

A liposomal hydrogel for the prevention of bacterial adhesion to catheters

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Abstract

The adhesion of bacteria to medical implants and the subsequent development of a biofilm frequently results in the infection of surrounding tissue and may require removal of the device. We have developed a liposomal hydrogel system that significantly reduces bacterial adhesion to silicone catheter material. The system consists of a poly (ethylene glycol)–gelatin hydrogel in which liposomes containing the antibiotic ciprofloxacin are sequestered. A poly (ethylene glycol)–gelatin–liposome mixture was applied to a silicone surface that had been pre-treated with phenylazido-modified gelatin. Hydrogel cross-linking and attachment to surface-immobilized gelatin was accomplished through the formation of urethane bonds between gelatin and nitrophenyl carbonate-activated poly (ethylene glycol). Liposomal hydrogel-coated catheters were shown to have an initial ciprofloxacin content of $185 \pm 16 \mu\text{g cm}^{-2}$. Ciprofloxacin was released over seven days with an average release rate of $1.9 \pm 0.2 \mu\text{g cm}^{-2} \text{h}^{-1}$ for the first 94 h. *In vitro* assays using a clinical isolate of *Pseudomonas aeruginosa* established the antimicrobial efficacy of the liposomal hydrogel. A modified Kirby–Bauer assay produced growth-inhibition zone diameters of 39 ± 1 mm, while bacterial adhesion was completely inhibited on catheter surfaces throughout a seven-day *in vitro* adhesion assay. This new antimicrobial coating shows promise as a prophylactic and/or treatment for catheter-related infection. © 1998 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Implanted medical devices are crucial in current clinical applications. However, infections arising from the use of indwelling biomedical devices, especially catheters, are associated with increased morbidity and mortality, prolonged hospitalization, patient discomfort and increased medical costs [1, 2]. The numbers of these devices used in North America are indeed astounding; for example 200 million catheters of all types, including 16 million urinary catheters, over five million central venous catheters, and approximately 250,000 ureteral stents are used on an annual basis [3]. Urinary catheters, in particular, are associated with a high rate of infection. The probability

of infection increases 7–10% per day of catheterization [4] with systemic sepsis occurring in a significant number of cases [5]. Despite many attempts to improve the design and surface properties of these liquid-flow conduits so as to eliminate bacterial biofilm formation [6–8], they remain a focus for bacterial adhesion, and a major source of nosocomial infections. It is generally accepted that no single method has yet emerged for the adequate and satisfactory management of catheter-related infection [9, 10]. New approaches need to be developed that would allow us to improve the outcome of episodes of biofilm-mediated infection in patients fitted with indwelling biomedical devices.

Our approach to controlling bacterial biofilm formation on urinary catheters involves the use of drug-loaded liposomes sequestered within a biocompatible matrix that is retained on the surface of the catheter. In several

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respects, liposomes are the ideal drug delivery vehicle. They have the potential to store both apolar and polar compounds via interactions with the lipid bilayer or compartmentalization within their aqueous core, respectively. Specific formulation of the liposome bilayer allows for drug release over periods ranging from days to weeks [11, 12]. Furthermore, the biocompatibility of liposomes ensures that they will be safely degraded and assimilated by the host after their supply of drug is exhausted [13].

There are two potentially critical problems associated with binding liposomes directly to the surface of a medical device. Our calculations indicate that if drug-loaded liposomes occupied all available surface area, insufficient antibiotic would be released to prevent bacterial adhesion for a significant period. In addition, the shear forces generated during the handling and insertion process would likely displace liposomes from the surface. Both concerns can be addressed if liposomes are entrapped within a biocompatible material such as a gelatin gel. A liposome-gelatin solution can be conveniently applied to a surface forming a solid coating within minutes with subsequent cross-linking stabilizing the gel to body temperature. The three dimensional gel matrix is capable of accommodating large quantities of drug-loaded liposomes [14], while simultaneously protecting the liposomes from membrane-disrupting shear forces encountered during handling and insertion of the device.

The liposomal hydrogel developed in this work was constructed of three principal components: gelatin, nitrophenyl carbonate-activated poly (ethylene glycol) (NPC-PEG), and liposomes loaded with the antibiotic ciprofloxacin (CIP). CIP was chosen as the model antimicrobial agent because it is effective against a broad spectrum of bacteria associated with catheter surfaces and urinary tract infections [15]. The PEG-gelatin-liposome mixture was applied to a silicone surface that had been previously coated with a thin layer of 4-azido-2,3,5,6-tetrafluorobenzoic acid-modified gelatin (AFB-gelatin). The pre-treatment involved shortwave UV light irradiation for a brief period and resulted in the immobilization of AFB-gelatin on the catheter surface. The attachment of the hydrogel to the modified silicone surface, as well as cross-linking of the hydrogel, was induced by immersion of coated catheters in alkaline solution. The release kinetics of CIP from different types of liposomal hydrogel were investigated in conjunction with determinations of the *in vitro* efficacy of CIP-loaded liposomal hydrogel against *P. aeruginosa*.

2. Materials and methods

2.1. General

The phospholipids dipalmitoylphosphatidylcholine (DPPC) and PEG-distearoylphosphatidylethanolamine

(PEG-DSPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Rhodamine dipalmitoylphosphatidylethanolamine (rhodamine-DPPE) and 4-azido-2,3,5,6-tetrafluorobenzoic acid (AFB) were purchased from Molecular Probes (Eugene, OR). Porcine gelatin-a (MW 50,000–10,000; 300 Bloom), poly (oxyethylene) bis (p-nitrophenyl carbonate) (NPC-PEG), and cholesterol were obtained from Sigma (St. Louis, MO). 1,3-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), and Sirius Red were purchased from Aldrich (Milwaukee, WI). Ciprofloxacin·HCl was provided by Bayer Healthcare Canada (Etobicoke, ON). Solvents were of analytical grade and all reagents were used without further purification. Deionized water (Milli-Q, Millipore, Bedford, MA), filtered through a 0.22 μm membrane, was used in all experiments. CIP was analyzed in a Perkin-Elmer (Beaconsfield, England) LS-50 fluorimeter. Sirius Red and p-nitrophenol concentrations were determined using a Hewlett-Packard 8452A spectrophotometer (Sunnyvale, CA).

Silicone Foley 10F catheters (Sherwood Medical, St. Louis, MO) were prepared for use by sectioning into cylinders (5 mm outer diameter). The lengths of the sections were either 20 mm (bacterial inhibition zone study) or 10 mm (all other experiments). The open ends of the sections were sealed with silicone rubber (RTV 108, GE, Pickering, ON). Silicone sections were cleaned prior to each experiment by refluxing in methanol for 6 h followed by rinsing in water.

2.2. AFB-gelatin preparation and surface immobilization

NHS-AFB was prepared as described in Keana and Cai [16] using the coupling agent DCC. AFB-gelatin was synthesized by the addition of 8.3 mg (25 μmol) of NHS-AFB in 0.5 mL methanol to 100 mL of 50 mM borate buffer (pH 8.6) containing 1% gelatin. Following overnight incubation at room temperature, the mixture was filtered and dialyzed against water for 24 h. All procedures involving AFB were performed in the dark or under dim light conditions.

Catheter sections were immersed in AFB-gelatin solution (5 mg mL⁻¹) for 1 h at room temperature and subsequently removed and dried for 2 h at 40°C. All samples were exposed to short wave (254 nm) UV light (Mineral-light Lamp, UVP, San Gabriel, CA) at a distance of 2 cm for 3 min. Adsorbed protein was removed by washing in 1% SDS solution at 80°C for 30 min. The gelatin fixed onto the catheters was quantitated by a Sirius Red staining and elution procedure [17]. In brief, the dye is bound to the immobilized protein by incubating coated catheters in an aqueous picric acid solution containing Sirius Red (73 mM). The samples are washed with 0.01 N HCl for 30 min to remove unbound dye. Subsequently, the bound dye is eluted from the gelatin using a dilute NaOH solution (0.1 N) and quantitated by spectrophotometry.

The amount of dye eluted is proportional to the quantity of cross-linked gelatin present, and, by comparison to standards, an accurate estimate of surface-immobilized gelatin can be obtained.

2.3. Liposomal hydrogel and catheter preparation

Liposomes were composed of DPPC/cholesterol/PEG-DSPE/rhodamine-DPPE in a 1:1:0.05:0.001 ratio. The lipids were dissolved in 4 mL of chloroform and the solvent was removed in vacuo for 2 h. The resulting lipid film was hydrated with 1 mL of 250 mM ammonium sulfate (pH 2.5) at 45°C. Liposomes were then frozen in liquid nitrogen and thawed in a 45°C water bath (5 ×), followed by high-pressure extrusion through two 100 nm-pore membranes (5 ×). External ammonium sulfate was removed by passing the suspension through a Sephadex G-50 column (1 × 10 cm) and eluting with a 10% sucrose solution (pH 4.0). Liposomes in suspension and those entrapped within PEG-gelatin gels were loaded with CIP according to the remote-loading technique described in Oh et al. [18]. Unencapsulated drug was removed by gel filtration for the liposome-only samples but not for the liposomal hydrogel samples. The drug-loading efficiency with respect to liposomal suspensions was determined by comparing the amount of CIP associated with liposomes before and after gel filtration. The loading efficiency for the liposomal formulation used in this study was $90 \pm 2\%$. Liposomal hydrogels were prepared for scanning electron microscopy using a malachite green-based procedure [19].

PEG-gelatin solutions consisted of 10% gelatin, 6% NPC-PEG and 10% sucrose at pH 4.0. When liposomes were required, they were added from a liposome suspension. The concentration of lipid in PEG-gelatin solutions was 15 mM with respect to DPPC. All solutions were heated at 45°C for 15 min to dissolve gelatin.

Catheter surfaces were modified with AFB-gelatin as previously described. Catheter sections were spin-coated with 60 μ L of fluid PEG-gelatin or PEG-gelatin–liposome mixture. All samples were subsequently incubated at 4°C for 15 min. Gels were polymerized by immersion in 200 mM Borate buffer (pH 8.5) for 1 h. Residual p-nitrophenol was removed from the gels by continual washing in 10% sucrose solutions (pH 4.0) until the absorbance of the solutions at 400 nm was negligible.

Dehydrated hydrogels were prepared by drying coated catheter sections in an oven at 35°C for 2.5 h. The dried gels were then rehydrated in Tris buffer (10 mM Tris, 110 mM NaCl, pH 7.4) or concentrated CIP solution (25 mg mL⁻¹) at 45°C.

2.4. Determination of drug efflux kinetics

The CIP release experiments were initiated by placing each catheter section or dialysis membrane (containing

Table 1

The total amounts of CIP incorporated into various hydrogel-coated catheter sections

Coating formulation	Ciprofloxacin (μ g cm ⁻²)
Hydrogel	42 \pm 12
Liposomal hydrogel	185 \pm 16
Dried liposomal hydrogel ^a	173 \pm 6
Dried liposomal hydrogel ^b	1298 \pm 48

^aHydrogels were loaded with CIP, dried, and rehydrated in buffered saline solution (pH = 7.4).

^bHydrogels were dried and rehydrated in concentrated CIP solution (25 mg mL⁻¹).

liposome suspension 2.7 mM in DPPC) into separate liquid scintillation vials filled with 15 mL of Tris buffer. Periodically, 3 mL was removed from each vial for CIP quantitation via a fluorescence assay using an excitation wavelength of 324 nm, an emission wavelength of 440 nm, and 5 nm slit widths. The amount of CIP present was determined by comparisons to a standard curve.

The drug-retention capacities of the various hydrogel samples (Table 1) were calculated from the data obtained in the CIP release experiment. The cumulative amount of CIP released after seven days was taken as a measure of the amount of drug initially present. Note that the amount of drug remaining in any of the samples after seven days was always less than 1 μ g.

2.5. Bacterial adhesion assay

A clinical isolate of *P. aeruginosa* obtained from a patient with peritonitis was used for all challenge assays. An 18 h nutrient broth culture was prepared from a primary isolate maintained at -70°C in a 50% (v/v) glycerol-phosphate buffered saline (PBS) solution.

For each coating formulation, 12 catheter sections were aseptically placed in 100 mL of sterile nutrient broth (Difco, Detroit, MI). The *P. aeruginosa* inoculum size was sufficient to yield $1.5 \pm 0.5 \times 10^7$ cfu mL⁻¹ in the 100 mL volume. The inoculated catheter suspensions were then placed in an incubator maintained at 37°C and agitated at a rate of 100 rpm. One-half of the 100 mL volume was aseptically removed from each beaker and replaced with a like volume of sterile nutrient broth on a daily basis. At time intervals of 1, 3, 5, and 7 days, triplicate catheter sections were removed from each of the beakers and viable bacteria were recovered from the catheter surfaces and counted as previously described [20]. The number of viable bacteria in nutrient broth samples was also determined.

2.6. Zone of inhibition study

The antimicrobial activity of coated and uncoated silastic catheter sections was assessed using a modified

Kirby-Bauer technique, previously described by Sherertz et al. [21]. *P. aeruginosa* (described above) was grown in nutrient broth for 18 h at 37°C. The cells were washed three times in PBS, pH 7.2, then resuspended to a concentration of 0.5 McFarland Units, corresponding to approximately 1×10^8 cfu mL⁻¹. Sterile cotton swabs were dipped briefly in this suspension, then uniformly spread across the surface of 100 mm diameter Mueller–Hinton agar plates. Two cm lengths of the coated and uncoated catheter sections described above were rinsed with approximately 2 mL of PBS, pH 7.2, to remove residual suspending antibiotic solution from the surfaces. The rinsed catheter sections were then carefully pressed into the center of each of the plates. Following incubation for 24 h at 37°C, the zones of inhibition surrounding each of the sections were measured at the aspects perpendicular to the long axes. Triplicate assays were performed for each catheter treatment.

2.7. Data analysis

The microbiological data are presented as the mean ± 1 standard deviation of viable bacteria cm⁻² of catheter surface area. The data were subjected to statistical analysis using non-paired Student's *t*-test. All CIP release experiments were conducted in quadruplicate.

3. Results and discussion

3.1. Surface pretreatment and gel preparation

The immobilization of AFB-gelatin onto the catheter surface was verified by a staining procedure using the collagen-specific dye Sirius Red. UV-exposed samples, and control samples kept in the dark, were washed in hot detergent solution in order to remove adsorbed gelatin. UV-treated samples were stained red by the dye, whereas control samples were stained yellow indicating that little or no protein remained after the detergent wash. Elution and quantitation of the gelatin-complexed dye indicated that 12.7 ± 2.3 μ g of gelatin had been affixed per cm² of UV-irradiated surface; no gelatin was detected on the control samples. The results demonstrate that exposure of surface-adsorbed AFB-gelatin to shortwave UV light can render it insoluble. Since each AFB-gelatin molecule contained approximately 14 molecules of AFB, corresponding to 55% substitution of the total number of ϵ -amino groups present in an average gelatin molecule (data not shown), it is likely that UV-exposure induced extensive cross-linking of the protein through reactions of the photo-generated nitrenes. Also, fluorinated aryl azides such as AFB can insert into carbon–hydrogen bonds upon exposure to UV light [22], thus, it is possible that some percentage of adsorbed AFB-gelatin became covalently linked to the polydimethylsiloxane network

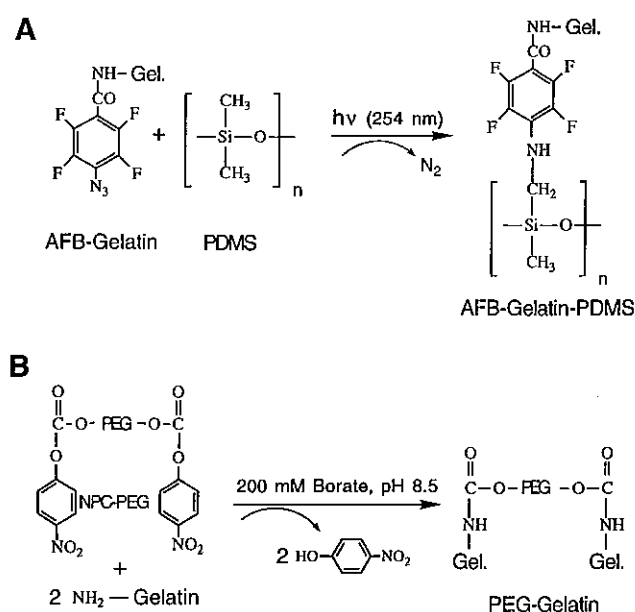


Fig. 1. (A) Hypothetical reaction sequence depicting the covalent attachment of AFB-gelatin to polydimethylsiloxane (PDMS) upon UV irradiation. (B) Reaction scheme outlining the crosslinking of gelatin by NPC-PEG in alkaline solution.

(Fig. 1A). The covalent coupling of aryl azide derivatives of PEG and albumin to dimethylsilane-coated glass is known to occur [23, 24].

While not absolutely necessary to obtain a stable and durable coating, we found that the pre-treatment of the surface with AFB-gelatin enhanced the adhesion of the hydrogel to the catheter surface. The modification of the surface with AFB-gelatin provides amino groups that can react with the activated PEG in the gel. Thus, the NPC-PEG included in the liposome and gelatin mixture performs the dual role of cross-linking the bulk gelatin gel and acting as a bridging molecule between gelatin in the bulk gel and surface-immobilized AFB-gelatin. The crosslinking process occurs under alkaline conditions and results in highly stable urethane bonds [25] between PEG and gelatin (Fig. 1B). A photograph of a catheter section coated with liposomal hydrogel is shown in Fig. 2A. The hydrogel was endowed with a pink colour from the presence of rhodamine-labelled lipid in the liposome bilayer. Visual inspection of hydrogel cross-sections revealed that the intensity of the colour did not vary with distance from the surface suggesting that liposomes were evenly distributed throughout the gel matrix. The hydrogel coating was conveniently produced by rotating the catheter as the mixture was applied, resulting in a homogeneous layer with relatively uniform thickness (0.3–0.4 mm). More detailed views of the hydrogel surface with numerous liposomes present are shown in Fig. 2B and C. The high density of liposomes on the surface may be partly artefactual, since the fixation and dehydration process required for preparing the hydrogel for SEM

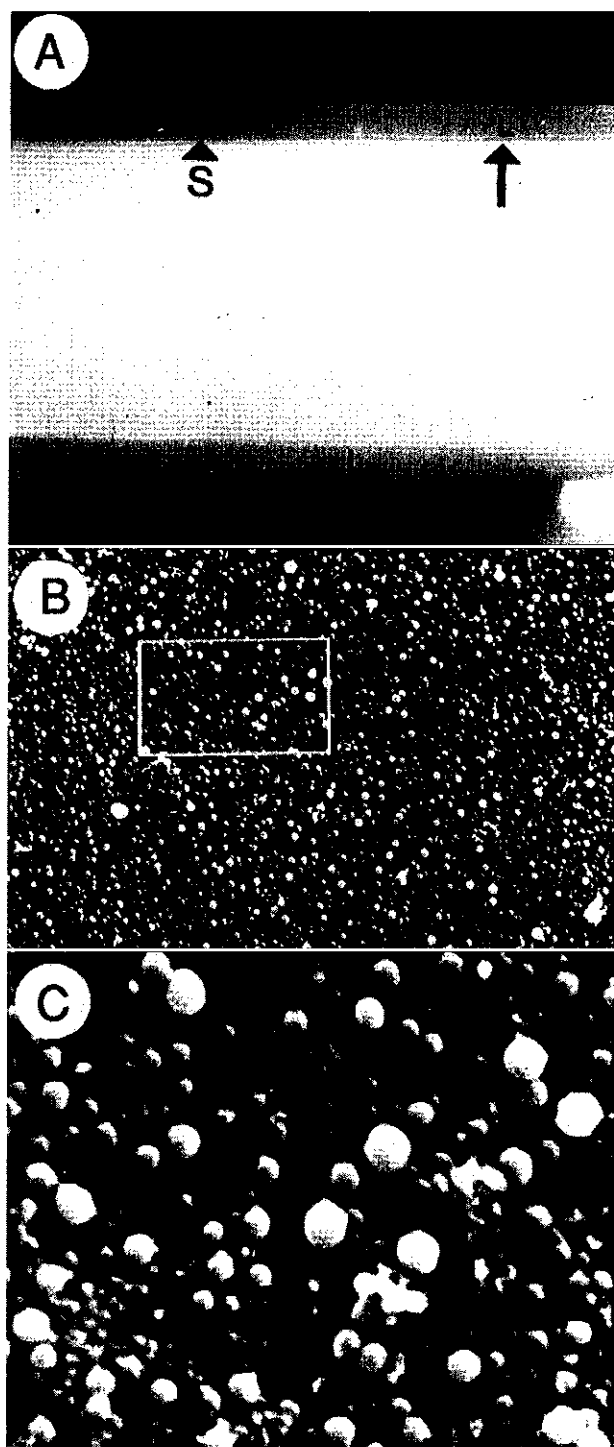


Fig. 2. (A) A catheter section coated with liposomal PEG-gelatin gel. The surface of the catheter is denoted by S and the hydrogel layer is indicated by L; $\times 15$. (B) The surface of a liposomal hydrogel as observed by SEM; $\times 2000$. (C) An area encompassed by the white rectangle in 2(B) viewed at higher magnification ($\times 9000$).

may have concentrated liposomes on the surface. SEM images of cross-sectioned liposomal hydrogels were similar in appearance (not shown). Undoubtedly, there are some liposomes present on the surface of fully hydrated

gels and this is desirable as the lipid in the liposomes may act as a boundary lubricant facilitating catheter insertion.

3.2. Ciprofloxacin efflux studies

Coated catheters were placed in buffered saline solutions (37°C) in order to monitor the release of CIP from liposomal hydrogels. At selected times, these solutions were assayed for CIP and subsequently replaced with an equal volume of saline. The amount of CIP released at a given time, expressed as a percentage of the total released, is shown in Fig. 3. There are two major trends that emerge from the data. The hydrogel-only and dried liposomal hydrogel treatments were not successful in retaining CIP for a sustained period as more than 99% of the initially incorporated drug was released within the first 4 h. In contrast, it took longer than 6.8 d (or 163 h) for greater than 99% of the initially incorporated drug to be released from liposomes and the non-desiccated liposomal hydrogel. Note that due to the drug-loading protocol used, liposomal hydrogels contained mainly encapsulated drug but also a small amount of unencapsulated drug. However, the contribution of unencapsulated CIP to the extended release of CIP from liposomal hydrogels was negligible since unencapsulated CIP is completely leached from the hydrogels within 4 h (Fig. 3). Zero order release kinetics were most closely approximated during the first 94 h of drug release with an average release rate of $1.9 \pm 0.2 \mu\text{g cm}^{-2} \text{h}^{-1}$.

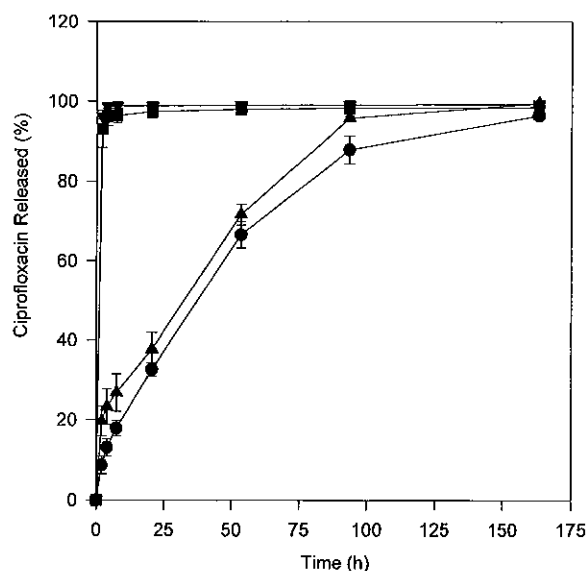


Fig. 3. The release of CIP from catheter sections coated with PEG-gelatin hydrogels expressed as a percentage of the total amount released at selected times. The treatments consisted of hydrogels loaded with CIP (■), liposomal hydrogels containing CIP (▲), and pre-dried liposomal hydrogels with CIP (▼). The release of CIP from liposomes in an aqueous suspension was also determined (●).

The similarity in the release profiles of liposomes in suspension and those sequestered within the hydrogel indicates that hydrogel-embedded liposomes maintain their integrity during the coating procedure and throughout the release period. The PEG-gelatin gel remained intact and its pink colour did not vary over the entire course of the experiment suggesting that the liposomes remained firmly sequestered within the gel. Also, in preliminary studies, analysis of the incubation medium did not reveal any rhodamine fluorescence.

The rapid release of CIP from the rehydrated liposomal hydrogel indicates that the air drying process disrupts the integrity of the hydrogel-incorporated liposomes. The result is consistent with the well-characterized destabilization of liposomes that occurs during desiccation [26]. In an effort to identify an effective air-drying/rehydration strategy, dried hydrogels were rehydrated in solutions of CIP·HCl (25 mg mL^{-1}). The total average amount of antibiotic entrapped within these hydrogels is listed in Table 1; for comparison, the total amounts of drug entrapped by the different hydrogels in the previous experiment are also included. As expected, the hydrogels rehydrated in concentrated CIP solution (25 mg mL^{-1}) retained large quantities of antibiotic (approx. 1.4 mg per catheter section). However, nearly all ($> 99\%$) of the hydrogel-associated CIP was released after the first 4 h of incubation (data not shown).

The optimal efflux profile in terms of prolonged release of antibiotic was obtained from liposomal hydrogel samples that were not previously dehydrated. Liposomal hydrogels were able to gradually release their initial load of CIP over 7 d. The amount of sequestered drug and the duration of its release may be improved by increasing the concentration of liposomes within the hydrogel and/or modifications to the liposome formulation. Alternatively, these goals may be met by increasing the quantity of hydrogel applied to the catheter's surface. A dried liposomal hydrogel with suitable sustained release properties should be achievable with the development of a lyophilization protocol. Numerous studies have shown that liposomes freeze-dried in the presence of sugars such as sucrose or trehalose can be rehydrated without substantial loss of their contents [26,27].

3.3. Bacterial challenge assays

Since the ultimate aim of this work is to develop a catheter coating capable of impeding bacterial adhesion in vivo, it was necessary to gauge the antibacterial activity of the liposomal hydrogel system. Two different in vitro experiments were conducted for this purpose. The first assay involved the placement of various hydrogel-coated sections and one set of uncoated sections on agar plates inoculated with a lawn of *P. aeruginosa*. After 24 h, both hydrogel-coated and uncoated sections that had been exposed to CIP released sufficient drug to

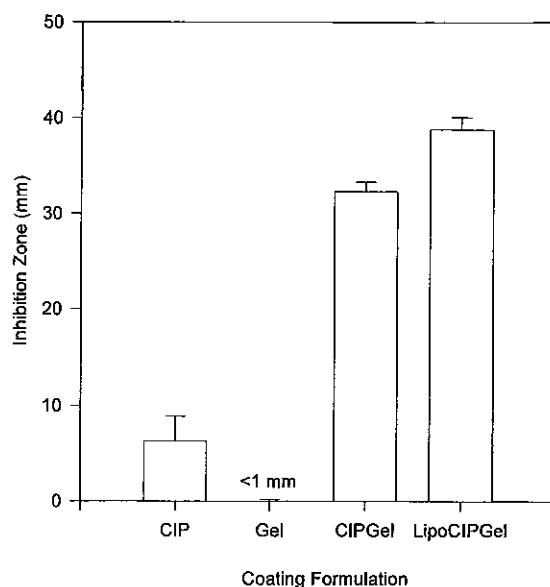


Fig. 4. *P. aeruginosa* growth-inhibition zones created by CIP-treated catheter sections (CIP) and various hydrogel-coated catheters. The hydrogel treatments consisted of hydrogel only (Gel), CIP-loaded hydrogel (CIPGel), and liposomal hydrogel containing CIP (LipoCIPGel). Zones of inhibition represent diameters of growth inhibition measured perpendicular to the long axes of the catheter sections.

prevent the growth of bacteria in their vicinity (Fig. 4). The zones of inhibition created by the CIP-loaded hydrogel samples were approximately five to sixfold larger than the inhibition zones of control catheter sections that had been treated with CIP only. As expected, the drug-free hydrogel-coated sections did not affect the growth of bacteria on the agar plate. These data emphasize the ability of the hydrogel formulation described herein to retain therapeutic concentrations of antibiotic.

The ability of hydrogel-coated catheters to resist bacterial colonization for a prolonged period was tested by exposing untreated, hydrogel-coated (with and without CIP), and CIP-containing liposomal hydrogel-coated sections to a clinical strain of *P. aeruginosa* known to form biofilms on silicone catheters. It is evident from the data in Fig. 5 that the hydrogel coating containing antibiotic-loaded liposomes was effective in preventing cells from adhering, as no bacteria were detected on these surfaces throughout the seven-day experiment. The absence of viable cells on the liposomal hydrogel surface was not simply due to the total elimination of the initial inoculum resulting from the burst release of drug during the first few hours, since bacteria were consistently present on CIP-loaded hydrogels without liposomes. Also, the number of viable bacteria in the broth containing the liposomal hydrogel sections was approximately $6.7 \times 10^2 \text{ cfu mL}^{-1}$ at the end of the seventh day. It is likely that the continuous release of CIP throughout the experiment was the major factor responsible for the

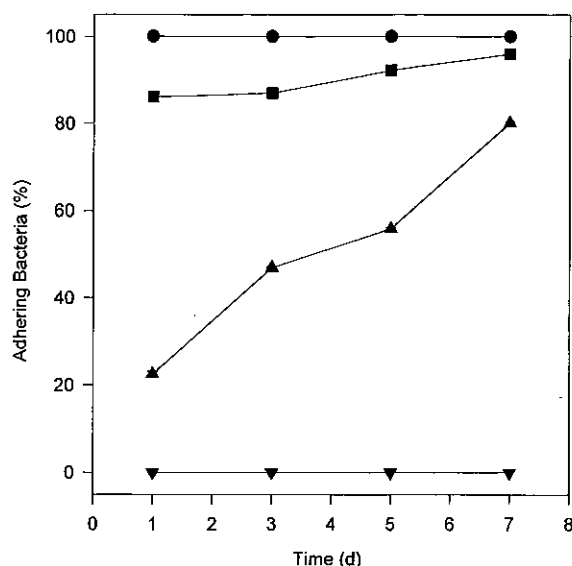


Fig. 5. The adhesion of viable bacterial to hydrogel-coated catheter sections expressed as the percentage of bacteria found on untreated catheters (●). Coating formulations consisted of hydrogel only (■), hydrogel with CIP (▲), and liposomal hydrogel with CIP (▼).

complete prevention of bacterial adhesion to liposomal hydrogels.

We have developed a method for sequestering liposomes onto the surface of a silicone catheter through their inclusion in a surface-coating PEG-gelatin hydrogel. Liposomal hydrogels were capable of encapsulating therapeutic amounts of CIP for prolonged periods as demonstrated by the complete inhibition of bacterial adhesion for up to seven days. Modifications of the technique should allow it to be applied to other medical devices, such as peritoneal dialysis catheters, vascular prostheses, and reconstructive implants. An attractive feature of this system is the possibility of sustained release of compounds having a range of chemical properties. Also, the lubricious nature of the PEG-gelatin hydrogel should enhance the biocompatibility of coated devices as hydrogel-coated catheters tend to minimize the tissue inflammation associated with other types of catheters [28].

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