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Plasma-deposited membranes for controlled release of antibiotic to prevent bacterial adhesion and biofilm formation

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Abstract

Bacterial infection on implanted medical devices is a significant clinical problem caused by the adhesion of bacteria to the biomaterial surface followed by biofilm formation and recruitment of other cells lines such as blood platelets, leading to potential thrombosis and thromboembolisms. To minimize biofilm formation and potential device-based infections, a polyurethane (Biospan) matrix was developed to release, in a controlled manner, an antibiotic (ciprofloxacinTM) locally at the implant interface. One material set consisted of the polyetherurethane (PEU) base matrix radiofrequency glow discharge plasma deposited with triethylene glycol dimethyl ether (triglyme); the other set had an additional coating of poly(butyl methacrylate) (pBMA). Triglyme served as a nonfouling coating, whereas the pBMA served as a controlled porosity release membrane. The pBMA-coated PEU contained and released ciprofloxacinTM in a controlled manner. The efficacy of the modified PEU polymers against *Pseudomonas aeruginosa* suspensions was evaluated under flow conditions in a parallel plate flow cell. Bacterial adhesion and colonization, if any, to the test polymers were examined by direct microscopic image analysis and corroborated with destructive sampling, followed by direct cell counting. The rate of initial bacterial cell adhesion to triglyme-coated PEU was 0.77%, and to the pBMA-coated PEU releasing ciprofloxacinTM was 6% of the observed adhesion rates for the control PEU. However, the rate of adherent cell accumulation due to cell growth and replication was approximately the same for the triglyme-coated PEU and the PEU controls, but was zero for the pBMA-coated PEU releasing ciprofloxacinTM.

Keywords

Pseudomonas aeruginosa; bacterial adhesion; biofilm formation; controlled release antibiotic; triglyme antiadhesion coating

INTRODUCTION

It is estimated that over 3 million artificial or prosthetic parts are implanted per annum in the United States alone.¹ However, over half of hospital-acquired infections are associated with implants or indwelling medical devices, with the case-to-fatality ratio of these infections ranging between 5% and 60%.^{1,2} Bacterial infections by adherent bacteria have been observed¹ on prosthetic heart valves (valve endocarditis), cardiac pacemakers, vascular prostheses, intravascular catheters, cerebrospinal fluid shunts, orthopedic implants, urinary catheters, ocular prostheses and contact lenses, and intrauterine contraceptive devices (IUCDs). These infections have the following unique characteristics: (a) they comprise indolent pathogenic patterns with alternating quiescent and acute periods, (b) there is an initial response to antibiotic therapy but relapses are frequent and infections more tolerant to drugs, and (c) whereas infections are often polymicrobial, the predominant species are members of the autochthonous skin or bowel flora or ubiquitous environmental microbes that are pathogenic only in immunocompromised patients.

Bacterial colonization and biofilm formation on any surface or substratum proceeds by the scenario depicted in Figure 1. The body reacts to prosthetic implants by coating them with a film composed of various proteins (e.g., fibronectin, laminin, fibrin, collagen, immunoglobulins) some or all of which can serve as receptors for colonizing bacteria. Bacteria, transported to the substratum by either molecular diffusion or convective transport, can adhere by either a nonspecific adhesion mechanism (governed by electrostatic forces acting between the cell and surface) or a specific adhesin binding reaction. Once attached to the substratum, bacteria can produce copious amounts of extracellular mucopolysaccharides that bind divalent cations,³ forming a tenacious three-dimensional matrix of cells and extracellular polymers termed a biofilm.^{4,5} These bacterial polymers can mix with those of other species, polymers of host cells, or blood platelets to form a mixed-cell line biofilm that is highly resistant to rigorous antibiotic challenges.^{6,7}

To prevent biomaterial-related infections, the current strategy is to treat the patients systemically with antibiotics at high concentrations, although this is seldom successful, with the only recourse being the surgical removal of the implant. A better approach would be to provide antibiotics immediately after implantation at the site where it is most required—namely, the material-body interface. Our goal was to develop and evaluate a biomaterial that would release in a controlled manner a small-molecular-weight antibiotic in the near-wall region to prevent bacterial colonization.

Biomedical-grade polyurethane (PEU), Biospan, was used as the base polymer because of its excellent mechanical properties, its widespread use as a bloodcontacting material, and its use as a model for many other types of polyurethanes employed biomedically. Another advantage of PEU in this work is that it is highly susceptible to bacterial colonization.^{1,2} CiprofloxacinTM was incorporated, along with polyethylene glycol (PEG) as pore-forming agent, in a PEU (Biospan) matrix. CiprofloxacinTM represents a class of fluoroquinolone antibiotics selected due to its wide antimicrobial spectrum and extremely low minimum inhibitory concentration (MIC₅₀), which, against suspended cells of the species *Pseudomonas aeruginosa*, is in the range 0.25–0.50 µg/mL.^{10,11} PEG [molecular weight

(M_w) = 4000] (Polysciences, Warrington, PA) was determined to be a superior pore-forming agent after extensive comparison with bovine serum albumin.⁸ PEG allowed a greater fraction of the ciprofloxacinTM to be released from the PEU compared with bovine serum albumin (BSA). Details of the matrix synthesis, formulation protocols, and ratios of drug to pore-forming agent are provided in Kwok et al.⁸ Biospan alone is prone to bacterial adhesion and colonization. To reduce the susceptibility of PEU to bacterial adhesion, the base polymer was radiofrequency glow discharge plasma deposition (RF-GDPD) coated with a polymerized layer of triethylene glycol dimethyl ether (triglyme). Johnston et al.¹² showed this method to practically eliminate bacterial adhesion to treated-glass versus controls. To eliminate the burst effects observed for ciprofloxacinTM release from untreated PEU and achieve zero-order ciprofloxacinTM release kinetics, a thin (~80 Å) layer of poly(*n*-butyl methacrylate) [p(BMA)] was RF-GDPD deposited over the ciprofloxacinTM-loaded PEU matrices. Details regarding the effects of various RF-GDPD conditions on the pBMA layer properties and on the release rates of ciprofloxacinTM are provided in Kwok et al.⁹

The objective of the research presented here was to ascertain the effectiveness of the three different formulations of a biomedical-grade PEU (Biospan)–polyethylene oxide mixture at inhibiting bacterial colonization under flow conditions. The scope of this work included:

1. Development of a flow cell system to evaluate the fabricated biomaterials for their potential for bacterial adhesion and biofilm formation, under controlled environmental and hydrodynamic conditions.
2. Development of a novel fluorescent staining technique to allow for the visualization and enumeration of bacterial cells against an opaque surface without interfering with normal cell behavior. This objective will be presented fully elsewhere.
3. Evaluation of the efficacy to prevent bacterial colonization of the three PEU (Biospan)–polyethylene oxide materials: the control PEU base polymer, PEU base material with a plasma-deposited triglyme coating, and PEU base material with a coating of pBMA containing and releasing the antibiotic ciprofloxacinTM.

We report here the time course of bacterial adhesion and biofilm formation on these materials and assess their efficacy in preventing bacterial colonization.

MATERIALS AND METHODS

Biomaterials fabrication

Biomedical-grade PEU (Biospan) was used as the basic polymer and PEG was used as the pore-forming agent in all fabrications. To load the base PEU material with antibiotic, the fluoroquinolone ciprofloxacinTM was premixed with PEG, both in powder form, then blended with 18% PEU in solution, using dimethylacetamide (DMAc) as solvent. The mixture was then cast in a tray and dried under a stream of dry air in a 60°C oven. CiprofloxacinTM-loaded PEU films were trimmed to standard microscope slide dimensions. Control PEU films were made as above without the antibiotic. All biomaterials examined in this study were developed and chemically analyzed at the University of Washington Engineered Biomaterials Center (UWEB). Details as to the fabrication of all variations of

these PEU materials and the rate of antibiotic release as a function of fabrication conditions are provided in Kwok et al.^{8,9}

All PEU base films were coated using RF-GDPD. PEU films were etched briefly in an argon plasma (typically 40 W, 0.175 mm Hg, 5 min) just prior to coating deposition. Monomers of either butyl methacrylate or triethylene glycol dimethyl ether (triglyme) were heated to 60°C to increase their vapor pressure. Plasma deposition conditions for the pBMA membrane coatings of the PEU were varied to affect a desired pBMA membrane porosity and thus a specific antibiotic release rate. Johnston et al.¹² reported that triglyme coatings served to reduce the random adhesion of macromolecules to the surface. After each reaction, coated PEU films were analyzed with X-ray photoelectron spectroscopy (XPS) to determine the degree of coverage and chemical nature of the coating. Static secondary ion mass spectroscopy (SIMS) analysis permitted evaluation of the surface crosslinking density.⁹ Both XPS and SIMS analyses were carried out on RF-GDPD-treated PEU samples before and 24 h after continual exposure to the bacterial suspension or sterile nutrient solution, each flowing at a wall shear stress of 20.3 dyne/cm². Results⁹ of XPS and SIMS analysis indicate the RF-GDPD surface layers remained intact and uniformly coated through an entire experiment.

Antibiotic release rates were determined using the zero-concentration infinite sink method. CiprofloxacinTM-loaded PEU films were submerged in gently mixed buffer solutions and samples were collected periodically to determine release rates as a function of the different materials fabrication procedures. A zero-concentration infinite sink condition was maintained throughout each test.

Antibiotic concentrations

Liquid-phase ciprofloxacinTM concentrations were determined spectrophotometrically at 280 nm.

Bacterial systems

Pseudomonas aeruginosa (ERC-1) was obtained from the National Science Foundation Engineering Research Center for Biofilm Engineering (Montana State University, Bozeman, MT). Cultures of *P. aeruginosa* were stored as frozen stocks maintained in a solution of 2% peptone and 20% glycerol at -70°C.

Culture medium

A minimal salts medium with glucose as the sole carbon and energy source was used to culture the *P. aeruginosa*. A 1-L solution consisted of 0.5 g glucose, 2.56 g Na₂HPO₄, 2.08 g KH₂PO₄, 1.0 g NH₄Cl, 0.1 g CaCl₂, 0.5 g MgSO₄, and 0.02 mL of a trace metal solution. The trace metal solution was composed of 0.5% CuSO₄ · 5H₂O, 0.5% ZnSO₄ · 7H₂O, 0.5% FeSO₄ · 7H₂O, and 0.2% MnCl₂ · 4H₂O, all in a weight per volume ratio dissolved in 10% concentrated HCl. The trace metal solution was autoclaved separately from the nutrient medium, as was a 5M CaCl₂ solution and a 2.5M MgSO₄ solution. The glucose, trace metals, CaCl₂, and MgSO₄ were all added to the sterile medium by injection through a septum after the medium had been brought to room temperature. All solutions were filter-

sterilized through a 0.2- μ m pore-size, sterile, syringe tip filter (Corning) before addition to the medium. All solutions were made using double-distilled deionized water and autoclaved in glass containers at 121°C for 15 min/L.

***À priori* bacterial cell fluorescent staining**

Oftentimes bacteria have the same index of refraction as their surroundings, which makes it difficult microscopically to discern cells without either cytometrically or fluorescently staining one or more cell components. Any number of fluorescent bacterial stains could be applied to bacterial cells once they are attached to a target surface, to visualize and digitally capture their images. Disadvantages of this approach include (a) interruption and cessation of the experiment to apply the stain; (b) the fact that most fluorescent stains kill cells, which prevents further experimentation; and (c) as in the case of PEU polymer substrata used here, the fact that most stains are absorbed by porous substrata, rendering the entire background fluorescent. For these reasons, an *à priori* staining procedure was developed that would fluoresce the bacterial cells before exposure to the PEU while not affecting the cells growth or adhesive characteristics. Hoechst stain 33342 was selected as a DNA stain because it had been previously reported not to kill bacteria or affect their adhesion characteristics.¹³ A series of batch *P. aeruginosa* growth studies was carried out to determine the maximum stain concentration that would (a) sufficiently stain the *P. aeruginosa* cells without (b) detrimentally changing their growth or adhesive characteristics. A concentration of 10 μ g/mL was determined as this optimum stain concentration.¹⁴ Details of the *à priori* staining procedure and its calibration are given in Hendricks.¹⁴

Hoechst DNA stain 33342 was obtained in 100-mg quantities and stored at 0°C until a fresh solution was needed. Ten milliliters of sterile water was added through a 0.2- μ m, sterile, syringe tip filter to dissolve the Hoechst stain powder. Once the Hoechst solution was made, it was stored at -2°C until used. All stains were stored in light-sensitive bottles and made fresh at least once a month.

Continuous suspended culture system

Most bacterial adhesion studies in the biomaterials community use (a) batch reactor conditions to develop the bacterial inocula for an adhesion experiment and (b) batch and static systems to carry out the actual colonization test of the biomaterial itself. Regrettably, from both a microbiological standpoint and a process analysis aspect, static batch culture adhesion tests suffer far too many disadvantages. While there is an inherent ease to their setup, batch cultures of suspended bacteria take place under unsteady-state conditions, with the ambient chemical and biological conditions changing in time. Thus, reproducing the same starting bacterial inoculum from such a system would be only accidental. Furthermore, carrying out a biomaterial-bacterial adhesion test under batch conditions creates a situation where the bacterial growth rate, cell concentration, physiology, and nutrient conditions at the biomaterial interface all change in time. Should the biomaterial release a toxic agent, its effect might be to kill suspended cells, which would give the erroneous impression there was an effect on adhesion. Under static fluid conditions, transport of bacterial cells to the target surface might be the rate-limiting step, thus obscuring the true bacterial adhesion kinetics. Thus, any differences between adhesion rates or extent seen between two experiments could

not necessarily be attributed to just differences in the biomaterials. Sampling a biomaterial submerged in a cell culture operated as batch static test usually requires manually withdrawing the sample, causing it to pass through an air–water interface. Busscher et al.¹⁵ showed that the shear stress produced in this process was sufficient to physically remove 60–90% of the adherent cells when a clean solution was employed.

Consequently, we chose to cultivate our bacterial inoculum in continuous culture at a known growth rate and physiological state. A chemostat is a continuously fed, constant reaction volume, well-mixed bioreactor.¹⁶ A chemostat was used here to provide a continual supply of *P. aeruginosa* at room temperature ($23 \pm 2.0^\circ\text{C}$) and at a known cell concentration and culture growth rate. The chemostat consisted of a 125-mL filter flask equipped with openings for nutrient inlet, effluent, aeration, and inoculation. Reactor liquid overflow was set to maintain reactor volume constant at 128 mL.

Table I lists the operating parameters of the steady-state chemostat. At steady-state operation, the specific growth rate of the *P. aeruginosa* culture is equal to the inverse of the reactor residence time τ which is the ratio of the reactor volume to influent flow rate V/F .

Effluent from the chemostat was connected to a waste jug, unless cells were needed for an adhesion experiment, in which case effluent from the chemostat was directed to a sterile collection vessel. Once sufficient cells were collected, they were stained with 10 $\mu\text{g/mL}$ of Hoechst 33342.¹⁴ Once the stain was added to the cells, the flask containing the mixture was wrapped in foil and put on an insulated stir plate for 1 h. Then, the suspension was centrifuged at $10,000 \times g$ for 15 min at 20°C (Model RC5C, GSA rotor; Sorvall Instruments, Dupont). The supernatant was then decanted and the cells were resuspended in sterile complete medium, minus the glucose, to a concentration of $\sim 5 \times 10^8$ cells/mL. This final cell suspension served as the bacterial challenge to the biomaterials in question.

Bacterial adhesion and biofilm study system

A schematic of the parallel-plate flow cell is shown in Figure 2. The PEU sample films under consideration were placed on the bottom polycarbonate base. The top half of the parallel plate assembly was fabricated with entrance and exit portals. A white silicone O-ring was used to seal the two halves of the flow cell and served to form the flow channel gap width between the sample plane and an overriding glass coverslip. A glass coverslip fitted into the upper plate was held in place using denture adhesive. The top half of the flow cell was made of clear polycarbonate and included a viewing area for the microscopic objective. The final dimensions of the flow cell were as follows: width 16 mm, length 44 mm, gap width 0.80 mm, surface area 12.8 mm^2 , hydraulic radius 0.4 mm, Reynolds number 2.1, wall shear stress 20.3 dynes/cm^2 . The velocity, set to affect a wall shear stress of 20.3 dynes/cm^2 , was primarily selected to ensure that the flow system was not externally mass transport rate limited; otherwise, data collected on bacterial adhesion would not be indicative of the true adhesion process, but rather the rate of cell transport to the surface. Fluid was delivered to the flow cell by peristaltic pump, passed across the sample in question, and then emptied into a sterile effluent container.

Before any experiment, the entire flow system was sterilized in 5% chlorine solution, followed by a rinse in 70% ethanol, then finally rinsed with distilled water, and exposed to ultraviolet (UV) light for 15 min. Experiments were carried out in two stages: first, an adhesion phase, followed by a growth-only phase. During the adhesion-only phase, the suspension of Hoescht-stained *P. aeruginosa* cells at $\sim 5 \times 10^8$ cells/mL was pumped through the flow cell for 6 h. For the growth-only stage, at 6 h, delivery of the cell suspension was terminated and only a sterile nutrient solution was delivered to stimulate growth of any viable *P. aeruginosa* cells attached to the surfaces of the sample.

At time 0, the flow cell was placed on the stage of an Olympus BH-2 epifluorescence UV upright microscope equipped with filter combinations for UV, violet, blue, and green regions of the spectrum. A $\times 40$ Nikon water-immersion objective was used for observation of the PEU sample. A minimum of three fields were captured by an Optonics OPDEI-4700 cooled CCD camera. Images were digitized by a Targa 64+ analog digital converter card and stored on computer until the images could be analyzed using Image Pro-Plus™. For the adhesion mode, images are reported at 2, 4, and 6 h, and for the subsequent growth mode, images are reported for 8, 20, and 24 h. During the early stages of an experiment, individual cells were counted directly from their images. If an image contained an excess number of cells (during growth periods on control PEU films) that did not allow direct counting, the total image fluorescence was determined and divided by the average image fluorescence for a model single cell. This calculated approach agreed well with direct cells counts taken by destructive sampling of the test PEU surface at the termination of each experiment.

Fluorescence created by the *à priori* Hoescht stain appeared to be stable throughout an experiment, even in those cases where growth of adherent cells was recorded. Unfortunately, there appeared to be no effect of the antibiotic on the fluorescence of an adherent cell, or else it could have been employed as a real-time indicator of cell response. Analyses for viable cells and total cells in the influent and effluent were performed periodically throughout an experiment; viable and total cell counts on the PEU films were taken only at the termination of an experiment, at time 24 h. The number of viable cells in any solution was determined using standard selective plate count methods. To obtain total cell counts of bacteria adherent to the PEU films, the PEU films were placed in a sterile buffered saline solution and sonicated for 30 s to remove the adherent bacteria. Since the cells were stained *à priori* with Hoescht stain, no additional stain was required. Samples of the resulting cell suspension were used to determine both the number of viable cell counts (plate counts) and total cell counts by the standard epifluorescence procedure of Hobbie et al.¹⁷ Liquid samples of the influent and effluent were also collected periodically to determine the total and viable cell counts entering and leaving the flow cell; these samples were processed as above, except the unnecessary sonication step was eliminated.

Viable cell counts were taken to ensure that live bacteria were in fact being used to challenge the test polymer films and monitor the efficacy of the ciprofloxacin™ release. Viable cell counts were carried out by the standard drop plate method. A portion of any cell suspension sample (influent, effluent, or resuspended biofilm) was used to make serial dilutions for plating onto plate count agar (Difco Laboratories). A 100-μL multipipetor (Rainin; epd 2) was used to dispense 10 10-μL drops of a specific dilution of cell suspension onto an agar-

coated plate. This procedure was performed in triplicate for each dilution plated. Plates were incubated at room temperature for 24 h. Plates containing between three and 30 colonies were counted and converted to colony-forming units (cfu/mL) by taking into account the correct dilution factor.

RESULTS

Sustained release of antibiotic

Figure 3 summarizes a series of release rate experiments for PEU base materials containing 0.40 g ciprofloxacinTM, as a function of conditions for the plasma deposition of the controlled-release pBMA coating.⁹ XPS and SIMS analysis confirm an increase in pBMA crosslinking (inversely proportional to membrane porosity) with an increase in plasma reaction time. The increase in crosslinking reduced porosity, which in turn decreased the rate of ciprofloxacinTM release rate, as shown in Figure 3 as a function of plasma deposition power (watts), duration of reaction (minutes), and plasma pressure (milliTorr). It was these latter pBMA-coated PEU samples (Fig. 3), loaded with ciprofloxacinTM, that were used in cell adhesion studies described below.

Bacterial colonization studies

Figure 4(A-G) is a representative series of digital images collected during a single experiment composed of an initial adhesion-only phase ($t = 0 \rightarrow 6$ h) followed by a growth-only phase ($t = 6 \rightarrow 24$ h); the test substratum seen in video images in Figure 4(A-E) is a pBMA-PEU control film without a release agent; Figure 4(F) is the image observed at $t = 24$ h for the triglyme-coated PEU, and Figure 4(G) is the video image at $t = 24$ h of the pBMA-PEU releasing ciprofloxacinTM. A minimum of three bacterial adhesion/colonization experiments were carried out for each material type. Data for adherent cell numbers per area versus time for each experiment were generated from the digital images obtained from a single experiment on one polymer sample. Each data point reported at a certain time point is the mean of measurements taken at eight different locations per material sample.

Figures 5 and 6 exemplifies the entire 24 h for three experiments comparing the cell adhesion and adherent cell growth periods on PEU controls, triglyme-coated PEU, and ciprofloxacinTM-releasing PEU. Figure 5 is all three experiments compared on the same adherent cell numerical scale. Because of the large differences in total cells attached between control PEU substrata and other treated materials, Figure 6 provides the same data as in Figure 5, but only for the triglyme-coated PEU and ciprofloxacinTM-releasing pBMA-PEU material. Figures 7 and 8 are, respectively, the same sets of data as Figures 5 and 6, but for only the first 6 h, comprising the adhesion phase of each experiment.

Figure 9 summarizes the direct epifluorescent cell counts taken destructively from the surface of the various substrata, at the end of each experiment. Total cell counts taken directly from the substrata were within $\pm 4\%$ of estimates collected from the image analysis of the microscopic video recordings (data not shown¹⁴).

From Figures 5, 6, and 9, it is obvious that either treatment—triglyme coating alone or the ciprofloxacinTM-release PEU—was successful in dramatically reducing the rate of bacterial adhesion and the extent of biofilm formation in these short-term experiments.

Initial bacterial cell adhesion rates were calculated for each experiment as the initial accumulation of adherent cells over the first 6-h period (i.e., the linear slope of adherent cells per unit time),

$$\left. \frac{dB(t)}{dt} \right|_{t=0 \rightarrow 6} = \text{Initial adhesion rate (cell numbers/area-time)}.$$

Net adherent cell accumulation rates during the growth stage (6–24 hrs) were calculated as the cell normalized accumulation rate, i.e.,

$$\frac{1}{B(t)} \frac{dB(t)}{dt} = \text{Net cell accumulation}$$

where $B(t)$ = adherent cell concentration (cell numbers/L²) at time = t . Alternatively by definition,

$$\frac{d \ln B(t)}{dt} = \text{Net cell accumulation}$$

Thus, data from 6–24 h can be replotted (Fig. 10) as the natural logarithm $\ln(B)$ versus time, where the slope of this data would be a rate constant for the net accumulation of cells.

Table II summarizes these two sets of calculations comparing both the adhesion rates and the net accumulation rates for the various materials considered. Clearly evident in the video images, quantitative data, and Table II, both the triglyme coating and the release of ciprofloxacinTM reduced the overall accumulation of bacteria and biofilm over the 24-h period by 93.5% and 99.91%, respectively. Examination of only the adhesion phase (Table II) also indicates the treated PEU films reduced cellular adhesion compared to the control PEU materials; surprisingly, all triglyme-coated PEU materials exhibited a larger reduction in adhesion rate (99.3%) versus control than did the pBMA-PEU materials releasing ciprofloxacinTM (94.1%). However, during the growth period, when only a sterile nutrient solution was supplied to adherent cells, net accumulation rate constants were approximately the same for the control and triglyme-coated PEU, but the ciprofloxacinTM-releasing pBMA-PEU exhibited a zero (and in some cases, negative) accumulation rate.

One advantage of sustained localized release of an antibiotic from a biomaterial is that the patient is not subjected to large systemic dosages of the drug. Samples taken during the first 6 h (when cells were in the effluent) indicated (data not shown¹⁴) that there was no significant difference between the viable suspended cell counts entering and leaving the flow cell, which suggests the concentration of released antibiotic was not sufficiently high to influence the viability of the suspended cells. Effluent concentrations of ciprofloxacinTM, taken throughout an experiment, were also found to be below detectable levels.¹⁴ However, at time = 24 h, direct samples of cells attached to ciprofloxacinTM-containing pBMA-PEU

films (data not shown¹⁴) indicated that all bacteria attached to these surfaces were nonviable, as determined by agar plate counts.

Overall, the bacterial colonization flow cells experiments showed that ciprofloxacinTM could be incorporated successfully into Biospan/PEG polymers and still maintain its efficacy upon sustained release over a 24-h period of continual exposure to flowing fluid. In addition, these experiments demonstrated that cell adhesion and colonization of opaque materials could be monitored continuously and noninvasively for up to 24 h under flow conditions.

SUMMARY

A series of poly(ether urethane) films was fabricated with the express purpose of reducing or eliminating bacterial adhesion and thus reducing potential bacterial infection of the material. One series of PEU films was coated with triethylene glycol dimethyl ether (triglyme) by way of a radiofrequency gas plasma deposition, whereas a second group was coated with pBMA, allowing the sustained release of a model antibiotic, ciprofloxacinTM.

Triglyme, as with other PEO-containing molecules, was chosen because it exhibits excellent nonfouling properties with regard to proteins and other organic macromolecules.¹⁸⁻²⁶ The protein resistance of PEO-modified surfaces has been attributed to excluded volume effects,²⁷ loss of conformational entropy,²¹ and osmotic effects as water is driven from the polymer²⁸ upon approach of a protein molecule. Hypotheses based on the low PEO/aq interfacial free energy,²² structuring of water at the PEO-water interface,²⁹ the absence of ionic groups,²⁹ and optimal polar-nonpolar interactions with proteins^{30,31} have been proposed. Most studies suggest that long PEO chains resist protein adsorption better than short chains, but recently, studies with self-assembled monolayers have indicated that surfaces with short-chain oligo(ethylene oxide) are adequate to reduce protein adsorption provided the packing density of chains exceeds a threshold value. Humphries and coworkers³² reported that bacterial attachment to PEO-modified graft copolymers was ~90% that observed on glass, stainless steel, and hydroxyapatite. Dunkirk et al.³³ studied bacterial attachment to photochemically immobilized PEO, and showed that long-chain (76 repeat units) PEO modifications reduced adhesion of *P. aeruginosa*, *Staphylococcus epidermidis*, and *S. aureus* by 86–97% relative to polystyrene. Bridgett et al.³³ showed that block copolymers with three PEO repeat units were sufficient to reduce *S. epidermidis* bacterial adhesion by about 90–97% relative to the untreated polystyrene control. Although informative, most of these studies were carried out as batch static adhesion tests, and were thus inherently unable to provide definitive data regarding PEO effects on the separate processes of adhesion versus growth. Johnston¹² carried out the most definitive study to date regarding the effects of PEO molecule size on protein adsorption and bacterial adhesion and subsequent biofilm formation. Johnston found that tri- and tetraglyme coatings, along with 12-crown and 15-crown ethyl ethers, completely reduced the adhesion of rod-shaped bacteria in a parallel orientation to the target substratum but did not prevent perpendicular adhesion of the bacterium *P. aeruginosa*. Johnston suggested that orientation and packing of the PEO molecules allowed the adhesion of *P. aeruginosa* by way of the bacterium's multiple pili or single flagellum, located at one end of the rod-shaped cell. Johnston's results also showed that subtle differences in PEO coating molecular surface structure and coating

composition affected bacterial adhesion and that crude measures such as wettability or hydrophobicity do not correlate to changes in bacterial adhesion.

The second series of PEU films were (a) fabricated with powdered PEO and the antibiotic ciprofloxacin™, incorporated within the PEU matrix, followed by (b) a plasma-deposited layer of poly(butyl methacrylate). Plasma deposition was optimized to provide a pBMA barrier coating of variable porosity to control the rate of antibiotic release. Ciprofloxacin™ release studies, along with XPS and TOF-SIMS examination of the pBMA coating (data not shown^{8,9}) indicate that pBMA porosity could be controlled to set a desired release rate from the PEU matrix.

A novel *à priori* cell-staining method allowed non-invasive microscopic estimation, in a flowing fluid half-cell system, of both initial cell adhesion rates and net biofilm accumulation rates. In general, both modified PEU films substantially reduced initial cell adhesion rates and the maximum extent of accumulated biofilm compared to the control materials. Compared to control PEU materials, PEU coated with plasma-deposited triglyme reduced the initial rate of bacterial adhesion by 97% compared to 94% observed for pBMA-PEU releasing the antibiotic. However, upon introduction of a sterile nutrient feed solution at 6 h, pBMA-ciprofloxacin™ releasing PEU exhibited a zero rate of accumulating adherent bacteria; thus, the maximum amount of biofilm at 24 h was significantly less compared to PEU controls and the triglyme-coated PEU.

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Processes Governing Biofilm Formation

Figure 1.

Processes governing biofilm formation. 1. Preconditioning of substratum by adsorbing organic molecules. 2. Transport of bacterial cells to the substratum from the bulk liquid, which can be by way of diffusion, convective transport, motility, or sedimentation. 3. Adhesion of bacterial cells to the surface. 4. Desorption of an adherent cell from the substratum. 5. Adherent cell metabolism of nutrients, cell growth and replication, and synthesis of extracellular polymers. 6. Biofilm detachment.

Figure 2.
(A) A once-through parallel plate flow cell system for bacterial adhesion studies. (B) Flow cell reactor.

Figure 3.

Comparison of ciprofloxacinTM release rates from pBMA-coated PEU materials as a function of BMA deposition conditions. Each curve is at a different deposition condition in plasma power (W), deposition reaction time (min), and pressure (mm Hg) which creates a different pBMA porosity barrier membrane. The amount of ciprofloxacinTM in each sample was 0.40 g.

Figure 4.

Still frames of a video recording of PEU materials exposed to a suspension of *Pseudomonas aeruginosa* at 5×10^8 cell numbers/mL and a wall shear stress of 20.3 dynes/cm². (A–E) Observations of the control PEU material at 0, 2, 4, 6, and 24 h, respectively. Cell suspensions were supplied from 0 → 6 h (adhesion mode); then, a sterile nutrient feed was supplied the flow cell from 6 → 24 h (biofilm formation). (F) For comparison, the amount of biofilm after 24 h on the triglyme-coated PEU. (G) Amount of biofilm after 24 h on pBMA/ciprofloxacin™ releasing PEU (original magnification $\times 40$).

Figure 5. Adherent cell numbers per unit area with elapsed time for three different materials: PEU control, PEU with triglyme only, and PEU with pBMA barrier membrane releasing ciprofloxacin™. Total experiment is shown.

Figure 6. Adherent cell numbers per unit area with elapsed time for just the PEU with triglyme only and PEU with pBMA barrier membrane releasing ciprofloxacin™.

Figure 7.
Adherent cell numbers per unit area with elapsed time for the first 6 h (adhesion stage) of experiments in Figure 5 for all three materials.

Figure 8.
Adherent cell numbers per unit area with elapsed time for the first 6 h (adhesion stage) of experiments in Figure 5 for just the PEU with triglyme only and PEU with pBMA barrier membrane releasing ciprofloxacin™.

Figure 9.
Direct cell counts taken from destructive samples recovered from the indicated material at termination, an experiment at 24 h.

Figure 10. Natural logarithm of the adherent cell numbers during the nutrient feed, biofilm accumulation stage. Slopes of these curves were considered as a cell-normalized biofilm net accumulation constant.

TABLE I**Suspended Culture Bioreactor Operating Conditions**

Reactor Parameter	Parameter Value
Reactor volume (mL)	128
Inlet nutrient flow rate (mL/min)	1.0
Temperature (°C)	23.0
p ^H	7.3
Reactor residence time (h)	2.1

TABLE II

Comparison of Initial Cell Adhesion and Net Adherent Cell Accumulation Rates

Biomaterial	Initial Adhesion Rate (cell no. – mm⁻² h⁻¹)	Net Accumulation Rate (h⁻¹)	Maximum Cell No. at 24 h (cell no. – mm⁻²)
Control PEU	2.2E + 04	0.22	7.9E + 05
Triglyme coated only	1.7E + 02	0.27	5.1E + 04
pBMA-coated PEU releasing ciprofloxacin™	1.3E + 03	0.0	7.4E + 02