
Biological characterization of a novel biodegradable antimicrobial polymer synthesized with fluoroquinolones

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Abstract: Biomaterial-related infections continue to represent a significant challenge to the medical community. Several approaches have been utilized to incorporate antimicrobial agents at the surface of implant devices in attempts to delay or eliminate the formation of biofilms. To date, most of these strategies have focused on drug conjugation or diffusion-limited systems for the delivery of such pharmaceutical agents. More recently, work has been presented on the feasibility of incorporating drugs into the backbone of polymers as a main-chain monomer. When sequenced into the backbone of the polymer with other monomers that are hydrolytically sensitive to enzyme-catalyzed breakdown, it is thought that drugs may be able to be selectively released. Specifically, degradable polyurethanes have been synthesized with fluoroquinolone antibiotics and have shown an ability to kill bacteria when released following degradation of the polymer chains by the macrophage-derived enzyme cholesterol esterase. However, specificity of the cleavage

sites in the polymer was difficult to control. Since cholesterol esterase has specificity for hydrophobic moieties, it is desirable to alter the formulation of the polyurethanes to incorporate long hydrophobic monomers immediately adjacent to the ciprofloxacin molecule. Hence, the current study focuses on evaluating the enzyme-catalyzed degradation of a degradable polyurethane synthesized with 1,12 diisocyanatododecane as a substitute for 1,6 diisocyanatohexane, which was used in previous work. Validation of specific ciprofloxacin release and the generation of antimicrobial are shown. A preliminary cell study to assess the cytotoxicity of this biodegradable antibiotic polymer shows that the material has no observable effects on cell proliferation or cell membrane structure. © 2001 John Wiley & Sons, Inc., *J Biomed Mater Res* 59: 35–45, 2002

Key words: ciprofloxacin; antimicrobial; polyurethane; biodegradation; enzyme; bacteria; cytotoxicity; drug polymer

INTRODUCTION

Infections associated with biomaterials represent a significant challenge to the more widespread application of medical implants.^{1,2} The presence of a foreign body such as a synthetic biomaterial provides a site for microbial attachment.³ The adhesion of bacteria to a biomaterial surface has been recognized as the critical event in a series of reactions that lead to biomaterial-

related infections. Following adhesion, bacterial cells begin to accumulate and produce extracellular polymeric substances (EPS). Bacteria growing within the EPS matrix form an adhesive "biofilm" and are afforded protection against the surrounding environment.⁴ The biofilm has been found to impair the activity of antimicrobial agents, phagocytic cells, and humoral components of the host immune system.^{5,6} Some common etiological agents associated with device-related infections include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Candida albicans*.^{3,7}

Bacteria growing within biofilms can withstand many times the dosage of antibiotic sufficient to completely eradicate planktonic (free-floating) bacteria.^{8–10} Consequently, there has been an effort towards the development of coatings that release antibacterial agents from the surface of the biomaterial. In this manner, it is thought that such a material can provide a

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local concentration of agent sufficient to eradicate bacteria and prevent the formation of a biofilm. Methods that have been explored include ionic binding techniques,^{11,12} the use of silver,¹³ and physico-chemical surface modification.¹⁴ However, frequent problems include the rapid release of antibiotic^{15,16} and the failure to show a reduced frequency of infection.^{17,18} In addition, the potential exists for emergence of antibiotic-resistant strains associated with the more widespread use of diffusible antibiotics.^{19,20}

Consequently, there remains a need for the development of new methods to prevent bacterial adhesion. An alternate approach to antimicrobial drug delivery would involve releasing the antimicrobial only in the presence of infection. This could be achieved by using an actively controlled drug delivery system that releases a drug based on an external stimulus. These systems previously have been developed using a number of different methods, including electric fields,²¹ magnetic fields,²² and enzyme reactions,²³ as triggering mechanisms.

Suzuki et al. recently proposed the use of an enzymatically degradable peptide link between the antibiotic and polymer carrier as an on-off switch for the delivery of antibiotic in a wound dressing.²⁴ The approach described in the current study utilizes an antimicrobial polymer that contains the antimicrobial agent linked covalently as a monomer into the backbone structure of the macromolecule. The polymer was designed specifically to be degraded by enzymes that are generated by cells present during the inflammatory response. Therefore, since the implantation of a biomaterial, in general, will trigger inflammatory processes,²⁵ the release of antibacterial agents would be triggered at the time of implant. As wound healing occurs, the level of enzyme production will decrease, and thus antibiotic release subsequently would decrease. Such an approach could provide an effective barrier to infection until tissue integration is complete.²⁶

Previous work has demonstrated the feasibility of this concept: ciprofloxacin, a fluoroquinolone antibiotic, covalently was incorporated into a polyurethane co-polymer²⁶ with hexane diisocyanate and polycaprolactone. The degradation of the polymer and the release of antibiotic in the presence of cholesterol esterase were demonstrated using an *in vitro* biodegradation assay.²⁶ The current study has focused on evaluating the enzyme-catalyzed degradation of a similar polymer, synthesized with 1,12 diisocyanatododecane, and has demonstrated the ability of acute inflammatory cells (i.e., neutrophils) to release antimicrobial activity from the drug polymer system. A preliminary cell cytotoxicity study also was carried out to assess the toxicity of this biodegradable antibiotic polymer.

MATERIALS AND METHODS

The detailed sequence of experimental procedures is outlined in Figure 1. The drug polymers were synthesized and characterized by gel permeation chromatography (GPC) and elemental analysis. Three assays were used to evaluate the materials: an enzyme biodegradation assay with cholesterol esterase (to model an aspect of the hydrolytic activity associated with human macrophages), a neutrophil cell culture technique,³⁵ and a mouse fibroblast model to assess cell toxicity.

Synthesis of drug polymers

The drug polymer was synthesized using 1,12-diisocyanatododecane (DDI; 97% purity), polycaprolactone diol, avg. MW 2000 (PCL), and dibutyltin dilaurate catalyst (97%), all of which were obtained from the Aldrich Chemical Company (Milwaukee, WI). The antibiotic ciprofloxacin hydrochloride (cipro) generously was provided by Bayer Healthcare Inc., Mississauga, ON, Canada, and was chosen due to its broad-spectrum activity as well as for the presence of reactive amine and carboxylic acid functional groups. A radiolabeled analog of the drug polymer was synthesized to monitor the biodegradation of the polymer by the enzyme. Radiolabeled DDI (¹⁴C-DDI) (NEN, DuPont Custom Synthesis, Billerica, MA) was supplied in amber glass ampoules, each containing 0.25 mCi dissolved in anhydrous toluene. 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) and counting in a liquid scintillation counter (Beckman LS500, Mississauga, ON, Canada).

Before the synthesis, DDI was vacuum distilled over CaH₂ (ACP Chemicals Inc., Montreal, PQ, Canada), sealed in glass ampoules under dry nitrogen, and stored at room temperature until use. PCL was dried at 55°C overnight in a vacuum oven. Dimethyl sulphoxide (DMSO) (HPLC grade, Aldrich Chemical Company) was vacuum-distilled over CaH₂ immediately before polymerization. Cipro was dried at room

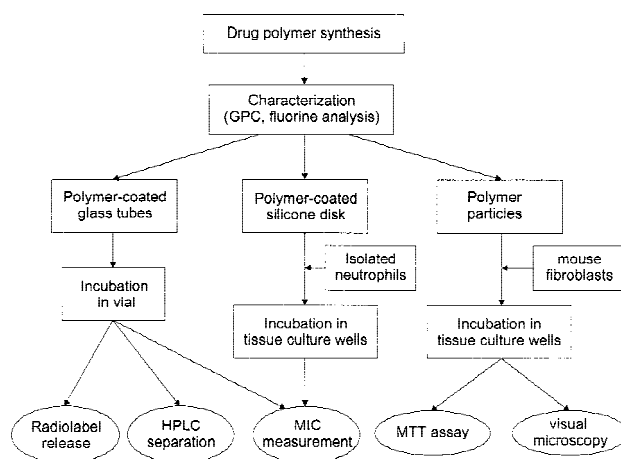


Figure 1. Experimental protocol.

temperature for 1 week in a vacuum oven over a bed of anhydrous calcium sulfate desiccant (W.A. Hammond Drierite Co., Xenia, OH).

The reaction consisted of a standard two-step solution polymerization reaction. The synthesis was carried out in a Labconco® glove box (Fisher Scientific, Unionville, ON, Canada) under a dry nitrogen atmosphere. The stoichiometry of the reactants was 2.5 : 1.6 : 0.625 of DDI : PCL : ciprofloxacin, respectively. In the first step of the synthesis, DDI, PCL, and dibutyltin dilaurate (0.01 mmol) were added to 10 mL of DMSO in a glass reactor. For the radiolabeled polymer, 0.25 mCi of ¹⁴C-DDI was mixed with the non-radiolabeled DDI. The prepolymer solution was stirred continuously by a magnetic stirrer and maintained between 55°–65°C using a temperature controller (VWR series 400 HPS, Mississauga, ON, Canada). This mixture was allowed to react for 3 h. In the second step of the reaction, 0.4 g of cipro was added along with an additional 10 mL of DMSO. Triethylamine (1.04 mmoles; Aldrich Chemical Company) was added to facilitate cipro dissolution. The polymer solution was allowed to react for an additional 22 h, then was cooled to 25°C. The polymer solution then was quenched with methanol and precipitated in distilled water. The solid was washed with distilled water using a soxhlet extractor for 2 days, followed by stirring in a beaker for 1 day to remove unreacted drug. Finally, the polymer was filtered and subsequently dried in a convection oven at 50°C for 24 h, followed by vacuum drying at 50°C for an additional 24 h.

The nomenclature of the synthesized polymers represents the agents used in their synthesis, that is, DDI/PCL/cipro. There also may be an alphanumeric code at the end of the name if reference is being made to a specific batch number. For the purpose of comparison to previous work,²⁶ the properties of DDI/PCL/cipro were compared to two other polymers. The first of these two materials was a polymer consisting solely of PCL and DDI synthesized in one step using 1:1 stoichiometry (DDI/PCL). This polymer enabled the assessment of toxicity for nondrug materials and was considered as a negative control in the antimicrobial activity assays. Also included was a polymer having similar chemistry to DDI/PCL/cipro except it was synthesized with hexane diisocyanate (HDI) rather than with DDI. The details of the synthesis for this material and its characterization were reported previously.²⁶ This latter polymer was assigned the label HDI/PCL/cipro.

Material characterization

GPC was used to determine the average molecular weight and polydispersity of the drug polymers. The equipment consisted of a Waters GPC system (Mississauga, ON, Canada) and was described in previous work.²⁷ The mobile phase consisted of 0.05M of lithium bromide in N,N-dimethylformamide (DMF) (HPLC grade, Aldrich Chemical Company); the columns were maintained at 80°C while the flow rate was 1 mL/min. Polymer samples were prepared by dissolving the sample in the mobile phase at a concentration of 0.5 wt/wt %. The solutions were filtered using a 0.5- μ m PTFE syringe filter prior to injection.

Polymer samples were sent to Galbraith Laboratories (Knoxville, TN) for fluorine elemental analysis. The sample was combusted in a Schoniger flask according to ASTM E442.²⁸ Following combustion, the sample was dissolved in TISAB (total ionic strength adjustment buffer) and analyzed with an Orion fluoride-specific ion electrode (90-01) and a Fisher Accumet-specific ion meter, MP825. The detection limit using this procedure was 0.002 wt/wt %.

Enzyme biodegradation experiment

The biodegradation of the drug polymer in the presence of enzyme was evaluated by coating the polymer onto small hollow glass tubes. This was accomplished by preparing polymer solutions made from 10% (wt/wt) polymer in dimethylacetamide (DMAC). The details of this procedure were described previously.²⁶

Cholesterol esterase (CE) was selected as the enzyme for the biodegradation tests. The ability of CE to degrade polyesterurethanes has been documented in previous studies.^{29,30} Cholesterol esterase (bovine pancreas, Genzyme Diagnostics, Cambridge, MA) solutions were prepared by dissolving the powder in 0.05M of phosphate buffer, pH 7.0. Solutions of 40 units/mL activity were prepared and frozen at -80°C until required. The enzyme activity was determined with methods described previously.^{30,31}

The biodegradation test was carried out by placing 14 segments of polymer-coated tubing into autoclaved 1-dram screw-cap vials and incubating them with either phosphate buffer (pH 7.0) or buffer with 40 units/mL of CE at 37°C. Samples were incubated for 3 weeks and aliquots of the incubation solution were removed every week for radiolabel counting and drug analysis. Enzyme activity was replenished daily by adding 100 μ L of concentrated enzyme solution (800 units/mL). Data were plotted as disintegrations per minute (dpm) versus time.

High performance liquid chromatography (HPLC)

The incubation solutions from the enzyme biodegradation tests were analyzed by HPLC methods. Prior to injection, the solutions were filtered using UF-CL (Millipore Corp., Bedford, MA) centrifugal filtration units with nominal molecular weight cutoffs of 5000. Samples were filtered for 0.5–1 h at 3000 rpm (IEC Clinical Centrifuge, Needham, MA). The effectiveness of this technique in removing residual enzyme was reported previously.²⁷

A gradient method was developed to analyze the degradation products. This method used a mobile phase consisting of methanol (solvent A), and 2 mM of ammonium acetate (Aldrich Chemical Company) adjusted to pH 2.7 with acetic acid (Solvent B, Aldrich Chemical Company). Solvent C consisted of HPLC grade H₂O (Caledon Labs, Georgetown, ON, Canada), which was used to flush the column of the buffer salts. The gradient program was described previously.²⁶ The hardware consisted of a Waters HPLC system (Mississauga, ON, Canada) with a fluorescence detector

(Waters model 474, Mississauga, ON, Canada) and a data acquisition and processing computer (Waters Millennium).²⁶ The flow rate was set at 1 mL/min.

Antimicrobial assay

A broth microdilution assay³² 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* or *E. coli* (clinical isolate, Toronto General Hospital) was washed three times in phosphate-buffered saline (PBS), pH 7.0, and resuspended in PBS. A Mueller-Hinton broth dilution series containing aliquots of the degradation media was inoculated with a solution of 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 alone was inoculated as a positive control. The MIC level of ciprofloxacin against *P. aeruginosa* previously was reported to be 0.5 µg/mL²⁶ whereas the MIC level in the presence of *E. coli* was determined to be 0.05 µg/mL.

Cell cytotoxicity test

Mouse fibroblasts (ATCC CCL1, NCTC clone 929, American Type Culture Collection, Rockville, MD) were cultured in minimum essential medium (MEM; Sigma) supplemented with Eagle's salts and 10% fetal bovine serum. Five serial subcultures were performed and no contamination was found. A seed stock was stored by freezing the cells in MEM and 5% DMSO.

Small particles of drug polymer (obtained after synthesis and purification) were sterilized by EtO and allowed to degas under ambient conditions for 3 days. A cell suspension of 1.5×10^6 cells/mL was prepared. Five drops of suspension were added to 2 mL of medium in each well of a six-well tissue culture plate. A portion of each polymer (0.1 g) was mixed with 1 mL of fresh medium and transferred to wells on the plate. The cells were incubated for 24 h and examined with an image analysis system (Northern Exposure, Empix Imaging, Inc., Mississauga, ON, Canada). A blank control consisted of DDI/PCL (no ciprofloxacin), and a positive control consisted of a 0.45 wt/vol % phenol solution in MEM. Duplicate cultures were carried out for each of the test materials.

Cell viability was determined by using the MTT (3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl tetrazolium bromide) assay.^{33,34} After 24 h of cell-polymer contact, 0.08 mL of MTT were added into each well. The cells were further incubated for 4 h. The supernatant of each well was discarded and then 1.0 mL of DMSO was added to dissolve the MTT crystals. The absorbances of each well were recorded at 570 nm. All determinations were carried out in triplicate.

Neutrophil degradation assay

A degradation assay that uses human neutrophils in culture previously was developed to study polyurethane bio-

degradation.^{35,36} This assay was used to assess whether or not protease activity associated with the acute inflammatory response could degrade the drug polymer.

PMNs were isolated from whole human blood by using a modification of the procedure described by Boyum.³⁷ Whole blood (70 mL) was collected into heparin-containing Vacutainers® (Becton-Dickinson, Franklin Lakes, NJ) from normal healthy volunteers at the Toronto Hospital. Histopaque-1119 (Sigma-Aldrich, Oakville, ON, Canada) (5 mL) was added into 15-mL conical centrifuge tubes. Histopaque-1077 (Sigma-Aldrich, Oakville, ON, Canada) (2.0 mL) carefully was layered on top of the Histopaque-1119 in each tube, was allowed to warm to room temperature, and then was centrifuged (Beckman TJ-6, Mississauga, ON, Canada) at 700 g for 30 min. Whole blood (5 mL) carefully was layered onto the Histopaque solutions in each tube. The tubes then were centrifuged at 700 g for 30 min at room temperature. The upper layers were aspirated and discarded. The Histopaque-1119 layer was transferred with a pipette to a new centrifuge tube and diluted in fresh RPMI-1640 (Gibco BRL, Burlington, ON, Canada) (10 mL) to wash out the Histopaque. This suspension was centrifuged at 700 g for 15 min at room temperature. The supernatant was aspirated and the pellet resuspended in 10 mL of RPMI and further centrifuged at 700 g for 10 min at room temperature. Sterile water (5 mL) at 4°C was added to suspend the pellet and to lyse any remaining red blood cells. After 30 s, an equal volume of saline solution (1.8%) was added to return the solution to isotonicity. The suspension then was centrifuged for 10 min at 250 g and 4°C. The cell lysis step was repeated when necessary. Final pellets were resuspended and combined in a total of 20 mL of RPMI-1640 in a sterile 50-mL tube. The final concentration was adjusted with RPMI to 1×10^6 cells/mL. The solutions were stored on ice and used within 1 h.

Samples were prepared by coating the drug polymers onto 2-cm round silicone disks. Before coating, the disks were washed in distilled water, dried, and autoclaved. The disks were coated with a polymer solution made up in DMAC (10% wt/wt) and dried in a convection oven at 55°C. After 24 h, the coating was repeated, followed by further drying at 55°C under vacuum. The polymer-coated disks were placed on the bottom of a 12-well tissue culture plate (VWR Canlab, Mississauga, ON, Canada). The PMN cell suspension (3.0 mL) was added to each well containing the disks. The culture plates then were placed in an incubator at 37°C, 5% CO₂, and 100% humidity. At each time interval (4, 12, 24, 48, and 72 h), the contents of the wells were withdrawn and centrifuged at 1000 g for 15 min. The supernatants then were analyzed for antimicrobial activity using an MIC assay (described in the antimicrobial assay section).

The neutrophil activator, phorbol myristate acetate (PMA), was used in one of the neutrophil groups to assess the effect of activated neutrophils on the release of antimicrobial activity. PMA, received as a lyophilized powder, was dissolved in DMSO at a concentration of 1 mg/mL and stored at -14°C. On the day of the experiment, the PMA solution was thawed and diluted to $1 \times 10^{-7}M$ with PBS. A PMA/PBS solution (750 µL) was added to the wells and the plates were returned to the incubator.

RESULTS

Polymer synthesis and characterization

The reaction of DDI with PCL proceeded for 3 h and the solution remained clear throughout this step. Following the addition of cipro, the reaction solution remained clear for the first 2 h; then a small amount of fine precipitate was observed over the next 20 h and remained suspended in solution. Prior to the recovery of the polymer, the suspension was centrifuged, and the remaining clear supernatant polymer solution was recovered for precipitation. The precipitated polymers consisted of small white particles that subsequently were recovered through filtration from the water/solvent mixture. Average molecular weights and fluorine contents are summarized in Table I. The polystyrene equivalent weight average molecular weights for the two DDI-based polymers were similar to those of the HDI polymer, as reported in previous work.²⁶ The fluorine content for the DDI-based polymers is similar to those previously reported for drug polymers synthesized with HDI. These fluorine values can be converted to the wt % drug (rather than remaining as a wt % elemental fluorine) contained within the polymers, based on the molecular weight of the whole drug molecule. This conversion yields % drug values of 9.1%, 7.0%, and 5.7% for DDI/PCL/cipro(m30), DDI/PCL/cipro(s12), and HDI/PCL/cipro(s14), respectively.

Biodegradation studies

In order to study the degradation, a radiolabeled analog of DDI/PCL/cipro was synthesized using ¹⁴C-DDI. The specific radioactivity of the polymer was 3.4×10^5 dpm. This polymer was incubated with a macrophage-associated enzyme, cholesterol esterase. The cumulative radiolabel release profiles, following incubation in buffer and enzyme solutions, are given in Figure 2 ($n = 3$). The radiolabel release was initially

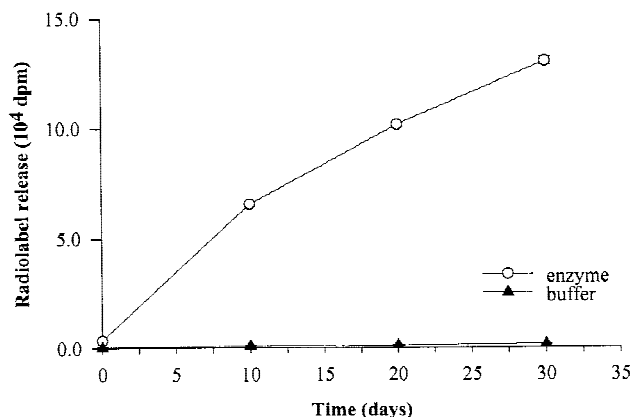


Figure 2. Cumulative radiolabel release.

high for the first 10 days and, with time, followed a slower rate of product release. At all time points, the accumulation of degradation product was greater ($p < 0.05$) for the enzyme-incubated samples than for the buffer-incubated groups.

HPLC analysis

Figure 3 shows the HPLC chromatograms for the drug polymer solutions from the biodegradation studies with buffer and cholesterol esterase (7-day data). All HPLC data were recorded at a wavelength of 280 nm, at which the maximum absorbance occurs for cipro-containing products. The injection of the ciprofloxacin standard produced a single peak with a retention time of 32 min (chromatogram c; Fig. 3). When comparing the enzyme- (chromatogram a) and buffer- (chromatogram b) incubated samples, it was seen that the enzyme did not induce the release of new drug-containing polymer degradation products other than those observed in the buffer sample. However, there are two peaks at approximately 7 and 16 min that are

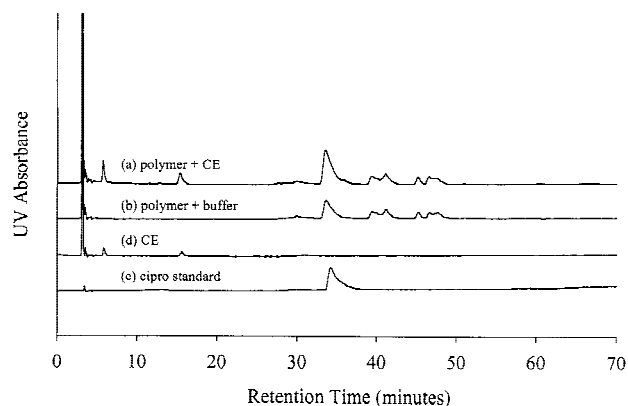


Figure 3. HPLC chromatograms of drug polymer biodegradation (7 day data).

TABLE I
GPC and Fluorine Content

Sample Name (Batch)	\overline{M}_w^a	PD ^b	Fluorine (wt %)
DDI/PCL	2.8×10^4	1.2	N/A ^c
DDI/PCL/cipro(m30)	2.0×10^4	1.6	0.52
DDI/PCL/cipro(s12)	2.4×10^4	1.6	0.4
HDI/PCL/cipro(s14)*	$2.4 \times 10^{4*}$	1.6*	0.33*

^a \overline{M}_w = polystyrene equivalent weight average molecular weight.

^bPD = polydispersity.

^cN/A = not measured.

*Reported from reference 26.

associated with the enzyme itself (chromatogram d). The drug peak at 32 min is the dominant peak for both the buffer- and the enzyme-treated groups although there is more of this product in the presence of the enzyme-treated polymer. The four smaller peaks appearing between 40 and 50 min are present in similar levels for both the buffer and the enzyme groups.

The 32-min peak in both the buffer- and enzyme-incubated solutions was confirmed by UV and mass spectrometry characterization methods to be ciprofloxacin, as previously reported on.²⁶ The quantitative analysis of the free drug release (i.e., the 32-min peaks) was carried out for the buffer and enzyme systems. Figure 4 shows the cumulative drug release per cm² of polymer surface area. Over the incubation period there is a significant increase in drug release ($p < 0.05$) for the enzyme-incubated samples compared to the buffer. The difference is most pronounced during the initial 7-day period.

Antimicrobial activity

Table II shows the results of the MIC assay for samples obtained from the biodegradation study. The buffer-incubated solutions showed no activity against *P. aeruginosa* (< MIC value of 0.5 $\mu\text{g/mL}$, determined experimentally using the pure drug) for the entire incubation time period. In contrast, the enzyme-incubated samples exhibited activities equivalent to the MIC value of 0.5 $\mu\text{g/mL}$ for days 7 through 28.

Cytotoxicity test

Figures 5 and 6 show light microscope images of the cells on the DDI and HDI drug polymers, along with

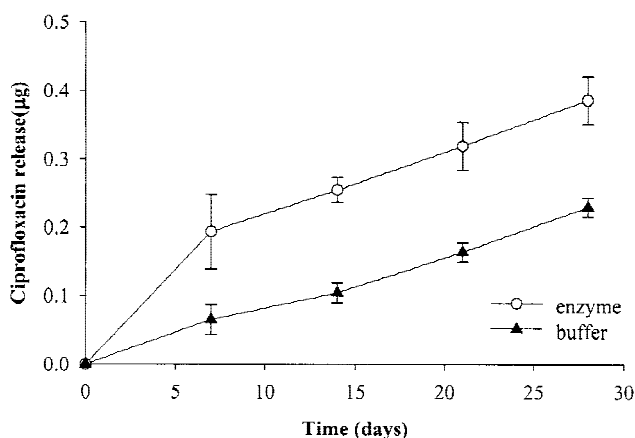


Figure 4. Cumulative ciprofloxacin release from drug polymer.

TABLE II
Minimum Inhibitory Concentration (MIC) Values for Ciprofloxacin Activity Associated with DDI/PCL/Cipro(s12) Incubation Solutions (Reported as $\mu\text{g/mL}$) in the Presence of *P. aeruginosa* (10^7 CFU/mL)*

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28
CE	<0.5	0.5	0.5	0.5	0.5
Buffer	<0.5	<0.5	<0.5	<0.5	<0.5

Note: based on the MIC protocol, 0.5 $\mu\text{g/mL}$ actually is the drug concentration range of 0.31 to 0.62 $\mu\text{g/mL}$.

* $n = 3$.

the controls. After 24 h of cell–drug polymer contact, the DDI/PCL/cipro shows no obvious changes in fibroblast cell density [Fig. 5(b)] or morphology [Fig. 6(b)] relative to the DDI/PCL control [Figs. 5(c) and 6(c)]. With the HDI/PCL/cipro polymer, no change in cell density was observed [Fig. 5(d)], but cell sloughing and granulation occasionally were found [Fig. 6(d)]. In contrast, the negative control (0.45% phenol) showed a clear reduction in cell density [Fig. 5(a)]; cell malformation, sloughing, and cell lysis also were observed [Fig. 6(a)]. The phenol-positive control sample shows clear evidence of cell toxicity, as anticipated.

Cell viability also was assayed by the MTT test. The results of this test are shown in Figure 7. Again, when compared to cells cultured on a polyurethane control (DDI/PCL) and a blank culture dish containing no polyurethane, similar cell viability was observed for each of the two drug polymer formulations. The positive control (phenol) showed approximately 40% viability relative to the two negative controls (i.e., blanks and nonciprofloxacin containing polymer).

Neutrophil biodegradation assay

The drug release from DDI/PCL/cipro following incubation with human neutrophils was followed for 72 h by measuring the antimicrobial activity in an MIC assay using *E. coli*. The data in Figure 8 show that the non-drug-containing polymer (DDI/PCL) showed no evidence of antimicrobial activity against the bacteria for all time points in the experiment. A similar observation was made for the drug polymer when incubated in only the RPMI solution (i.e., buffer control). In the absence of cell stimulation, the PMN system was able to induce the release of drug activity from the polymer within 72 h. The stimulation of the cells by PMA induced an accelerated release of antimicrobial activity within 48 h.

DISCUSSION

The current trend in biomaterial development has been to engineer surfaces to produce a desired bio-

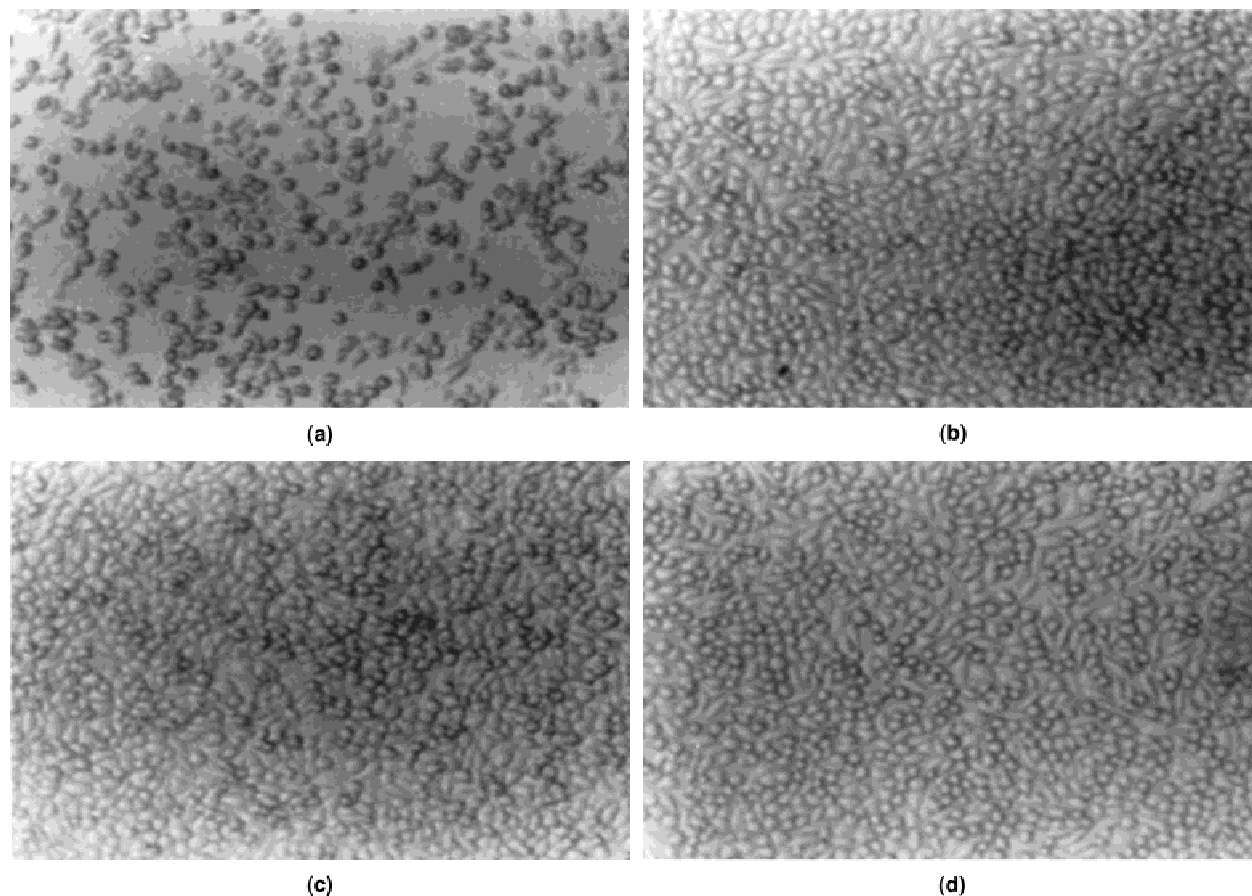


Figure 5. Light micrographs of mouse fibroblasts seeded on polymer surfaces (low magnification): (a) control (0.45% phenol); (b) DDI/PCL/cipro(m30); (c) DDI/PCL; (d) HDI/PCL/cipro(s14).

logic response.³⁸ The biodegradable drug polymers currently being investigated represent a type of bioresponsive material that may be able to be used as an antimicrobial coating to address the problem of surface-adhered bacteria associated with medical devices, particularly during the wound-healing period when host tissue integration is incomplete. The polymer is designed to degrade in response to inflammatory cell-derived enzymes, such as those associated with PMNs^{35,36} and macrophages.³⁰ The enzymes associated with PMNs will be prevalent when the biomaterials are first implanted, as the inflammatory response is activated.²⁵ Since initially there will be a significant number of PMNs present in the acute phase following implantation of the device, the degradation studies with the human-derived neutrophils represent a good first model for the assessment of antibiotic release around implant devices. During this early period, polymer degradation and antibiotic release will be triggered, affording biomaterial protection against bacterial adhesion. As wound healing occurs, cellular activity will be reduced, thus diminishing both polymer degradation and antibiotic release.

It has been shown in previous work that it is possible to synthesize polymers from drugs such as

cipro.²⁶ In the current study, new formulations were synthesized and tested in an attempt to increase the enzyme specificity of the degradation. The incorporation of a ¹⁴C radiolabel into polyester and polyether urethanes previously has been shown to be a sensitive marker for the measurement of biodegradation by enzymes.^{29,30} The data shown in Figure 2 indicate that incubation of the drug polymer with the enzyme produces a significantly higher radiolabel release than the buffer-incubated sample. This effect was most pronounced at the 10-day time point, where approximately 80 times more radioactivity was detected in the enzyme incubated sample versus the buffer samples. This result confirms that the degradation of the polymer can be catalyzed by the presence of enzyme. However, based on previous work, this polymer is considerably more stable than the HDI analog [HDI/PCL/cipro(s14)].²⁶

HPLC analysis of the incubation medium was carried out with both enzyme and buffer systems. A typical chromatogram is shown in Figure 3. The only observed difference between the two chromatograms was the larger ciprofloxacin peak in the enzyme solution. The smaller peaks appearing between 40 and 46 min are not related to ciprofloxacin, as based on an

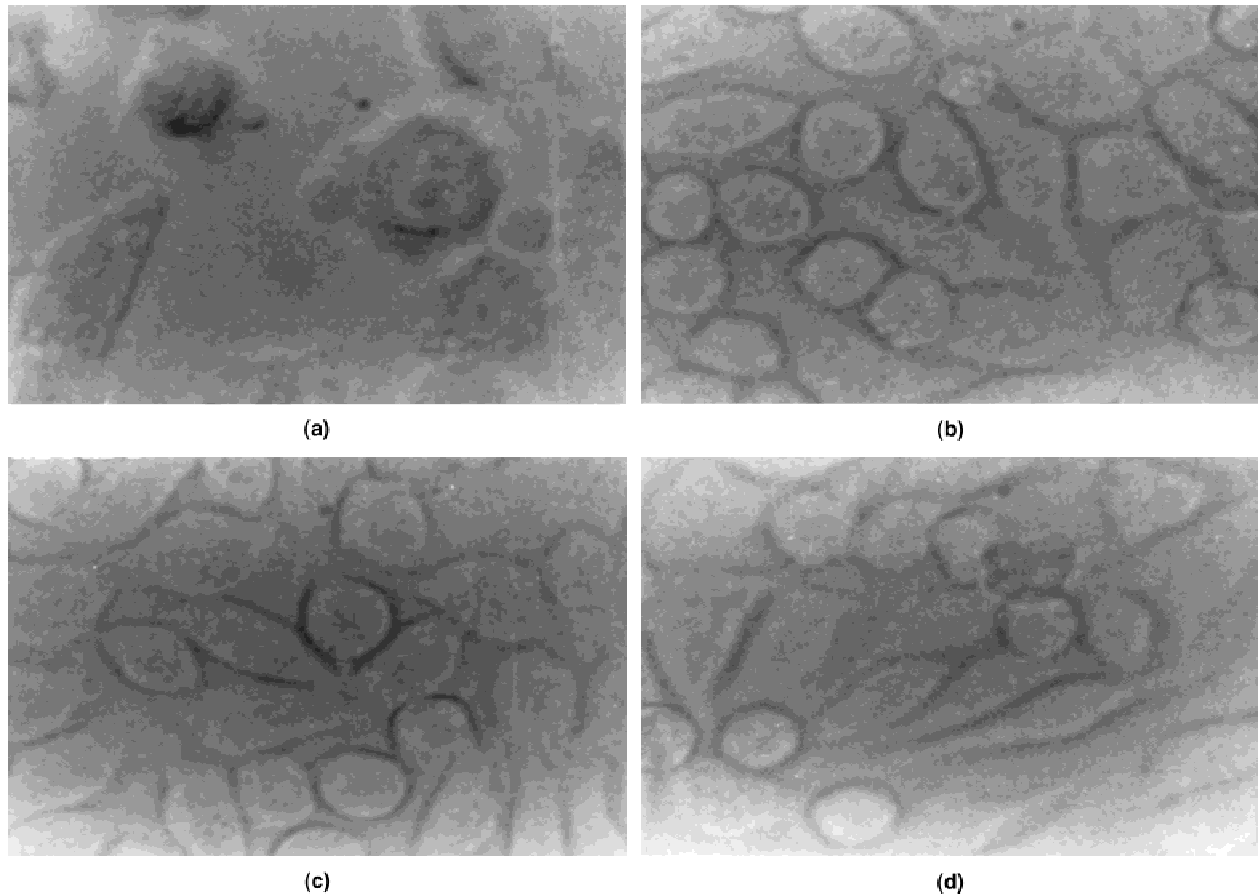


Figure 6. Light micrographs of mouse fibroblasts seeded on polymer surfaces (high magnification): (a) control (0.45% phenol); (b) DDI/PCL/cipro(m30); (c) DDI/PCL; (d) HDI/PCL/cipro(s14).

examination of their full UV spectra, which did not show the characteristic features of the drug. Since the other components of the polymer do not absorb at 280 nm, it was concluded that the absorbance associated with the peaks between 40–60 min were related to unknown compounds acquired in the degradation solutions during the incubation process. The results of the HPLC analysis are in contrast to the previous work with the HDI analog.²⁶ The latter work revealed that the enzyme was able to liberate a number of products that contained the drug in significantly higher quantities than to those of the DDI-based polymer, but it was not able to cause a specific release of free ciprofloxacin relative to that of the buffer controls.²⁶ There could be several explanations for the differences between these two materials. It is possible that the configuration of the polymer chains is providing the enzyme with preferential access to soft segment areas. In such a situation, the initial degradation products that would be produced would exhibit low UV absorption at 280 nm, since they would not contain the drug, and lower radiolabel release since they would not contain the radioactive DDI moiety. In order to assess this possibility, future work would need to probe for the dominant presence of PCL-related prod-

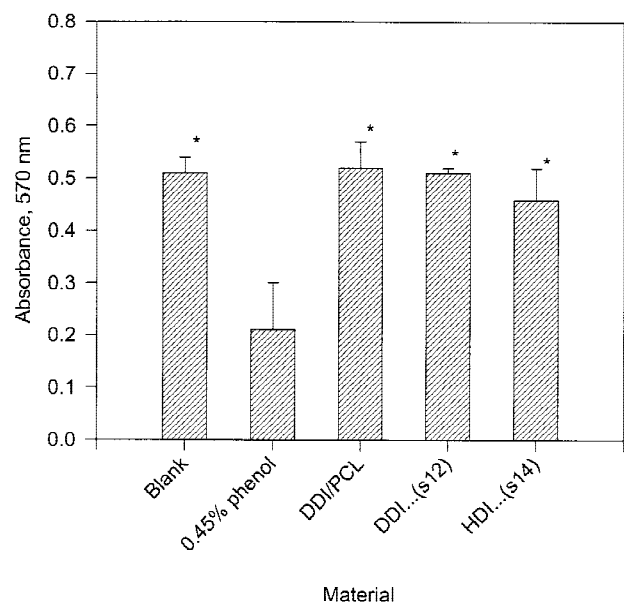


Figure 7. MTT assay. *Indicates statistical significance from phenol control ($p < 0.05$).

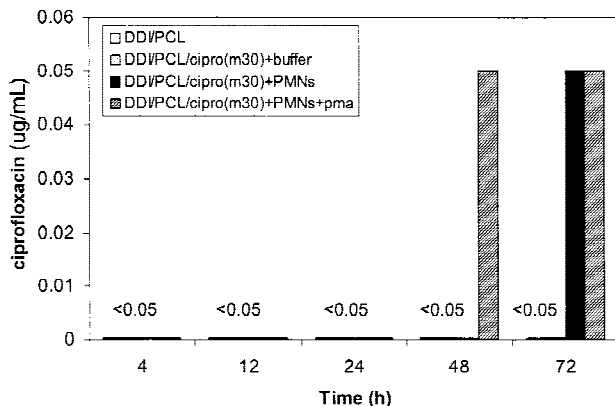


Figure 8. Ciprofloxacin release from various drug polymer materials incubated in the neutrophil cell culture assay. Values determined by MIC assay.

uct using LC/MS methods. In the latter scenario, the subsequent release of cipro/HDI or cipro/DDI segments remaining on the surface would be limited by their solubility. It may be expected that the solubility of the former would be greater than that of the cipro/DDI segment that contains the longer hydrophobic chain. Hence, less cipro/DDI product would result, leading to the lower drug and radiolabel release levels previously observed for cipro/HDI.²⁶

The fact that more free ciprofloxacin product was observed for the enzyme versus buffer solution also must be explained (Fig. 4) because this was not previously observed for HDI/PCL/cipro. Since the results differ substantially from those of the previous work with the HDI molecule, it is suggested that CE cleavage may be influenced by the length of the diisocyanate monomer; that is, HDI versus DDI. This was the only parameter that varied between the two polymeric materials. Cholesterol esterase catalyzes the hydrolysis of long-chain fatty acid esters of cholesterol.³¹ Furthermore, CE exhibits little or no substrate specificity.^{39,40} It catalyzes the hydrolysis of water-soluble carboxylic esters, including *p*-nitrophenyl acetate.^{41,42} One of the natural substrates of CE, cholesterol oleate,³⁰ is shown in Figure 9. The similarity of a DDI/cipro and HDI/cipro polymer segment to that of the natural substrate can be seen; that is, a large rigid ring structure is attached to a long linear hydrocarbon chain, both segments being coupled by hydrolyzable groups. These common features may explain why the DDI polymer shows a preferred cleavage of the drug from the adjacent DDI unit versus the HDI unit. HDI is a shorter chain that brings the polar urethane groups in closer proximity to one another [Fig. 9(c)] and may not provide the appropriate binding site 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 that binds to the large, hydrophobic rings

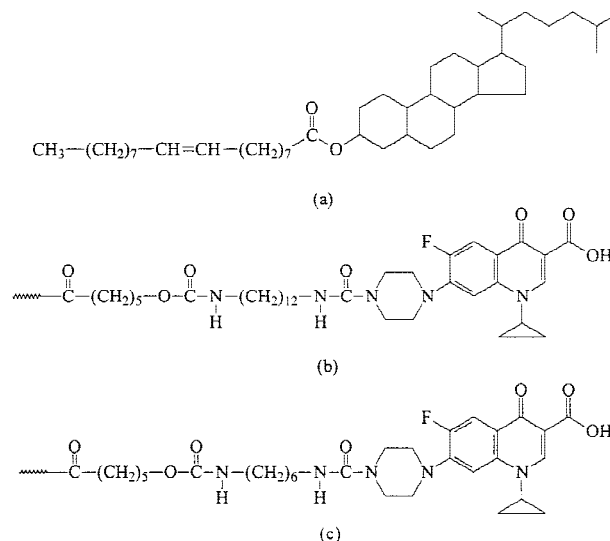


Figure 9. (a) Cholesterol oleate; (b) drug polymer segment.

and the other that binds to the hydrocarbon chains of the fatty acid.^{43,44} The specificity of the latter region for straight, aliphatic hydrocarbon chains was determined by using a series of CE inhibitors.⁴⁴ A number of *n*-alkylboronic acid inhibitors, ranging from one to eight carbons, were shown to inhibit the hydrolysis of *p*-nitrophenylbutyrate. The results showed that *n*-hexaneboronic acid was the most potent inhibitor and emphasized the importance of chain length. Hence the length of the hydrocarbon segment of the aliphatic diisocyanate very well may influence how CE binds to the drug polymers synthesized in this study.

The incubation solutions also were tested for its antimicrobial activity by measuring minimum inhibitory concentrations (MIC). The results of this assay (Table II) show that the enzyme-incubated solutions were able to inhibit the growth of bacteria at levels similar to 0.5 $\mu\text{g/mL}$ against *P. aeruginosa*. In contrast, the buffer samples did not exhibit antimicrobial activity. These data support the HPLC results (Fig. 4) that indicate more free drug release with the enzyme-treated group of DDI/PCL/cipro(s12). While there are some differences in absolute values when the MIC and HPLC data are compared, these may be expected since the MIC assay is dependent on assessing a complex biologic response and is an indirect measurement of the actual presence of the drug.⁴⁵ The results of this assay are important in that they demonstrate that the ciprofloxacin was not inactivated during the synthesis procedure or subsequent polymer hydrolysis even though the antibiotic was exposed to harsh solvents, heat, and various other chemical factors prior to its release by enzyme.

A human neutrophil culture system has been shown to be effective in evaluating the susceptibility of biomedical polyurethanes to biodegradation.^{35,36} This assay was used to assess whether neutrophils cultured

on the drug polymer surface would degrade the polymer and release antibiotics. The results show that PMNs were able to degrade the materials within 96 h (Fig. 8). The addition of PMA was shown to accelerate the process (see Fig. 8, which shows the presence of inhibitory effects after 48 h that were not present in the other samples). This assay shows that the presence of human-neutrophil-derived activities are able to enhance the release of active ciprofloxacin since the drug was tested using a biological assay.

One of the important factors in determining the biocompatibility of prospective biomaterials is the cytotoxicity of the material and degradation products. The results obtained in the present study show that the base materials themselves did not induce cytotoxic effects in the mouse fibroblast system. However, further tests are required to assess chronic and acute toxicity, particularly under postdegradation conditions.

CONCLUSIONS

This study has demonstrated the feasibility of using a polymer containing monomers that possess antibiotic nature as a bioresponsive and biodegradable antibiotic delivery system. It was demonstrated that the release of antibiotic is enhanced in the presence of cholesterol esterase, and the use of different chain lengths in the diisocyanate appears to have incorporated some degree of specificity for cipro release. The antibiotic released was shown to exhibit antimicrobial activity, both against *P. aeruginosa* and *E. coli*, following its release in the presence of enzymes from inflammatory cells. The addition of a cell activator (PMA) accelerated the release of drug to 48 h as compared to 72 h in the absence of the activator. In addition, the polymers did not induce cytotoxic effects in a mouse fibroblast model. Further work is needed to determine the structures of other degradation products released. This latter information will be helpful in tailoring the design of polymers to release antibiotic at desired rates.

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