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# Synthesis and characterization of a novel biodegradable antimicrobial polymer

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## Abstract

Bacterial infection is a frequent complication associated with the use of medical devices. In an effort to address this problem, antibacterial agents have been incorporated or applied directly onto the surfaces of numerous types of medical devices. This study assessed the feasibility of using a novel biodegradable polymer to release antibiotic drugs in response to inflammatory related enzymes. A model drug polymer was synthesized using 1,6-hexane diisocyanate (HDI), polycaprolactone diol (PCL), and a fluoroquinolone antibiotic, ciprofloxacin. Polymers were characterized by size-exclusion chromatography (SEC), and elemental analysis. Biodegradation studies were carried out by incubating the polymers with solutions of cholesterol esterase (CE) or phosphate buffer (pH 7.0) for 30 days at 37°C. The degradation was assessed by high-performance liquid chromatography (HPLC), mass spectrometry (MS) and <sup>14</sup>C radiolabel release. Subsequently, the activity of the released antibiotic was assessed against a clinical isolate of *Pseudomonas aeruginosa*. HPLC analysis showed the release of multiple degradation products which were identified, by tandem MS, to include ciprofloxacin and derivatives of ciprofloxacin. The microbiological assessment showed that the released ciprofloxacin possessed antimicrobial activity; 1 µg/ml was measured after 10 days. The results of this study suggest that these novel bioresponsive antimicrobial polymers or similar analogs show promise for use in the control of medical device associated infections. © 2000 Elsevier Science Ltd. All rights reserved.

**Keywords:** Biodegradable polymers; Antimicrobial; Infection; Ciprofloxacin; Quinolones; Polyurethanes

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## 1. Introduction

Infection is a major medical complication associated with the use of transcutaneous and totally implanted medical devices [1–3]. Serious complications which may result from these infections include tissue destruction, premature device failure, and the spread of the infection to other areas [4]. The ability of microorganisms to adhere to biomaterials is a key factor in the initiation of disease processes. Local and systemic factors also play an important role in the host's response to a colonized foreign body, and the immune response can be modulated by the presence of some forms of bacterial

biofilms [5]. In addition to the altered host response to the presence of bacteria, device-associated bacteria often exhibit a differential resistance to antimicrobial agents relative to their planktonic (free-floating) counterparts [6,7]. This resistance is broad spectrum, and includes disinfectants [8] and biocides [9]. The combination of these two factors—host-mediated immune modulation and antimicrobial resistance—accounts for the often times severe consequences of device-related microbial infections. Bacteria colonizing medical devices exhibit a number of phenotypic and genotypic characteristics that are distinct from planktonic organisms and provide them with an adaptive advantage in an otherwise hostile environment replete with antibiotics, along with cellular and humoral antagonistic agents. Clearly, treatment of biofilm-associated infections presents a significant challenge, and the development of effective prophylactic approaches is indicated.

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The prevention and control of biomaterial-related infections has been the subject of many investigations. The majority of these studies have used methods of incorporating antimicrobial agents into the medical device itself. A common method involves coating a device such as a catheter or vascular graft with antibiotics via an ionic bonding process [10–12]. Other methods which have been attempted include physically entrapping the antibiotic in the polymer matrix [13] and bonding the antibiotic via a textile heat process [14]. Silver, a potent antibacterial agent, has also been coated on the surface of catheters to prevent infection [15,16]. A few studies have attempted to modify the surface of polymers or medical devices to obtain surfaces which are anti-adhesive to bacteria [17–19]. However, due to the complex nature of bacteria–biomaterial interactions, it is unlikely that the adhesion of bacteria can be completely prevented by physico-chemical surface modification alone [20]. A variety of other strategies have included the destruction of the biofilm by enzymes followed by administration of antimicrobials [21], and electrical enhancement of antimicrobial penetration [22]. Although some of these strategies show promise, none of them have been accepted for widespread clinical use.

In vitro experiments have shown that bacteria on the surface of a biomaterial can withstand many times the concentration of antibiotic required to eradicate planktonic bacteria [23]. Therefore, the application of antimicrobial agents directly onto medical devices may be beneficial for two reasons: (1) bacteria around the implant may be killed before they have a chance to form biofilms, and (2) coated devices typically improve the ability to deliver higher antibiotic concentrations locally at the site of infection. However, the current limitation of this treatment is the rapid loss of antibiotic which limits long-term effectiveness [11,24–26]. In addition, the application of medical devices pretreated with actively diffusing antibiotics may be compromised by the emergence of resistant bacterial strains [27,28].

An ideal drug delivery system releases drug at specific locations at the required time. Many antibiotic-treated medical devices release antimicrobials continuously at high dose levels, irrespective of whether infection is present or not. In this study, it was desired to design a new antibiotic delivery system which could accelerate the delivery of drug containing products under conditions present during an infection. The implantation of a synthetic biomaterial elicits a number of biological responses, one of which is inflammation [29]. This reaction is characterized by the presence of leukocytes and macrophages, which have been shown to release various hydrolytic enzymes [30,31]. Recently, Suzuki et al. have taken advantage of the use of enzymes to degrade a polymer matrix containing a depot of antimicrobial agents [32]. The polymeric drug delivery system proposed in this study takes advantage of the enzymes released dur-

ing the inflammatory process to trigger the degradation of the polymer. Subsequently, antibiotic drugs are released which were originally used as actual monomers in the synthesis of the polymer itself. Since the inflammatory response is activated in the presence of bacteria or injury, which may predispose a patient to infection [1,41], antibiotic could be released, proportionate to the magnitude of the response. Since polyester-urethanes have been shown to be susceptible to degradation by inflammatory enzymes [33], a polymer containing similar chemistry was used as an initial model for this study. The feasibility of this concept will be shown using a polymer, whereby a fluoroquinolone antimicrobial drug is incorporated as a monomer unit into the backbone of a polyurethane, which is synthesized using an aliphatic diisocyanate and a polyester diol.

## 2. Materials and methods

### 2.1. Drug polymer synthesis

The materials used for the synthesis of the polymer include 1,6-hexane diisocyanate (HDI), polycaprolactone diol (PCL), average molecular weight 2000, and dibutyltin dilaurate catalyst, all of which were obtained from the Aldrich Chemical Company, Milwaukee, WI. [ $^{14}\text{C}$ ]-1,6-hexane diisocyanate (NEN, DuPont Custom Synthesis, Boston, MA) was supplied in glass ampoules, each containing 0.25 mCi dissolved in anhydrous toluene. The radioactive label was used as a marker for the detection of degradation products. Ciprofloxacin hydrochloride in powder form was supplied by Bayer Healthcare Inc. (Mississauga, ON, Canada).

Prior to use, HDI was vacuum distilled at 93°C and 1 Torr, while PCL was vacuum degassed overnight at 60°C and 0.2 Torr. The drug was dried at 60°C under vacuum for 24 h to remove residual moisture. Synthesis of the drug polymers was carried out in a Labconco® glove box (Fisher Scientific, Unionville, ON, Canada) under a dry nitrogen atmosphere. The solvent, dimethylsulphoxide (DMSO, Aldrich Chemical Company), was distilled at 0.05 Torr and 35°C within 24 h of the synthesis. The reaction was carried out with a stoichiometry of 2 : 1 : 1 of HDI : PCL : ciprofloxacin. In the first step of the synthesis, 2.08 mmol of HDI was continuously stirred with 1.04 mmol of PCL in 10 ml of DMSO. For the radiolabeled polymer, 0.25 mCi of  $^{14}\text{C}$ -HDI was mixed with the non-radiolabeled HDI. Dibutyltin dilaurate catalyst was then added (0.1 mmol). This mixture was allowed to react between 60 and 70°C for 3 h. A solution of 1.04 mmol (0.4 g) of ciprofloxacin in 10 ml of DMSO was heated to 70°C and 1.04 mmol of triethylamine (Aldrich Chemical Company) was added to aid dissolution. This solution was subsequently added to the reactor. The entire reaction scheme is depicted in Fig. 1.

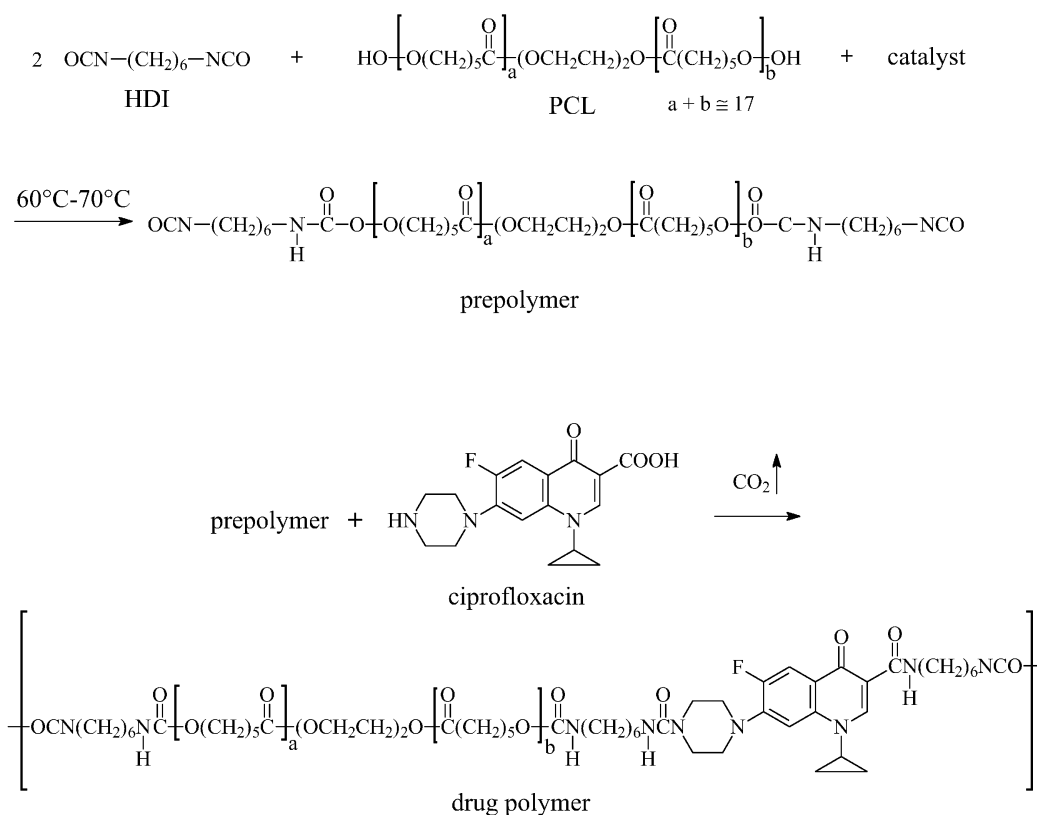


Fig. 1. Reaction scheme for the synthesis of the drug polymer.

The polymer solution was allowed to stir overnight, and was subsequently cooled and precipitated in distilled water. The wet polymer was stirred over a period of 3 days in distilled water, with intermittent water changes each day, in order to remove unreacted drug and monomers. Finally, the polymer was dried in a convection oven at 50°C for 24 h, followed by vacuum drying at 50°C for an additional 24 h. The specific activity for the radiolabeled polymer was determined by dissolving approximately 1 mg of polymer in 1 ml of dimethylacetamide (DMAC, Aldrich Chemical Company, Milwaukee, WI) and counting in a liquid scintillation counter (Beckman LS500, Mississauga, ON, Canada).

## 2.2. Molecular weight analysis

The molecular weight of the drug polymer was measured by size-exclusion chromatography (SEC). The equipment used consisted of a solvent delivery system (Waters 510 pump), a 200 µl fixed loop injector (Rheodyne 7125), a differential refractive index detector (Waters 410), a bank of three columns (Waters Styragel<sup>®</sup> HT3, HR2, HR1) in a thermostatted oven at 80°C, and a data acquisition computer (Waters Millennium<sup>™</sup> Software v2.1). The mobile phase was dimethylformamide (DMF, Aldrich Chemical Company) containing 0.05 M

LiBr. The injection volume was 200 µl and the polymer concentration was approximately 0.2% (w/w). The flow rate was set to 1 ml/min. Polystyrene standards (Varian, Sunnyvale, CA) were used for calibration. Subsequently, molecular weight data was reported as polystyrene-equivalent molecular weights.

## 2.3. Elemental analysis

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, 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 [34]. This work was carried out by Guelph Chemical Laboratories (Guelph, ON, Canada).

## 2.4. Enzyme preparation

Cholesterol esterase (E.C. 3.1.1.13) was used for the degradation studies since it has been found to be specifically isolated from inflammatory cells [35] and has been shown to degrade biomedical polyurethanes [33,36,37]. Cholesterol esterase (CE, bovine pancreas, Genzyme

Diagnostics, Cambridge, MA) solutions were prepared by dissolving the powder in 0.05 M phosphate buffer, pH 7.0. 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 or 1 N NaOH.

The activity of CE was determined using a modified version of the *p*-nitrophenyl acetate assay developed by Labow et al. [38]. The substrate was prepared by dissolving 22 mg of *p*-nitrophenyl acetate (Aldrich Chemical Company) in 1 ml of methanol and adding this to 100 ml of 0.1 M sodium acetate buffer, pH 5.0. CE activity was determined by adding 100  $\mu$ l of enzyme solution into a 3 ml cuvette containing 1 ml of 0.05 M phosphate buffer and 2 ml of the substrate. UV spectrophotometer (Ultraspec II, LKB Biochrom Ltd., Cambridge, UK) measurements were taken at room temperature for a 5 min period (immediately after addition of enzyme) at a wavelength of 410 nm. One unit of enzyme activity was defined as 1 nmol of substrate hydrolyzed per minute, calculated from the molar absorptivity of the *p*-nitrophenyl acetate ion as 16 300 l mol<sup>-1</sup> cm<sup>-1</sup>, at pH 7.0 [38]. Stock solutions of 40 units/ml activity were prepared and frozen at -80°C until required.

### 2.5. Biodegradation of drug polymers

The drug polymers were coated onto hollow glass tubes cut into 0.5 cm lengths (4 mm OD, 2 mm ID). Prior to coating, the tubes were cleaned with 20 ml/l Decon® 75 (BDH Laboratories, Toronto, ON, Canada) in an ultrasonic bath for 30 min, rinsed thoroughly with deionized water and dried upright at 110°C for at least 24 h. Polymer solutions were prepared by dissolving the polymer at a 10% (w/v) concentration in DMAC. Each tube was dip-coated with a pair of tweezers. Excess solution was removed by placing the tubes on an absorbent towel 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 h. The coating procedure was repeated each day for four days (4 coats total), with the final coat dried in a vacuum oven at 50°C for 24 h.

Under a sterile laminar flow hood, the tubes were placed into autoclaved glass screw cap vials (4 ml). Fourteen tubes were stacked in each vial regularly packed in two layers, which corresponded to a total surface area of 15.8 cm<sup>2</sup>.

The drug polymers were tested by incubating the vials with CE (40 units/ml) and phosphate buffer only (pH 7.0) at 37°C. Each of the groups consisted of three replicate vials. Initially, 2 ml of solution was added to each vial, and a 1 ml aliquot was immediately withdrawn at the time zero sample point. At each subsequent sampling point, a 300  $\mu$ l aliquot was withdrawn for microbiological analysis, and a 700  $\mu$ l aliquot was archived for high performance liquid chromatography (HPLC) and mass

spectrometry (MS) analysis. For experiments with the radiolabeled polymer, an additional 200  $\mu$ l aliquot was withdrawn for scintillation counting. Radioactive solutions were counted with 10 ml of scintillation cocktail (Formula 989, Packard Instrument Co., Montreal, PQ, Canada). Samples for HPLC and bioassay were frozen in liquid nitrogen and stored at -80°C until analysis. Each day, a 100  $\mu$ l aliquot (120  $\mu$ l for the radiolabeled polymer) of a 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 10 day time intervals and the experiment was run for 30 days.

### 2.6. High-performance liquid chromatography

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., Bedford, MA) centrifugal filtration units with a nominal molecular weight cutoff of 5000. Samples were filtered for 2–4 h at 3000 rpm (IEC Clinical Centrifuge, Needham, MA). The effectiveness of this technique in removing residual enzyme was previously reported on by Wang et al. [39].

A gradient method was developed to analyze the degradation products. The gradient method used a mobile phase consisting of methanol (solvent A), and 2 mM ammonium acetate (Aldrich Chemical Company) adjusted to pH 2.7 with acetic acid (Solvent B, Aldrich Chemical Company). Solvent C consisted of HPLC-grade H<sub>2</sub>O (Caledon Labs, Georgetown, ON, Canada), which was used to flush the column of the buffer salts. The gradient program is described in Table 1. The flow rate was 1 ml/min with an initial system pressure of approximately 600 psi.

### 2.7. Mass spectrometry

Product peaks were collected for mass spectrometric analysis. A Perkin-Elmer/Sciex (Concord, ON, Canada) API-III triple quadrupole, ionspray mass spectrometer was used. The mobile phase was pumped at a flow rate of 0.02 ml/min using an LKB Bromma (Sweden) HPLC pump. The mobile phase was a solution of 50%

Table 1  
HPLC gradient program<sup>a</sup>

Time (min)	Flow (ml)	% A	% B	% C	Curve
0	1	10	90	0	
90	1	100	0	0	Linear
100	2	0	0	100	Linear
110	2	100	0	0	Linear

<sup>a</sup>A = methanol; B = 2 mM ammonium acetate, pH = 2.7; C = H<sub>2</sub>O.

acetonitrile and 50% 1 mM ammonium acetate, and 0.1% acetic acid. The voltage applied to the tip of the ion spray needle was 5 kV and the voltage applied to the orifice was 80 V. Sample volumes of 5–20  $\mu\text{l}$  were injected directly, depending on the concentration of the products.

The ion spray spectrometer gives a spectrum of protonated molecular ions ( $\text{MH}^+$ ) in the sample. Additional adducts of the molecular ions were produced ( $\text{MNa}^+$ ,  $\text{MK}^+$ ,  $\text{MNH}_4^+$ ) as they were present in the mobile phase or sample. Tandem Mass Spectrometry (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 100–2000 amu is better than  $\pm 0.5$  amu.

Preparation of the degradation product samples for mass spectrometry was carried out by the following procedure. Each HPLC product peak was collected in 15 ml polypropylene vials (Corning, Fisher Scientific, Unionville, ON, Canada). Evaporation of the organic phase was carried out in a  $\text{N}_2$  stream, and the remaining mobile phase was freeze-dried overnight. The product was reconstituted with 5–20  $\mu\text{l}$  of mobile phase prior to injection into the mass spectrometer.

### 2.8. Antimicrobial activity of degradation solutions

A broth microdilution assay [40] was used to determine the antimicrobial activity of the degradation solutions by determination of minimum inhibitory concentrations (MICs). An 18 h nutrient broth culture of *P. aeruginosa* was washed three times in phosphate-buffered saline (PBS), pH 7.0, and resuspended in PBS. A Mueller-Hinton broth dilution series containing the degradation solutions was inoculated with a solution of  $2 \times 10^7$  colony-forming units (CFU)/ml. The suspensions were incubated at 37°C for 18–24 h. Each well was recorded as turbid (growth) or clear (no growth) visually. A well containing broth only was inoculated as a positive control. Using the measured MIC value for ciprofloxacin with *P. aeruginosa* (i.e. 0.5  $\mu\text{g}/\text{ml}$ ) it was possible to estimate the ciprofloxacin-like activity associated with the samples.

## 3. Results

### 3.1. Polymer synthesis and characterization

The weight average molecular weight ( $\overline{M}_w$ ; polystyrene equivalent) of the drug polymer was determined to be  $2.4 \times 10^4$  with a polydispersity of 1.6. Bulk fluorine content was determined by Quelp Chemical Laboratories to be 0.33 wt% by elemental analysis. The dry polymer

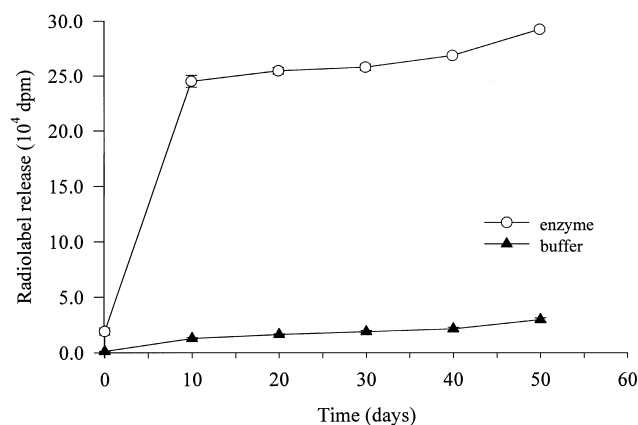


Fig. 2. Cumulative radiolabel release of HDI containing products.

was slightly yellow in colour and the yield for the polymer synthesis was approximately 60%.

### 3.2. Radiolabel release experiment

In order to confirm polymer degradation, a radiolabeled analog of the polymer was synthesized with  $^{14}\text{C}$ -HDI, and biodegradation testing was performed. The radiolabeled drug polymer had a similar  $M_w$  (i.e.  $2.0 \times 10^4$ ) to the non-radiolabeled polymer. The specific radioactivity of the polymer was determined to be  $2.09 \times 10^5$  disintegrations per min (dpm)/mg polymer. The cumulative radiolabel release profiles, following incubation in buffer and enzyme solutions, are given in Fig. 2. The radiolabel release with enzyme was initially high within the first ten days and then followed a slower but increasing release of product with time.

### 3.3. HPLC separation and tandem mass spectrometry of biodegradation products

Fig. 3 shows the HPLC chromatograms for the drug polymer incubated at pH 7.0 for 10 days. As shown in chromatogram a, 7 major peaks were detected in the CE incubated sample. In contrast, these peaks are present in significantly smaller quantities for the buffer incubated polymer (chromatogram b), with the exception of peak 1, which corresponds to free ciprofloxacin, as shown by the injection of the standard (chromatogram c). Chromatogram d shows the injection of a mobile phase blank, which demonstrates the presence of an impurity peak which partially overlaps with peak 3 in chromatogram a. The UV spectrum of the impurity peak (Fig. 4) for the blank differs from that of peak 3 from chromatogram a (Fig. 3).

Fig. 4 shows UV spectra, obtained from the photodiode array detector for each of the product peaks detected, for the CE incubated samples. The spectra of the

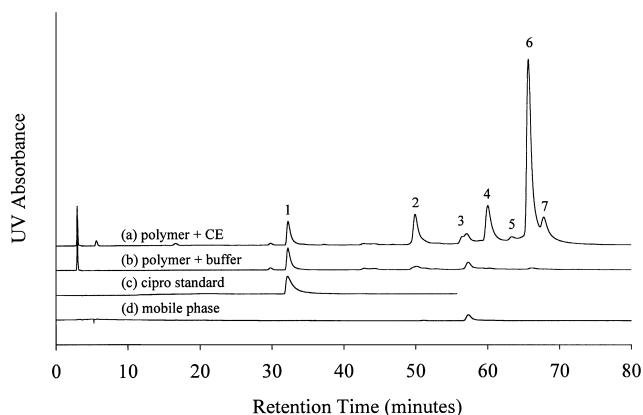


Fig. 3. HPLC chromatograms of drug polymer following biodegradation.

peaks appear to have similar characteristics to the spectrum of pure ciprofloxacin; however, some do contain additional absorbance maxima not found in that of the

pure drug. This suggests that these are likely derivatives from the polymer which contain the drug component.

The radiochromatograms of the 10 day incubation samples in CE and buffer are shown in Fig. 5. Elevated levels of radioactivity are associated with peaks 2, 3, 4, 6, and 7 in the CE incubated sample. 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. As shown in Fig. 5a, there are retention times where there is a significant radiolabel release, but a weak UV absorbance (specifically, retention times of 48 and 54 min). The buffer control (Fig. 5b) yielded background levels (i.e. 20–30 dpm) of radioactivity, indicating significantly less degradation of the polymer that was incubated with buffer as compared to treatment with cholesterol esterase. An additional fraction was collected for identification (fraction R in Fig. 5a) by mass spectrometry, since it contained a strong radiolabel signal but a weak UV signal. Each of the eight HPLC fractions (shown in chromatogram a, Fig. 5) was collected and analyzed via

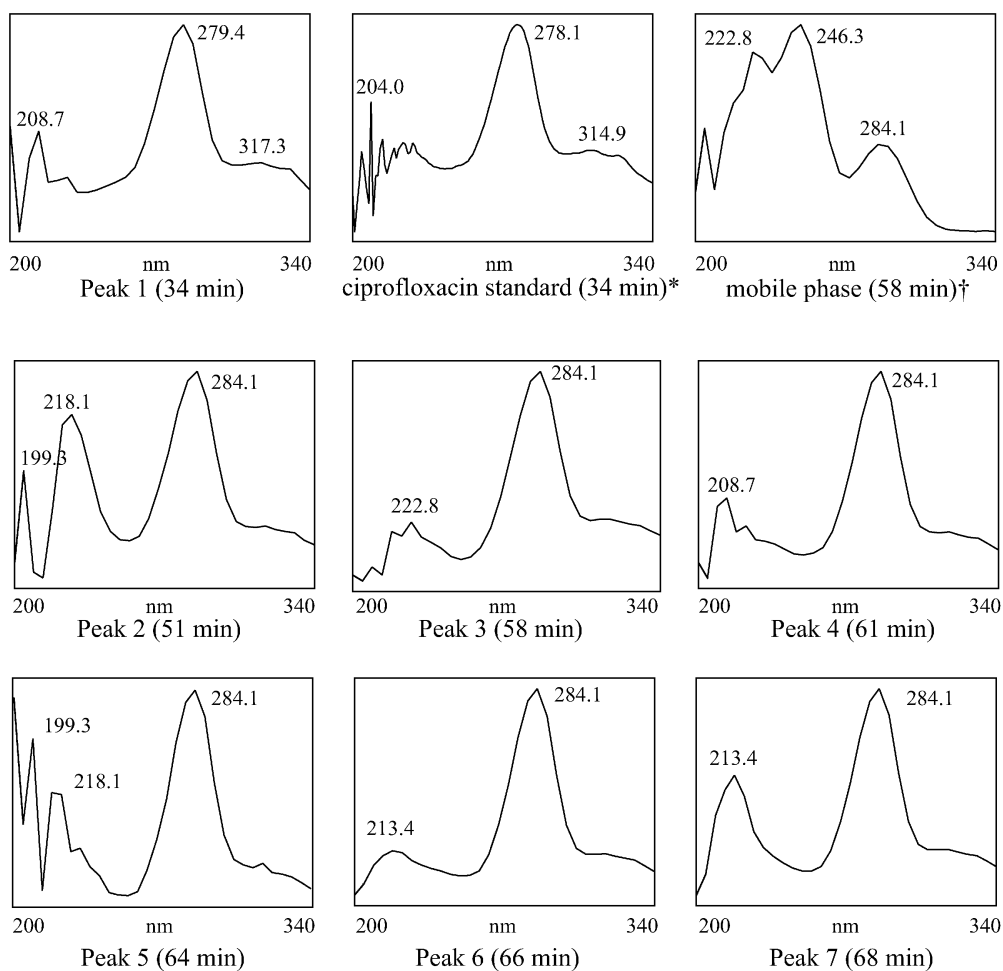


Fig. 4. UV spectra of degradation products from enzyme incubation (\* denotes ciprofloxacin standard. † denotes impurity peaks associated with the mobile phase, chromatogram d, Fig. 3).

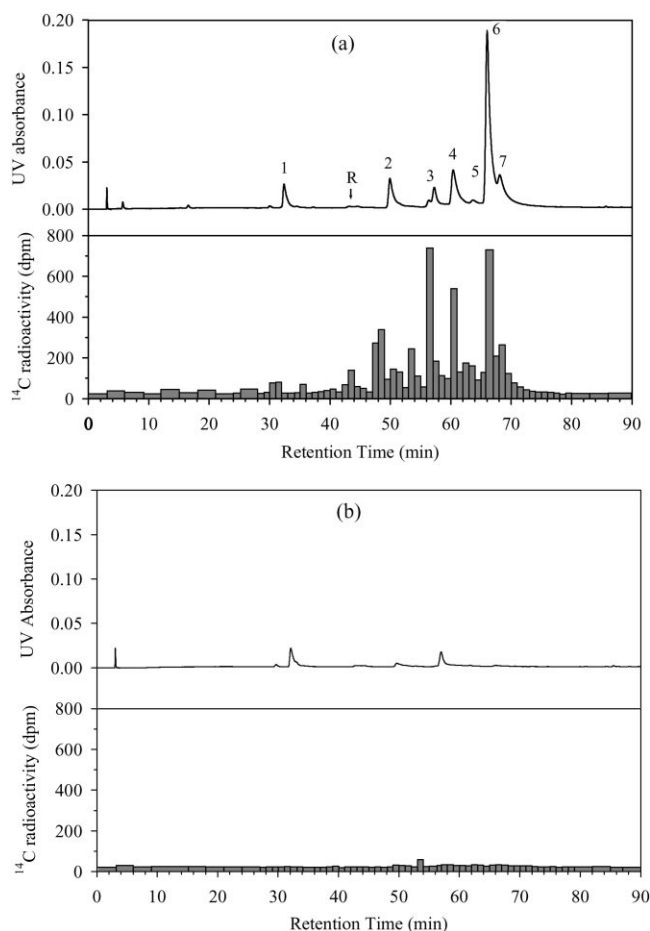


Fig. 5. Radioactivity of separated products for (a) [ $^{14}\text{C}$ ]-drug polymer incubated for 10 days, with CE, pH = 7.0, (b) [ $^{14}\text{C}$ ]-drug polymer incubated for 10 days, without CE, pH = 7.0.

tandem mass spectrometry. Table 2 shows the proposed structure of each of the products detected. This information shows that the mass spectra of peaks 3, 4, 5, and 7 contained more than one molecular ion. For the purpose of nomenclature, each of the products found within one of these peaks was designated with a lowercase letter, as shown in Table 2. The proposed chemical structures of several products include the drug coupled to HDI and fragments of PCL. In addition, three of the products show allophanate linkages (products 4b, 7, R), which indicate that cross reactions had likely occurred during the synthesis.

#### 3.4. Quantification of biodegradation products and ciprofloxacin release profile

The ciprofloxacin release profile was determined by calculating the area under the ciprofloxacin peak (retention time: 34 min, Fig. 3) and the cumulative release of ciprofloxacin is shown in Fig. 6. No significant difference ( $P > 0.05$ ) in cumulative ciprofloxacin release between enzyme and buffer controls was observed. This result was

also reproduced for the radiolabeled analog (data not shown).

Fig. 7 shows a plot of the cumulative release of the degradation products for the drug polymer at 10, 20 and 30 days. The products show an increasing trend over the 30 days for all compounds with the exception of fractions 5 and 7. However, it should be noted that fractions 6 and 7 are not completely resolved in the HPLC chromatogram (Fig. 3) and therefore the exact trend for these two products may differ somewhat from that shown in Fig. 7.

#### 3.5. Antimicrobial activity of incubation solutions

The concentration of antimicrobial activity for the drug polymer are shown in Table 3. A value of  $1\ \mu\text{g}/\text{ml}$  (representing a range of  $0.62\text{--}1.24\ \mu\text{g}/\text{ml}$ , based on the MIC calculation) was determined for both buffer and enzyme incubated samples for 10, 20 and 30 days. The concentration ranges determined by the MIC calculation, although slightly lower than the concentration values for the HPLC analysis, show similar trends when comparing data for the buffer vs. enzyme incubated samples.

## 4. Discussion

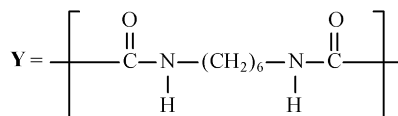
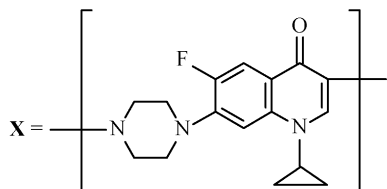
The majority of medical devices that contain antibiotics are impregnated systems relying on simple diffusion of the antibiotic either from the bulk of the polymer or directly from the surface of the device. The antimicrobial drug polymer synthesized in this study, released the drug in both the active and non-active form, in the presence of enzymes associated with inflammatory cells. Since inflammation occurs during the initial surgical implantation [41], enzymes will be present to release the drug. In addition, the presence of bacteria contributes to further inflammation and therefore further stimulation for the release of enzymes. In this study, polyurethane chemistry (diisocyanates) was used in conjunction with a fluoroquinolone antibiotic as a model polymer system. In theory, it is possible to use other chemistries, provided that the antibiotic can be polymerized into the backbone of the polymer, and that physiologically relevant enzymes present in a clinical situation are capable of cleaving the antibiotic from the backbone.

The drug polymers were synthesized by first forming a diisocyanate-PCL prepolymer. The traditional chain extender was replaced by the antibiotic, ciprofloxacin. In acid form, the ciprofloxacin powder is difficult to dissolve, and the solution must be heated to  $60^\circ\text{C}$ . In order to facilitate dissolution, triethylamine was added as a scavenger for the hydrogen ions present on the amine group of the ciprofloxacin molecule.

It was noted that the final reaction mixture contained small insoluble precipitates. Since the weight average

Table 2  
Proposed structures of biodegradation products

Product	<i>m/z</i>	Structure
1	332	<b>X</b>
2	474	$\text{H}_2\text{N}-(\text{CH}_2)_6-\overset{\text{H}}{\underset{ }{\text{N}}}-\overset{\text{O}}{\parallel}{\text{C}}-\text{X}-\text{COOH}$
3a	616	$\text{H}_2\text{N}-(\text{CH}_2)_6-\overset{\text{H}}{\underset{ }{\text{N}}}-\overset{\text{O}}{\parallel}{\text{C}}-\text{X}-\text{Y}-\text{OH}$
3b	632	$\text{HO}-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_5\text{O}-\text{Y}-\text{O}-(\text{CH}_2)_5-\overset{\text{O}}{\parallel}{\text{C}}-\text{OH}$
4a, 5b	606	$\text{HO}-(\text{CH}_2\text{CH}_2\text{O})_2-\text{Y}-\text{X}-\text{COOH}$
4b	575	$\text{HO}-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_5-\text{O}-\overset{\text{O}}{\parallel}{\text{C}}-\overset{\text{H}}{\underset{ }{\text{N}}}-\text{H}-(\text{CH}_2)_6-\overset{\text{O}}{\parallel}{\text{C}}-\text{O}-(\text{CH}_2)_5-\overset{\text{O}}{\parallel}{\text{C}}-\text{OH}$ $\begin{array}{c}   \\ \text{C}=\text{O} \\   \\ \text{N}-\text{H} \\   \\ (\text{CH}_2)_6 \\   \\ \text{NH}_2 \end{array}$
5a	748	$\text{HO}-(\text{CH}_2\text{CH}_2\text{O})_2-\text{Y}-\text{X}-\text{Y}-\text{OH}$
6, 5c, 7b	632	$\text{HO}-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_5-\text{O}-\text{Y}-\text{X}-\text{COOH}$
7	774	$\text{HO}-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_5-\text{O}-\overset{\text{O}}{\parallel}{\text{C}}-\overset{\text{H}}{\underset{ }{\text{N}}}-\text{H}-(\text{CH}_2)_6-\overset{\text{H}}{\underset{ }{\text{N}}}-\overset{\text{O}}{\parallel}{\text{C}}-\text{X}-\text{COOH}$ $\begin{array}{c}   \\ \text{C}=\text{O} \\   \\ \text{N}-\text{H} \\   \\ (\text{CH}_2)_6 \\   \\ \text{NH}_2 \end{array}$
R	417	$\text{H}_2\text{N}-(\text{CH}_2)_6-\overset{\text{O}}{\parallel}{\text{N}}-\overset{\text{O}}{\parallel}{\text{C}}-\text{O}-(\text{CH}_2)_5-\overset{\text{O}}{\parallel}{\text{C}}-\text{OH}$ $\begin{array}{c}   \\ \text{C}=\text{O} \\   \\ \text{N}-\text{H} \\   \\ (\text{CH}_2)_6 \\   \\ \text{NH}_2 \end{array}$



molecular weights were significantly lower than conventional polycaprolactone-ethylene diamine chain extended polyurethanes [33], it was suspected that the

reaction was perturbed by side reactions, possibly branching or cross-linking, which may have been promoted by the presence of the tin catalyst. This possibility

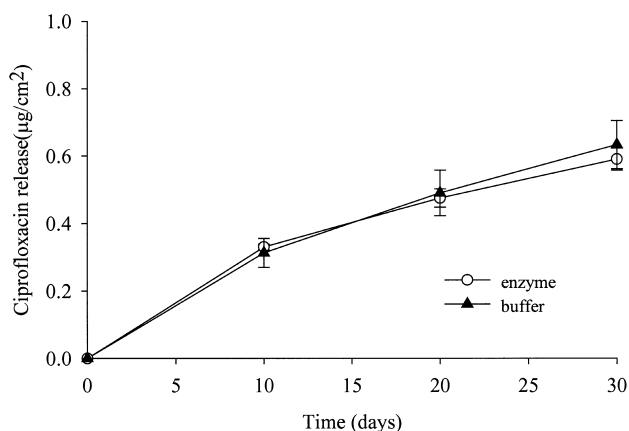


Fig. 6. Cumulative ciprofloxacin release from drug polymer. Error bars represent  $\pm 1$  standard deviation.

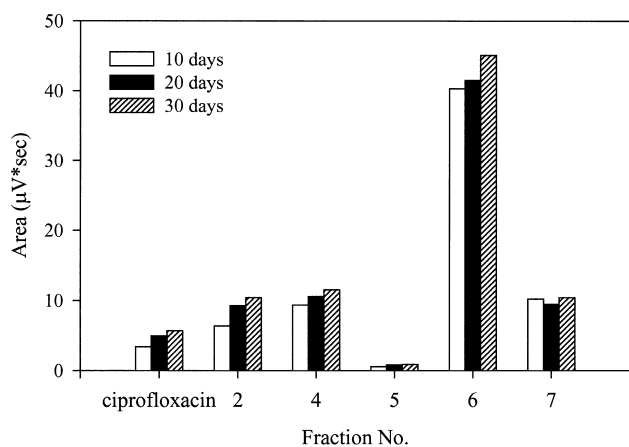


Fig. 7. Cumulative HPLC peak areas associated with product release from [ $^{14}\text{C}$ ]-drug polymer incubated with CE, 37°C, pH 7, for 10, 20 and 30 days.

was supported by the less than theoretical value of fluorine content (i.e., 0.33 vs. 0.71 wt% (theoretical based on reagent stoichiometry)) as analyzed by elemental analysis, since isocyanate groups involved in these cross-reactions would not be available for reaction with ciprofloxacin. Further evidence for the formation of cross-linked components in the synthesis was provided by the mass spectrometry data (Table 2), which revealed that products R, 4b, and 7 contained branched diisocyanate derivatives. On-going work is being conducted in order to probe the kinetics of the reactions and investigate alternative reaction strategies that could minimize the possibility of cross-reactions.

The results shown in Fig. 2 illustrate that CE is able to cause a greater amount of radiolabel release than buffer alone. The pattern of radiolabel release is similar to that previously reported for a polyesterurethane consisting of 2,4-toluene diisocyanate, polycaprolactone, and ethylene diamine [33]. Although the radiolabel tracer technique shows that the drug polymer is degraded by the action of

Table 3

Ciprofloxacin concentrations determined by MIC calculations and HPLC

Treatment	Day 0	Day 10	Day 20	Day 30
<i>MIC calculations (ciprofloxacin, µg/ml)<sup>a</sup></i>				
CE	< 0.5	1	1	1
Buffer	< 0.5	1	1	1
<i>HPLC (ciprofloxacin, µg/ml)<sup>b</sup></i>				
CE	< 0.57	2.73 $\pm$ 0.058	2.57 $\pm$ 0.15	2.17 $\pm$ 0.15
Buffer	< 0.57	2.63 $\pm$ 0.21	2.77 $\pm$ 0.35	2.7 $\pm$ 0

<sup>a</sup>0.5 = (>0.31 and <0.62 µg/ml); 1 = (>0.62 and <1.24 µg/ml).

<sup>b</sup>MIC for ciprofloxacin with *P. aeruginosa* was 0.5 µg/ml (determined in laboratory). The value of 0.57 µg/ml was the lowest concentration point obtained for the HPLC calibration curve.

the enzyme, it was necessary to measure degradation by HPLC in order to determine the extent of ciprofloxacin release and the form of its release.

The HPLC analysis of the biodegradation solutions showed that the drug polymer produced a significant number of degradation products in addition to free ciprofloxacin. The determination of their structures not only provided information on how CE was cleaving the drug polymer, but also provided clues as to how the drug reacted with the other reagents during the polymerization. Proposed structures for these products were generated based on the molecular masses of the fragments detected in the MS/MS spectra. Products 2 and 3a (Table 2) consist of ciprofloxacin bonded to HDI while products 4a and 5a possess a diethylene glycol unit, which is a component in the synthesis of PCL [39,42]. Products 6 and 7a contain a caproic acid segment, which is the primary monomer of PCL. Products 5b and 4a are structurally identical, while products 5c and 7b are identical to product 6. This demonstrates the need for further refinement of the separation methods, since the HPLC analysis did not fully resolve these latter products in separate peaks (chromatogram a, Fig. 3).

From the analysis of the HPLC results over time (see Fig. 7), 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 polycaprolactone, at either the caproate or diglycol group. This also corresponds to HPLC data reported by Wang et al. [42], 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 (Table 2). The results also show the presence of allophanate linkages, products 4b, 7a, and R, which as mentioned earlier, suggest that side reactions of the polymer occurred during the synthesis. This is consistent with the fact that the polymers

were difficult to dissolve in DMSO, DMAC, and DMF, and that the molecular weights were lower than anticipated.

The dominant drug-containing product, based on the highest UV absorbance in Fig. 3, was associated with product 6 ( $m/z$  632). The product was produced by the cleavage of one of the ester bonds of the PCL molecule (Table 2). 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. 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 (Fig. 7). It should be considered that the presence of unreacted terminal ends possibly resulted from the fact that –NCO groups were being consumed in the branching or cross-linking reactions. These considerations suggest that either the reaction of ciprofloxacin at the carboxylic acid end may not have been favored or that this end was the most susceptible to hydrolysis during the incubation periods.

Fig. 6 shows the release of free ciprofloxacin over time as calculated by HPLC. Unlike the radiolabel release profiles, there was no significant difference in free ciprofloxacin release between the polymer incubated with either CE or buffer. It is clear from these data that although the polymer is degrading, the enzyme is unable to specifically cleave the polymer segments required to release free ciprofloxacin. 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. 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 (Figs. 2 and 3).

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 Fig. 6). It is interesting to note that the radiolabel release also shows a high initial release for both enzyme and buffer treatments (Fig. 2). 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 [33] and CE diffusion into the polymer is limited [43]. A third possibility is that since the enzyme must be continuously replenished during the experiment, there may be significant adsorption of enzyme breakdown products onto the surface of the polymer, thus blocking available hydrolysis

sites [43]. Smith et al. [44] 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.

The results of the broth dilution assay showed that the ciprofloxacin released from the drug polymer possessed antimicrobial activity against *P. 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. Table 3 shows that the MIC calculations did not precisely yield the same values as the HPLC analysis. However, differences in concentrations obtained from biological and chemical assays of antibiotics sometimes reflect availability (i.e., solubility) of the analyte to the bioassay organism [45].

Based on the data obtained by the antimicrobial activity assay, these ciprofloxacin derivatized degradation products are unable themselves to inhibit the growth of *P. aeruginosa*. This can be concluded based on the fact there was no significant difference in antimicrobial activity between the CE and buffer-incubated samples (see Table 3). This may be rationalized by considering the mechanism of action of ciprofloxacin. Quinolones, including ciprofloxacin, inhibit the activity of the bacterial enzyme DNA gyrase, which leads to bacterial cell death [46,47]. In order for this to occur, the drug must bind to specific portions of the bacterial DNA during protein synthesis [46]. Shen et al. [49] have hypothesized that the quinolone molecules form supermolecules through hydrogen bonding. The carboxylic acid group is important for this reaction to occur, as it is part of the minimum pharmacore (structure required for pharmacological activity) of the antibiotic [48]. 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. The results of this study suggest that complete degradation of the polymer is necessary for the ciprofloxacin to exhibit antibacterial activity.

## 5. Conclusions

In summary, this study showed that an antibiotic could be polymerized into the backbone of a polymer, and that the polymer could be degraded by an inflammatory cell-derived enzyme, cholesterol esterase. Analysis of the solutions showed that ciprofloxacin was released and able to inhibit the growth of *P. aeruginosa*. Although the enzyme was able to cleave the polymer and produce multiple antibiotic-containing degradation products, an enhanced release of free ciprofloxacin was not exhibited with this polymer formulation. The degradation products containing ciprofloxacin bonded to

fragments of PCL and HDI did not display antimicrobial activity. In order to enhance the release of active drug, a number of possibilities can be further explored. The use of other inflammatory cell enzymes in conjunction with CE may be able to further degrade the degradation products to produce free drug. Furthermore, the alteration of the polymer backbone structure may allow CE to have greater specificity and result in a further generation of free ciprofloxacin. Currently, the use of 1,12-dodecane diisocyanate is being investigated, and preliminary results show a more specific enzyme-catalyzed release of free drug [50]. Since the presence of degradation by-products, other than that of the free drug were observed, it also becomes important to consider toxicity issues and these experiments are being carried out in conjunction with the development of new polymer formulations.

It should become intuitively obvious that formulations could be synthesized to take advantage of other enzyme systems than those of the inflammatory response. One could even capitalize on enzymes generated by bacteria themselves. An example of bacteria generating significant levels of enzymes are those associated with periodontal disease. Trypsin-like activities are generated by *Porphyromonas gingivalis* [51] and this activity has been documented to degrade polyurethane type structures [44,52,53]. Hence, one needs only to work out an appropriate sequence of chemical function in the polyurethane or other polymer type which would be sensitive to this enzyme system. It would not be inconceivable that multiple drug polymer types could be co-processed into the form of a device, thereby endowing the device with multiple sensitivity. Just as the sensitivity to enzyme degradation is important, so would be the selected drugs. Ciprofloxacin has a limited range of function and other fluoroquinolones or families of drugs may need to be used to provide a broad spectrum of protection. As well, while this paper has focused on the example of delivering antibiotics it is not inconceivable that other therapeutic drugs could be delivered by such systems.

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