as a raw material in the construction of a biomedical device, or could be used as a coating for an existing device.

The design of this polymer is based upon the knowledge that certain polyurethane elastomers (polyester urethanes) are susceptible to degradation by a number of inflammatory-cell derived enzymes (Santerre et al., 1994). A polymer drug, synthesized with polyurethane chemistry and with an antibiotic as a repeat unit in the backbone of the polymer, could release antibiotic during the degradation of the polymer. It is hypothesized that the release of antibiotic in a drug polymer of this type could be self-regulating, by responding to a biological stimulus. Since the implantation of a biomaterial causes tissue injury to the local area, the inflammatory response would be triggered. A product of this response is the presence of inflammatory cells, which can release a number of lysosomal enzymes, in an attempt to degrade the foreign body. Since the polymer drug would be degraded by these enzymes, the release of antibiotic would occur and be proportional to the magnitude of the enzyme release. As previously mentioned, this initial period immediately after implantation is critical, when the biomaterial is most susceptible to bacterial adhesion. Thus the local implant area would be protected from bacteria during the wound healing process, which may allow the tissue cells to integrate with the biomaterial. Once the wound healing has progressed, the release of enzymes would decrease, turning off delivery of the antibiotic.
The scope of this thesis was to demonstrate the feasibility of synthesizing such a drug delivery system. Various polymer drug formulations were produced using a model antibiotic, ciprofloxacin, with 1,6-hexane diisocyanate, 1,12-diisocyanatododecane, polycaprolactone diol, and a poly(oxyethylene) diamine. Preliminary polymer characterization was carried out by measuring the molecular weights of the polymer by gel permeation chromatography. The polymers were tested in a simulated physiological environment, which contained a model inflammatory-cell derived enzyme, cholesterol esterase. The release of ciprofloxacin and degradation products into the incubation solutions was monitored by high performance liquid chromatography. In order to confirm the structure of the drug and polymer derived products, ion-spray mass spectrometry was used. Finally, the biological activity of the released drug was tested using the broth dilution method to assay antimicrobial activity against a clinical isolate of *Pseudomonas aeruginosa*. 
2.0 REVIEW OF LITERATURE

2.1 Polyurethane Chemistry

Polyurethanes are a family of block copolymers which have been used extensively as biomaterials over the past few decades, due to their excellent physical and mechanical properties, relatively good blood compatibility, and hydrolytic stability (Lelah and Cooper, 1986; Szycher, 1991). Polyurethanes are composed of alternating blocks of soft and hard segment units linked together by a urethane or urea group. Although polyurethanes may be composed of many different components, traditional elastomers are composed of a long chain polyol (soft segment), typically a polyester or polyether, a diisocyanate, and a chain extender consisting of a short chain diol, water, or diamine. The polymer is formed via reaction of the diisocyanate with an amine or alcohol functional group. Figure 2.1 is a schematic representation of a typical polyurethane.

![Figure 2.1 Structure of a segmented polyurethane (Lamba et al., 1998).](image)

2.1.1 Isocyanates

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:

\[
\text{R-} \overset{\text{N}}{\overset{-}{\text{C=O}}} \longleftrightarrow \text{R-} \overset{\text{N}}{\overset{\text{=\text{C-}}}{\overset{-}{\text{O}}}} \longleftrightarrow \text{R-} \overset{\text{N}}{\overset{\text{=\text{C-}}}{\overset{+}{\text{O}}} \rightleftharpoons (2.1)}
\]
The presence of these various states allows for several classes of reactions. Important in polyurethane synthesis are reactions involving functional groups with active hydrogens (Saunders and Frisch, 1965). Since the resonance structures indicate that the highest net positive charge is on the carbon atom, this centre becomes susceptible to nucleophilic attack. The rate of reaction depends on the type of structure associated with both the nucleophile and isocyanate. The presence of electron-withdrawing substituents on the nucleophile will draw electrons away from the active hydrogen group and hinder its reactivity with the isocyanate. For example, these compounds have been observed to have the following order of reactivity (Wright, 1969):

\[
\text{CH}_3\text{NH}_2 > \text{C}_6\text{H}_5\text{NH}_2 > \text{CH}_2\text{OH} > \text{C}_6\text{H}_5\text{OH} > \text{CH}_3\text{SH} > \text{N} \quad (2.2)
\]

In general, the more basic the nucleophile, the greater its reactivity (Saunders and Frisch 1965). Substituents on the isocyanate molecule will have a similar, but opposite effect than substituents on the nucleophile. Electrophilic groups adjacent to the isocyanate will draw electrons from the carbon, making it more positive and more susceptible to nucleophilic attack. The increase in activity with various substituents is as follows (Wright, 1969):

\[
\text{tert.-butyl} < \text{cyclohexyl} < \text{n-alkyl} < \text{benzyl} < \text{phenyl} < \text{p-nitrophenyl} \quad (2.3)
\]

In addition, stereochemical factors also play an important role. Ortho substituents in aromatic compounds, branching in aliphatic compounds, or bulky substituents close to the reactive site will retard reaction rates. These steric factors not only affect the reactivity of isocyanate and nucleophile, but may also hinder the effectiveness of any catalysts used.
2.1.2 Principal Isocyanate Reactions

The reaction of an isocyanate with an alcohol is the most important reaction in the formation of urethane polymers. The mechanism is as follows (Lelah and Cooper, 1986):

\[
\begin{align*}
&\text{R} - \text{N} = \text{C} = \text{O} \quad \text{H}_2\text{O} \\
&\text{H} - \text{O} \\
&\text{R} - \text{N} - \text{C} - \text{O} - \text{R}' \\
\end{align*}
\]

(2.4)

The ensuing bond is called a carbamate ester linkage, commonly known as a urethane bond. The reaction is catalyzed by a variety of compounds, the most common being tertiary amines and organometallic compounds (Wirpsza, 1993). The effect of steric hindrance is pronounced with alcohols, so while primary alcohols react readily at 25-50°C, secondary and tertiary alcohols react much more slowly (Saunders and Frisch, 1965).

The reaction of water is a special case of the isocyanate/hydroxyl reaction, and produces an unstable intermediate which breaks down into an amine and a carbon dioxide. In some cases, water is a desirable component in the synthesis, as in the production of foams, but in other cases may act as an impurity, reducing the molecular weight and mechanical properties of the polymer.

\[
\begin{align*}
&\text{R} - \text{NCO} + \text{H}_2\text{O} \\
&\text{R} - \text{N} - \text{C} - \text{OH} \\
&\text{R} - \text{NH}_2 + \text{CO}_2 \\
\end{align*}
\]

(2.5)

Amines can also react with isocyanates to form urea linkages:

\[
\begin{align*}
&\text{R} - \text{N} = \text{C} = \text{O} + \text{R'} - \text{NH}_2 \\
&\text{H} - \text{O} - \text{H} \\
&\text{R} - \text{N} - \text{C} - \text{N} - \text{R'} \\
\end{align*}
\]

(2.6)
This reaction proceeds faster than the hydroxyl reaction. The more basic amines react faster, unless steric hindrance is excessive (Saunders and Frisch, 1965). Primary aliphatic amines are extremely reactive, even at 0-25°C. Secondary aliphatic and primary aromatic amines react similarly, but less readily than primary aliphatic amines. Polymers which contain both urethane and urea linkages are sometimes referred to as polyurethane ureas (Lelah and Cooper, 1986).

The urethane and urea linkages which are formed during the reaction of isocyanates with alcohols and amines can themselves react with isocyanates. The formation of these products, allophonates and biurets, respectively, result in the chemical crosslinking of the polymer chains. The urethane reacts to form allophonates, requiring elevated temperatures of approximately 120°-140°C in uncatalyzed systems (Saunders and Frisch, 1965):

\[
R-N=C=O + R'-N-C-N-R'' \rightarrow R-N-C-N-C-O-R'' \quad (2.7)
\]

Ureas react with isocyanates to form biurets at temperatures greater than 100°C in uncatalyzed systems (Saunders and Frisch, 1965):

\[
R-N=C=O + R'-N-C-N-R'' \rightarrow R-N-C-N-C-N-R'' \quad (2.8)
\]

Other types of side reactions can produce dimers (uretidine diones) and trimers (isocyanurates).
Carboxylic acids, possessing a hydroxyl group, react readily with isocyanates, but less readily than primary alcohols and water (Saunders and Frisch, 1965). Aliphatic isocyanates and aliphatic acids give anhydrides which decompose to amides:

\[
RNCO + R'COOH \rightarrow \left[ \begin{array}{c}
O \\
RNHC\cdots O \cdots CR'
\end{array} \right] \rightarrow RNHCOR' + CO_2 \quad (2.9)
\]

The initial addition product is dependent of the structure of the isocyanate, acid, and reaction conditions. For example, the reaction of aromatic isocyanates and aliphatic acids would yield anhydrides and eventually acid anhydride, urea, and carbon dioxide (Saunders and Frisch, 1965):

\[
\text{ArNCO} + R'\text{COOH} \rightarrow \left[ \begin{array}{c}
O \\
\text{ArNHC}\cdots O \cdots CR'
\end{array} \right] \rightarrow \left[ \begin{array}{c}
O \\
\text{ArNHC}\cdots O \cdots \text{CNHAr}
\end{array} \right] + R'C\cdots O\cdots CR' \quad (2.10)
\]

\[
\left[ \begin{array}{c}
\text{ArNHCONHAr}
\end{array} \right] + CO_2
\]

2.1.3 Reagents

The most common diisocyanates used in polyurethanes are 2,4 toluene diisocyanate (TDI) and methylene di-\textit{p}-phenyl-4,4'-diisocyanate (MDI) (Figure 2.2). Since polymers produced using aromatic isocyanates are somewhat light sensitive, aliphatic isocyanates have also found wide use. These isocyanates are characterized by a lower reactivity, but improved light stability and hydrolytic and thermal resistance. However, this comes at the expense of weaker mechanical properties.
2,4 toluene diisocyanate (2,4 TDI)  methylene bis(p-phenyl diisocyanate) (MDI)

OCN—(CH₂)₆—NCO  OCN—CH₂—CH₂—O—NCO

1,6 hexane diisocyanate (HDI)  methylene bis(p-cyclohexyl isocyanate) (H₁₂MDI)

**Figure 2.2** Some commonly used diisocyanates.

The choice of soft segment is critical in determining the ultimate physical and biological properties of biomedical polyurethanes (Szycher, 1991). Soft segments typically used include hydroxyl-terminated polyesters, polyethers, hydrocarbon polymers, or polydimethyl siloxanes. Some commonly used ones are shown in Figure 2.3.

**Figure 2.3** Some commonly used soft segments.

Polyester urethanes possess good mechanical properties, but are susceptible to hydrolytic cleavage (Pinchuk, 1994; Santerre et al., 1993). Polyethylene oxide based polyurethanes possess poor water resistance, due to the hydrophilic nature of the polyl, while
polypropylene oxide based materials are soft (Lelah and Cooper, 1986). Polytetramethylene oxides produce polyurethanes with good physical properties, including good hydrolytic stability and water resistance (Lelah and Cooper, 1986). However, they have recently been shown to be easily oxidized (Meij et al. 1993; Sutherland et al., 1993). Polycarbonates have been reported to be more resistant to oxidative degradation than the polyethers, however their susceptibility to hydrolytic degradation is in question (Tang et al., 1998). Other soft segments are being used in new applications, including amino acids (Pkhakadze et al., 1996), and natural polymers (Saad et al., 1997).

Chain extenders serve to increase the hard segment size of polyurethanes, and are sometimes associated with improving the mechanical properties of the polymer. Some classical chain extenders include ethylene glycol, 1,4 butane diol, hexane diol, and ethylene diamine. Recent studies have reported the use of functionalized chain extenders and end-capping agents, possessing sulphonated (Santerre and Brash, 1991) and fluorinated groups (Tang et al., 1996; Yoon and Ratner, 1986).

2.1.4 Synthesis of Polyurethanes

The reaction conditions may affect structure, physical properties, molecular weight, and processability of polyurethanes. As previously mentioned, isocyanates can react with the urethane and urea linkages produced during the reaction, and with contaminants such as water or oligomeric hydroxyls. The relative rate at which each of these species reacts can be controlled by the manipulation of variables such as temperature, stirring, the use of catalysts or inhibitors, isocyanate chemistry, solvents, and the sequence of monomer addition.

The most common method applied in laboratory research for the synthesis of biomedical polyurethanes is the two step or prepolymer method. In the first step, an excess of diisocyanate is reacted with the polylol to produce an isocyanate terminated oligomer of molecular weight 1000-5000. The prepolymer is converted into a high molecular weight polymer by reaction with a diol or diamine chain extender. Alternately, one-step reactions

13
may be employed where all reagents are mixed together simultaneously. Syntheses by two-step methods are preferred due to greater control of reaction chemistry (Lamba et al., 1998). In addition, two-step reactions typically produce narrower molecular weight distributions than single-step polymerization (Lelah and Cooper, 1986).

Most laboratory syntheses are carried out in solution. The choice of solvent may affect the rate of the uncatalyzed reaction, as well as the effectiveness of any catalyst used (Lelah and Cooper, 1986). For example, solvents which complex with the active hydrogen compound or the catalyst will result in a slower reaction than a solvent which does not associate. Commonly used solvents include N,N-dimethylacetamide (DMAC), dimethylformamide (DMF), tetrahydrofuran (THF), and dimethylsulphoxide (DMSO) (Gogolewski, 1989). Commercially produced polymers typically use solvent-free, bulk polymerization techniques.

The removal of impurities present in the monomers is important, due to the highly reactive nature of the isocyanates. Oligomeric hydroxyls and other contaminants present in reagents may lead to the lowering of molecular weights. As well, water is a common contaminant that should be eliminated. Reagents can be purified by distillation or degassing, and moisture can be avoided by conducting reactions in a dry, inert gas.

Reaction temperature can influence the balance between polymer chain growth and crosslink formation (Lamba et al., 1998). At relatively low temperatures of up to 50°C, reactions of diisocyanates with amines and alcohols yield urethanes and ureas. At higher temperatures, allophanates and biuret formation begin to become important. In order to prevent crosslinking and produce linear polyurethanes, polymerization should be conducted at temperatures below 80°C (Lamba et al., 1998).

### 2.2 Biodegradable Polymers

Biodegradable or bioerodible polymers are materials which readily breakdown and are metabolized in the biological environment. A biomaterial implant composed of such a
material is of considerable value in many applications, since the surgical removal of the device is unnecessary. The first major clinical application was the degradable suture, introduced in the 1970s (Frazza and Schmitt, 1971). Since then, many other applications have been explored (some of which are commercially available), including drug delivery devices (Chasin, 1995; Chasin and Langer, 1990; Heller, 1988), fracture fixation devices (Gogolewski, 1992), tissue engineering (Langer, 1993), adhesion barriers (Chowdhury et al., 1996) and temporary vascular grafts and stents (Kohn and Langer, 1996). The design of degradable polymers is considerably more challenging than biostable polymers. The degradation and erosion rate must match the requirements of the process (such as drug release), and must also be controlled to ensure that the mechanical stability of the device is maintained for the appropriate lifetime of the device. In terms of biocompatibility, biodegradable polymers have to satisfy more stringent requirements than nondegradable materials. In addition to the possibility of toxic contaminants leaching from the polymer (residual monomers, stabilizers), the toxicity of released degradation products and metabolites must also be considered (Kohn and Langer, 1996). The difficulty in meeting these requirements has limited the number of degradable polymers in current biomedical use (Kimura, 1993). Figure 2.4 represents some examples of polymers currently being used or investigated. A number of papers have reviewed the use of these polymers for biomedical applications (Engelberg and Kohn, 1991; Piskin, 1995).
Figure 2.4  Chemical structures of various degradable polymers.

A number of terms are found in the literature which describe the breakdown of a polymer in vivo. In this thesis, the term biodegradation will be used when a biological agent (enzyme or microbe) plays a significant role in the degradation process (Williams, 1987). Bioerosion refers to the process by which a water-insoluble polymer is converted to water soluble materials under physiological conditions (Heller, 1987). The degradation of polymers in vivo is a complex process which is not entirely understood. In the literature, there is a consensus that hydrolysis is a dominant mechanism that governs degradation (Goperich, 1996; Coury, 1996; Williams, 1992). However, there are clearly other processes, as some materials have different degradation rates depending upon implantation conditions (Williams, 1992). Other
processes which have been implicated include oxidation, mineralization, and mechanical loading (Stokes, 1993).

2.2.1 Hydrolytic Degradation

Hydrolysis is the scission of chemical functional groups by reaction with water. Essentially, this may be considered the "reverse of polycondensation" (Smith et al., 1987b). Hydrolysis may be catalyzed by acids, bases, salts, or enzymes (Williams, 1982). The main factor governing the rate of hydrolysis is the type of bond in the polymer backbone (Gopferich, 1996). Heterochain polymers containing oxygen or nitrogen are generally susceptible to hydrolysis. Some linkage types include ester, amide, urethane, anhydride, and orthoester. Several classifications for ranking the reactivity exist, which are based on hydrolysis of polymers (Gopferich, 1996), or extrapolated from the hydrolysis of low molecular weight compounds. However, the relative reactivity may change drastically with catalysis or the presence of substituent groups (Baker, 1987). For example, aromatic polyesters such as poly(ethylene terephthalate) are hydrolytically stable under physiological conditions, while aliphatic polyesters such as poly(glycolic acid) degrade rapidly in vivo (Williams, 1982).

In addition to chemical structure, several other factors influence the rate of hydrolysis, including polymer morphology, dimensions, hydrophilicity, and the surrounding environment (Coury, 1996). For example, poly (glycolic acid) erodes faster than the more hydrophobic poly(lactic acid), although the ester bonds have about the same chemical reactivity toward water (Kohn and Langer, 1996). The rate of erosion of polyanhydrides composed of hydrophobic bis(carboxy phenoxy) propane and hydrophilic sebacic acid (SA) can be increased by increasing the SA content (Tamada and Langer, 1992). Polycarbonates and polyacetals are relatively stable at physiological pH, in part due to their low water absorption (Stokes, 1993). Highly hydrophobic polymers such as silicone and poly(tetrafluoroethylene) are very resistant to hydrolysis (Gopferich, 1996).
Polymer morphology can also have an effect on hydrolysis. Polymers may be classified as crystalline, amorphous-glassy, and amorphous rubbery. In crystalline form, polymer chains pack more densely, thus inhibiting water penetration. Thus amorphous polymers exhibit higher rates of backbone hydrolysis (Gopferich, 1996). A good example is the hydrolysis rates of poly(l-lactic acid) versus poly(dl-lactic acid). These polymers have identical backbone structures, and identical degrees of hydrophobicity, yet poly(l-lactic acid) has been shown to degrade much slower than poly (dl-lactic acid) (Vert et al. 1981). This is due to the fact that poly (l-lactic acid) is semicrystalline, while poly(dl-lactic acid) is amorphous. For amorphous polymers, in the glassy state they are less permeable to water than in the rubbery state. This may be an important consideration when the polymer has a glass transition temperature not far above body temperature.

2.2.2 Biodegradation

Implantation of a polymeric material in the in vivo environment invokes an array of specific host responses, each which may contribute to biodegradation of polymers. Inflammation, wound healing, and the foreign body response are all components of the cellular host response to tissue injury. Figure 2.5 illustrates the sequence of events which occur. Fluid, proteins, and blood cells are released from the vascular system into the tissue in a process called exudation (Anderson, 1988). Following this, chemicals which are released from plasma, cells and injured tissue mediate the cellular response (Anderson, 1988). The cell types found during the inflammatory response varies depending on the age of the injury. Neutrophils usually predominate during the first few days following injury. The major role of these cells is to phagocytose microorganisms and foreign materials. Neutrophils are gradually replaced by monocytes, which in turn can differentiate into macrophages which can live for months. Monocyte emigration may continue for days to weeks, depending on the injury and implanted biomaterial. This period of monocyte activity is known as chronic inflammation.
Figure 2.5  The sequence of events following implantation of a biomaterial. (Reprinted with permission from Anderson, "Inflammation, Wound Healing, and the Foreign Body Response", in Biomaterials Science, B.D Ratner et al., eds., 167, Copyright 1997 by Academic Press).

Although implanted biomaterials are generally too large to be phagocytosed by neutrophils or macrophages, a phenomenon known as "frustrated phagocytosis" may occur (Anderson, 1993). In this process, the biomaterial is not engulfed, but instead extracellular release of leukocyte products occurs in an attempt to degrade the material. Neutrophils may release enzymes such as cathepsins, collagenase, elastase, and esterases (Labow et al., 1995). Salthouse (1976) showed that lysosomal hydrolases are most prominent during the subacute and chronic phases of the tissue response near the tissue biomaterial-interface. These enzymes may serve as catalysts in the hydrolysis of polymeric materials.

As well as being able to synthesize enzymes, macrophages and polymorphonuclear leukocytes (PMN) metabolize oxygen and may generate superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals, and hypochlorous acid (HOCl) (Coury, 1996). These substances can potentially oxidize synthetic polymers, and have been implicated in the degradation of biomaterials (Stokes, 1993; Anderson, 1996).
2.2.3 Degradation of Polyurethanes

Biomedical polyurethanes were originally intended to be used in applications requiring biostability. However, it is now known that many polyurethanes degrade *in vivo* (Lamba, 1998). Polyester urethanes have been found to be very susceptible to hydrolytic degradation (Griesser, 1991; Szycher, 1991). Poly(ether urethanes), on the other hand, are more resistant to hydrolysis, but are readily susceptible to oxidation (Gogolewski, 1989). The hydrolysis reactions of some of the bonds involved in polyurethane degradation are shown in Figure 2.6.

\[
R-C-O-R' + H_2O \rightarrow R-C-OH + HO-R'
\]

ester

\[
H \quad O
R-N-C-O-R' + H_2O \rightarrow R-NH_2 + R'-OH + CO_2
\]

urethane

\[
H \quad O \quad H
R-N-C-N-R' + H_2O \rightarrow R-NH_2 + R'-NH_2 + CO_2
\]

urea

Figure 2.6 Hydrolysis reaction of ester, urethane, and urea linkages.

The urethane and urea linkages are less susceptible to hydrolysis than the ester linkage (Lelah and Cooper, 1986). However, the rate of hydrolysis will depend on any catalysts present, and the hydrophilicity of the polymer (Lamba et al., 1998). The biodegradation of polyurethanes, involving enzymes and oxidants, has been well documented in the literature (Griesser, 1991; Lamba et al., 1998).

A number of investigators have shown enzymes can degrade polyurethanes *in vitro*. Many of these studies used papain (a protease) and trypsin (a hydrolase). Ratner et al. (1988) treated a
Pellethane 2363-80AE and other polyurethanes with papain, chymotrypsin, and a number of other enzymes. Degradation was detected by observing shifts in molecular weight by gel permeation chromatography. Bouvier et al. (1991) also observed the release of low molecular weight material from Pellethane 2363-80AE when treated with trypsin. Marchant et al. (1987) and Zhao et al. (1987) observed changes in surface chemistry and mechanical properties when poly(ether urethanes) were treated with the proteolytic enzyme papain over a period of one month. Similar results were shown by Phua et al. (1987), who incubated Biomer® with papain and urease. Polyurethanes with poly(ethylene oxide) soft segments were also found to be degraded by papain (Takahara et al. 1992).

Smith et al. (1987) studied polyurethane degradation by synthesizing 14C-labelled poly(ether urethanes) and exposing them to solutions of esterase, papain, and lysosomal liver enzymes. They found that polyurethanes incubated with esterase, cathepsin C, and a liver homogenate released higher concentrations of 14C labelled species than buffer controls. Santerre et al. (1993) also found polyurethane degradation was enzyme and material specific. 14C labelled poly(ester urethanes) were degraded over a 30 day period by cholesterol esterase, but not collagenase, cathepsin B and xanthine oxidase. In a second study, poly(ether urethanes) were also found to degraded by cholesterol esterase, but to a significantly lesser degree (Santerre, 1994). In other studies using radiolabelled tracers, elastase (Labow et al., 1995) and carboxyl esterase (Labow et al., 1994) were found to degrade polyester urethanes.

The mechanism of enzyme degradation appears to vary according to the enzyme and the structure of the polyurethane (Lamba et al., 1998; Greisser, 1991). Several studies have attempted to address this issue. It has been hypothesized that papain can hydrolyze urethane and urea linkages, while urease can only attack urea linkages (Phua et al., 1987). Takahara et al. (1992) also provided evidence of urethane bond dissociation by papain by using X-ray photoelectron spectroscopy (XPS). One hypothesis is that papain attacks the urethane and urea bonds at the interface between soft and hard regions as these linkages are more accessible (Zhao et al., 1987). The structure of the polyurethane has also been observed to affect degradation. For example, the molecular weight of the polyether soft segment has been
shown to affect degradation. Polymers with polyether soft segment molecular weights of 1000 were susceptible to papain and cathepsin C, while polyethers with molecular weights 650 and 2000 were unaffected (Smith et al., 1987). Hergenrother and Cooper (1993) showed the effect of crystallinity of the hard segment domains affects biodegradation. H₁₂MDI based polyurethanes, which do not form crystalline hard segment domains, showed greater degradation in vivo than MDI analogs. Santerre and Labow (1997) have shown a correlation between the size of the hard segment size and the degradation of polyether urethanes. They hypothesized that polyurethanes which formed hard segment micro-domains are able to shield the urethane and urea bonds from hydrolytic attack. Wang et al. (1997) have shown that the urethanes and ureas are relatively stable as compared to the ester bond in polyester urethanes degraded by cholesterol esterase.

The degradation of polyether urethanes has been attributed to oxidative mechanisms as opposed to hydrolysis (Lamba et al., 1998). Some mechanisms which have been identified include autooxidation (Stokes et al., 1987), environmental stress cracking (ESC) (Zhao et al., 1990), and metal ion induced oxidation (Stokes, 1990). Studies have shown a variety of phagocyte-derived oxidants can degrade polyether urethanes, including hydrogen peroxide (Meijs et al., 1993), hypochlorous acid, and nitric oxide (Sutherland et al., 1993). Oxidation appears to be caused by attack of the polyether soft segment (Stokes et al., 1987; 1990), at the α-methylene position (Zhao et al., 1993). Although oxidation has been shown to contribute to polyether urethane degradation in vitro, the in vivo mechanisms are complex and believed to be influenced by many factors. For example, degradation has been shown to not be caused directly by oxidative enzymes (Santerre et al., 1994; Sutherland et al., 1993). One mechanism that has been proposed begins with macrophage adhesion and activation, followed by the release of superoxide anion radicals and enzymes, which acidify the local environment (Anderson et al., 1992). The superoxides combine with protons to form hydroperoxide radicals, which subsequently attack the α-carbon of the polyether soft segment. The ether is oxidized to an ester linkage, which is not stable in the presence of esterases and acid, and is hydrolyzed to a carboxylic acid and alcohol.
Bacteria and fungi have also been found to degrade polyurethanes (Filip, 1979; Shuttleworth, 1986; Darby and Kaplan, 1968). Jansen et al. (1991b) found that survival rates of \textit{S. epidermidis} in the presence of synthetic polyurethanes were prolonged as compared to control experiments conducted in the absence of any nutrients. The bacteria were found to exhibit urease activity which was hypothesized to cause the degradation. Jayabalalan and Shunmugakumar (1994) designed polyurethanes resistant to fungal attack by incorporating crosslinks. Fungi have also been identified in the biodegradation of silicone (Busscher et al., 1997).

\subsection*{2.2.4 Bioerodible Polyurethanes}

Although polyurethane degradation is undesirable in many biomedical applications, some investigators have taken advantage of the fact that certain polyurethanes degrade \textit{in vivo}, and have developed designed-to-degrade polyurethanes. These polyurethanes incorporate degradable soft segments, such as those shown in Figure 2.4. These investigations are preliminary, as the majority of these studies do not present detailed \textit{in vitro} and \textit{in vivo} degradation data. Further studies on degradation rates, toxicology, and mechanical properties are needed before these materials can be used in biomedical applications.

A number of investigations have synthesized polyurethanes with poly(lactide), poly(glycolide) or poly(caprolactone) soft segments (Bruin et al., 1988; de Groot et al., 1997) Kobayashi et al. (1991) synthesized a water curable biodegradable polyurethane for use as a bioadhesive. Soft segments based on copolymers of D,L-lactide, D,L-glycolide-\(\varepsilon\)-caprolactone, and polyethylene glycol were synthesized with diisocyanates to form a water curable prepolymer. The mechanism of degradation was concluded to be hydrolysis (nonenzymatic) based on \textit{in vitro} and \textit{in vivo} experiments. The polymers degraded into small pieces within 16 weeks subcutaneously in rats, and were completely degraded at 12 months. Bichon et al. (1984) developed an air cured polyurethane, which was used to coat catgut sutures. Polyester soft segments were synthesized with diethylene glycol, diacids or anhydrides. Sutures implanted subcutaneously in rats degraded over a period of 24 days.
More recently, Saad et al. (1997) used poly(hydroxy butyrate-cohydroxy valerate), and polycaprolactone diol to synthesize a biodegradable polyurethane. In vitro and in vivo testing showed good biocompatibility, and a 50% weight average molecular weight loss after 12 months implantation in rats.

Enzymatically degradable poly(amide-urethanes) with HDI and dicarboxylic acid chlorides have also been proposed (Huang et al., 1986). The polymers were found to undergo slow hydrolysis in buffer but were degraded readily by the enzyme subtilisin. Phakadze et al. (1996) also synthesized enzyme degradable materials based on amino acids and polyesters. They observed degradation by measuring intrinsic viscosity, tearing strength, and modulus after incubation with chymotrypsin, pepsin, and papain. Skarja and Woodhouse (1997) synthesized polyurethane elastomers containing polycaprolactone, polyethylene oxide, and a phenylalanine chain extender. Incubation with chymotrypsin up to 56 days showed significant mass loss, change in molecular weight, and surface alteration (by scanning electron microscopy).

2.3 Polymer Drug Delivery

Drug delivery systems for the controlled release of therapeutic agents is becoming as important as the design of new drugs themselves. The incorporation of bioactive agents into solid polymers is one way to accomplish this, and has been the focus of much research in recent years.

The rationale for the development of drug delivery systems can be explained by considering the problem of dose-delivery. Figure 2.7 illustrates blood plasma concentrations following a single dose of a bioactive agent. The drug concentration profile is characterized by a rapid initial increase in concentration, followed by a period of decay as the drug is metabolized and excreted (Figure 2.7a). Problems may arise since many drugs have a therapeutic window, below which level the drug is ineffective and above which the drug may become toxic (Langer and Peppas, 1981). With a single dose, the only way to lengthen the time which the
drug is effective is to increase the size of the dose. However, this may lead to plasma concentrations above the toxic level (Figure 2.7b). Therefore, an implantable polymeric drug delivery device that can deliver and maintain effective plasma levels is of great interest (Figure 2.7c).

A number of potential advantages exist with drug delivery devices. Some of these include: 1) plasma drug levels are continuously maintained in the desirable range; 2) the drug can be delivered locally to the target site, thus increasing the effectiveness and minimizing side effects; 3) drugs that have short in vivo half lives may be protected from degradation; 4) drug delivery by this method may be less expensive and less wasteful of drug; 5) patient compliance may be improved.

Figure 2.7 Drug concentration in plasma as a function of time following administration.

There are a number of methods of incorporating bioactive agents into polymers to produce a controlled release system. These systems have been classified according to the mechanism of release (see Table 2.1) (Heller et al., 1996). The simplest type of device is the diffusion controlled device, where the drug is physically dispersed within a polymer matrix (monolith) or surrounded by a film (membrane). The rate of release is controlled by the diffusion of the
drug through the matrix or membrane. More complex devices under investigation use a stimulus to control the amount of drug release, such as magnetically controlled systems (Hsieh et al., 1989), electrically controlled systems (Ly et al., 1993; Woo, 1994), or enzyme triggered systems (Pitt et al., 1985; Pitt, 1986).

Table 2.1 Classification of Controlled Release Systems. (Reprinted with permission from Heller, "Drug Delivery Systems", in Biomaterials Science, B.D. Ratner et al., eds., 347, Copyright 1996 by Academic Press).

<table>
<thead>
<tr>
<th>Type of System</th>
<th>Rate-control mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion controlled</td>
<td></td>
</tr>
<tr>
<td>Reservoir Devices</td>
<td>Diffusion through membrane</td>
</tr>
<tr>
<td>Monolithic devices</td>
<td>Diffusion through bulk polymer</td>
</tr>
<tr>
<td>Water penetration controlled</td>
<td>Osmotic transport of water through Semipermeable membrane</td>
</tr>
<tr>
<td>Osmotic systems</td>
<td></td>
</tr>
<tr>
<td>Swelling systems</td>
<td>Water penetration into glassy polymer</td>
</tr>
<tr>
<td>Chemically controlled</td>
<td>Either pure polymer erosion (surface Erosion) or combination of Erosion and diffusion (bulk erosion)</td>
</tr>
<tr>
<td>Monolithic systems</td>
<td>Combination of hydrolysis of pendant group and Diffusion from bulk polymer</td>
</tr>
<tr>
<td>Pendant chain systems</td>
<td></td>
</tr>
<tr>
<td>Regulated systems</td>
<td>External application of magnetic Field or ultrasound to device</td>
</tr>
<tr>
<td>Magnetic or ultrasound</td>
<td>Use of competitive desorption or enzyme-substrate reactions. Rate control is built into device</td>
</tr>
<tr>
<td>Chemical</td>
<td></td>
</tr>
</tbody>
</table>

2.3.1 Biodegradable Drug Delivery Systems

As previously mentioned, biodegradable polymers have been widely used in drug delivery systems. Figure 2.8 shows a few common approaches to the design of degradable devices (Ranade, 1996). The most common approach is to disperse the drug physically within a biodegradable matrix (Figure 2.8, Type I, II, III). As the matrix erodes, the entrapped drug diffuses and is released. The rate of release is governed by the relative rate of polymer
erosion and drug diffusion. There are numerous reviews of this type of system in the literature (Heller, 1985, 1996; Langer and Peppas, 1981).

Figure 2.8 Biodegradable drug delivery types (Reprinted with permission from Controlled Drug Delivery Vol. I, S.D. Bruck, ed., Copyright 1983 by CRC Press).

An alternate approach is to covalently bond the drug via biodegradable linkages either pendant to the polymer or as part of the backbone (Types IV-V, Figure 2.8). These are termed polymeric drugs, polymeric prodrugs, or polymeric-drug conjugates (Akashi, 1993; Ottenbrite, 1991). The use of these polymer carriers can modify the pharmacokinetics, the mode of cell uptake, drug permeability, toxicity, and the rate of elimination from the body (Ottenbrite, 1991; Poinani et al., 1994). Polymer drug targeting to a specific biological site may also be achieved by attaching a specific moiety, such as an antibodies or other
biomolecules (Ottenbrite, 1991; Ringsdorf, 1975). In some cases, activity of the drug is lost with conjugation, and the removal of the drug is necessary, while in other cases, activity is preserved.

Polymer drug conjugates have frequently been synthesized to deliver anticancer agents. One macromolecule carrier which has been extensively investigated is N-(2-hydroxypropyl) methacrylamide (HPMA). The anthracyline antibiotics (daunomycin, adriamycin) have been attached to HPMA by oligopeptide side chains (Duncan et al., 1988; Kopecek, 1990). Hydrolysis of the peptide chains was shown to be catalyzed by lysosomal enzymes, including cysteine proteineases (Kopecek, 1990). In vivo experiments in a rat model showed decreased toxicity (Rihova et al., 1988) and enhanced effectiveness of the conjugate in reducing tumor growth over the free daunomycin (Cassidy et al., 1989). Another macromolecule under investigation is poly(PEG-Lys), a water soluble poly(ether urethane). Poly(PEG-Lys) is an alternating copolymer of poly(ethylene glycol) (PEG) and the amino acid L-lysine. The covalent attachment of antibiotics (Nathan et al., 1993), anticancer agents (Nathan et al., 1994), and antifibrotic agents (Poinia et al., 1994) via biodegradable ester linkages to Poly(PEG-Lys) has been described. The polymer conjugates possessing the antifibrotic agent cis-4-hydroxy-L-proline were able to inhibit fibroblast growth in vitro and collagen accumulation in an in vivo rat model (Poinia et al., 1994).

Other polymers used for conjugation which have been investigated include poly(α-amino acids) (Bennett et al., 1988; Li et al., 1991), divinyl ether-maleic anhydride (DIVEMA) (Hirano et al., 1986; Kaneda et al., 1997), polyanions (Yashima et al., 1990), and polysaccharides such as dextrans and chitin (Ohya et al., 1996; Ouchi et al., 1991).

Another approach taken is to self polymerize a biologically active compound in order to modify pharmacokinetic properties. Usually the drug molecule must be chemically modified to produce the desired monomer. An example of this method was proposed by Ghosh et al. (1988, 1990). Nalidixic acid, a quinolone antibiotic, was modified to form a new monomer
which could be subsequently polymerized. Initial studies showed rapid hydrolysis in vivo, with 50% hydrolysis at approximately 100 hours.

2.4 Biomaterial Associated Infection

The processes and interactions which ultimately lead to infection in a patient with a biomaterial implant are complex, and not completely understood. However, studies have determined that biomaterial associated infections are uniquely characterized by a number of features (Gristina 1996): (1) the presence of a foreign body (i.e. a biomaterial or damaged tissue), (2) the adhesion of bacteria to these surfaces (3), the resistance of bacteria to host defenses and systemic antibiotic therapy (4) the appearance of characteristic bacteria (5) the presence of multiple bacterial species (6) the persistence of the infection until the removal of the biomaterial (7) and specific phenomena related to the type of device, location of implantation, and organisms present. An understanding of the characteristics of the interactions between bacteria and biomaterials is necessary in developing new methods of treatment. This section will describe some of these processes and discuss current research into methods of treatment.

2.4.1 Adhesion Mechanisms

The adhesion of bacteria to the surface of the biomaterial is the first event which leads to a biomaterial-related infection (Mittelman, 1996). Bacterial adhesion is the process in which bacteria firmly associate with the surface. Once the bacteria has adhered, energy is required to cause a separation. Adhesion can be classified as non-specific, which is through physiochemical interactions, or specific, which is by a receptor-ligand interaction.

Nonspecific adhesion involves physiochemical interactions which are dependent on the properties of both the bacteria and substrate. As bacteria move toward the surface of the biomaterial, various types of forces may influence whether adhesion will ultimately occur. At the molecular level, these forces include van der Waals, electrostatic, and hydrophobic
(Fletcher, 1996). A number of studies have determined some of the factors which may influence these interactions, including the chemical composition of the material (Oga et al., 1988; Barth et al., 1989), surface charge (Hogt et al., 1985), hydrophobicity (Hogt et al., 1983), and surface topography or roughness (Chu and Williams, 1984). Two theories, based on colloid and surface chemistry, have been developed in attempt to understand the interactions which control adhesion. The “DLVO theory” (Derjaguin and Landau, 1941; Verwey and Overbeck, 1948) takes into account van der Waals and electrostatic forces. In the thermodynamic approach, adhesion is treated in terms of surface free energies of the bacterium, substrate, and surrounding liquid (Absolom et al., 1979, 1982). Although the application of these models has provided some insights into which parameters are significant, they are not adequate to explain many experimental results (Fletcher, 1996).

Specific adhesion involves adhesins, which are surface macromolecules produced by bacteria which allow attachment to substrates with stereochemical specificity. Pathogenic bacteria typically possess specific receptors for eukaryotic host cells. Some species of bacteria possess fimbria or pili which allow them to adhere to specific biological substrates such as cell membranes. Several investigators have found evidence that adhesins may also play a role in the attachment to synthetic surfaces. Timmerman et al. (1991) described a S. epidermidis biomaterial adhesin which appears to mediate attachment to polystyrene. Jansen et al. (1990) have identified surface lectins (carbohydrate-binding proteins) on S. saprophyticus and P. aeruginosa which may contribute in the adhesion to polyurethanes. Despite these and other findings, specific adhesion to synthetic biomaterials remains somewhat controversial (Mittelman, 1996). Experimental conditions can significantly influence the outcome of experiments. As mentioned previously, implanted biomaterials rapidly become coated with proteinaceous or polysaccharide based conditioning films. It is likely that the constituents of these films have an effect on adhesin-receptor studies. Christensen et al. (1989) suggested that the specific interactions are more likely mediated by the conditioning film, rather than the substrate itself. For instance, staphylococcal adhesion has been found to be mediated by plasma proteins such as fibrin, fibrinogen, and fibronectin (Cheung and Fischetti, 1990, Hermann et al., 1988, Delmi et al.; 1994).
2.4.2 The Bacterial Biofilm

Once attached the surface of the biomaterial, if conditions are favourable, bacteria may secrete extracellular polymeric substances (EPS) (also referred to as glycocalyx or slime) which form a matrix surrounding the bacterial cells (Costerton et al., 1981). The EPS is composed of a wide variety of monosaccharides, including neutral hexoses, polyols, uronic acids, and amino sugars (Sutherland, 1978). The exact composition is both strain-specific and nutrient dependent. The accumulation of bacterial colonies and extracellular material is defined as a biofilm (Gristina and Naylor, 1996). The formation of a biofilm is believed to be a significant factor contributing to the persistence and notorious difficulty in eradicating biomaterial centred infections.

Biofilm bacteria are afforded a number of benefits versus their planktonic (free floating) counterparts. The EPS acts as an ion-exchange resin between the bacterial cells and the environment which facilitates nutrient uptake (Costerton et al., 1987). Biofilm bacteria have been shown to be protected from complement opsonic factors, phagocytic cells, and antimicrobial agents (Costerton et al., 1987; Hoyle et al., 1990). In addition, components of the EPS can modulate the cellular immune response (Deighton and Borland, 1993; Jones et al., 1992; Stiver et al., 1988).

A number of studies have shown that bacteria within biofilms are several times more resistant to antibiotics and antimicrobials (Reid et al., 1993, Khoury et al., 1993). The biofilm may acting as a diffusion barrier which impedes the penetration of antibiotics (Suci et al., 1994). Other postulated mechanisms include slow growth rate (Evans et al., 1991) and the production of antibiotic-degrading enzymes (Giwerzman et al., 1991).

2.4.3 Characteristic Microorganisms

According to clinical surveys, the most frequently isolated microorganisms in biomaterial associated infections are Staphylococcus epidermidis (coagulase-negative), and Staphylococcus aureus (coagulase-positive) (Gristina, 1987; Christensen et al., 1989; An et
The staphylococci, which include both *S. epidermidis* and *S. aureus*, are normal components of the skin microflora (Kloos and Musselwhite, 1975). *S. aureus* is a classic human pathogen, which is fully capable of causing disease in the absence of a foreign body (Musher and McKenzie, 1977). On the other hand, *S. epidermidis* is normally nonpathogenic, but has the ability to cause infection in the presence of a biomaterial surface (Christensen et al., 1989). Other bacteria that have been isolated clinically include, *Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis*, β-hemolytic streptococci, and enterococci, *Candida albicans* (Christensen et al., 1985; Gristina et al., 1987, Sugarman and Young, 1984).

*S. epidermidis* is frequently associated with polymeric biomaterials such as vascular grafts, catheters, shunts, total joints, and total artificial hearts (Gristina et al., 1987b). *S. aureus* is the major pathogen isolated from metallic biomaterials in bone, joint, and soft tissue infections (Gristina, 1987). *P. aeruginosa* and *S. epidermidis* are the primary causes of infection involved with extended-wear contact lenses (Slusher, 1987). *E. coli* is the most common pathogen found with urinary catheter related infections, although *P. aeruginosa*, *Proteus* species, and enterococci have also been found (Warren, 1987). In many cases, biomaterial associated infections are caused by more than one species of microorganism (Gristina and Naylor, 1996).

### 2.4.4 Prevention of Device Related Infections

A variety of strategies have been employed to combat biomaterial associated infections. Infection rates have been reduced through improved operating room techniques, such as the ultraclean air system, and the proper use of prophylactic antibiotics (Lidwell et al., 1982; Brady et al., 1985). The use of antiseptics or antimicrobial ointments applied topically to the exit site of percutaneous devices (catheters) has also been investigated, but studies have shown this may only delay infection (Goldmann and Pier, 1993; Maki and Band, 1981). The incorporation of antimicrobials on the devices themselves has been the subject of much
research. Various antibiotics and antiseptics have been coated on surfaces of devices such as vascular grafts, stents and catheters.

Silver is known to have antibacterial properties with low toxicity and favourable biocompatibility in humans (Williams, 1989). Silver coatings have been applied to medical devices such as catheters (Gabriel 1995, 1996), cuffs (Maki et al., 1988), and stents (Leung et al., 1992). Silver coated peritoneal dialysis (PD) catheters have been shown to reduce infection in a long term rabbit model (Dasgupta, 1994). Silver coatings have also been used on central venous access, (Maki 1988), urinary (Tackeuchi et al., 1993; Lundeburg 1986), and peritoneal dialysis (Vas, 1995; Mittelman, 1994) catheters in human clinical trials. However, some clinical trials have failed to demonstrate long term efficacy (Babycos et al. 1993, Riley et al., 1995, Groeger et al. 1993; Hasiyani et al., 1996). There is also the problem of bacterial resistance to silver emerging over time (Williams 1989, Silver and Misra 1993), which can lead to multiple antibiotic resistance. Despite these problems, the use of silver as an antibacterial agent continues. One recent study used an iontophoretic catheter which electrically generates silver ions (Raad et al., 1996). Silver has also been used in combination with other antibiotics (Modak et al., 1987; White et al., 1984; Benvenisty et al., 1988).

Various antibiotics have been coated on catheters or vascular grafts using an ionic bonding process. Trooskin et al. (1985, 1987) described a method which used a cationic surfactant, tridodecylammonium chloride (TDMAC), to bind penicillin G to silicone catheters. Antibiotic release was demonstrated over a short period of time (days). PTFE vascular grafts have also been coated using TDMAC or benzylalkonium chloride and penicillin G (Harvey et al., 1982; Modak et al., 1987). Antibiotic coated grafts implanted in a canine model showed significantly fewer infections than control grafts (Greco et al., 1985). More recently, chlorhexidine and silver-sulphadiazine were used to coat central venous catheters using a proprietary process, (Maki et al., 1991; Bach et al, 1994, 1996; Schmidt et al., 1996), and are now commercially available in the USA. Several investigators have shown the catheter was effective in preventing bacterial colonization and subsequent infection (Maki et al., 1991;
Ramsay et al., 1994). However, other clinical trials have failed to show any differences (Ciresi et al., 1996, Pemberton et al., 1996). Another coated catheter which has become commercially available is a minocycline/rifampin coated central venous catheter, which uses the TDMAC ionic bonding process (Raad et al., 1996). Initial clinical trials have shown a reduced risk for catheter colonization and bloodstream infections (Raad et al., 1997).

In the development of antibiotic coated devices, a significant problem that is encountered is the rapid release of the antibiotic over a short period of time. Consequently, improved methods of bonding or incorporating antibiotics have been tried. Ciprofloxacin has been bonded to Dacron® using a textile processing technique (Phaneuf et al., 1993). In a subcutaneous rabbit model, animals with the implants resisted infection by Staphylococcus aureus as compared to plain and ciprofloxacin dipped Dacron® for a period of 1 week (Ozaki et al., 1993). Karck et al (1993) combined gentamicin with a fibrin sealent which was coated onto vascular grafts. As the fibrin biopolymer was degraded, the gentamicin was released. In vitro elution studies showed a peak release at 3 days, followed by a significant decrease. In a porcine model, 50% of the fibrin-gentamicin specimens were contaminated with S. aureus upon retrieval one week later. Chervu et al. (1991) described a rifampin/collagen release system with Dacron® vascular grafts in dogs. The grafts actively prevented infection significantly for 7 days (Chervu et al., 1991b).

Incorporation of antibiotics into polymers as a diffusion-controlled drug delivery system has also been proposed. Ciprofloxacin has been dispersed into polyurethanes in a number of studies (Jansen et al., 1992; Schierholz et al., 1997). However, similar problems were encountered, as some of the formulations released the drug too quickly. Golomb et al. (1991) incorporated parabens in polyurethane matrices by a solvent casting method. S. epidermidis adhesion was significantly reduced in vitro. A currently accepted application of antibiotic drug delivery is in the treatment of osteomyelitis, a type of orthopaedic infection. A number of such systems have been developed, including poly(methyl methacrylate) (PMMA) beads (Nelson et al., 1994; DiMaio et al., 1994), polylactic-co-glycolic acid (Price et al., 1996), polyanhydrides (Laurencin et al., 1993) and biodegradable bone cements (Gerhart et al.,
Antibiotics which have been incorporated include ciprofloxacin, gentamicin, vancomycin, and amikacin.

An alternate method of designing an infection resistant material is to modify the surface of the biomaterial to prevent bacterial adhesion. Currently methods under investigation include techniques such as plasma modification (Mittelman, 1996b), ion-implantation (Sioshansi, 1994), or gamma irradiation (Jansen et al. 1995). Jansen et al. (1995) used 2-hydroxyethylmethacrylate to render the surface of a polyurethane hydrophilic, since it has been shown that bacteria prefer hydrophobic surfaces. *S. epidermidis* adhesion was shown to be strongly reduced compared to polyurethane controls. Another study based on the same premise immobilized polyethylene oxide on polyethylene terephthalate (PET) (Desai et al., 1992). Significant reductions (between 70% and 95%) in adherent bacteria were observed compared to native PET.

Some investigators have attempted to overcome the resistance of biofilm encased bacteria by enhancing the penetration of antimicrobial agents. Khoury et al. (1992), found that weak electric fields applied with antibiotic administration enhanced the killing of *Pseudomonas aeruginosa* biofilms by tobramycin. It was assumed that the electric field enhanced the penetration of the antibiotic through the biofilm by electrophoresis (Blenkinsopp et al., 1992).

### 2.5 Quinolone Antimicrobial Agents

Quinolone antimicrobial agents, (also called fluoroquinolones, 4-quinolones, quinolone carboxylic acids) are synthetic antibiotics which are derivatives of an earlier developed agent, nalidixic acid, which was first isolated by Lesher et al. (1962) as a by-product of chloroquine synthesis. Nalidixic acid demonstrated activity against gram negative bacteria and was used to treat urinary tract infections (Norris, 1988). Chemical modifications of the basic nalixidic acid structure have led to thousands of analogues which are significantly more potent *in vitro* and possess a wider spectrum of activity. Some of these agents include ciprofloxacin, lomefloxacin, norfloxacin, and peflacin, sparflloxacin, and ofloxacin (Figure 2.9).
Figure 2.9  Some quinolone antibacterial compounds.

The pharmacore of a drug is defined as the minimum structure required for significant physiological activity. Typically, this unit by itself possesses a low potency and specificity. The function of the rest of the drug molecule (the auxopharmacore), is to provide tighter receptor fit, greater selectivity, desirable pharmacokinetic properties (absorption, excretion, distribution, metabolism, etc.), and solubility (Mitscher, 1993). In the quinolones, the minimum pharmacore consists of a 4-pyridone ring with a 3-carboxylic acid group (Figure 2.10). The reduction of the 2,3 double bond eliminates activity (Remers and Bear, 1997). The auxopharmacore is usually a fused aromatic ring with pendant substituents (Figure 2.9). The variation of the substituents allows for the synthesis of thousands of analogues. However, certain substituents are key in providing enhanced activity. The presence of a fluorine atom at C6 dramatically enhances activity. The fluorine group is present on most of
the highly active quinolones (Remers and Bear, 1997). Additionally, many of the analogues have a piperazino group on carbon 7, which serves to broaden the spectrum of the antibiotic, especially against gram-negative organisms.

\[ \text{Required Pharmacore} \]

\[ \begin{array}{c}
\text{(F)} \\
\text{R}_2 - \text{N} \quad \text{N} \\
\text{R}_3
\end{array} \]

\[ \begin{array}{c}
\text{O} \\
\text{COOH}
\end{array} \]

\[ \text{X} \]

\[ \text{N}_1 \]

\[ \text{R}_1 \]

\[ \text{2} \]

\[ \text{3} \]

\[ \text{4} \]

\[ \text{5} \]

\[ \text{6} \]

\[ \text{7} \]

\[ \text{8} \]

\[ \text{Required Pharmacore} \]

Figure 2.10  Quinolone pharmacore and auxopharmacore.

The mechanism of action of quinolones is through the inhibition of DNA synthesis within bacterial cells. This is accomplished by inhibiting the action of bacterial DNA gyrase, which is responsible for the ordered uncoiling of DNA strands (Crumplin, 1986). Due to the differences in human DNA chromosomal structure, quinolones do not inhibit DNA gyrase in humans. A number of models have been developed which explain how DNA gyrase is inhibited. One such model is the cooperative quinolone-DNA binding model (Shen et al., 1989). Based on studies of [\(^{3}\text{H}\)]norflxacin binding to DNA and DNA gyrase, the model hypothesized that quinolone molecules bind to a DNA–DNA gyrase complex. The quinolones self associate into a supermolecule (containing 4 molecules of drug) which is able to bind to the DNA by multiple hydrogen bond receptors. Three functional binding domains on the quinolone were suggested (Figure 2.11): the DNA-binding domain, the drug self-association domain, and the drug-enzyme interaction domain.
Ciprofloxacin is one of the best analogues in clinical use today (Mitschcer, 1993). Ciprofloxacin includes a fluorine at the C-6 position, the piperazino group at C-7, and the cyclopropyl at C-1. Ciprofloxacin is a wide spectrum antibiotic, exhibiting bactericidal activity against a wide range of both gram-negative and gram positive bacteria. Typical clinical uses include the treatment of urinary tract infections (UTI), respiratory infections, and bone and joint infections (Norris, 1988). Some *in vitro* Minimum Inhibitory Concentrations (MIC) of ciprofloxacin against various bacteria are provided in Table 2.2.

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>ATCC 25922</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>ATCC 29212</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>ATCC 27853</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>ATCC 29213</td>
</tr>
</tbody>
</table>
3.0 MATERIALS AND EXPERIMENTAL METHODS

3.1 Synthesis of Drug Polymers

3.1.1 Materials Used for Drug Polymer Synthesis

Drug polymers were synthesized using the chemicals listed in Table 3.1. The supplier information for each of the compounds is given in Appendix A. Several polymers were synthesized with varying chemistries in order to manipulate molecular weight and drug release rates. Aliphatic isocyanates were used in place of traditional aromatics such as 2,4 toluene diisocyanate (TDI) or 4,4'-diisocyanatodiphenyl methane (MDI), which have been shown by studies to release toxic biodegradation products under simulated physiological conditions (Wang et al., 1998; Szycher et al. 1991). Two types of soft segments were used, poly(oxyethylene) diamines (formerly known as Jeffamines) and polycaprolactone diols, to give polymers of varying hydrophobicity. The antibiotic, ciprofloxacin hydrochloride (Cipro®) was chosen as a model antibiotic, due to its broad spectrum antimicrobial properties, clinical efficacy, and chemistry. Cipro® possesses two functional groups, a secondary amine and a carboxylic acid, which can potentially polymerize with the diisocyanates. A catalyst was required due to the use of aliphatic isocyanates, which are less reactive than their aromatic counterparts (Gogolewski, 1989). Dibutyltin dilaurate was chosen due to its high catalytic activity (Lelah and Cooper, 1986).
### Table 3.1 Materials Used in Drug Polymer Synthesis

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Chemical Structure</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,6-diisocyanatohexane (98%) FW 168.20</td>
<td>OCN—(CH₂)₆—NCO</td>
<td>HDI</td>
</tr>
<tr>
<td>[1,6-¹⁴C] diisocyanatohexane FW 168.20</td>
<td>OCN—(CH₂)₆—NCO</td>
<td>¹⁴C-HDI</td>
</tr>
<tr>
<td>1,12-diisocyanododecane (97%) FW 252.36</td>
<td>OCN—(CH₂)₁₂—NCO</td>
<td>DDI</td>
</tr>
<tr>
<td>Cipro® (ciprofloxacin hydrochloride) FW 385.8</td>
<td><img src="image" alt="Ciprofloxacin" /></td>
<td></td>
</tr>
<tr>
<td>Poly(oxyethylene) diamines:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jeffamine-ED900 b=20.5,a+c=3.5, avg. M₀ 900</td>
<td>CH₃(CH₂)₉CO₂Sn[(CH₂)₉CH₃]₂</td>
<td>PPE-900</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPE-2001</td>
</tr>
<tr>
<td>Jeffamine-ED2001 b=45.5,a+c=3.5, avg. M₀ 2000</td>
<td>CH₃(CH₂)₁₉CO₂Sn[(CH₂)₁₉CH₃]₂</td>
<td></td>
</tr>
<tr>
<td>Dibutyltin dilaurate (95%) FW 631.56</td>
<td>[CH₃(CH₂)₁₀CO₂]₂Sn[(CH₂)₁₀CH₃]₂</td>
<td>DBTDL</td>
</tr>
<tr>
<td>Triethylamine (99 + %) FW 101.19</td>
<td>(CH₃)₃N</td>
<td>TEA</td>
</tr>
<tr>
<td>Nitrogen (Prepurified)</td>
<td>N₂</td>
<td>N₂</td>
</tr>
<tr>
<td>Dimethyl Sulphoxide (HPLC grade) FW 78.13</td>
<td>(CH₃)₂SO</td>
<td>DMSO</td>
</tr>
</tbody>
</table>
3.1.2 Reagent Purification

Prior to the synthesis, all reagents were purified by distillation, drying, or degassing to remove moisture and other undesirable components. This step is particularly important since isocyanates are very reactive with water and low molecular weight hydroxyls (Gogolewski, 1989). A diagram of the distillation apparatus used is shown in Appendix B.

Diisocyanates were vacuum distilled to separate dimers from the monomers, and to remove lower boiling impurities. 1,6-diisocyanatohexane (HDI) was distilled at 1 torr and 93°C while 1,12-diisocyanatododecane (DDI) was distilled at 0.12 torr and 140°C. A 10 cm micro-distillation column wrapped with glass wool insulation and heating tape was used. The product was collected and separated into 0.5 ml aliquots and immediately sealed into small glass ampoules to keep the isocyanates moisture free.

The oligomeric soft segments polycaprolactone diol (PCL) and poly(oxyethylene) diamine (PPE) were degassed under vacuum for 24 hours at 0.2 torr at 60°C. Cipro® (Bayer Inc., Healthcare Division) was supplied as a powder in vacuum sealed vials. However, to remove the excess water, the drug was dried for 24 hours under vacuum (30 in. Hg) at 60°C before use. The reaction solvent, dimethylsulphoxide (DMSO, HPLC grade), was distilled prior to use to remove any residual moisture at 0.05 torr and 35°C less than 24 hours before each reaction.

Several solvents were considered, including N,N-dimethylformamide (DMF), tetrahydrofuran, and N,N-dimethylacetamide (DMAC). However, DMSO was the only solvent capable of dissolving ciprofloxacin at a temperature of 70°C.
3.1.3 Synthesis Procedures

The methods of synthesis developed for the drug polymers were adaptations of standard one and two-step polyurethane solution polymerization techniques (Gogolewski, 1989). The reaction schemes for the two procedures are shown in Figure 3.1. In the 2-step method, the diisocyanate was reacted with the diol or diamine to form a prepolymer solution. The ciprofloxacin was added as a chain extender. This “method” was also run in reverse, with a diisocyanate-drug prepolymer, followed by coupling of the oligomeric diol or diamine.

**one-step synthesis**

\[
\text{diisocyanate} + \text{diol or diamine} + \text{drug} \\
\text{dimethylsulphoxide} + \text{dibutyltin dilaurate} \\
60°C 12-22 hrs \\
\rightarrow \\
drug polymer
\]

**two-step synthesis**

\[
\text{diisocyanate} + \text{diol or diamine} \\
\text{dimethylsulphoxide} \\
dibutyltin dilaurate \\
60°C 3 hours \\
\rightarrow \\
\text{prepolymer} + \text{drug} \\
60°C 12-22 hours \\
\rightarrow \\
drug polymer
\]

\[
\text{diisocyanate} + \text{drug} \\
\text{dimethylsulphoxide} \\
dibutyltin dilaurate \\
60°C 0.5-3 hours \\
\rightarrow \\
\text{prepolymer} + \text{diol or diamine} \\
60°C 12-22 hours \\
\rightarrow \\
drug polymer
\]

**Figure 3.1** Reaction schemes for the synthesis of the drug polymers.
Synthesis of the drug polymers was carried out in a glovebox (Labconco® Protector® Model 50600) under a nitrogen atmosphere. Immediately before the synthesis, the reagents were weighed (Mettler AT201 analytical balance) into separate beakers, sealed with parafilm, and placed in the glovebox. The glovebox was pumped and purged at least four times to obtain a dry nitrogen atmosphere. All reactions were run with a stoichiometry of 2:1:1 of diisocyanate: ciprofloxacin : oligomeric component.

The reaction was carried out in a three-neck 50 ml glass flask (Corning), with a Teflon® magnetic stir bar. A 110°C thermometer was placed into one of the side necks, and a glass thermometer well was fitted into the other. The middle neck, used for reagent addition, was kept stoppered during the reaction. A microprocessor controlled hotplate/stirrer (VWR Series 400 HPS) was used, with a constant stirring rate of 450 rpm. The thermocouple probe was inserted into the glass thermometer well to isolate it from the reactor solvent. There was a 7°C offset between the thermometer and thermocouple due to the insulating glass well. A complete diagram of the apparatus is shown in Figure 3.2.

![Diagram of apparatus](image)

**Figure 3.2** Apparatus for synthesis of drug polymers.
In the two-step prepolymer method, 1.04 millimoles of the diol or diamine, dissolved in 5 ml of DMSO, was added to 2.07 millimoles of diisocyanate in 5 ml of DMSO. For the synthesis of radiolabelled polymers, 0.25 mCi of \(^{14}\)C radiolabelled HDI was mixed with non-radiolabelled HDI. Dibutyltin dilaurate was added at a concentration of 0.3% or 3% (6 or 60 mg) based on the work of Tang et al. (1996). The resulting solution was allowed to react at a temperature range of 60°-70°C for 3 hours. Since ciprofloxacin is light sensitive, the reactor was covered with aluminum foil. Following this, 1.04 millimoles (0.4 g) of ciprofloxacin hydrochloride was dissolved in 10 ml of DMSO along with 1.04 mmoles (0.144 ml) of triethylamine and heated to 70°C. The triethylamine was added to scavenge the HCl bound to the ciprofloxacin salt, thus enabling the amine group to react with the diisocyanate. The entire drug solution was then added to the reactor in one shot. The reaction was allowed to stir for 12-22 hours, at which time the polymer was precipitated dropwise into distilled water. A diagram of a typical reaction is shown in Figure 3.3.

For some of the reactions, ciprofloxacin was allowed to first react with the diisocyanate to form the prepolymer, followed by further reaction with the oligomeric component. In these cases, prepolymer reaction times were between 1.5-3 hours.

In the one-step reactions, both the ciprofloxacin and the diol or diamine was added at the beginning of the reaction.

Precipitation of all polymers was carried out by slowly adding the reaction solution dropwise into a beaker of distilled water while gently mixing with a magnetic stirrer. When the solution turned cloudy, the water was decanted and replaced. This process was repeated until all the polymer was precipitated. If the polymer was too fine to settle and allow for decanting, the solution was centrifuged for 10 min at 3000 rpm before replacing the distilled water. The precipitated polymer was allowed to stir overnight. The next day, a 40 ml sample of the wash solution was freeze-dried and kept for residual drug analysis by high performance liquid chromatography (HPLC). The remaining wash solution was replaced with fresh water and the procedure was repeated 3 times in order to ensure adequate removal of unreacted
drug and monomer. The polymer was dried in a Teflon® petri dish at 60°C for 24 hours using a convection oven (Fisher Isotemp® Forced Draft Oven). Further drying was then carried out under a vacuum of 30” H₂O (Fisher vacuum oven model 285) at 60°C for a period of 24 hours. The drug polymer was stored in a dessicator and wrapped in aluminum foil to protect it from light. For the radiolabelled polymers, the specific activity of each polymer was determined by dissolving approximately 1 mg of polymer in 1 ml of N,N-dimethylacetamide (DMAC) and counting in a liquid scintillation counter (Beckman LS500, Mississauga, ON).

\[
\begin{align*}
2 \text{OCN}-(\text{CH}_2)_6-\text{NCO} & \quad \text{HDI} \\
& \quad \begin{cases}
\text{PCL} & \\
\text{prepolymer} & \\
\text{ciprofloxacin} & \\
\text{drug polymer} &
\end{cases}
\end{align*}
\]

Figure 3.3 Reactions in the synthesis of a typical drug polymer.
3.1.4 Drug Polymer Nomenclature

The nomenclature used to identify the drug polymers in this thesis are based on the reactants their molecular weights, and the batch number of the reaction. An example of drug polymer nomenclature is shown in Table 3.1. The first sequence of letters refers to the diisocyanate, while the second sequence of letters refers to the oligomeric soft segment. The next numerical sequence refers to the average molecular weight of the oligomeric soft segment, while the numerical sequence in parentheses refers to the batch number of the series of polymers. The presence of radiolabelled HDI was indicated by $[^14\text{C}]$. Since each of the polymers contains ciprofloxacin, it was not specified in the nomenclature.

<table>
<thead>
<tr>
<th>Example: DDI/PCL-2000(12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDI</td>
</tr>
<tr>
<td>PCL</td>
</tr>
<tr>
<td>2000</td>
</tr>
<tr>
<td>(12)</td>
</tr>
</tbody>
</table>

3.2 Characterization of Drug Polymers

3.2.1 Gel Permeation Chromatography

Gel permeation chromatography is a method for determining the molecular size distribution of a polymer. A dilute polymer solution is injected into a column packed with porous beads, typically made of crosslinked polystyrene. The porosity of these beads is critical, and is usually between 50 and $10^6$ Å. Small polymer molecules can penetrate these pores and thus have a long flow path through the column. Larger polymer molecules, however, cannot penetrate the smallest pores and thus have a much shorter flow path since they travel around
the packing and not through it. Hence, they and elute from the column at earlier retention times. Since these molecules are effectively excluded, GPC is a form of *size-exclusion* chromatography, where the largest molecules elute first. The polymer concentration, or parameter which is proportional to concentration, is continuously monitored at the exit of the column, and a plot of concentration vs. elution volume is recorded.

The components of the GPC apparatus used in this study are shown in Figure 3.4. The system consists of: (i) a Waters 510 HPLC pump, (ii) a Rheodyne manual injector with a 200 μl sample loop, (iii) a bank of Waters Styrage columns (HT3, HT4, HT5) (Milford, MA), (iv) a Waters 410 differential refractometer, (v) a PC data acquisition system (Waters Millenium 2.1). A 0.05 M Lithium bromide (LiBr) in N,N-dimethylformamide (DMF) mobile phase was used. DMF was chosen for its ability to dissolve drug polymers and is based on methods previously developed for polyurethanes (Santerre et al., 1993). LiBr is an additive to prevent hydrogen bonding between polymer chains, thus leading to erroneously high molecular weight measurements. The use of LiBr has been documented in a number of papers (Dubin et al., 1977; Booth et al., 1980; Scheung, 1984). Prior to use, the mobile phase was filtered through a 0.45 μm fluorocarbon filter (Millipore FHUP, Bedford, MA). The flowrate of the mobile phase was 1 ml/min and the column bank was heated to 80°C. Samples were prepared to a concentration of 0.2% (wt/vol) in mobile phase, and were filtered through 0.45 μm polytetrafluoroethylene syringe filters (Chromatographic Specialties, Brockville, ON) prior to injection.

Absolute molecular weight data for polyurethanes and other copolymers are difficult to obtain, since in addition to a molecular weight distribution, copolymers exhibit a distribution of compositions (Speckhard et al., 1986). For polyurethanes, additional complications include their poor solubility in common solvents, the use of three monomers instead of two, and the possibility of side reactions such as allophonate cross-linking (Lee et al., 1986). Therefore, molecular weights values were reported as polystyrene equivalent weight average molecular weights ($\bar{M}_w$), number average molecular weights ($\bar{M}_n$), and polydispersities. Calibration curves were constructed by injecting polystyrene standards (Varian, Sunnyvale
Ca.) of narrow molecular weight distributions. A typical calibration curve is given in Appendix C.

![Diagram of GPC setup](image)

**Figure 3.4 Instrumentation for GPC.**

### 3.2.2 Elemental Analysis

Polymer samples were sent to Guelph Chemical Labs (Guelph, ON) for bulk elemental analysis. The methods for each element were summarized as follows:

**Carbon, Hydrogen, Nitrogen**

The samples were analyzed using a Carlo-Erba 1108 Elemental Analyzer, where weighed samples were combusted in an oxygen atmosphere at 1000°C. The samples were then reduced by passing through copper at 600°C. The resulting gases namely N₂, CO₂, H₂, O₂ were then separated by a gas chromatographic column and detected by a thermal conductivity detector. The % carbon, hydrogen, and nitrogen were deduced from calibration using NBS standards of known % carbon, nitrogen and hydrogen.
Tin

The samples were analyzed using an ICP-AES spectrometer where a weighed sample was digested in acid until dissolution was complete then diluted in an appropriate solvent or gas. The instrument response for the sample solution was then measured and the concentration of analyte in the sample was calculated.

Fluorine

The samples were combusted with sodium peroxide in a Schodinger oxygen flask in an oxygen-rich atmosphere using distilled water as the absorbing media. The resulting solution was then diluted to a specific volume. An aliquot was taken, of which the pH was adjusted, then titrated with thorium nitrate using Alizarin Red S as an indicator. The fluorine content was then calculated using the volume of the titrant that was consumed.

3.3 Biodegradation Experiments

In vitro biodegradation tests were based on a method developed by Santerre et al. (1994). Cholesterol esterase (CE), a lysosomal enzyme produced by macrophages during the in vivo inflammatory response, was used as a model enzyme. Previous work has shown that CE can readily degrade polyester and polyether based urethanes (Santerre et al., 1994, 1997). Ciprofloxacin and other degradation products released during the incubation period were isolated using high performance liquid chromatography (HPLC), and structures were confirmed by ionspray mass spectroscopy (MS). Drug polymers synthesized with $^{14}$C radiolabelled HDI were used in conjunction with HPLC to monitor specific release of HDI containing components. A microbiological assay was used to determine the activity of the released ciprofloxacin and ciprofloxacin containing degradation products.
3.3.1 Preparation of Polymer Coated Glass Tubes

Antimicrobial polymers were coated onto hollow glass tubes cut into 0.5 cm (4 mm OD, 2 mm ID) or 1 cm (3 mm OD, 2 mm ID) long pieces. Prior to coating, the tubes were cleaned in an ultrasonic bath for 30 minutes, rinsed thoroughly with deionized water and dried upright at 110°C for at least 24 hours. Polymer solutions were prepared by dissolving the polymer at a 10% (wt/vol) concentration in N,N-dimethylacetamide (DMAC) (Aldrich, Milwaukee, WI). Each tube was dip casted with a pair of tweezers. Excess solution was removed by placing the tubes on a kimwipe for a few minutes, and then transferring them to a dry Teflon® plate. The coated tubes were dried in a convection oven at 50°C for 24 hours. The coating procedure was repeated each day for a total of four times, with the final coat dried in a vacuum oven at 50°C for 24 hours. Specific radioactivity was determined for 14C radiolabelled polymers by dissolving one coated tube in 1 ml of DMAC and counting the solution in a liquid scintillation counter.

Under a sterile laminar flow hood, the tubes were placed into autoclaved 1 dram glass screw cap vials or sterile 10 ml Vacutainers (Becton-Dickinson, Franklin Lakes, NJ). Fourteen tubes were stacked in each vial, in two layers, regularly packed (Figure 3.5) corresponding to a total surface area of 13.2 cm².

![Diagram of coated glass tube in a vial with enzyme or buffer solution](image)

**Figure 3.5 Incubation of drug polymers.**
3.3.2 Preparation of Incubation Solutions

Cholesterol esterase (bovine pancreas, E.C. 3.1.1.13, Genzyme Diagnostics, Cambridge, MA) solutions were prepared by dissolving the powder in 0.05M phosphate buffer, pH 7.0, (20 units/ml). The buffer was prepared by dissolving 2.68 g of sodium dihydrogen phosphate and 4.2 g of sodium hydrogen phosphate in 1 L of deionized water. The pH was adjusted to 7.0 with 1 N HCl of 1N NaOH.

The activity of CE was determined using a modified version of the p-nitrophenyl acetate assay developed by Labow et al. (1983). The substrate was prepared by dissolving 22 mg of p-nitrophenyl acetate (Aldrich, Milwaukee, WI) in one ml of methanol and adding this to 100 ml of 0.1M sodium acetate buffer, pH 5.0. CE activity was determined by adding 100 µl of enzyme solution into a 3 ml cuvette containing two ml of 0.05M phosphate buffer and two ml of the substrate. UV spectrophotometer (Ultraspex II, LKB Biochrom Ltd., Cambridge, UK) measurements were taken at room temperature for a 5 minutes period (immediately after addition of enzyme) at a wavelength of 410 nm. The activity of the enzyme was expressed as nmol substrate hydrolyzed per minute, calculated from the molar absorptivity of the p-nitrophenyl acetate ion as 16 300 litre . mol⁻¹ . cm⁻¹ at pH 7.0 (Labow et al., 1983). Solutions of 40 units/ml activity were prepared.

3.3.3 Enzyme Stability Testing

The stability of cholesterol esterase was determined in buffer and in the presence of the drug polymer. Vials containing the coated polymer tubes were incubated with 1 ml of enzyme solution (40 units/ml) along with vials with no polymer (control) at 37°C. At time zero, 100 µl of solution was removed and assayed as described above (Section 3.3.2). Enzyme activity was assayed at 2, 4, 6, and 24 hours. The results of the test are shown in Appendix D. A drop in activity was noted after only 2 hours, but subsequently maintained this level over the remaining sample points up to 24 hours. The enzyme was replenished daily to maintain sufficient levels as described in the Section 3.3.4. Ciprofloxacin was also incubated with the enzyme, and was found to have no effect on enzyme activity.
3.3.4 Incubation of Drug Polymers

Each of the drug polymer formulations was tested by incubating the vials with CE (20 units/ml) and phosphate buffer (pH 7.0) only at 37°C. For HPLC purposes, additional vials containing uncoated glass tubes were incubated with enzyme. Each of the groups consisted of three replicate vials to determine the error. Initially, 2 ml of solution was added to each vial, and a one ml aliquot was immediately withdrawn as the time zero sample point. At each subsequent sampling time, 300 µl was withdrawn for microbiological analysis, and 700 µl was kept for HPLC and MS analysis. For the radiolabelled polymer, and additional 200 µl was withdrawn for scintillation counting. Radioactive solutions were counted with 10 ml of scintillation cocktail (Formula 989, Packard Instrument Co., Ontario). Samples for HPLC and microbiology were frozen in liquid nitrogen and stored at -80°C until analysis. Each day, a 100 µl aliquot (120 µl for the radiolabelled polymer) of the concentrated CE solution (800 units/ml) was added to each vial in order to maintain enzyme activity. Buffer controls were also replenished to match volumes. Samples were withdrawn at time intervals ranging from 3 to 10 days, and each experiment was run for 10 to 40 days.

3.4 Drug Release, Degradation Product Isolation, and Compound Identification

3.4.1 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a technique for physically separating a mixture of two or more compounds. Separation is achieved by injecting a sample into a stream of liquid flowing through a column packed with a stationary phase. The sample distributes between the liquid and the stationary phase. As the mobile phase elutes through the column, the sample is washed out. Separation occurs due to the differential affinity of each compound for the stationary phase.

The components of the HPLC used are shown in Figure 3.6. A Waters U6K injector (Milford, MA) is connected to a Waters µbondapak C₁₈ steel cartridge column or a Waters
μbondapak radial compression cartridge column. The mobile phase is degassed by a helium sparge, elevated above the Waters 600 pump and controller, which mixes the solvent and programs the gradient run. The analytes are detected via a Waters 996 photodiode array detector, which scans the entire UV spectrum. Data is recorded and processed via PC with Waters Millenium Software. The columns are protected from fouling by use of a 0.22 μm stainless steel inlet frit and a guard column (Waters Guard-Pak™).

![Diagram of HPLC instrumentation](image)

**Figure 3.6** Instrumentation for HPLC.

### 3.4.1.1 Chemicals

Ciprofloxacin hydrochloride, and ciprofloxacin intravenous (IV) solution were obtained from Bayer Inc., Healthcare Division (Etobicoke, ON). All solvents were glass distilled, HPLC grade. Methanol was obtained from Mallinckrodt (Paris, KY) and acetonitrile from Caledon labs (Georgetown, ON). Acetic acid (double distilled) and Tetrabutylammonium hydroxide (40% solution in water) were purchased from Aldrich (Milwaukee, WI). Potassium dihydrogen phosphate (ACS grade) was obtained from BDH Chemicals (Mississauga, ON). Orthophosphoric acid (85%, HPLC grade) was obtained from Fisher Scientific (Unionville, ON).
3.4.1.2 Ciprofloxacin Quantification by Isocratic Methods

Ciprofloxacin levels in the polymer synthesis wash solutions were quantified via an isocratic separation. Numerous HPLC methods for detection of ciprofloxacin have been published in the literature (Fasching et al., 1985; Krol et al., 1986, Gau et al., 1985), and the method used was based on the method of Fasching et al. (1985). The mobile phase consisted of 12% Acetonitrile and 88% 0.025M phosphoric acid adjusted to pH 3.0 with tetrabutylammonium hydroxide. Prior to use, acetonitrile was filtered through 0.45 μm fluoroarbon filter (FHUP, Millipore, Bedford, MA) and the buffer was filtered with a 0.22 μm polyvinylpyrrolidone filter (GV, Millipore, Bedford MA). This quaternary ammonium salt acted as an ion-pairing reagent, since ciprofloxacin contains a basic amine group an exhibits severe peak tailing in the absence of such additives. Samples and standards were filtered using 0.2 μm syringe filters (MV, Millipore, Bedford, MA). Standards of concentrations ranging from 1 to 10 μg/ml were prepared from ciprofloxacin hydrochloride powder. 5 μl of solution was injected with a mobile phase flow rate of 1.0 ml/min.

3.4.1.3 Gradient Separation of Degradation Products

Incubation solutions from the degradation of the drug polymer contained enzyme and enzyme breakdown products. Prior to injection, the solutions were filtered using UF-CL (Millipore Corp.) centrifugal filtration units with nominal molecular weight cutoff of 5000. Samples were filtered for 2-4 hours at 3000 rpm (IEC Clinical Centrifuge, Needham, MA). The effectiveness of this technique in removing enzyme was reported by Wang et al. (1997). Two gradient methods were developed to analyze the degradation products.

The gradient A method used a mobile phase consisting of acetonitrile (solvent A), and 0.1M KH₂PO₄ adjusted to pH 2.5 with 85% orthophosphoric acid (Solvent B). Solvent C consisted of HPLC grade H₂O, which was used to flush the column of the buffer salts. The gradient program is shown in Table 3.3. The flowrate was 1 ml/min with a back pressure of approximately 600 psi.
Table 3.3  HPLC Gradient Method A

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (ml)</th>
<th>% A</th>
<th>% B</th>
<th>% D</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>10</td>
<td>90</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>70</td>
<td>30</td>
<td>0</td>
<td>Linear</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
<td>70</td>
<td>30</td>
<td>0</td>
<td>Linear</td>
</tr>
<tr>
<td>41</td>
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<td>0</td>
<td>100</td>
<td>Linear</td>
</tr>
<tr>
<td>51</td>
<td>2</td>
<td>100</td>
<td>0</td>
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<td>1</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>Linear</td>
</tr>
</tbody>
</table>

In order to isolate fractions for mass spectrometry, a different mobile phase was required since phosphate salts would contaminate the spectra. The mobile phase was prepared as follows, acetonitrile (solvent A), and 2mM Ammonium Acetate adjusted to pH 2.7 with acetic acid (solvent B), and H₂O (solvent C). However, preliminary mass spectroscopy analysis showed multiple ions for some of the chromatogram peaks obtained with the HDI based polymers. Therefore, in order to resolve these peaks, the mobile phase was adjusted to methanol (solvent A), 2mM Ammonium Acetate adjusted to pH 2.7 with acetic acid (solvent B). The conditions for this method, which will subsequently be referred to as gradient B, are shown in Table 3.4.

Table 3.4  HPLC Gradient Method B

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (ml)</th>
<th>% A</th>
<th>% B</th>
<th>% C</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>10</td>
<td>90</td>
<td>0</td>
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<td>100</td>
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<td>2</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>Linear</td>
</tr>
</tbody>
</table>
3.4.2 Mass Spectrometry

Mass Spectrometry is an analytical technique used for determining the structure and molecular mass of chemical compounds. The application of this highly sensitive method has been enhanced by its coupling with chromatographic techniques, initially gas chromatography (GC/MS), and more recently with liquid chromatography (LC/MS).

An LC coupled mass spectrometer separates analytes by their mass-to-charge ratio. The compound eluting from the end of the liquid chromatograph is first vapourized at the inlet of the mass spectrometer. The molecules are ionized by a variety of techniques, such as ion spray, where the mobile phase is pumped through a capillary and the liquid is evaporated off. The ions are then accelerated through a potential, and are separated by focusing with an electromagnet, such as a quadrupole. The entire molecular spectrum can be scanned and recorded by tuning the quadrupole. In tandem mass spectrometry (MS/MS) which was used in this study, a single parent ion is selected and is induced to fragment further by collision with a gas (collision induced dissociation, or CID). The resulting mass spectrum of ion fragments yields a “fragmentation pathway” which can be used to deduce the structure of the original molecular ion. This arrangement is particular useful with soft ionization techniques such as ion spray, where little fragmentation occurs initially (Johnstone and Rose, 1996). A schematic diagram of a triple quadrupole mass spectrometer is given in Figure 3.7.

![Schematic of a triple quadrupole tandem mass spectrometer](image)

Figure 3.7 Schematic of a triple quadrupole tandem mass spectrometer (Yost and Enke, 1983).

Mass spectrometry was carried out at the Mass Spectrometry Laboratory at the Carbohydrate Research Centre, University of Toronto. A Perkin-Elmer/Sciex (Concord, ON) API-III triple
quadrupole, ionspray mass spectrometer was used. The mobile phase was pumped at a flowrate of 0.02 ml/min using an LKB Bromma (Sweden) HPLC pump. The mobile phase was a solution of 50% acetonitrile and 50% 1 mM ammonium acetate, and 0.1% acetic acid. The voltage applied to the tip of the ion spray needle (where the ions are produced) was 5 kvolts and the voltage applied to the orifice (where the ions pass into the mass spectrometer) is 80 volts. 1 to 20 µl were injected directly, depending on the concentration of the products.

The ion spray spectrometer gives a spectrum of protonated molecular ions (MH+) in the sample. Additional adducts of the molecular ions were produced (MNa+, MK+, MNH4+) as they were present in the mobile phase or sample.

Tandem Mass Spectroscopy (MS/MS) was also performed on each selected parent ion. The pressure of the argon collision gas in the second quadrupole of the system was set to 200 torr. Mass spectrum results were plotted as relative ion intensity vs. mass-to-charge (m/z) ratio. The accuracy of m/z measurements in the mass range 100 to 2000 amu is better than ±0.5 amu.

3.4.2.1 Sample Preparation

Mass spectroscopy of the degradation products was determined by the following procedure. HPLC separation was carried out according to section 3.4.1.3. Each product peak was collected in 15 ml polypropylene vials (Corning, Fisher Scientific, Unionville, ON). Evaporation of the organic phase was carried out in a N2 stream, and the remaining mobile phase was freeze-dried overnight. The product was reconstituted with 5 to 20 µl prior to injection into the mass spectrometer.

3.4.3 Antimicrobial Activity Assay

Antimicrobial sensitivity testing is necessary to determine the in-vitro activity of new antibacterial agents. Standardized procedures have been developed and two such methods
are the agar diffusion method, and the dilution method (broth or agar) (NCCLS standard, 1993). In the agar diffusion test, the antibacterial agent is allowed to diffuse from a point source, typically a paper disc, into an agar medium which has been seeded with the test organism. A zone of inhibition will be formed around the disk, and the size of the zone can be correlated to minimum inhibitory concentration (MIC), i.e., the minimum concentration of agent required to inhibit the growth of the microorganism (Amsterdam, 1998). However, there are limitations associated with the agar diffusion method. The size of the zone can be affected by such properties such as solubility, ionic charge, and molecular size (Greenwood). The growth rate of the bacteria may affect the zone. For these reasons the broth dilution technique is preferred and was selected for use in this study.

In the broth dilution assay, serial two-fold dilutions of the antibacterial agent are prepared in Mueller-Hinton broth medium (a standard in the USA). In the macrodilution assay, one ml volumes are used, but a micro-method is also commonly applied with 100 μl volumes in a microtitration tray. The test solution is incubated at 37°C overnight and the end-point is read as the concentration of antibiotic in which no turbidity is visually observed. This concentration is reported as the MIC value for that particular antibiotic versus the test organism. Three controls are prepared, an uninoculated tube containing broth plus antibiotic and broth alone as sterility controls, and an antibiotic free tube inoculated with the test organism to check for growth.

The broth dilution assay was applied to each aliquot of incubation solution removed from the biodegradation testing of the drug polymers. The assay was carried out in the laboratory of Dr. Marc Mittelman at the Centre for Infection and Biomaterials Research, Toronto Hospital, General Division. *Pseudomonas aeruginosa* was used as the test organism, due to its clinical relevance in medical device infections, and its susceptibility to ciprofloxacin (see Table 2.2). Two or three colonies of a clinically isolated *Pseudomonas aeruginosa* (Toronto Hospital, General Division) were transferred to 25 ml of Tryptic Soy Broth (Difco Laboratories, Detroit, MI) in a sterile 50 ml centrifuge tube, and incubated on a shaking incubator for 18-24 hours at 37°C. The ensuing culture was centrifuged for 10 minutes at 3200 rpm (Beckman)
and the broth discarded. The culture was washed three times with phosphate buffered saline (PBS), pH 7.4, by centrifuging and replacing the buffer between each wash. The bacteria were resuspended in PBS and diluted to 0.5 McFarland turbidity standard visually. The exact number of colony forming units (CFU) per ml was determined by conducting a plate count. Six 10-fold dilutions in PBS were prepared from the 0.5 McFarland bacterial cell suspension. Three drops of 20 µl of each dilution were plated on nutrient agar (Difco Laboratories) and incubated for 18-24 h at 37°C. The number of colonies were determined from the dilution which exhibited discrete colonies. The initial concentration of bacteria in the 0.5 McFarland standard can then be calculated. The desired concentration range is between $8 \times 10^6$ and $2 \times 10^7$ CFU/ml.

In the next step, 100 µl of Mueller-Hinton broth (Difco Laboratories) was dispensed into each well of a 96-well Microtiter plate (Costar, Cambridge, MA). 100 µl of the unfiltered incubation solution was added to the first well. Six serial two-fold dilutions were made with a 100 µl micro pipettor (Eppendorf, Brinkmann, Westbury, NY). Each well was mixed during the dilutions by withdrawing and replacing the solution 10 times with the pipettor. 10 µl of the 0.5 McFarland cell suspension was added to each well. An additional well of 100 µl of broth alone was innoculated to serve as a control. Two control wells were run consisting of 100 µl of broth alone and broth plus 10 µl of the inoculum. The trays were wrapped in parafilm and enclosed in a sealed container with wet paper towel to prevent drying. The containers were incubated for 18-24 hours at 37°C. Each well was recorded as turbid (growth) or clear (no growth) visually by using the control well as the measure of turbidity.
4.0 RESULTS

4.1 Drug Polymer Synthesis

The synthesis of the drug polymers required experimentation with reagents and reaction conditions in order to assess the feasibility of incorporating ciprofloxacin into the polymer backbone. A series of drug polymers was synthesized based on two diisocyanates, HDI and DDI, and two oligomers, polycaprolactone diol (PCL) and poly(oxyethylene) diamine (PPE). Although the primary objective of the synthesis procedure was to develop a formulation which was susceptible to enzymatic degradation and allow for the release of ciprofloxacin, consideration was also given to developing a material that could be used as a stable coating or formed object for subsequent testing under in vitro and in vivo conditions. Table 4.1 summarizes the reagents and reaction conditions of the twenty drug polymers synthesized.

While a variety of different conditions and formulations were attempted, many of the reactions were visually similar, with only a few differences (Table 4.1). In each case, the addition of ciprofloxacin produced a yellow reaction solution. Triethylamine (TEA), which was used in some of the reactions (see discussion), was added to assist in the dissolution of ciprofloxacin, and caused the reaction solution to instantly turn a deep yellow colour upon mixing with the drug. The two-step reactions produced clear, colourless reaction solutions during the first step for the reaction of the diisocyanate with both PCL or PPE. A noticeable increase in solution viscosity was observed over the 3 hour prepolymer step.

In many of the reactions, precipitates were observed in the reaction solution, indicating insoluble products (most probably polymeric in nature) were being generated. Reactions carried out with a drug—diisocyanate first step turned cloudy after approximately 1.5 hours. Reactions with a diisocyanate—soft segment first step remained in solution for the prepolymer step. However, for the second stage of the latter reaction, solution cloudiness was observed for systems with DDI at a reaction temperature of 60°C overnight. HDI
<table>
<thead>
<tr>
<th>Polymer Formulation</th>
<th>Reagents, (Reaction time, temperature)</th>
<th>Catalyst Amount (mg)</th>
<th>Yield, g (Yield, %)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDI/PPE-900(1)</td>
<td>I: HDI/cipro (2 hrs, 70°C) II: PPE (overnight, 25°C)</td>
<td>6.5</td>
<td>0.235 (12.3%)</td>
<td>I: solution precipitates after 1.5 hrs</td>
</tr>
<tr>
<td>HDI/PPE-900(2)</td>
<td>I: HDI/PPE (2 hrs, 45°C) II: cipro (2 hrs, 60°C)</td>
<td>5.7</td>
<td>0.041 (0.02%)</td>
<td>I: solution remains clear II: precipitates upon cooling</td>
</tr>
<tr>
<td>HDI/PPE-900(3)</td>
<td>I: HDI/PPE (2 hrs, 45°C) II: cipro (13 hrs, 60°C)</td>
<td>6</td>
<td>0.245 (12.9%)</td>
<td>I: solution remains clear II: precipitates form &gt; 2 hrs</td>
</tr>
<tr>
<td>HDI/PPE-900(4)</td>
<td>I: HDI/PPE/cipro (22 hrs 65°C)</td>
<td>6</td>
<td>N/A</td>
<td>I: solutions remains clear, precipitates upon cooling</td>
</tr>
<tr>
<td>DDI/PPE-900(5)</td>
<td>I: DDI/PPE/cipro (15 hrs 65°C)</td>
<td>6</td>
<td>0.709 (33.9%)</td>
<td>I: precipitates form &gt; 2hrs</td>
</tr>
<tr>
<td>DDI/PPE-900(6)</td>
<td>I: DDI/PPE/cipro (26 hrs 65°C)</td>
<td>60</td>
<td>N/A</td>
<td>I: precipitates form &gt; 1.5-2 hrs</td>
</tr>
<tr>
<td>DDI/PPE-900(7)</td>
<td>I: DDI/PPE/cipro (19 hrs 60°C)</td>
<td>60</td>
<td>1.17 (55.8%)</td>
<td>I: precipitates form at 2 hours</td>
</tr>
<tr>
<td>DDI/PPE-900(8)</td>
<td>I: DDI/PPE/cipro/TEA (19 hrs 65°C)</td>
<td>60</td>
<td>1.10 (57.9)</td>
<td>I: TEA turns cipro solution yellow precipitates form &gt; 5hours</td>
</tr>
<tr>
<td>DDI/PCL-2000(9)</td>
<td>I: DDI/PCL/cipro/TEA (19 hrs 65°C)</td>
<td>60</td>
<td>1.97 (65.8%)</td>
<td>I: precipitates form after 1.5 hrs</td>
</tr>
<tr>
<td>DDI/PCL-2000(10)</td>
<td>I: DDI/cipro/TEA (1.5 hrs, 65°C) II: PCL (20.5 hrs, 65 C)</td>
<td>60</td>
<td>1.97 (65%)</td>
<td>I: precipitates form after 1.25 hrs</td>
</tr>
</tbody>
</table>
Table 4.1  continued…

<table>
<thead>
<tr>
<th>Formula</th>
<th>Reagents (Time, hours ; temperature,°C)</th>
<th>Catalyst Amount (mg)</th>
<th>Yield (%)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDI/PCL-2000(11)</td>
<td>I: DDI/PCL (3 hrs, 65°C) II: cipro/TEA (20.5 hrs, 65°C)</td>
<td>60</td>
<td>1.08</td>
<td>I: clear colourless solution II: precipitates form after cooling to room temperature?</td>
</tr>
<tr>
<td>DDI/PCL-2000(12)</td>
<td>I: DDI/PCL (3 hrs, 65°C) II: cipro/TEA (40 hrs, 65°C)</td>
<td>60</td>
<td>1.86</td>
<td>I: clear colourless solution II: cloudy next day</td>
</tr>
<tr>
<td>DDI/PCL-2000(13)</td>
<td>I: DDI/PCL (3 hrs, 65°C) II: cipro/TEA (20 hrs, 65°C)</td>
<td>60</td>
<td>1.88</td>
<td>I: clear colourless solution II: cloudy next day</td>
</tr>
<tr>
<td>HDI/PCL-2000(14)</td>
<td>I: HDI/PCL (3 hrs, 65°C) II: cipro/TEA (22 hrs, 65°C)</td>
<td>60</td>
<td>1.82</td>
<td>I: clear colourless solution II: precipitates form upon cooling to room temperature</td>
</tr>
<tr>
<td>[14C]-HDI/PCL-2000(15)</td>
<td>I: HDI/PCL (3 hrs, 65°C) II: cipro/TEA (22 hrs, 65°C)</td>
<td>60</td>
<td>2.265</td>
<td>I: clear colourless solution II: precipitates form upon cooling to room temperature</td>
</tr>
<tr>
<td>HDI/PPE-900(16)</td>
<td>I: HDI/PPE (3 hours 65°C) II: cipro/TEA (22 hrs, 65°C)</td>
<td>60</td>
<td>N/A</td>
<td>I: clear colourless solution II: turns cloudy upon cooling to room temperature</td>
</tr>
<tr>
<td>HDI/PPE-900(17)</td>
<td>I: HDI/PPE-900 (3 hrs, 65°C) II: cipro/TEA (22 hrs, 65°C)</td>
<td>60</td>
<td>0.505</td>
<td>I: clear colourless solution II: turns cloudy upon cooling to room temperature</td>
</tr>
<tr>
<td>HDI/PPE-2000(18)</td>
<td>I: HDI/PPE-2000 (3 hrs, 65°C) II: cipro/TEA (22 hrs, 65°C)</td>
<td>60</td>
<td>0.389</td>
<td>I: clear colourless solution II: turns cloudy upon cooling to room temperature</td>
</tr>
<tr>
<td>HDI/PCL-1250(19)</td>
<td>I: HDI/PCL-1250 (3 hrs, 65°C) II: cipro/TEA (20 hrs, 65°C)</td>
<td>60</td>
<td>1.11</td>
<td>I: clear colourless solution II: turns cloudy upon cooling to room temperature</td>
</tr>
<tr>
<td>[14C]-HDI/PCL-2000(20)</td>
<td>I: HDI./PCL-2000 (3 hrs, 65°C) II: cipro/TEA (22 hrs, 65°C)</td>
<td>60</td>
<td>0.924</td>
<td>I: clear colourless solution II: turns cloudy upon cooling to room temperature</td>
</tr>
</tbody>
</table>
based polymers remained clear at 60°C for the whole reaction period, but turned cloudy when cooled to room temperature. All the one-step reactions became cloudy, regardless of which combination of diisocyanate or oligomeric segment was used. DDI/PPE-900 polymers became cloudy after approximately 2 hours, while this was extended to 5 hours with the use of TEA (DDI/PPE-900(8)). The DDI/PCL-2000(10) reaction in the presence of TEA became cloudy after approximately 1.25 hours.

The physical appearances of each class of polymer are highlighted in Table 4.2. There were very few visual differences from batch to batch synthesis of a specific formulation. PCL based drug polymers appeared softer, while the PPE based drug polymers appeared harder, and had a darker yellow colour. There was a slight difference between DDI and HDI based polymers, with the DDI ones appearing to be physically stronger, although no quantitative measurement of these features was assessed.

**Table 4.2   Physical Appearance of Drug Polymers**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Physical appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDI/PPE-900</td>
<td>deep yellow, dense, sticky, brittle</td>
</tr>
<tr>
<td>DDI/PPE-900</td>
<td>dark yellow, dense, brittle</td>
</tr>
<tr>
<td>HDI/PCL-2000</td>
<td>pale yellow, elastomeric</td>
</tr>
<tr>
<td>DDI/PCL-2000</td>
<td>pale yellow, elastomeric</td>
</tr>
<tr>
<td>HDI/PCL-1250</td>
<td>pale yellow, slightly rubbery</td>
</tr>
<tr>
<td>HDI/PPE-2001</td>
<td>dark yellow, dense, brittle</td>
</tr>
</tbody>
</table>

**4.1.2 Polymer Precipitation and Residual Free Drug Analysis**

Polymers synthesized with the PPE oligomer were difficult to recover from the reaction solution. As the reaction mixtures were added to the washing solution (i.e. double distilled water), a cloudy yellow suspension was formed, composed of very fine particles. PCL based polymers formed larger white precipitates in the water. Some of the latter reaction mixtures
precipitated into larger pellets which subsequently broke down into finer particles after stirring in the wash water.

For the materials synthesized with higher batch numbers than (8) (i.e DD1/PPE-900(8)), HPLC analysis of the wash water for free ciprofloxacin was conducted on each day of the washing period (i.e. three days). Table 4.3 reports the residual drug values. While this value does not precisely define the actual amount of free drug remaining in the polymer, it does provide an approximate indication of the relative presence of residual drug. For most of the polymers, the residual drug decreased marginally over the three day period, however, some increased on the second day (batches 8 and 17). HDI/PCL-2000(14) showed a much higher residual drug concentration in comparison to the other polymers. It should be noted that HDI/PCL-2000(20) is a repeat synthesis of HDI/PCL-2000(14). Since this former polymer showed residual levels within the range of the other polymers, it can be concluded that the high levels of drug for HDI/PCL-2000(14) are unique to the washing samples of this particular batch and not to the formulation in general. Factors that were suspected to contribute to this observation will be discussed on in Section 5.2.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Residual ciprofloxacin (µg/g polymer)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 1</td>
</tr>
<tr>
<td>HDI/PPE-900(17)</td>
<td>14.16</td>
</tr>
<tr>
<td>HDI/PPE-2001(18)</td>
<td>18.10</td>
</tr>
<tr>
<td>DDI/PPE-900(8)</td>
<td>12.03</td>
</tr>
<tr>
<td>DDI/PCL-2000(9)</td>
<td>6.79</td>
</tr>
<tr>
<td>DDI/PCL-2000(10)</td>
<td>6.93</td>
</tr>
<tr>
<td>DDI/PCL-2000(11)</td>
<td>115.5</td>
</tr>
<tr>
<td>DDI/PCL-2000(12)</td>
<td>6.36</td>
</tr>
<tr>
<td>DDI/PCL-2000(13)</td>
<td>6.19</td>
</tr>
<tr>
<td>HDI/PCL-2000(14)</td>
<td>10688</td>
</tr>
<tr>
<td>HDI/PCL-2000(20)</td>
<td>17.79</td>
</tr>
<tr>
<td>HDI/PCL-1250(19)</td>
<td>8.18</td>
</tr>
</tbody>
</table>
4.1.3 Gel Permeation Chromatography

Gel permeation chromatography data are provided in Table 4.4, as polystyrene equivalent molecular weights. Weight average molecular weights ($\bar{M}_w$) of the soluble materials ranged from $1.3 \times 10^4$ to $1.6 \times 10^4$. In general, the PPE based polymers showed broader distributions, as indicated by polydispersity values of 1.3-1.5 for PPE polymers versus 1.2 - 1.6 for the PCL series.

Several of the polymers were difficult to dissolve (See Table 4.4) at the selected concentration for GPC analysis (0.2 wt%), and had to be heated and/or vigourously stirred. The PCL based polymers dissolved more easily than the PPE based polymers, although there were still some remaining insoluble particles. Since filtering was carried out prior to injecting the samples, it should be noted that the molecular weight values reflect only the soluble polymer segments.

### Table 4.4 Molecular Weights of Drug Polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$\bar{M}_n$ ($10^4$)</th>
<th>$\bar{M}_w$ ($10^4$)</th>
<th>Polydispersity</th>
<th>Solution appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDI/PPE-900(17)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>insoluble</td>
</tr>
<tr>
<td>HDI/PPE-2000(18)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>insoluble</td>
</tr>
<tr>
<td>DDI/PPE-900(5)</td>
<td>1.3</td>
<td>1.8</td>
<td>1.3</td>
<td>cloudy</td>
</tr>
<tr>
<td>DDI/PPE-900(7)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>insoluble</td>
</tr>
<tr>
<td>DDI/PPE-900(8)</td>
<td>2.0</td>
<td>3.0</td>
<td>1.5</td>
<td>cloudy</td>
</tr>
<tr>
<td>DDI/PCL-2000(9)</td>
<td>1.3</td>
<td>1.6</td>
<td>1.3</td>
<td>cloudy</td>
</tr>
<tr>
<td>DDI/PCL-2000(10)</td>
<td>1.3</td>
<td>1.7</td>
<td>1.2</td>
<td>cloudy</td>
</tr>
<tr>
<td>DDI/PCL-2000(11)</td>
<td>2.0</td>
<td>2.8</td>
<td>1.4</td>
<td>slightly cloudy</td>
</tr>
<tr>
<td>DDI/PCL-2000(12)</td>
<td>1.5</td>
<td>2.4</td>
<td>1.6</td>
<td>slightly cloudy</td>
</tr>
<tr>
<td>DDI/PCL-2000(13)</td>
<td>1.4</td>
<td>2.3</td>
<td>1.3</td>
<td>slightly cloudy</td>
</tr>
<tr>
<td>HDI/PCL-2000(14)</td>
<td>1.5</td>
<td>2.4</td>
<td>1.6</td>
<td>slightly cloudy</td>
</tr>
<tr>
<td>[14C]-HDI/PCL-2000(20)</td>
<td>1.3</td>
<td>2.0</td>
<td>1.6</td>
<td>slightly cloudy</td>
</tr>
<tr>
<td>HDI/PCL-1250(19)</td>
<td>1.0</td>
<td>1.6</td>
<td>1.6</td>
<td>solution</td>
</tr>
</tbody>
</table>

-- = not detected
Figure 4.1 illustrates the chromatograms obtained for PPE based polymers. DDI/PPE-900(5) exhibits a bimodal peak, while DDI/PPE-900(7) synthesized with 10 times more catalyst, did not dissolve in the mobile phase. Filtration and injection of this polymer produced a flat chromatogram (Figure 4.1). DDI/PPE-900(8), synthesized with triethylamine, shows the highest molecular weight (i.e. shortest retention time for peak and onset) of the PPE series of polymers. Injection of pure mobile phase shows that the sharp peak at 25 minutes followed by the negative deflection are caused by mobile phase impurities or moisture. Figure 4.2 illustrates several of the PCL based chromatograms. HDI/PCL-2000(12) and DDI/PCL-2000(14) (2-step syntheses) exhibit skewed distributions, while DDI/PCL-2000(9) and (10) (one-step syntheses) show more gaussian shaped distributions.

Figure 4.1  GPC chromatograms of PPE based drug polymers.
4.1.4 Elemental Analysis

Elemental analysis for carbon, hydrogen, nitrogen, fluorine and tin was conducted on selected polymers and the data are given in Table 4.5. The fluorine content, representative of the drug incorporation, ranged from 0.1% to 2.64%. It was noted that the nitrogen content for the PPE polymers was higher, which reflects, in part, the presence of nitrogen groups in the PPE oligomer. Oxygen content could not be determined by Guelph Chemical Laboratories (GCL) due to the interference of fluorine with their analysis protocol. HDI/PCL-2000(12) and DDI/PCL-2000(14), which were analyzed twice (Table 4.5), show that the fluorine values can deviate by about 0.3%. For example, the fluorine content measurements for DDI/PCL-2000(12) exhibits values of 0.72% and 0.4% respectively. The deviation represents approximately a 50% error in the values and therefore puts into question the quantitative value of the GCL data. This large variation may reflect either inhomogeneity in the polymer sample or variability of the analytical technique. Guelph Chemical Labs, where the polymers were sent for analysis, reported that the polymer samples appeared inhomogenous. Elemental
analysis for each of the polymers should have been repeated; however, the high cost of this service prohibited this.

Tin content was analyzed for a few polymers in order to determine the presence of residual catalyst. There was some residual tin, associated with the catalyst, for polymers HDI/PPE-900(17) and HDI/PPE-2000(18) (0.2% and 0.19% respectively).

Table 4.5  Elemental Analysis of Selected Polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Carbon %</th>
<th>Hydrogen %</th>
<th>Nitrogen %</th>
<th>Fluorine %</th>
<th>Tin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDI/PPE-900(17)</td>
<td>42.48</td>
<td>7.69</td>
<td>10.00</td>
<td>2.64</td>
<td>0.2</td>
</tr>
<tr>
<td>DDI/PPE-900(5)</td>
<td>56.22</td>
<td>8.75</td>
<td>6.75</td>
<td>0.76</td>
<td>-</td>
</tr>
<tr>
<td>DDI/PPE-900(7)</td>
<td>59.59</td>
<td>10.54</td>
<td>7.71</td>
<td>0.83</td>
<td>-</td>
</tr>
<tr>
<td>DDI/PPE-900(8)</td>
<td>59.46</td>
<td>9.47</td>
<td>7.33</td>
<td>0.66</td>
<td>-</td>
</tr>
<tr>
<td>HDI/PPE-2000(18)</td>
<td>47.20</td>
<td>7.90</td>
<td>10.77</td>
<td>2.52</td>
<td>0.19</td>
</tr>
<tr>
<td>DDI/PCL-2000(12)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.72</td>
<td>-</td>
</tr>
<tr>
<td>*DDI/PCL-2000(12)</td>
<td>64.05</td>
<td>9.12</td>
<td>5.50</td>
<td>0.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HDI/PCL-2000(14)</td>
<td>48.70</td>
<td>9.44</td>
<td>2.64</td>
<td>&lt;0.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>*HDI/PCL-2000(14)</td>
<td>61.33</td>
<td>8.98</td>
<td>3.06</td>
<td>0.33</td>
<td>-</td>
</tr>
<tr>
<td>HDI/PCL-1250(19)</td>
<td>60.38</td>
<td>8.12</td>
<td>5.45</td>
<td>1.00</td>
<td>-</td>
</tr>
</tbody>
</table>

-= not analyzed, * = repeat analysis, <0.05 = not detected

4.2  Biodegradation Studies and Isolation of Polymer Breakdown Products

4.2.1  High Performance Liquid Chromatographic (HPLC) Separation

Numerous methods for the HPLC analysis of ciprofloxacin and its metabolites have been published (Gau et al., 1985; Mack, 1993; Fasching et al.; 1986; Krol et al., 1986). However, incubation solutions from the biodegradation experiment contained two potentially interfering products, the enzyme and other polymer degradation products. Therefore, several HPLC methods were developed in parallel with the biodegradation studies. As described in section 3.4.1.3, three separation methods were optimized for these studies. An isocratic method was used to quantify free drug levels during the purification step of the synthesis.
(Table 4.3). However, this method was inadequate for the analysis of the biodegradation samples, due to the presence of interfering enzyme peaks (Figure 4.3). Therefore a gradient method was developed as described in section 3.4.1.3 on page 54 (gradient A). An additional gradient method, gradient B, was used to provide enhanced peak separation for the selection of samples during identification of biodegradation products by mass spectrometry. For each HPLC method, standards were run with pure ciprofloxacin powder, and curves were linear in the 0.1-80 µg/ml range (see Appendix E for sample calibration curves).

![Representative chromatogram of the isocratic separation of ciprofloxacin from degradation solutions. DDIPPE-900(8) was incubated with cholesterol esterase (CE), 37°C, pH 7.0, for 17 days.](image)

While biodegradation studies were carried out for several polymers, only a select number of these studies underwent extensive HPLC analysis due to limitations in time and resources. HPLC analysis was conducted for the following three formulations, using gradient A. DDI/PPE-900(8), DDI/PCL-2000(12), and HDI/PCL-2000(14); gradient B was used for [14C]-HDI/PCL-2000(20).
Figure 4.4 shows representative chromatograms of DDI/PPE-900(8) after 10 days incubation with and without CE. A ciprofloxacin standard and a CE control are also shown. The retention time of ciprofloxacin is approximately 17 minutes. There are two peaks associated with the enzyme treated control samples which appear at 3 and 11 minutes. The chromatograms show no significant difference between enzyme and buffer treated samples.

![Figure 4.4](image)

**Figure 4.4**  HPLC chromatograms of DDI/PPE-900(8) polymer incubated for 10 days, $37^\circ$C, pH 7, in (a) enzyme, (b) buffer, (c) ciprofloxacin standard, (d) enzyme control.

Figure 4.5 shows representative chromatograms of a DDI drug polymer (DDI/PCL-2000(12)) after 7 days incubation with and without CE. The results are similar to DDI/PPE-900, exhibiting similar peaks, although at different intensities. It is noted that when the polymer is incubated in the presence of CE there is a significantly enhanced release of ciprofloxacin as compared to when incubation is only carried out in buffer.
Figure 4.5  HPLC chromatograms of (a) enzyme control, DD/PCL-2000(12) polymer incubated for 7 days, 37°C, pH 7, in (b) enzyme, (c) buffer, (d) ciprofloxacin standard.

UV absorbance spectra of the products (DD/PCL-2000(12)) under the peak at 17 minutes in enzyme (chromatogram a, Figure 4.5) and buffer (chromatogram b, Figure 4.5) treated samples along with the ciprofloxacin standard (chromatogram c, Figure 4.5), are shown in Figure 4.6. The spectra are identical, thus confirming that the 17 minute peak is likely related to pure ciprofloxacin. There were no other breakdown products (peaks) detected for either DD/PPE-900(8) (Figure 4.4) or DD/PCL-2000(12) (Figure 4.5).

Figure 4.6  UV absorbance spectra for products from DD/PCL-2000(12), (a) enzyme treated sample (17 min.) (b) buffer treated sample (17 min.) (c) ciprofloxacin standard (17 min).
Representative chromatograms for HDI/PCL-2000(14) are shown in Figure 4.7. As in the case of the DDI based polymer (Figure 4.5), the retention time of ciprofloxacin appears at 17 minutes. This system differed from DDYPCL-2000(12) in that additional peaks were detected for HDI/PCL-2000(14) samples which were incubated with CE. These products were found at retention times of 22, 23, and 30 minutes as well as an unresolved twin peak at 26 minutes. None of these products were isolated in the polymer sample incubated with buffer only.

![HPLC Chromatograms](image)

**Figure 4.7**  HPLC chromatograms for HDI/PCL-2000(14) incubated at 37°C, pH 7, for 10 days analyzed by gradient method A.

Figure 4.8 shows a representative chromatogram of HDI/PCL-2000(14) using gradient method B. A comparison of this chromatogram with Figure 4.8 reveals that several products were co-eluting at 30 minutes in Figure 4.7. A total of seven products were found using gradient B. These products are labelled as fractions 1-7 in Figure 4.8. Figure 4.8 also contains the UV spectra for each of these products. While the different spectra (Figure 4.8) have similar characteristics to the spectrum of pure ciprofloxacin, some do contain additional absorbance maxima at 213, 218 or 246 nm. This suggests that these are derivatives from the polymer which contain the drug component. Figure 4.8 also shows a chromatogram of a
mobile phase blank injection (bottom chromatogram). From this, it can be seen that peak 3 is, in part, related to a mobile phase or sample buffer impurity. The UV spectrum of peak 3 is also identical to the UV spectrum of the peak appearing in the blank injection (Figure 4.8). However, as will be shown in Section 4.2.4 (page 86), mass spectrometry of this fraction showed that in addition to the impurities that were contained under the peak, there were also products which were related to the polymer. These latter products were not present upon analysis of the mobile phase impurity peak.
Figure 4.8  HPLC separation (Gradient B) of products associated with the incubation of DDI/PCL-2000(14) in CE, pH=7.0, 10 days. Samples were collected for subsequent mass spectroscopic analysis.
4.2.2 Release Profiles for Ciprofloxacin and Other Degradation Products

The amount of free drug released for each of the incubation experiments is shown in Figures 4.9-4.12. Ciprofloxacin release is expressed as the cumulative total drug released per total surface area of the polymer. The data for DDI/PPE-900(8) (Figure 4.9) show no significant difference between enzyme and buffer treated samples. The highest release was over the first 2 days, at a level of 3.52 µg/cm² (CE) and 3.33 µg/cm² (buffer). This was followed by a gradual increase to a level of 5.17 µg/cm² (CE) and 5.21 µg/cm² (buffer) over an additional 15 days. Visual inspection of the samples during the biodegradation experiment showed that the films were peeling off the coated glass tubes. There were small particles of polymer visible at the bottom of each vial. This lack of adherence to the glass tubes was attributed in part to the hydrophilic nature of the PPE-900 oligomeric component.

![Graph](image)

Figure 4.9  Cumulative drug release for HDI/PPE-900(8).

The results of the incubation experiment for DDI/PCL-2000(12) (Figure 4.10) show a significant enzyme specific effect on the release of free ciprofloxacin. At the first time point (7 days), the amount of drug released was 0.19 µg/cm² for CE incubated samples compared to 0.07 µg/cm² for buffer alone ($P < 0.05$). A lower release rate was subsequently monitored.
up to 28 days. It was noted that the ratio of enzyme to buffer release decreased for the duration of the experiment going from approximately 3 at day 7 to 1.7 for day 28. Upon inspection of the coated tubes, there was no visible erosion of the polymer, only a noticeable yellowing of the solution. This was likely due to a build-up of enzyme associated breakdown products, since the non-polymer enzyme controls showed a similar discolouration.

![Graph](image)

**Figure 4.10** Cumulative drug release for DDI/PCL-2000(12).

The incubation of HDI/PCL-2000(14) (Figure 4.11) showed no significant difference between enzyme and buffer controls ($P > 0.05$) although the level of drug release was higher than that of the DDI analog (Figure 4.10). This result was reproduced for the radiolabelled analog (HDI/PCL-2000(20), Figure 4.12).
Figure 4.11  Cumulative drug release for HDI/PCL-2000(14).

Figure 4.12  Cumulative drug release for $^{14}$C-HDI/PCL-2000(20).
Figure 4.13 shows a plot of the cumulative release of the degradation products of HDI/PCL-2000(20) for 10, 20 and 30 days (fractions correspond to labelled fractions in Figure 4.8). The amounts of products are increasing over the 30 days for all compounds with the exception of fraction 7. It should be noted that fractions 6 and 7 are not completely resolved in the HPLC chromatogram (Figure 4.8), and hence the apparent lack of change for fraction 7 may have been caused by the inaccuracy in integrating and quantifying the peaks by computer analysis.

![Figure 4.13](image)

**Figure 4.13** Cumulative peak areas associated with product release from $[^{14}C]$-DDI/PCL-2000(20) incubated with CE, 37°C, pH 7, for 10, 20 and 30 days.

**4.2.3 $^{14}$C-radiolabelled Biodegradation and HPLC Separation**

In order to confirm polymer degradation, a radiolabelled analog of HDI/PCL-2000(14) was synthesized with $^{14}$C-HDI (HDI/PCL-2000(20)), and biodegradation testing was performed as described in Section 3.3. HDI/PCL-2000(20) had a similar $M_w$ to HDI/PCL-2000(14) (See Table 4.4). The cumulative radiolabel release profiles, following incubation in buffer and enzyme solutions, are given in Figure 4.14. The radiolabel release is initially high within the
first ten days and then increases more slowly. This trend parallels the observed trends in the release of ciprofloxacin with time (See Figure 4.12).

![Graph showing radiolabel release over time](image)

**Figure 4.14** $[^{14}C]$-HDI/PCL-2000(20) radiolabel release.

The radiochromatograms of HDI/PCL-2000(20) for the 10 day incubation samples in CE and buffer are shown in Figures 4.15 and 4.16 respectively. Elevated levels of radioactivity were related to fractions 2, 3, 4, 6, and 7, which were previously defined for HDI/PCL-2000(14) (Figure 4.8), when treated with CE. This indicates the presence of the HDI component in these products. This information was valuable for subsequent product identification studies of low UV absorbing products (Figure 5.12 in Section 5.3). As shown in Figure 4.15, there are retention times where there is a significant radiolabel release, but a weak UV absorbance (specifically, retention times of 48 and 54 minutes). The buffer control yielded background levels (i.e. 20-30 dpm) of radioactivity, thus showing that there is significantly less erosion of the polymer incubated with buffer as compared to treatment with cholesterol esterase. An additional fraction was collected an identified (fraction R in Figure 4.15) by mass spectrometry (Figure 5.12).
Figure 4.15  \[
[^{14}\text{C}]-\text{HDI/PCL-2000(20)} \text{ incubated for 10 days, with CE, pH}=7.0, \text{ radioactivity of separated products.}
\]

Figure 4.16  \[
[^{14}\text{C}]-\text{HDI/PCL-2000(20)} \text{ incubated for 10 days, without CE, pH}=7.0, \text{ radioactivity of separated products.}
\]
4.2.4 Identification of Products by Mass Spectrometry

The degradation products released during the biodegradation of HDI/PCL-2000(14) were identified by mass spectrometry (carried out by Doreen Wen at the Carbohydrate Research Centre, University of Toronto). HPLC gradient B (Section 3.4.1.3, page 54) was specifically used to minimize the interference of potassium phosphate ions with the generation of mass fragments in the mass spectrometer, as well as to improve the separation of products. References made to the product fractions in this section correspond to the fraction labels in Figure 4.8. The term “M” will be used to designate the molecular ion being analyzed.

The mass spectrum of the ciprofloxacin standard is shown in Figure 4.17. The protonated molecular ion [M+H]⁺ has a mass-to-charge (m/z) ratio of 332, while other complexed ions were produced at, [M+23] (associated with Na⁺), [M+41] (associated with acetonitrile), and [M+332] (self association). In order to assist with the identification of degradation product structure, tandem mass spectroscopy was conducted, and the MS/MS spectrum of the protonated ciprofloxacin peak at m/z 332 is shown in Figure 4.18. There are strong signals at m/z 314, 288, 245, 231, and 204. The fragment at m/z 314 corresponds to the loss of 18 atomic mass units (amu) (equivalent to a water molecule) from 332. The spectrum is characteristic of that previously reported for ciprofloxacin molecules (Gau et al., 1986).
Figure 4.17  Mass spectrum of ciprofloxacin standard, m/z 332.

Figure 4.18  MS/MS spectrum of ciprofloxacin standard m/z 332.
The mass spectra of the seven fractions from Figure 4.8 are shown in Figures 4.19-4.34. In comparison with the ciprofloxacin standard, the molecular ion intensities in each spectra are low, thus some background contamination peaks also appear. Table 4.6 lists the principle molecular ions associated with the polymer for each fraction. Multiple ions were detected as a result of the association of different cations found in the sample and mobile phase. Specifically, several products were associated with NH$_4^+$ (18) or Na$^+$ (23), and these are also listed in Table 4.6. In addition, the spectra of fractions 3, 4, 5, and 7 contained more than one molecular ion. In the latter case, each of these are designated by lowercase letters. For example, fraction 3 contained two compounds, labelled as product 3a and 3b.

**Table 4.6  Principle Ions of Degradation Products of HDI/PCL-2000(14)**

<table>
<thead>
<tr>
<th>Product</th>
<th>[M+H]$^+$</th>
<th>[M+NH$_4^+$]$^+$</th>
<th>[M+Na]$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>332</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>474</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3a</td>
<td>616</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3b</td>
<td>433</td>
<td>450</td>
<td>455</td>
</tr>
<tr>
<td>4a</td>
<td>606</td>
<td>--</td>
<td>628</td>
</tr>
<tr>
<td>4b</td>
<td>575</td>
<td>592</td>
<td>597</td>
</tr>
<tr>
<td>5a</td>
<td>749</td>
<td>766</td>
<td>771</td>
</tr>
<tr>
<td>5b</td>
<td>606</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>5c</td>
<td>632</td>
<td>--</td>
<td>654</td>
</tr>
<tr>
<td>6</td>
<td>632</td>
<td>654</td>
<td>--</td>
</tr>
<tr>
<td>7a</td>
<td>774</td>
<td>--</td>
<td>796</td>
</tr>
<tr>
<td>7b</td>
<td>632</td>
<td>--</td>
<td>654</td>
</tr>
</tbody>
</table>

-- = not detected

The mass spectrum of fraction 1 (Figure 4.19) which had an identical retention time to the ciprofloxacin standard (32 minutes), contains a single molecular ion with $m/z$ 332. The MS/MS spectrum of this product (Figure 4.20) confirms it to be ciprofloxacin, since the same dominant fragments that were associated with the pure drug (Figure 4.18) were also found at $m/z$ 314, 288, 245, 231.
Figure 4.19  Mass spectrum of fraction 1 (32 minutes).

Figure 4.20  MS/MS spectrum of product 1, m/z 332.

Fraction 2, shown in Figure 4.21, has a molecular ion (MH\(^+\)) with an m/z of 474. The MS/MS spectrum of the molecular ion is shown in Figure 4.22. The principal fragment ions
appear at \( m/z \) 456 (MH\(^+\)-H\(_2\)O), 438, 332, and 313. Again, the presence of the 332 molecular ion would suggest the presence of the drug component in this product.

**Figure 4.21** Mass spectrum of fraction 2.

**Figure 4.22** MS/MS spectrum of product 2, \( m/z \) 474.
The mass spectrum of fraction 3 shows two principal parent ions with \( m/z \) values of 616 and 433 (Figure 4.23). The MS/MS spectrum of product 3a (\( m/z \) 616) is shown in Figure 4.24. Major fragment ions appear at \( m/z \) 598, 474, and 456. The MS/MS spectrum of the second molecular ion, product 3b (\( m/z \) 433) is shown in Figure 4.25. Major fragment ions appear at \( m/z \) 97, 105, 115, 133, and 143.

![Mass spectrum of fraction 3](image1)

**Figure 4.23** Mass spectrum of fraction 3.

![MS/MS spectrum of product 3a](image2)

**Figure 4.24** MS/MS spectrum of product 3a, \( m/z \) 616.
The mass spectrum of fraction 4 also yields two principal ions. The m/z values of these two compounds are 606 and 575 (Figure 4.26). The MS/MS spectrum of product 4a (m/z of 606) is shown in Figure 4.27. Major fragments are present at m/z 588 (-H₂O), and 500 and 482, with a low abundance of fragments at m/z 332, 314 and 294. The MS/MS spectrum of the second molecular ion, product 4b (m/z 575) is shown in Figure 4.28. The major fragment ions appear at m/z 97, 115, 133, and 143, which are some of the same fragments found in product 3b. Additional fragments for the latter product also appear at m/z 443, 311, 275.
Figure 4.26  Mass spectrum of fraction 4.

Figure 4.27  MS/MS spectrum of product 4a, m/z 606.
Fraction 5 (Figure 4.29) contains three principal products, the largest parent molecular ion is $m/z$ 748. The MS/MS spectrum of product 5a is shown in Figure 4.30. The major fragments are found at $m/z$ of 731 ($M_s+H^+-H_2O$), 642, 500, 474, and 313. The other two molecular ions, 5b, $m/z$ 606, and 5c, $m/z$ 632 (654 is its Na$^+$ analog) are suspected to be residual products associated with fractions 4 and 6, since their MS/MS spectra were identical to those of product 4a (Figures 4.27) and 6 (Figure 4.32). These were collected since the peaks were not fully resolved by the HPLC separation (Figure 4.8).
Figure 4.29  Mass spectrum of fraction 5.

Figure 4.30  MS/MS spectrum of product 5a, m/z 749.
The MS spectrum of fraction 6 is shown in Figure 4.31. The product has an m/z of 632. Figure 4.32 shows the MS/MS spectrum, which contains major fragments at m/z 614, 596, 500, 482, 332, 314, 288 and 231. The presence of the latter four peaks imply the existence of the drug as a molecular component of this molecule, since the drug standard (Figure 4.18) contains the same fragments.
The mass spectrum of fraction 7 is shown in Figure 4.33. This fraction generated two molecular ions with m/z values of 774 and 632. The MS/MS spectrum of 7a (m/z 774) is shown in Figure 4.34. The fragments of interest appear at m/z 756, 642, 500, 474, 456, 332, 313 and 311. Again, the presence of 332 suggest the inclusion of ciprofloxacin in the molecules. The fragment at m/z 756 is associated with the loss of water. Product 7b was found to have an identical MS/MS spectrum to that of product 6 (Figure 4.32).

Figure 4.33  Mass spectrum of fraction 7.
Figure 4.34  MS/MS spectrum of product 7a, m/z 774.
4.3  Antimicrobial Activity Assay

A microtiter broth dilution assay was conducted to determine whether the ciprofloxacin released from the drug polymer possessed antimicrobial activity (conducted with the assistance of Selva Sinnadurai, the Toronto Hospital). The ciprofloxacin concentrations from each of the polymers incubated were calculated based on the MIC of ciprofloxacin (see Tables 4.7-4.10), which was determined by conducting the dilution assay with pure ciprofloxacin. The MIC of ciprofloxacin against *P. aeruginosa* was determined to be 0.31 μg/ml and 0.62 μg/ml. According to the biodegradation sampling protocol (Section 3.3.4, page 52), 1 ml of incubation solution was withdrawn for each time point, thus leaving 1 ml of solution remaining in the vial. Therefore, concentrations given in this assay for each time point represent both residual ciprofloxacin activity from the previous time point and the ciprofloxacin accumulated during the time interval of interest. For comparison, ciprofloxacin concentrations as analyzed by HPLC are also given in Tables 4.7-4.10.

Table 4.7 shows ciprofloxacin concentrations determined by MIC and HPLC calculations for DDI/PPE-900(8). The MIC calculation showed a peak value of 16 μg/ml at 2 days for both enzyme and buffer. As can be seen in the data, the concentrations determined by the MIC calculation do not precisely match the values determined by HPLC. However, it should be noted that the trends are in agreement, with the peak value occurring at 2 days, and both assays showing no difference between enzyme and buffer treated samples. The discrepancy in the two methods is suspected to be due to a number of factors that will be discussed in Section 5.5.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>day 0</th>
<th>day 2</th>
<th>day 10</th>
<th>day 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>2</td>
<td>16</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>buffer</td>
<td>2</td>
<td>16</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>glass-CE</td>
<td>&lt; 0.5</td>
<td>&lt; 0.5</td>
<td>&lt; 0.5</td>
<td>&lt; 0.5</td>
</tr>
</tbody>
</table>

*0.5 = (>0.31 and < 0.62) 4 = (>2.48 and < 4.96) 16 = (>9.92 and < 19.84) 2 = (>1.24 and < 2.48) 8 = (>4.96 and < 9.92)
Table 4.7...continued

<table>
<thead>
<tr>
<th>Treatment</th>
<th>day 0</th>
<th>day 2</th>
<th>day 10</th>
<th>day 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>6.04</td>
<td>54.52</td>
<td>32.13</td>
<td>16.32</td>
</tr>
<tr>
<td>buffer</td>
<td>8.17</td>
<td>51.6</td>
<td>27.88</td>
<td>16.25</td>
</tr>
<tr>
<td>glass-CE</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.8 shows the drug concentrations for DDI/PCL-2000(12). Sub MIC levels were shown for the buffer controls up to 28 days incubation. On the other hand, enzyme treated samples showed a 0.5 μg/ml level for up to 28 days. As with DDI/PPE-900(8), the HPLC results are similar but not precisely the same as the MIC assay.

### Table 4.8 Ciprofloxacin Concentrations Determined by MIC Calculations and HPLC for DDI/PCL-2000(12), n=3

#### MIC calculations, (ciprofloxacin, μg/ml)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>day 0</th>
<th>day 7</th>
<th>day 14</th>
<th>day 21</th>
<th>day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>&lt;0.5</td>
<td>0.5†</td>
<td>0.5†</td>
<td>0.5†</td>
<td>0.5†</td>
</tr>
<tr>
<td>buffer</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

Note: 0.5 = > 0.31 and < 0.62 μg/ml
† contained one replicate with < 0.5 μg/ml

#### HPLC, (ciprofloxacin, μg/ml)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>days 0</th>
<th>day 7</th>
<th>day 14</th>
<th>day 21</th>
<th>day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>0</td>
<td>1.33 ± 0.40</td>
<td>0.87 ± 0.058</td>
<td>0.73 ± .15</td>
<td>2.17 ± .15</td>
</tr>
<tr>
<td>buffer</td>
<td>0</td>
<td>0.53 ± .18</td>
<td>0.40 ± 0.0058</td>
<td>0.41 ± .0058</td>
<td>0.43 ± .0058</td>
</tr>
</tbody>
</table>

Results for the HDI/PCL-2000(14) polymer are shown in Table 4.9. A value of 1 μg/ml (MIC calculation) was determined for both buffer and enzyme incubated samples for 10, 20 and 30 days. The concentrations determined by the MIC assay, although slightly lower than those for the HPLC analysis, show the same pattern in that equal concentrations are found in the buffer vs. enzyme incubated samples.
Table 4.9  Ciprofloxacin Concentrations Determined by MIC Calculations and HPLC for HDI/PCL-2000(14), n=3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MIC calculations, (ciprofloxacin, μg/ml)</th>
<th>HPLC, (ciprofloxacin, μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>days 0</td>
<td>day 10</td>
</tr>
<tr>
<td>CE</td>
<td>&lt;0.5</td>
<td>1</td>
</tr>
<tr>
<td>buffer</td>
<td>&lt;0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: 0.5 = (>0.31 and < 0.62 μg/mL)  
1 = (>0.62 and <1.24 μg/ml)

Data for polymer [14C]-HDI/PCL-2000(20) are reported in Table 4.10. The concentrations determined by MIC are slightly higher than the HDI/PCL-2000(14) values. This is in agreement with the concentrations as analyzed by HPLC. The enzyme and buffer treated samples for HDI/PCL-2000(20) show similar values, which are in agreement with the non-radiolabelled analog, HDI/PCL-2000(14) (Table 4.9).

Table 4.10  Ciprofloxacin Concentrations Determined by MIC Calculations and HPLC for [14C]-HDI/PCL-2000(20), n=1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MIC calculations, (ciprofloxacin, μg/ml)</th>
<th>HPLC, (ciprofloxacin, μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 0</td>
<td>day 10</td>
</tr>
<tr>
<td>CE</td>
<td>&lt;0.5</td>
<td>4</td>
</tr>
<tr>
<td>buffer</td>
<td>&lt;0.5</td>
<td>4</td>
</tr>
<tr>
<td>glass-CE</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

Note: 0.5 = (>0.31 and < 0.62 μg/mL)  
2 = (>1.25 and <2.5 μg/ml)
5.0 DISCUSSION

The objective of this study was to demonstrate the feasibility of synthesizing a new biodegradable polymer which gradually releases an antibiotic in order to prevent the proliferation of microorganisms on the surface of implantable medical devices. As discussed in the literature review, antibiotic impregnated or coated medical devices have been previously investigated, including catheters (Raad, 1996), vascular grafts (Phaneuf et al., 1993), and bone cement (Gerhardt et al., 1993). However, the potential drawbacks with the use of these devices include the development of antibiotic resistance (Raad et al., 1995) and the rapid elution of antibiotic, which limits long term use (Bach et al., 1994). The drug polymers examined in this study may provide a manner to reduce these problems.

The polymers in this study were synthesized by covalently linking the antibiotic directly into the backbone of the macromolecule. It is hypothesized that the molecular chain structure and material morphology will allow the host inflammatory response or the presence of bacteria to enzymatically degrade the polymer (Santerre et al., 1996). As the polymer chains are hydrolyzed, the antibiotic may be subsequently released. The release would be sustained until enzyme activity decreases as a result of the diminished host response (Santerre et al., 1996). Since ciprofloxacin is a potent antibiotic with relatively low MICs (Crumplin, 1986), a large depot of drug is thus available to allow for prolonged release. In addition, since elevated levels of drug are only released during the period of inflammation, it is possible that the problem of antibiotic resistance due to a continuous dose of antibiotic may be reduced.

In the first part of this chapter, the results of the drug polymer synthesis will be discussed and evaluated. The second part the chapter will elucidate structures from the mass spectra of the degradation products of HDI/PCL-2000(14), while the third part will discuss possible release mechanisms and the antimicrobial activity of the released ciprofloxacin and degradation products.
5.1 Development of Synthesis Procedures

Polyurethanes exhibit a wide range of physical and chemical properties, due to the many options available in selecting the reagents and molecular weights of each of the components and their relative ratios. Additionally, the synthesis conditions can significantly influence the final properties of the polymer. In this study, a number of variables were modified in order to synthesize a polymer that could be selectively degraded by enzymes. However, it should be noted that a thorough investigation of each of the reaction variables was not explored, and was beyond the scope of this thesis. Although the production of a high molecular weight polymer was considered to be a practical design consideration, this was not a primary goal of this study, since the polymer could be used as a coating, or grafted onto the surface of a substrate by a number of methods, including reactive gas plasma (Mittelman, 1996b).

One of the critical factors in the synthesis is the ability of the ciprofloxacin molecule to react with the diisocyanate. In comparison to diamine and diol functional groups, ciprofloxacin possesses functional groups of relatively low reactivity toward diisocyanates. The carboxylic acid group is less reactive than the amines on PPE or the hydroxyls on PCL. The piperazine group on the ciprofloxacin molecule is a secondary amine, which is also less reactive than primary amines (Saunders and Frisch, 1965).

5.1.1 Poly(oxyethylene) Diamine (PPE) Based Polymers

In order to ensure sufficient ciprofloxacin incorporation into the polymers, the initial reaction was carried out by reacting ciprofloxacin and HDI in the first step, followed by adding the PPE oligomer in the second step. In this manner, it was anticipated that the ciprofloxacin molecules would be able to undergo sufficient incorporation within the growing polymer chains. However, it was observed that the ciprofloxacin-diisocyanate prepolymer precipitated out of solution after approximately 1.5 hours into the reaction (see HDI/PPE-900(1) in Table 4.1). This insolubility may reflect the inherent poor solubility of the ciprofloxacin itself, since the drug required heating to 70°C in DMSO for dissolution. It may
be possible that the ciprofloxacin component in the polymer is self associating by strong van der Waals interactions. Evidence for this was found by examining the mass spectrum of the ciprofloxacin standard, which showed a peak at m/z 663 (Figure 4.17), corresponding to ciprofloxacin self associating. The precipitation of polymer chains during polyurethane synthesis has been shown to hinder the growth of the polymer (Lyman, 1960). In order to prevent precipitation, a one step synthesis, where all reagents were added at once, was attempted. Since the PPE segments were relatively hydrophilic and readily soluble, polymer chains containing PPE units were expected to keep the polymer in solution. This was moderately successful (see HDI/PPE-900(4) in Table 4.1), and increased the length of time before the polymer came out of solution.

In addition to precipitation problems, the processing and characterization of PPE drug polymers was difficult. During the purification step, the precipitation of the polymer in distilled water formed extremely fine particles which stuck to the washing vessel and were difficult to recover. Other solvents were substituted for water during the precipitation step, including acetone, methanol, hexane, methylene chloride, chloroform, and ethyl acetate. Each solvent simply swelled the polymer, and did not improve the recovery. In addition to low recovery, PPE based polymers had poor physical properties (Table 4.1) and were very difficult to dissolve in DMAC casting solutions.

1,12 diisocyanatododecan (DDI) was substituted for HDI in polymer batches 5-8. Since the length of the DDI hydrocarbon segment was longer, it was expected that it may reduce the degree of the association between ciprofloxacin units making up the polymer chains. Although precipitation of the polymer chains during the reaction was hastened (compare HDI/PPE-900(4) to HDI/PPE-900(5)-(8) in Table 4.1), the polymers formed larger precipitates in water, were less tacky, and easier to recover (see Table 4.2).

A number of difficulties were encountered during the molecular weight analysis of the PPE materials. All the polymers contained insoluble gels. Since these were removed during the GPC pre-injection filtration, the final concentration of polymer was low and in some cases
non-measurable (DDI/PPE-900(7) in Figure 4.1). In addition, the presence of mobile phase associated contaminants near the retention time of 25 minutes interfered with some of the chromatograms. Despite these problems, molecular weight information was still obtained for some PPE containing polymers (Table 4.4). The PPE based polymers exhibited relatively low molecular weights, as compared to those of conventional polyurethane elastomers (Lelah and Cooper, 1986). A possible explanation for the low values may be attributed to the fact that all the high molecular weight material was contained in the gels which were not analyzed. These gels are believed to be associated to a cross-linking side reaction of the drug polymer with free isocyanate groups (to be discussed later in this section). Although temperatures below 80°C were used, the presence of the tin catalyst may have promoted some cross-linking reactions (Lelah and Cooper, 1986). As mentioned in section 2.1.2, isocyanates may react in a number of ways. Biurets may be formed from the reaction of ureas with isocyanates; isocyanurates may form by trimerization of isocyanate groups. While cross-linking is one possible explanation for the low $M_w$ values, it should be considered that the anticipated low reactivity of ciprofloxacin may not have allowed the polymer chains to grow and therefore would also have resulted in low molecular weights.

Although the effect of catalyst was not investigated thoroughly, two different concentrations were used. The initial concentration was based on the work of Tang et al. (1996). Since the initial reactions failed to produce polymers with adequate physical character, the concentration was increased ten fold for polymer DDI/PPE-900(6) and for subsequent polymers. The effect of this change was unclear for the PPE based polymers. DDI/PPE-2000(7), synthesized with 10 times more catalyst, was practically insoluble in the GPC mobile phase. Consequently, the GPC chromatogram exhibited no significant polymer peak. The fact that the polymer was poorly soluble in the 0.5 M LiBr/DMF mobile phase suggests that extensive cross-linking was occurring during the reaction. However, the addition of triethylamine during polymer synthesis (DDI/PPE-900(8)) appeared to lessen the crosslinking. Evidence for this includes: the observation that the reactants of DDI/PPE-900(8) remained in solution for 5 hours as compared to 2 hours for the DDI/PPE-900(7), and the fact that DDI/PPE-900(8) was more soluble in the GPC mobile phase. The purpose of
using triethylamine was to scavenge the HCl from the ciprofloxacin hydrochloride monohydrate. Although drying was carried out to remove excess water in the drug (section 3.1.2), the remaining HCl in the ciprofloxacin may shield the functional groups and hinder the reaction. Thus triethylamine was added on an equimolar basis to the reaction immediately after the addition of ciprofloxacin. Additionally, triethylamine was also anticipated to have the added benefit of acting as a mild catalyst (Saunders and Frisch, 1965).

The importance of TEA is illustrated by the molecular weight of chromatograms in Figure 4.1. The GPC chromatogram of DDI/PPE-2000(5) synthesized without TEA, exhibits a bimodal distribution, thus indicating an incomplete reaction. This bimodal peak was not present with DDI/PPE-2000(8), a polymer synthesized with TEA. This difference may have resulted from the fact that the added TEA produced a higher ciprofloxacin reactivity.

The theoretical fluorine and nitrogen content of the drug polymers was calculated based on the molecular masses of the monomers, and assuming an ideal 2:1:1 stoichiometry. These values are tabulated in Tables 5.1 and 5.2, along with the experimental values for comparison. Since fluorine is only present on the drug molecule, fluorine content is indicative of the degree of drug incorporation. For PPE based polymers, the nitrogen content is indicative of the isocyanate and PPE content. The theoretical fluorine values for DDI/PPE-900(5) and (8) are both higher than the measured values. According to these values, only 70% and 61% of the initial drug was incorporated into DDI/PPE-900(5) and DDI/PPE-900(8) respectively. This is evidence that ciprofloxacin was not fully incorporated, and also supports the theory of possible cross-linking reactions occurring during the synthesis, since NCO groups could be consumed in side reactions competing with that of drug incorporation.

Interestingly, the fluorine content for HDI/PPE-900(17) actually shows a value greater than the theoretical value itself. In contrast to DDI/PPE-900(5) and (8), these former polymers were synthesized in a two step process with incorporation of the drug in the last step. Since the polymer did not precipitate during the reaction, it would be expected that more ciprofloxacin could be incorporated than for DDI/PPE-900(5) and (8) which precipitated
easily. Since the nitrogen levels were higher than theoretical for HDI/PPE-900(17) and the polymer was made up almost entirely of insoluble gels, it is suspected that cross-linking must be occurring. This matrix could be limiting the removal of unreacted drug during the washing phase and thus result in artificially high drug content. Another possible explanation is that there was concern with the elemental analysis technique. Guelph Chemical Labs (GCL) reported that the polymer samples sent for analysis were inhomogenous. This may have resulted in the analysis of a ciprofloxacin rich sample, which was not indicative of the overall average elemental composition of the polymer. Furthermore, as shown in Table 4.5, the fluorine content of DDI/PCL-2000(12), which was analyzed twice, showed significantly different values (0.72 wt% and 0.4 wt%). As a result of the latter observation, the problem may reside with the accuracy of GCL's technique. Unfortunately, this could not be verified due to the high cost of this analysis.

Table 5.1 Theoretical and Experimental Fluorine and Nitrogen Content for PPE Based Polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Theoretical F wt %</th>
<th>F wt%</th>
<th>Theoretical N wt %</th>
<th>N wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDI/PPE-900(5)</td>
<td>1.09</td>
<td>0.76</td>
<td>7.26</td>
<td>6.75</td>
</tr>
<tr>
<td>DDI/PPE-900(7)</td>
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<td>0.83</td>
<td>7.26</td>
<td>7.71</td>
</tr>
<tr>
<td>DDI/PPE-900(8)</td>
<td>1.09</td>
<td>0.66</td>
<td>7.26</td>
<td>7.33</td>
</tr>
<tr>
<td>HDI/PPE-900(17)</td>
<td>1.21</td>
<td>2.64</td>
<td>8.04</td>
<td>10.0</td>
</tr>
<tr>
<td>HDI/PPE-2000(18)</td>
<td>0.70</td>
<td>2.52</td>
<td>4.80</td>
<td>10.77</td>
</tr>
</tbody>
</table>

5.1.2 Polycaprolactone (PCL) Based Polymers

Poly(caprolactone) diol was used as an alternate oligomer in order to compare the effect of a more hydrophobic segment, and also to take advantage of ester linkages, which have been shown to be susceptible to cleavage by cholesterol esterase (Santerre et al., 1994). Reactions involving the PCL oligomer produced polymers which displayed better physical character (see Table 4.2). The more hydrophobic oligomer also produced drug polymers which were
more easily recovered since they formed larger precipitates and thus gave higher yields (see Table 4.1). The polymers were easier to dissolve for casting purposes and this improved the ability to carry out molecular weight analysis by GPC chromatography.

Two-step reactions were attempted in order to further increase molecular weights of the materials (see Table 4.1, polymer batches 10-20) and reduce the onset of precipitation during polymerization. To compensate for the lower reactivity of ciprofloxacin, longer reaction times (up to 22 hours) were used during the ciprofloxacin coupling (second step). These changes resulted in materials with increased elastomeric character (Table 4.2). Extensive biodegradation testing was conducted on polymers synthesized with this approach. It was originally hypothesized that this method of synthesis may lead to a low amount of ciprofloxacin incorporation in the polymer. However, the fluorine contents (Table 5.2) of DDI/PCL-2000(12) and DDI/PCL-2000(14) show that approximately 50% of the original drug added was incorporated, which is similar to the PPE one step formulations.

Figure 4.2 contained a few PCL based GPC chromatograms. The polymers DDI/PCL-2000(9) and DDI/PCL-2000(10), which were synthesized via one step, exhibit a gaussian shaped peak with a lower $M_w$ than the two-step polymers, DDI/PCL-2000(12) and HDI/PCL-2000(14). The two step reactions show a distribution which has a skewed peak, having a longer tail on the low molecular weight side. Peebles (1974, 1976) predicted sequence length distributions and molecular weights of polyurethanes based on kinetic considerations for one and two-step polymerization methods. Using analytical and numerical methods to solve the kinetic equations, it was shown that the sequence length distributions of hard blocks and molecular weights could be varied by alteration of the polymerization sequences and the method of mixing the components. In this study, the effect of changing the order of monomer addition was demonstrated by observing the molecular weight distributions of the PCL based polymers (Figure 4.2). It is difficult to specifically explain the cause of the skewed versus normal distribution at this time, due to the complex nature of the side reactions which appear to be occurring in this polymer system.
Despite the long reaction times, insufficient drug was reacted to produce high molecular weight polymers. While there could be many reasons why higher molecular weight polymers were not produced, the most likely one was suspected to be the low reactivity of ciprofloxacin. The fluorine analysis for the HDI/PCL-2000(12) and DDI/PCL-2000(14) (Table 5.2) both showed less than theoretical values, supporting this hypothesis. Another consideration was that the stoichiometry of the reagents could have been mismatched. Due to the limited availability of purified ciprofloxacin, only small polymer batches (2 gram) were synthesized, thus increasing the potential for error in the reagent concentration.

Table 5.2 Theoretical and Experimental Fluorine and Nitrogen Content for PCL Based Polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Theoretical F wt %</th>
<th>F wt%</th>
<th>Theoretical N wt %</th>
<th>N wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDI/PCL-2000(12)</td>
<td>0.67</td>
<td>0.40</td>
<td>3.46</td>
<td>5.50</td>
</tr>
<tr>
<td>HDI/PCL-2000(14)</td>
<td>0.71</td>
<td>0.33</td>
<td>3.67</td>
<td>3.06</td>
</tr>
<tr>
<td>HDI/PCL-1250(19)</td>
<td>1.02</td>
<td>1.00</td>
<td>5.24</td>
<td>5.45</td>
</tr>
</tbody>
</table>

5.2 Polymer Purification

Purification of the polymers was carried out by stirring the fine wet precipitates in distilled water for 3 days. This was done in an attempt to remove unreacted ciprofloxacin, which would interfere with the ability to assess if drug was released from the macromolecular chain at the time of the polymer's hydrolysis.

The residual levels for most of the polymers were in the ppm range, and did not decrease greatly over the three day period. While this may indicate that there is a finite level of free drug still leaching from the polymer it could also reflect the possibility that the polymer is slowly hydrolyzing and releasing this small amount of drug.
Data in Table 4.3 also indicated that HDI/PCL-2000(14) contained high levels of residual free drug. This amount decreased by about ten fold over the three day washing period. It was suspected that the elevated levels of drug were remaining as a result of the much larger precipitates formed during the work-up of the polymer. It was noted that these precipitates broke up into finer particles over the 3 day period. This most likely improved the diffusion and permitted a more effective washing of the polymer. Future work should consider the modification of the washing procedure in order to improve the removal of unreacted drug. Perhaps this could be achieved by redissolving and precipitating the polymers several times.

5.3 Structural Elucidation of Degradation Products

The analysis of the biodegradation solutions for the various formulations showed that only HDI/PCL-2000(14) produced a significant number of degradation products other than free ciprofloxacin. Their presence was important since it provided a means of confirming the reaction of the drug with the other reagents during the polymerization. Possible structures of these products were constructed based on the molecular masses of the fragments detected in the MS/MS spectrums (Figure 4.20-4.34).

Figure 5.1 shows the fragmentation pattern assigned with the ciprofloxacin molecule in relation to its MS/MS spectrum (Figure 4.18). The principal fragments are in agreement with electron ionization mass spectra found in the literature (Gau et al., 1986).
The HPLC peak of Fraction 1 had an identical retention time and UV spectrum to the ciprofloxacin standard (see Figure 4.8). The fragmentation ions found in the MS/MS spectrum (Figure 4.20) were virtually identical to the MS/MS spectrum of ciprofloxacin (Figure 4.18), which confirms product 1 to be the free drug. Although it was noted that MS/MS spectrum of product 1 (Figure 4.20) contains ions not found in the standard MS/MS spectrum (Figure 4.18), these have been assigned to sample impurities related to the incubation and processing solutions.

The MS/MS spectrum of product 2 (Figure 4.22) contains fragments at m/z 332 and 313, which suggest the product is a ciprofloxacin derivative. The UV spectrum obtained by the photodiode array detector (Figure 4.8) shows that fraction 2 and fraction 1 (ciprofloxacin) both have similar absorbance spectra at higher wavelengths (near 280 nm) but differ slightly at the lower wavelengths (200-220 nm). The differences at lower wavelengths are suspected to be associated to residual HDI/HDA segments coupled to the ciprofloxacin molecule since the HDA molecules absorb at the low wavelengths (see Figure 5.2). A probable structure for
product 2 is shown in Figure 5.3. It consists of one molecule of HDI bonded to ciprofloxacin at the piperazine moiety. Without further chemical analysis, such as NMR, this cannot be absolutely confirmed at this time.

**Figure 5.2** UV spectrum of hexane diamine and ciprofloxacin.

**Figure 5.3** Proposed structure and fragmentation pattern of product 2, m/z 474.
The proposed structure of product 3a (m/z 616) is shown in Figure 5.4. Fragment ions m/z 474 and 456 shown in the MS/MS spectrum of product 3a (Figure 4.24) have been associated with different fragments than the corresponding peaks found in product 2 (Figure 4.22). It is proposed that product 3a was hydrolyzed from the polymer chain. This new product corresponds to product 2 with the addition of an HDI unit on the carboxyl end (Figure 5.4). The MS/MS spectrum of product 3a (Figure 4.24) does not contain fragments with m/z 332 and 313. The fragment m/z 332, which corresponds to free ciprofloxacin would not appear since the carboxyl end of ciprofloxacin was eliminated in the polymer synthesis, and thus the subsequent fragmentation cannot duplicate that of pure ciprofloxacin. The disappearance of fragment m/z 313 may be attributed to the low abundance of fragment m/z 474 that was generated. This is rationalized based on the observation that the ratio of the m/z 313 fragment to the m/z 474 fragment in Figure 4.22 for product 2 was very low. Since the m/z 474 fragment in Figure 4.24 is low itself, the m/z 313 would be anticipated to be undetectable at the current concentration of product 3a.

Figure 5.4  Proposed structure and fragmentation pattern of product 3a, m/z 616.
The structure of product 3b, isolated from the HPLC chromatogram in Figure 4.8, and identified by an $m/z$ 433 (Figure 4.23), is given in Figure 5.5. The ion with $m/z$ 115 found in the MS/MS spectrum (Figure 4.25) corresponds to caproic acid, the repeat unit of the PCL oligomer. The ions at $m/z$ 97 and $m/z$ 69 were produced by the loss of H$_2$O and CO$_2$ respectively from the caproic acid fragment. The three ions ($m/z$ 115, 97, 69) were previously confirmed to be associated with caproic acid in a study by Wang et al. (1997) that had analyzed the degradation products of a polyurethane synthesized with poly(caprolactone) oligomer. Therefore, the structure of product 3b is given as caproic acid—HDI—caproic acid. Since there is no ciprofloxacin in this structure, its contribution to the UV absorbance of fraction 3 at 280 nm is minimal. Hence, fraction 3 contains two products, one ciprofloxacin derived, and one containing no ciprofloxacin moiety. The presence of a non-ciprofloxacin containing product with an HDI unit is further supported by comparing the ratio of $^{14}$C-labelled product to UV absorbance for fraction 3 versus that observed for any of the other fractions. The data in Figure 4.15 clearly show that fraction 3 has the strongest radiolabelled signal but one of the weakest UV absorption signals for the drug conjugates. The presence of this product confirms the degradation of the non-ciprofloxacin containing segments of the polymer.

![Proposed structure and fragmentation pattern of product 3b, $m/z$ 433.](image)

Figure 5.5  Proposed structure and fragmentation pattern of product 3b, $m/z$ 433.
Product 4a which has an \( m/z \) 606 (Figure 4.27) is shown in Figure 5.6. Major fragments present in Figure 4.27 are defined at \( m/z \) 588, 596 (-H\(_2\)O from each end of the degradation product), and 500, 482, and 332. The fragment at \( m/z \) 500 is associated with the loss of diethylene glycol -(CH\(_2\)CH\(_2\)O)\(_2\)- which is covalently linked to ciprofloxacin via HDI. The diglycol is used in the synthesis of poly(caprolactone) diol (Aldrich, personal communication). The fragments at 332 correspond again to the ciprofloxacin segment.

Figure 5.6 Proposed structure and fragmentation pattern of product 4a, \( m/z \) 606.
The proposed structure for product 4b ($m/z$ 575 in Figure 4.26 and the MS/MS spectrum in Figure 4.28) is shown in Figure 5.7. Differences of 132 amu between fragments suggests the presence of caproic acid units. It is proposed that this structure contains an aliphonate group (see Section 2.1.2), resulting from a possible side reaction in the polymer synthesis. In a similar manner to the situation for fraction 3, fraction 4 contains two products, one ciprofloxacin derived, and one containing no ciprofloxacin moiety. Again, this is consistent with the radiochromatograms for HDI/PCL-2000(20), shown in Figure 4.15. The identification of this product is important since it confirms the previous discussion that suggested the occurrence of cross-linking reactions during the polymer synthesis.

![Proposed structure and fragmentation pattern of product 4b, $m/z$ 575.](image-url)

**Figure 5.7** Proposed structure and fragmentation pattern of product 4b, $m/z$ 575.
There were three products isolated from fraction 5 (Figure 4.29). A proposed structure for product 5a (m/z 748) is shown in Figure 5.8 and is based on the MS/MS spectrum shown in Figure 4.30. The structure is similar to product 4a, except that it contains an additional HDI segment bonded to the carboxyl end of ciprofloxacin. The fragments with m/z 474 and 456 correspond to the same fragments found in product 3a, which indicate the presence of the HDI segment bonded to the carboxylic acid end of ciprofloxacin. As with product 3a, m/z 332 cannot be found; however, the m/z 313 ion is present in the MS/MS spectrum of product 5a (Figure 4.30). Product 5b (m/z 606) is the same product as 4a, while product 5c is the same as product 6 (discussed in the next paragraph).

Figure 5.8  Proposed structure and fragmentation pattern of product 5a, m/z 748.
The proposed structure for product 6 (m/z 632, MS/MS spectrum shown in Figure 4.32) is shown in Figure 5.9. This product is similar to product 4a (Figure 5.6), but includes a caproic acid unit instead of the diethylene glycol segment. The similarity of these two products is indicated by the presence of the common fragments at m/z 500 and 482 indicating the presence of HDI bonded to ciprofloxacin at the piperazine end. The presence of fragments having 114 m/z units apart (i.e. difference between m/z 614 and m/z 500, in Figure 4.32), indicates the loss of the caproic acid unit from the molecular ion. The strong presence of m/z values at 332 and 314 in Figure 4.30 is indicative that the carboxyl end is free.

Figure 5.9 Proposed structure and fragmentation pattern of product 6, m/z 632.
Analysis of the MS/MS spectrum of product 7a (m/z 774) in Figure 4.34 yields the proposed structure shown in Figure 5.10. Although the fragments m/z 642, 500, 482, 474, 456 appear as in the MS/MS spectrum of product 5a (Figure 4.30), this spectrum also contains prominent fragments at m/z 332 and 313. This suggests that the carboxylic acid end of the ciprofloxacin is open and not coupled to HDI. Based on this information, a structure was proposed which consisted of an allophanate linkage along with a caproic acid unit. Note that there are two possible locations for the allophonate link, as indicated by the asterisk in Figure 5.10. As with product 4b, this new molecule provides further evidence that cross-linking would have taken place during the synthesis.

*alternate position for allophanate linkage

Figure 5.10  Proposed structure and fragmentation pattern of product 7a, m/z 774.
Analysis of the HPLC radiochromatograms of HDI/PCL-2000(20) (Figure 4.15) showed areas where there was almost no UV absorbance, but a significant radiolabel peak, reflecting contributions from the $^{14}$C-HDI component. The analysis of these fractions yielded an additional product at a retention time of 53 minutes (Fraction R in Figure 4.15). The MS/MS spectrum of fraction R is shown in Figure 5.11. Major fragment ions appear at $m/z$ 275 and 143. This spectrum has similar fragments to that of product 4b (Figure 5.7), with the exception that it has one less caproic acid segment (Figure 5.12). Here again, there is evidence that the polymer underwent cross-linking during the polymerization step.

Figure 5.11 MS/MS spectrum of product R.

Figure 5.12 Structure of radiolabelled product (53 minutes) from HDI/PCL-2000(20).
From the analysis of the HPLC results over time (see Figure 4.13), the products released from the enzyme incubated solutions appear to be relatively stable, as their amounts increase over the 30 day incubation period. The chemical structures of these products indicate that during the polymer synthesis, HDI has reacted with poly(caprolactone), at either the caproate or diglycol group. This corresponds to HPLC data reported by Wang et al. (1997), for reactions between polycaprolactone diol and 2,4 toluene diisocyanate. Most importantly, HDI appears to be able to react to both ends of the ciprofloxacin molecule, as shown by products 3a and 5a. The results also show the presence of allophanate linkages, products 4b, 7a, and R (Figures 5.7, 5.10, and 5.12), which suggest that cross-linking of the polymer occurred during the synthesis. This is consistent with the fact that the polymers were poorly soluble in DMSO and DMF, and that the molecular weights of the polymers did not match that of conventional linear polyurethanes. As a result of the product analysis for HDI/PCL-2000(14), a schematic of a degradation pathway is proposed in Figure 5.13.

The dominant drug containing product, based on the highest UV absorbance in Figure 4.8, was associated with product 6 (m/z 632). This HPLC fraction was relatively pure since no other products appeared to be contributing to the absorbance for this retention time. The product was produced by the cleavage of one of the ester bonds of the PCL molecule (Figure 5.13). The free carboxyl end of the ciprofloxacin molecule has either been degraded by cleavage of the amide bond (NH—CO), or was unreacted during synthesis (Figure 5.13). While the presence of product 5a (m/z 748) and product 3a (m/z 616) provide evidence that ciprofloxacin can react at both ends, the amount of these products was lower than the other products (Figure 4.8). This suggests that the reaction of ciprofloxacin at the carboxylic acid end may not have been favoured or that this end was the most susceptible to hydrolysis during the incubation periods.

The structure of product 4a and 5b is similar to product 6, except that the caproate group is replaced by a diethylene glycol unit. The slightly longer retention time of the caproate containing segment is not surprising, since it is a more hydrophobic moiety than the
Figure 5.13 Degradation path to ciprofloxacin from polymer segments of DDI/PCL-2000(14)
diethylene glycol segment. The larger quantities of products 5c and 6 versus fraction 4a and 5b likely reflects the fact that the commercial oligomer contains more caproate end groups than diethylene glycol end groups, since the diethylene glycol is purposely added to couple poly(caproate) segments during the synthesis of the oligomer (Aldrich, personal communication). The cleavage of the HDI segment bonded to ciprofloxacin in product 5a would yield product 4a (Figure 5.13).

While the abundance of terminal carboxylic acid groups in the products were possibly produced by the cleavage of ester linkages, the presence of some of the terminal groups on products 2 and 3a, and 5a were produced by the hydrolysis of urethane, urea, or amide linkages in the polymer. However, these products were found in lower quantities according to UV absorbance (Figure 4.8). This may be due to the fact that the ester linkages are more susceptible to cleavage than urethane linkages, which is consistent with reports on polyurethane biodegradation (Stokes, 1993; Greisser, 1991).

5.4 Ciprofloxacin Release Mechanisms

The data obtained by the HPLC and radiolabel analysis show that the degradation of PCL containing drug polymers was catalyzed by cholesterol esterase. Of particular interest was DDI/PCL-2000(12), which released a greater amount of free ciprofloxacin in the presence of CE as compared to buffer (Figure 4.10). HDI/PCL-2000(14), although releasing the same amount of free ciprofloxacin in CE and buffer (Figure 4.11), produced multiple degradation products which were identified by MS (Figure 5.13). The fact that these results differ for each of the two polymers suggest that CE cleavage is influenced by the length of the diisocyanate monomer, i.e., HDI vs. DDI, since this was the only parameter that varied between the two materials.

Cholesterol esterase is an enzyme which catalyzes the hydrolysis of long chain fatty acid esters of cholesterol (Labow et al., 1983). Furthermore, CE exhibits little or no substrate specificity (Zahalka, 1987; Brockerhoff et al., 1974). It catalyzes the hydrolysis of water-
soluble carboxylic esters, including $p$-nitrophenyl acetate (Erlanson, 1970; Lynn et al., 1982). One of the natural substrates of CE, cholesterol oleate (Labow et al., 1994), is shown in Figure 5.14. The similarity of a DDI/ciprofloxacin polymer segment to that of the natural substrate can be seen, i.e., a large ring structure is attached to a linear carbon chain, both segments being coupled by a hydrolyzable group. These common features possibly explain why DDI/PCL-2000(12) shows a preferred cleavage of the drug from the adjacent DDI unit versus the HDI unit. HDI is a shorter chain and may not provide the appropriate binding that is necessary for cleavage of the drug. Studies of CE binding have supported the hypothesis that the active site of CE is composed of two binding regions, one which binds to the large, hydrophobic rings, and the other which binds to the hydrocarbon chains of the fatty acid (Sutton et al., 1986, 1990). The specificity of the latter region to bind straight, aliphatic hydrocarbon chains was determined by using a series of CE inhibitors (Sutton et al., 1990). A number of $n$-alkyl boronic acid inhibitors, ranging from 1 to 8 carbons, were shown to inhibit the hydrolysis of $p$-nitrophenylbutyrate. The results showed that $n$-hexaneboronic acid, was the most potent inhibitor. Since this inhibitor is an analog of $n$-heptanoic acid, this supported the fact that the active site of CE binds a fatty acyl function of seven carbons in length. Hence the length of the hydrocarbon segment of the aliphatic diisocyanate may very well influence how CE binds to the drug polymers synthesized in this study. Further studies will have to investigate such a dependence in more detail. If found to exist, then this could be a parameter by which to optimize the specificity of the drug release from the polymer.

![Figure 5.14](image)

Figure 5.14 a) Cholesterol oleate, b) DDI/ciprofloxacin polymer segment.
Another factor which may influence how CE binds to the drug polymer is the polymer morphology. Several studies have shown that the micro-domain formation in polyurethanes may affect biodegradation. Santerre and Labow (1997) found that polyurethane degradation by CE was dependent on the size of the hard segment micro-domains which are formed. Hergenrother and Cooper (1993) synthesized PTMO based polyurethanes with 4,4'-diphenylmethane diisocyanate (MDI) and dicyclohexylmethane diisocyanate (H12MDI). Following implantation in rats, the H12MDI materials showed a greater loss in molecular weight and mechanical properties than the MDI materials. This was attributed to the differences in hard segment domain size and the reduced crystalline state of H12MDI polyurethanes.

Based on the results of these studies, it can be hypothesized that the structural morphology of the DDI/PCL-2000(12) drug polymer compared to HDI/PCL-2000(14) may influence CE binding and subsequently the degradation of the polymer. There is evidence that a small change in diisocyanate structure can have a considerable effect on thermal properties (and thus polymer morphology). A recent study synthesized polyurethane ureas with PCL, 1,4-butanediamine, and two diisocyanates, 1,4 butane diisocyanate (BDI), and HDI (de Groot et al., 1997). The study showed that changing the diisocyanate from BDI to HDI led to tighter packing of hard segments. Final confirmation of the above hypothesis will require further characterization methods for polymer structure, such as differential scanning calorimetry (DSC) and small angle X-ray scattering.

The incubation of DDI/PCL-2000(12) in CE produced approximately 1.5 times more free drug than the buffer incubated polymer over a 7 day period. A number of possibilities may explain the absence of other degradation products which would be analogous to those found with HDI/PCL-2000(14). It is possible that the products are either not present in sufficient quantities to be detected, or are too hydrophobic and thus are not released into the aqueous incubation solution. If the products released did not contain ciprofloxacin segments, they would have a low UV absorbance and thus be undetectable with the PDA detector. It should be mentioned that a radiolabelled analogue of DDI/PCL-2000(12) has recently been
synthesized (unpublished) which showed a 100-fold decrease in radiolabel release compared to HDI/PCL-2000(20) (see Figure 5.15). It should be also noted that the specific radioactivity of this DDI polymer was $3.42 \times 10^5$ dpm/mg of polymer, which was higher than the value for HDI/PCL-2000(20), $2.09 \times 10^5$ dpm/mg. From this information, it is hypothesized that along with ciprofloxacin, degradation products containing only diisocyanate or PCL segments were released. Since these products have lower UV absorbances, they could not be detected by the HPLC methods applied in this study.

Figure 5.15  Cumulative radiolabel release for $[^{14}\text{C}]-\text{DDI/PCL-2000}$ (synthesized by Meilin Yang).

Aside from chemical cleavage, it cannot be ruled out that the polymers are, in part, leaching free drug from their matrices. Although during the synthesis of the polymers, a purification procedure was undertaken, there is a possibility that unreacted drug and other monomers may still be present. As shown in Table 4.3, there was still drug detected in the washing solutions. Therefore, in all the biodegradation experiments, a portion of the assayed drug may have been caused by leaching. However, if leaching was the dominant mechanism for the release of drug, one would expect to see a higher release of free ciprofloxacin in the enzyme solution due to the breakdown of the polymer matrix, which was shown to be extensive relative to buffer. Evidence of the latter was provided by the HPLC data (Figure 4.15) and the radiolabel release for HDI/PCL-2000(20) (Figure 4.14).
For each of the biodegradation experiments, the initial release of free drug was high, followed by a steady increase for the remaining sample points (see Figures 4.9-4.12). This phenomena is typically called the “burst” effect in the drug delivery literature (Baker, 1986). As well, it is interesting to note that the radiolabel release with HDI/PCL-2000(20) also shows a high initial release for both enzyme and buffer treatments (Figure 4.14). The initial release may be the result of a number of factors. The diffusion gradient may be decreasing as drug is released, reflecting a drug concentration increase over time and the establishment of a boundary layer. Another possibility is that the hydrolysis is initially fast due to readily available bonds on the surface of the material. Polyurethane degradation has been shown to be primarily surface mediated (Santerre et al., 1994) and CE diffusion into the polymer is limited (Duguay et al., 1995). A third possibility is that since the enzyme must be continuously replenished during the experiment, there may be significant adsorption of enzyme breakdown products to the surface of the polymer, thus blocking available hydrolysis sites (Duguay et al., 1995). Smith et al. (1987) demonstrated this effect in a biodegradation study of poly(ether urethanes), by showing the initial burst could be reestablished if the polymer sample was cleaned ultrasonically and reincubated.

DDI/PPE-900(8) showed the fastest rate of free ciprofloxacin release, but again this release was virtually the same for polymer incubated in CE and buffer. It is believed that this elevated release could have been related to the hydrophilic nature of the PPE oligomer as compared to PCL. In the case of DDI/PPE-900(8), the estimated residual drug levels in the polymer sample (based on data in Table 4.3) are considerably lower than the amount of drug released over 2 days (Figure 4.9). Considering that the values in Table 4.3 represent the leaching of the entire batch of the polymer which consisted of fine particulate (i.e. large surface area), this provides some doubt that leaching is a major contributor to the release from the cast film, and therefore that hydrolysis is significant. In order to further demonstrate this, a radiolabelled polymer could be synthesized in order to show the presence of the hydrolyzed diisocyanate moieties. Biodegradation studies have been previously carried out using radiolabelled polyurethanes containing poly(ethylene oxide)/poly(propylene oxide)
segments (Santerre et al., 1993). This work showed that the polyethylene oxide containing polymers were hydrolyzed via a non-enzymatic mechanism.

5.5 Antimicrobial Activity of Incubation Solutions

The results of the microtiter broth dilution assay showed that the ciprofloxacin released from the drug polymer possessed antibiotic activity against *Pseudomonas aeruginosa*. This shows that the activity of ciprofloxacin was not lost following exposure to the solvents, heat, and chemicals encountered during the synthesis and processing of the drug polymer. Data in Tables 4.7-4.10 show that ciprofloxacin concentrations as determined by MIC calculations were in good agreement, but not precisely the same as the HPLC analysis data. These differences could be explained by a number of factors. The microtiter broth dilution method, as described in Section 3.4.3 (page 57), consists of a series of two-fold dilutions. Therefore, the concentration calculated is a range between two integral multiples of the MIC of ciprofloxacin, and not an absolute value. Experimental variability may contribute to error in the assay. For example, environmental conditions may affect the growth of bacteria, such as the incubator temperature and the composition of the media. Since small volumes (microliters) of media, sample, and inoculum were used, pipetting errors may also explain some of the discrepancy.

The biodegradation of HDI/PCL-2000(14), in comparison with the other tested formulations, produced significant ciprofloxacin derived breakdown products. However, the antibiotic activity of the incubation solutions was not correspondingly higher (Table 4.9), indicating that these products do not possess antimicrobial activity. This can be rationalized by considering the mechanism of action of ciprofloxacin. As discussed in the Section 2.5, quinolones, including ciprofloxacin, inhibit the activity of the bacterial enzyme DNA gyrase, which leads to bacterial cell death (Shen et al., 1993; Hooper and Wolfson, 1993). In order for this to occur, the drug must bind to specific portions of the bacterial DNA during protein synthesis (Remers and Bear, 1997). In fact, researchers have hypothesized that the quinolone molecules form supermolecules through hydrogen bonding (Figure 2.10) (Shen et al., 1989).
The carboxylic acid group is important for this to occur, as it is part of the minimum pharmacore of the drug (Figure 2.9). The results of this study suggest that complete degradation of the polymer is necessary for the ciprofloxacin to exhibit antibacterial activity. Another possible reason for the inactivity of the drug-derivatives may be that the large size of the molecules hinders their diffusion through the bacterial cell membranes.

In this study, the rate of drug release was determined by measuring the concentration of antibiotic in small volumes of simulated physiological solution in contact with the drug polymer. Although the rate of release was determined to be sufficient to inhibit bacterial growth, it is difficult to project whether these concentrations would be adequate to prevent bacterial colonization in vivo for a prolonged period of time. Further microbiological assays need to be performed to determine whether the release rates obtained are sufficient to inhibit bacterial adhesion and subsequent colonization. Studies of antibiotic impregnated or coated materials have used a number of different in vitro models under both static and flow conditions. Tests which have been used include placing the substrate in broth inoculated with bacteria (Price et al., 1993), substrates placed on petri dishes (disk diffusion) (Schmitt et al., 1996), or the modified Robbins device (Raad et al., 1995). These tests are better suited methods than the broth dilution or disk diffusion tests, since the biofilm-embedded organisms adherent to biomaterial surfaces may become resistant to various antimicrobial agents (Raad et al., 1995). However, the design of a better in vitro test for the drug polymers in this study is complicated by the fact that the release of the antibiotic is dependent on the presence of inflammatory-cell derived enzymes. The influence of the type and concentration of enzymes on the rate of release must also be determined.

It should also be mentioned that before this new drug polymer can be used in a medical device, many other considerations must be addressed. The location of the medical device is a significant factor which may influence whether antibiotic concentrations are sufficient to prevent infection. For example, devices such as catheters and vascular grafts may be exposed to flowing blood, which may rapidly deplete the surface concentration. Urinary catheters are exposed to a considerably different environment in terms of pH, fluids, and cell types present.
In this study, the release of antibiotic is dependent on the presence of inflammatory enzymes; therefore, this material may not function in locations where there is little inflammation. However, one could conceive the synthesis of the polymer such that it would respond to different enzyme types.

5.6 Summary

The problem of medical device related infections is a challenging one which is largely related to the great adaptability of bacteria to colonize surfaces in hostile environments. Biomaterial related infections are directly responsible for hundreds of thousands of infections and tens of thousands of deaths each year (Mittelman, 1996). Despite the notable amount of research conducted toward solving this problem, relatively little is known about the mechanisms of bacterial adhesion to synthetic biomaterials, the effect of bacteria on the immune system, and the antibiotic resistance of biofilm laden bacteria (Mittelman 1996). Modification of surfaces have to date, been unsuccessful in preventing adhesion. Antibiotic containing materials has been recognized as a promising solution to reducing the risk of infection, however, the current materials are plagued with problems such as rapid release rates and lack of long term activity. The new biodegradable materials proposed in this study may provide an alternative solution to controlling biomaterial related infections.
6.0 CONCLUSIONS

1. Bulk fluorine analysis and HPLC/UV spectroscopy show that ciprofloxacin was incorporated into the polymer chain structure. Furthermore, the structures of the degradation products identified by mass spectrometry indicate that the diisocyanate reacted with both the amine and carboxylic acid groups of ciprofloxacin.

2. The PCL based drug polymers exhibited better physical character than PPE based drug polymers, which were difficult to precipitate and dissolve in solvents for casting and GPC. The difficulty in precipitating the PPE polymer in water was related to the hydrophilic nature of the PPE oligomer. The solubility problems were hypothesized to be due to the formation of cross-linked gels during the synthesis. The PPE oligomer contained amine functional groups, which are considerably more reactive with diisocyanates than the hydroxyl groups of the PCL oligomer. For this reason, it is anticipated that cross-linking would be a greater problem with the PPE diamines.

3. Cholesterol esterase catalyzed the hydrolysis of DDI/PCL-2000, as shown by a greater release rate of free ciprofloxacin in enzyme compared to buffer solution. However, no other degradation products were detected by the HPLC methods used in this study. It is believed that the degradation products were either below the detection limit, or possessed low UV absorbance.

4. Cholesterol esterase catalyzed the hydrolysis of HDI/PCL-2000, as shown by the HPLC analysis and the results of the radiolabelled experiment. However, the HDI/PCL-2000 drug polymer did not release a significantly different amount of ciprofloxacin when incubated with CE as compared to buffer. However, the CE incubated solutions did produce multiple degradation products. MS/MS analysis of these products show that they consist of the drug bonded to HDI, caproic acid, and diethylene glycol segments (associated with PCL). Since these products were found in the solutions up to 30 days, it is hypothesized that cholesterol esterase was unable to further catalyze the hydrolysis of these lower molecular weight products to pure ciprofloxacin.
5. Two of the structures identified by MS contained allophonate cross-links. This evidence, in addition to the presence of precipitates in the reaction solution, and the difficulty in solubilizing the polymers for GPC, shows that it is highly probable that urethane cross-linking was occurring during the polymer synthesis.

6. As a result of conclusions 3-5, it was hypothesized that the structure of the diisocyanate could influence the binding of cholesterol esterase, and subsequently, the structures cleaved from the drug polymer. Therefore, changing the chemistry of the diisocyanate could be used to alter the specificity of enzymatic cleavage, and subsequently the release pattern of ciprofloxacin.

7. The ciprofloxacin released from the incubation of each drug polymer exhibited antimicrobial activity against *Pseudomonas aeruginosa*, which shows that the of synthesis and processing steps carried out for the generation of the final drug polymer did not inactivate the drug’s biological activity.

8. The degradation products produced by HDI/PCL-2000 polymer did not exhibit antimicrobial activity against *Pseudomonas aeruginosa*. This was shown by the fact that the large amounts of degradation products released into the enzyme incubated solution did not produce a corresponding increase in antimicrobial activity. Therefore, it is suggested that complete hydrolysis of the polymer to produce pure drug is necessary for ciprofloxacin to exhibit antimicrobial activity.
7.0 RECOMMENDATIONS

1. Modifications of the synthesis procedure should be made in order to increase the polymer molecular weights. Titration of free isocyanate groups would assist in determining the extent of the reaction and the selection of optimum reaction conditions. Factors such as reagent stoichiometry, catalyst concentration, temperature, and time could be changed, since all of these factor have been reported to influence polymerization processes.

2. The effect of the diisocyanate chemistry should be further investigated, by using aliphatic diisocyanates with varying hydrocarbon segment lengths. If a relation between the chemistry and biodegradation rate can be determined, it may be possible to synthesize polymers with varying enzymatic degradation rates.

3. Further polymer characterization should be completed in order to determine the effect of structural morphology on the degradation of the polymer. Knowledge of the effect of morphology on polymer degradation would assist in optimizing the release rate of the antibiotic.

4. It was found in this study that HDI/PCL-2000 released multiple degradation products, which could not be hydrolyzed by CE in the solution over the 30 day incubation period. Further biodegradation tests should be performed using other enzymes to determine if these products can be further hydrolyzed to produce free drug.

5. Polymer degradation products were not detected with the DDI/PCL-2000 formulation. Further HPLC method development should be done to determine whether these products were really not generated, or were just undetectable using the methods in this study. The use of other HPLC detectors, such as an electrochemical detector may be helpful. Concentrating the recovered incubation solutions may also help.

6. Identification of the degradation product structures was performed using the HPLC-MS technique. Although chemical structures consistent with the mass spectra collected were
proposed, further chemical analysis using nuclear magnetic resonance (NMR) should be conducted to confirm product structure.

7. In conjunction with the use of alternate monomers and degradable bond types, other inflammatory cell derived enzymes should be tested for their potential to release antibiotic. Testing at acidic pH's may be physiologically relevant in the vicinity of macrophages and other inflammatory cells.

8. Biocompatibility testing should be done, such as testing the toxicity of the degradation products.

9. A better assay for bacterial adhesion should be developed to more closely mimic the in vivo environment. The dilution assays used in this study are more suited to planktonic (free-floating) rather that biofilm laden bacteria which are associated with biomaterial associated infections. A series of clinically relevant microorganisms should be tested, which include species such as Staphylococcus epidermidis, Staphylococcus aureus, and Escherichia coli, since these are frequently isolated from infected medical devices.

10. The use of other antibiotics should be explored, since combinations of antibiotics are frequently used to treat infections.
8.0 REFERENCES


Heller J, “Controlled release of biologically active compounds from bioderodible polymers”, *Biomaterials*, 1, 51-57 (1980).


## APPENDIX A  SUPPLIERS OF CHEMICAL REAGENTS

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APPENDIX B  DISTILLATION AND DEGASSING APPARATUS

Diagram of distillation and degassing apparatus:
- Heating mantle
- Reboiler
- Column
- Condenser with cooling water
- Receiver
- Vacuum pump
- Liquid nitrogen trap
- Pressure gauge
- Cold finger
APPENDIX C  GPC CALIBRATION CURVE

GPC_Cal_Plot_And_Table Calibration Curve

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APPENDIX D  ENZYME STABILITY TEST

![Graph showing enzyme activity over time for different conditions.]

- Control (no polymer)
- HDI/PCL-2000
- DDI/PCL-2000

**Graph Data:**
- Y-axis: Enzyme Activity (units/mL)
- X-axis: Time (hours)

Time (hours)

0 5 10 15 20 25
APPENDIX E  CIPROFLOXACIN CALIBRATION CURVE FOR HPLC