Published in final edited form as:

J Control Release. 2013 December 28; 172(3): . doi:10.1016/j.jconrel.2013.10.005.

Development of a poly (ether urethane) system for the controlled release of two novel anti-biofilm agents based on gallium or zinc and its efficacy to prevent bacterial biofilm formation

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Abstract

Traditional antibiotic therapy to control medical device-based infections typically fails to clear biofilm infections and may even promote the evolution of antibiotic resistant species. We report here the development of two novel antibiofilm agents; gallium (Ga) or zinc (Zn) complexed with protoporphyrin IX (PP) or mesoporphyrin IX (MP) that are both highly effective in negating suspended bacterial growth and biofilm formation. These chelated gallium or zinc complexes act as iron siderophore analogs, surplanted the natural iron uptake of most bacteria. Poly (ether urethane) (PEU; Biospan®) polymer films were fabricated for the controlled sustained release of the Ga- or Zn-complexes, using an incorporated pore-forming agent, poly (ethylene glycol) (PEG). An optimum formulation containing 8% PEG (MW=1450) in the PEU polymer effectively sustained drug release for at least 3 months. All drug-loaded PEU films exhibited *in vitro* 90% reduction of Gram-positive (*Staphylococcus epidermidis*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria in both suspended and biofilm culture versus the negative control PEU films releasing nothing. Cytotoxicity and endotoxin evaluation demonstrated no adverse responses to the Ga- or Zn-complex releasing PEU films. Finally, *in vivo* studies further substantiate the anti-biofilm efficacy of the PEU films releasing Ga- or Zn- complexes.

Keywords

Anti-biofilm biomaterials; interrupting iron metabolism, gallium and zinc siderophores, poly (ether urethane); drug release; *Staphylococcus epidermidis*; *Pseudomonas aeruginosa*

1. Introduction

It is estimated that over 3 million artificial or prosthetic devices are implanted annually in the United States [1]. Biomaterial-related infections of implanted devices are a significant clinical problem caused by bacterial adhesion and biofilm formation (three-dimensional matrices of bacterial cells and bacterial secreted extracellular polymers) at the implantation

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site [2]. An implant is especially susceptible to surface colonization, with the adherent bacteria being capable of forming biofilm at the implant-tissue interface [3]. Biofilms on a device surface are difficult to eradicate and are less susceptible to antibiotic challenges than their planktonic counterparts. Bacterial colonization of medical devices can lead to sepsis or thrombosis, potential failure or removal of the device, or death of the patient [4].

The current strategy to prevent biomaterial-related infections is to treat patients systemically with high antibiotic concentrations, which studies have shown has limited efficacy [5-7]. Moreover, the risk of antibiotic resistance development is drastically increased under the current standard use of systemic antibiotic treatment of medical-device infections. As a consequence, implant removal or even amputation resulting from such infections is increasingly more prevalent [8]. Therefore, developing a means to prevent bacterial colonization and biofilm formation is imperative. One strategy would be to deliver such treatments immediately after implantation at the interface where the biomaterial interacts with the body and the colonizing bacteria.

In most pathogens, iron (Fe) is essential for growth and the functioning of key enzymes, such as those involved in DNA synthesis, electron transport, and oxidative stress defense [9]. We hypothesize that elemental iron analogs (*e.g.*, Ga or Zn) chelated to *siderophores* (bacterially secreted iron chelators) will competitively inhibit and disrupt iron metabolism in bacteria. Siderophores are small, high-affinity iron chelating compounds secreted by microorganisms in low iron environments. In our approach, Zn and Ga complexed to synthetic siderophores are used in a “Trojan horse” approach to replace Fe and disrupt bacterial Fe metabolism [8]. While iron undergoes redox cycling within a cell, gallium and zinc cannot. Zn is selected because it is already present in all parts of the body, particularly in the red and white blood cells. Zn also aids in wound healing and enhancing immune responses [10]. While Zn can be toxic at high concentrations, its toxicity can be reduced through complexation with meso/protoporphyrins (ZnMP/ZnPP). It has been reported that ZnPP acts efficiently as a photodynamic therapeutic (PDT) agent against different forms of cancer *in vivo* [11-13]. ZnPP can also act as photodynamic antimicrobial at high concentration when exposed to illumination [14, 15]. It is well documented that both ZnPP and ZnMP, at concentrations ranging between 25 and 100 μ M, exhibit selective toxicity on erythroid and myeloid progenitor cells, *in vivo* [16, 17]. Transition metal gallium has an ionic radius nearly identical to that of Fe, and many biological systems are unable to distinguish Ga^{3+} from Fe^{3+} [18]. Ga is FDA-approved to treat hypercalcemia in malignant cancers [19]. Here, Zn- and Ga-meso and -protoporphyrins (ZnMP, ZnPP, GaMP, and GaPP) were developed as anti-microbial treatments.

In conventional systemic or parenteral drug delivery, drug concentrations will peak (burst effect) and then decline, achieving the required therapeutic dose for a momentary period [20]. Controlled-release drug delivery approaches seek to maintain the systemic drug concentration in the desired therapeutic range with negligible burst effect, over the required duration. The initial burst release is negligible if it does not cause local systemic toxicity and shorten the release profile significantly [21]. Here we will develop a model poly(ether urethane) (PEU) film that will release either Ga- or Zn-complexes for a sustained time period; such loaded polymer systems could be developed into entirely new implants (catheters, shunts, tissue engineering scaffolds) or as outer coatings applied to existing indwelling devices.

A segmented biomedical-grade poly (ether urethane) PEU (FDA accepted as Biospan®), was used as the base polymer because of its excellent mechanical properties. PEU has a two-phase microstructure, where the hard segment domains are distributed in a soft segment matrix. The hard segment provides great mechanical strength, while the soft segment

improves the ionic conductivity [22]. PEU is an FDA-approved blood-contacting material, and is commonly used in devices such as heart valves and spinal implants. Poly (ethylene glycol), PEG, was chosen as a pore-forming agent because it dissolves upon hydration, creating pores in the PEU through which drugs can escape. PEG was determined to be a superior pore-forming agent after extensive comparison with bovine serum albumin (BSA). This was also previously shown by Kwok *et al.* [23], in which PEG as a porogen was shown to release a greater fraction of loaded antibiotic versus BSA.

Gram-positive *Staphylococcus epidermidis* is the most prevalent bacterial strain of the human skin and mucous membrane microflora, and the epitome of an opportunistic pathogens [24]. *S. epidermidis* have emerged as a major nosocomial pathogen associated with infections of biomedical-device implants and responsible for persistent infections in individuals with compromised immune systems [24]. *S. epidermidis* seems to prevail on polymeric materials and is responsible for up to 60% of prosthetic hip implant infections since the 1980s, with these infections being persistent and often relapsing. *Pseudomonas aeruginosa*, a Gram negative bacterium, is also another common species that is responsible for biomedical-device infections. Both bacterial strains thrive not only in normal atmospheres, but also in environments with little oxygen, and therefore are able to colonize surfaces in artificial and natural environments [25, 26]. Treatments of post-operative infections are further complicated by the emergence of antibiotic-resistant mutants; the *S. epidermidis* and *P. aeruginosa* strains used in this study are resistant to a large range of antibiotics [27, 28].

The goal of this project was to develop biopolymer systems that would release a novel *non-antibiotic* therapy (ZnMP, GaMP and GaPP complexes) in a controlled manner to prevent biofilm formation. PEU films were fabricated using the pore-former PEG at various molecular weights and mass percentage (w/w) to achieve optimal release rates. The effectiveness of the various PEU biomaterials in reducing bacterial colonization and infection by *S. epidermidis* and *P. aeruginosa* was quantified *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Materials

The four drugs used in this study are: (1) ZnMP (MW= 630.06), (2) ZnPP (MW= 626.03), both purchased from Frontier Scientific (Logan, UT), (3) GaMP, and (4) GaPP; the latter two were synthesized in-house as described below. PP, MP and Gallium (III) chloride (GaCl_3) were purchased from Sigma-Aldrich (St. Louis, MO). Biospan®, a segmented poly(ether urethane) (PEU) was purchased from Polymer Technology Group Inc (Emeryville, CA). Poly (ethylene glycol) (PEG: MW=1450, 3400, 4600, and 8000) was purchased from Sigma (St. Louis, MO). Dimethyl formamide (DMF, 500mL) was purchased from EMD Chemicals (Gibbstown, NJ). Lysogeny broth (LB) was purchased from Fischer Scientific (Logan, UT). Tryptic soy broth (TSB) was purchased from Becton, Dickinson and Company (Sparks, MD).

2.2. Synthesis of Gallium complexes

GaMP and GaPP were synthesized using a chelation reaction (Fig. 1). PP or MP (0.2 mmol) dissolved in a mixture of 20 ml DMF/DMSO (2:1) was added to a solution of GaCl_3 in ethanol (0.5 mmol). The mixture was refluxed for 8 hours with a gentle stream of argon gas bubbled through the solution. After reaction, the DMF/DMSO solvent was removed by vacuum distillation at 80°C and 200 mmHg. The residual precipitate was washed with diH_2O three times to remove the excess GaCl_3 , and then lyophilized. The purity of

compounds was monitored by absorption spectroscopy [29]: GaPP: UV-Vis $\lambda_{\max}$ (nm) DMF: 412, 542, 580; GaMP: UV-Vis $\lambda_{\max}$ (nm) DMF: 400, 535, 571.

2.3. PEU film preparation and characterization

PEU was used as the base polymer and was designed to release the four drugs described above. First, the PEU polymer matrix was synthesized as follows: a variable amount of PEG pore-former (4, 8, 18, 30, and 40% w/w) was completely dissolved in 8.4 mL DMF solvent. A metal complex (ZnMP, GaPP or GaMP, 0.5% (w/w)) was introduced into the PEG/DMF mixture, then 7.70 g of 24% Biospan® was added to the mixture, shaken vigorously, and left overnight on a rotary shaker to eliminate air bubbles and allow complete mixing.

The resultant PEU/PEG/drug mixture was cast into an 8.5cm × 4.0cm rectangular Teflon fluoropolymer mold. The solvent DMF was allowed to evaporate at 55°C for 3 days. Vacuum was then applied at 55°C for another day to ensure complete solvent removal and to further eliminate air bubbles. Thickness of the resultant dry film was 1.0±0.03mm. The mass ratio of pore-former to PEU was varied and optimized in order to attain a desired release rate with a negligible “burst effect”. A second series of PEU/PEG films were loaded with nothing to act as negative controls. The PEU films were then punched into 10 mm diameter circular specimens to be used in release rate and anti-bacterial efficacy studies.

Scanning electron microscopy (SEM) was used to visualize the surface topography of the PEU polymer films. Oven dried samples were coated with gold for 60sec at 18 mA and imaged with a JEOL 7000F SEM (JEOL Ltd., Japan) at an accelerating voltage of 5-10 kV; working distance =10 mm.

2.4. *In vitro* release kinetics

The dry weight of all PEU films used in the metal complex release studies was determined. The “net amount of drug-loaded” in each circular specimen was calculated, with the assumption that the drug was uniformly distributed. To determine the release kinetics of each sample, individual 10 mm diameter drug-loaded PEU specimens were placed in the wells of a 24-well plate; each well containing 2 mL phosphate buffer saline (PBS) as the elution medium. The plate was then placed on gyratory shaker operated at 150 RPM and 37°C. To prevent drug degradation from light exposure, each well-plate was wrapped with aluminum foil. At each sampling time point (0.5, 1, 2, 4, 8, 12h for the first day, then at intervals of 24 hrs), the entire contents (2 mL) were withdrawn from each well and replaced with the same amount of fresh medium. The concentration of released metal complex was determined from absorbance readings of the collected samples measured using a UV spectrophotometer at wavelength of 576 nm. Solutions of known drug concentrations were used to generate a calibration to absorbance. Drug release experiments were repeated in triplicates per PEU film formulation. Sampling of the release medium continued for approximately 3 months.

The total amount of cumulative drug released over the time was calculated from:

$$M(t) = \sum_{t=0}^t V_s \cdot C_{drug}(t) \quad (1)$$

where M_t = total amount of cumulative drug released at any time t , [μg]; V_s = volume of elution medium (2mL); C_{drug} = concentration of drug at time t , [$\mu\text{g/mL}$].

The cumulative percent drug release over time is calculated using equation (2):

$$\text{Cumulative \% Release} = \sum_{t=0}^t [M_{acc}(t) / M_{total}] \times 100\% \quad (2)$$

where M_{total} = the total amount of drug in each sample which is determined from the weight of each individual 10 mm diameter circular specimen and the percentage of loaded drugs during the fabrication. Each experiment was repeated at least 2 times, and each sampling time represents at least 3 triplicate specimens.

Gallium complex release kinetics from the PEU matrices with PEG as porogen were assumed to conform to an unsteady-state mass transfer model as per Saltzman [30]. Starting with a continuity equation for unsteady state mass transport in a one-dimensional cartesian coordinate systems, then assuming (a) constant Fickian diffusion of the gallium complex, (b) no convective transport within the PEU matrix, (c) using the initial concentration of gallium complex loaded, (d) no chemical reaction of the gallium complex within the PEU, and (e) the two boundary conditions of (1) no flux at the center of the disc and (2) a zero gallium concentration at the outer boundary of the disc, the governing equation is as follows:

$$M_{acc}(t) / M_{total} = 1 - \sum_{n=0}^{\infty} \left(\frac{8}{(2n+1)^2 \pi^2} \right) \exp \left(-D_{eff} (2n+1)^2 \pi^2 t / 4w^2 \right) \quad (3)$$

where

M_{acc} = accumulative gallium complex released up to time (t), (M)

M_{total} = total gallium complex loaded, (M)

D_{eff} = effective diffusivity of gallium complex in PEU matrix, (L^2/time)

w = half the thickness of the PEU matrix, (L)

Equation (1) can be simplified for $M_{acc}/M_{total} < 0.60$ into the following:

$$M_{acc}/M_{total} \approx 4 \left(D_{eff} t / \pi w^2 \right)^{0.5} \quad (4)$$

Knowing M_{acc} from gallium complex release studies, the only unknown in Equation (2) is D_{eff} .

Assuming that

$$D_{eff} / D_{GAC-Water} = \varepsilon / \tau \quad (5)$$

where ε = porosity or void fraction of the PEU (assumed = to the stated PEG loading) and τ = tortuosity of the PEU pores. With $D_{GAC-Water}$, the diffusion coefficient of gallium complex in water, assumed = $5.7 \times 10^{-7} \text{ cm}^2/\text{sec}$ [31], the tortuosity values for the different PEU-PEG formulations were calculated.

Each polymer film was weighed at the beginning and the end of a release experiment to evaluate the weight loss during the long-term drug release. After the release experiments, the polymer films were dissolved into DMF solvent and the remaining percentage of the drugs was estimated by the same method described above.

2.5. Anti-bacterial assessment of drugs and drug delivery PEU films

Two model medical device colonizing bacteria strains were used in this study. The Gram-positive, *Staphylococcus epidermidis* RP62A (ATCC 35984) is a slime-producing strain isolated during the 1979 to 1980 Memphis, Tennessee, outbreak of intravascular catheter-associated sepsis. *S. epidermidis* RP62a is capable of accumulated growth and subsequent biofilm formation and is a methicillin-resistant biofilm isolate, all of which contribute to its pathogenicity in foreign-body infections. The Gram-negative, *Pseudomonas aeruginosa* O1 (generously provided by Dr. Pradeep Singh at University of Washington) is a cystic fibrosis isolate and widely studied laboratory strain of *P. aeruginosa* used in biofilm formation studies and implant colonization studies, for which the entire genome sequence is known [27, 32], *Ps. aeruginosa* PAO1 is known for its extensive resistance to numerous antibiotics, including imipenem, gentamicin, tobramycin, ciprofloxacin, and cotrimoxazole. Prior to use, all cells were maintained in 15% (v/v) glycerol stock at -80°C . Bacterial cultures from glycerol stocks were streaked on agar plates supplemented with 10g/L tryptic soy broth (TSB) and incubated at 37°C until the size of individual bacterial colonies reached ~ 1.5 mm in diameter. For suspended culture inocula, a single, isolated colony from a streaked plate was collected by a sterile loop and added to 25 ml of 10 g/L TSB medium, then incubated at 37°C under shaking conditions for ~ 16 hours.

To quantify the effects of Ga- or Zn-complex release on bacterial adhesion and biofilm formation, UV-sterilized circular “no-drug” (controls) and different drug-loaded PEU films (10 mm diameter) were placed in triplicates into 24-well polystyrene plates. Bacteria from overnight cultures were centrifuged, washed and then re-suspended into 0.3 g/L TSB medium to a final bacterial concentration of 10^6 cells/ml. A 2 mL volume of bacterial suspension was seeded into each wells, then the entire plate was placed in an incubator shaker at 37°C at 125 rpm. At various time points after incubation (0, 2, 4, 8, 12 and 24 hours), 100 μL of the bacterial suspension in each well was collected, serial diluted and placed on agar plates to determine the number of colony forming units (CFU) per volume (colony #/ml = colony # on plate $\times$ dilution factor / (0.01 mL sample volume)). In addition, at 8 and 24 hours, film samples were removed from plates and washed twice with PBS, and then placed into 10 mL polystyrene tubes containing 1 mL PBS. To remove adherent biofilm embedded bacterial cells, a probe sonicator at a frequency of 20 kHz was applied to PEU films on both sides; 5 seconds per side and three times per sample. The bacterial concentration in the resultant suspension was then enumerated and reported as CFU per film area (colony count on plate $\times$ dilution factor $\times$ 1 mL total volume / (0.01 mL sample volume $\times$ 0.785 cm^2 film surface area)). A 1/1000 $\times$ dilution of Live/Dead BacLight™ (Invitrogen) fluorescent nucleic acid stain was used to count live and dead cells. Cell suspensions were then filtered through a 0.22 μm pore size black polycarbonate membrane (Whatman, Piscataway, NJ). Five random fields (140 μm by 110 μm) of each membrane were imaged using a Zeiss Axioskop2 epi-microscope with a 100X oil objective (Carl Zeiss, Jena, Germany) and analyzed using “Image J” software (NIH, Bethesda, MD). The number of dead cells was determined as cells that stained red color and the total number of cells are a sum of those that stained both red and green.

The minimum inhibitory concentration (MIC) of the various Ga- and Zn-complexes were determined in batch culture conditions. GaPP, GaMP, ZnPP and ZnMP were dissolved in pH 8.0 diH₂O to make stock solutions at 1 mg/mL. Bacteria from overnight culture were diluted to 5×10^4 cells/ml in 0.3g/L TSB medium placed 15 ml polystyrene tubes. Different amounts of above antimicrobial agents from stock solutions were added to these diluted bacteria to final concentrations of 0, 2, 5, 10 and 20 $\mu\text{g/ml}$, respectively. After incubated in an air shaker at 37°C for 24 hours, the bacterial concentrations in solutions were determined by plating dilutions on TSB plate.

2.6. *In vitro* cytotoxicity testing of PEU films

Mouse NIH-3T3 fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Fibroblasts were maintained in an incubator at a controlled atmosphere of 37°C and 5% CO₂. Confluent fibroblasts were trypsinized and diluted in DMEM complete medium to a final concentration of 10⁵ cells/ml. A 1 mL fibroblast cell suspension (100,000 cells) was seeded to each well of a 24-well plate containing various UV-sterilized PEU films and incubated at 37°C and 5% CO₂. After 24 hours, an Alamar Blue® cell viability assay was performed (DAL1025, Invitrogen). Adherent cells were detached by trypsinization from the PEU films, washed with PBS, centrifuged and re-suspended in PBS to a concentration of 2.5 × 10⁵ cells/ml. A volume of 100 µL cell solution was placed to each well of a 96-well plate and 10 µE of Alamar Blue reagent added at 37°C for 2 hours. The fluorescence measured at 585 nm is proportional to the concentration of viable cells in each well. Fibroblast viability exposed to drug containing films was normalized to the viability of fibroblasts cultured on tissue-culture-treated polystyrene (TCPS) surfaces.

2.7. *In vitro* endotoxin testing of PEU films

The bacterial endotoxin concentrations of drug-loaded PEU films were quantified *in-vitro* by an endpoint chromogenic Limulus Amebocyte Lysate (LAL) assay (QCL-1000™, Lonza, Switzerland). PEU/PEG drug-loaded samples were serially washed in ethanol solutions decreasing in concentration from 100%, 75%, 50%, 25% and 0%. Each wash step was 15 minutes long followed by UV-sterilization for an additional 15 minutes on each side. 10 mg of the sterilized PEU sample films cut into 2 × 2 mm pieces were incubated in a tube containing 600 µL of pyrogen-free water, and then agitated in an incubator shaker at 37°C 125 rpm for 24 hours. 50 µL of the extract solutions were placed into a 96-well plate, and then incubated with 50 µL/well of LAL reagent at 37°C for 10 minutes. 100 µL of LAL substrate provided in the assay kit were loaded into each well and incubated at 37°C for 6 minutes. Stop solutions of 25% acetic acid were then added and mixed immediately. The absorbance of each well was measured at 405 nm and converted to concentration of endotoxin using a linear correlation determined from endotoxin standard solutions. Product inhibition test were performed to confirm that no factors were contained in extracts that would interfere with the LAL reaction.

2.8. *In vivo* PEU film implantation and bacterial challenges

All animal experiments adhered to federal guidelines and were approved by the University of Washington Animal Care and Use Committee. The PEU film disks pre-inoculated in *PA* O1 bacteria solutions for 6 hours were implanted subcutaneously in mice (Fig. SI). *S. epidermidis* RP62A was not selected for the *in vivo* bacterial challenges; our prior experience and that of others shows that aside from biofilm formation, *S. epidermidis* does not secrete any virulence factors or toxins, unlike as *S. aureus* or *Ps. aeruginosa*. Most immune robust mice are able to withstand a systemic injection of *S. epidermidis* [33] and we did not want to employ a immune-compromised animal model. The implantations were performed as previously described [34], Six-weeks-old C57B/6J female mice (Jackson Laboratories) were used for all *in vivo* tests. Each mouse received one implant pre-inoculated with bacteria subcutaneously. Each sample (drug-loaded or blank PEU films) had 5 replicates to provide statistical significance. Each mouse received one implant pre-inoculated with bacteria subcutaneously. Each PEU film sample had 5 replicates to provide statistical significance. Mice were anesthetized using isoflurane and then shaved. Surgical scissors were used to create a longitudinal incision on the central dorsal surface (Fig. SI, the short dash line). A blunt forceps was used to create a subcutaneous pocket on one side of the incision for the implantation of the disks, the position of the disk is shown in Figure SI. The

dorsal skin incision on the mouse is no longer than 1.5 cm. The incision is the full thickness through the skin to enable access to the subcutaneous space. After implantation, metallic wound clips were used to close the incisions. Mice were returned to their housing facility for observation until recovery. Blood samples were collected from saphenous vein.

2.9. Statistics

A Student's t test (2-tailed) was performed for statistic analysis. P-values less than 0.001 designate a significant difference.

3. Results

3.1. Ga- and Zn-Siderophore complex effects on planktonic bacteria

Before the fabrication of drug-loaded PEU films, the antimicrobial activities of four Ga- and Zn-siderophore based agents were determined using a Gram-positive *S. epidermidis* RP62A and a Gram-negative *P. aeruginosa* PAO1 in batch suspended cultures. A low concentration of TSB medium (0.3 g/L, low Fe levels) was used to mimic since the low free Fe levels seen *in vivo*. As shown in Fig. 2, ZnPP under the concentrations 30 μ M did not inhibit the growth of either *SE* or *PA* bacteria. Thus, ZnPP was excluded from further consideration. However, the other zinc complex, ZnMP reduced bacterial counts in a dose-dependent manner: a 15 μ M concentration produced a 3 log₁₀ reduction of bacteria counts; and a higher level of ZnMP (30 μ M) completely inhibited *SE* growth and reduced *PA* growth by approximately 10⁶-fold; a 6 log₁₀ reduction in bacteria counts. In comparison, the growth inhibitory action of the two Gallium complexes was more pronounced since 8 log₁₀ reduction of *SE* strain and 4 log₁₀ reduction of *PA* bacteria were observed at a much lower concentrations of GaPP and GaMP (2.5 μ M). Inhibition results at 48 hours were also similar to these above obtained at 24 hours (Data not shown).

3.2. PEU/PEG Ga- and Zn-complex releasing polymers

Polymer matrix formulations were optimized to achieve smooth, homogeneous flat films that resulted in sustained drug release. To produce desired sustained release kinetics, the PEU matrix formulation was optimized using just one complex, ZnMP as model since all three drugs used in this study share similar chemical structures and properties.

To determine which PEG molecular weight would produce the desired release rates, *in vitro* drug release studies were carried out. The effect of varying PEG molecular weight on ZnMP release rates was studied by adding 8% of PEG (w/w) to the PEU matrix, along with 0.5% of ZnMP (w/w). The molecular weight of the PEG added was varied from: 1450, 3400, 4600 and 8000.

Fig. 3A shows that increasing the molecular weight of the PEG pore-former resulted in decreasing ZnMP release rates in phosphate buffer, pH = 7.2 and temperature 37°C. ZnMP-loaded PEU specimens containing PEG at MW=1450 had the highest sustained release rates as compared to the other PEG MW. PEG (MW=1450) had a maximum cumulative percent release of approximately 80% (800 μ g) of total drug after 300 days. Release kinetics for PEG MW=3400 and 4600 were not significantly different from each other. In comparison, PEU with PEG (MW=8000) released approximately 55% (550 μ g) of the total loaded drug by 300 days. These data suggest that varying PEG molecular weight can control the total accumulative drug released from the PEU films. Unlike the lower molecular weight PEG porogens, PEG at MW (8000) was more difficult to dissolve into the PEU matrix, resulting in clumping and uneven distribution within PEU films. To obtain smooth PEU films with the desired release rates, the polymer formulation with higher cumulative release (MW=1450) was selected for further *in vitro* drug release studies.

Release studies were carried out for six different types of PEU films containing a constant 0.5% ZnMP and varied PEG (MW =1450) amounts from 0, 4, 8, 18, 30, and 40 % (w/w), in Triplicates at 37°C and pH = 7.2. The effect of increasing PEG loading on ZnMP release profile was quantified (Fig. 3B). Increasing PEG loadings increased drug release rates, since one is adding more pore-former to the PEU films. PEU films containing the two highest PEG amounts (30% and 40%) exhibit more cumulative drug release (approximately 80% and 96% of the total loading drugs) at 200 days compared to other samples. In contrast, PEU without PEG or 4% PEG showed about 55% to 65% of drug release after 330 days.

Release rate data from Fig. 3B was processed as described earlier using Eq. (3-5), allowing one to estimate the pore structure “tortuosity”, τ , for the different PEU/PEG formulations. Results are given in Table 1, which suggest that the pores structure within the PEU/PEG matrices becomes more complex and potentially interconnected as the PEG content increases, thus allowing a faster release rate of the gallium complex. SEM images of PEU surfaces with varied weight percentages of PEG also confirmed this result (Fig. SII). Further, the release rate required for achieving the minimum concentration of Ga complexes drugs to inhibit bacterial growth was calculated by using a diffusion model [35] (Fig. SIII). To obtain a sustained longer release profile yet produce the desired bacterial inhibitory concentration, PEU films containing 8% PEG formulation was chosen for further studies of Ga- and Zn-complex releasing PEU films and their antibacterial efficacy.

3.3 Efficacy of PEU/PEG films releasing Ga- and Zn-complexes

Two biofilm-forming pathogens, a Gram-positive (*S. epidermidis*) and a Gram-negative (*P. aeruginosa*) species were used to evaluate the anti-biofilm properties of Zn- and Ga-siderophores released from PEU films. As shown in Fig. 4, the three drugs released from PEU films (GaMP, GaPP and GaPP) only slightly inhibited the batch growth of both *SE* and *PA* strains at different levels in the liquid phase exposed to the PEU films. Compared to GaMP and GaPP (mean of 2 log₁₀ killing), the inhibition efficacy of ZnMP was greatest against the Gram-positive *S. epidermidis*, with complete inhibition (CFU=0) after 24 hours incubation. *P. aeruginosa* exposed to PEU-releasing films for 24 hrs resulted in only one log₁₀ decrease in *P. aeruginosa* using GaPP; ZnMP and GaMP appeared to have no effect on suspended cell numbers after 24 hours. ZnMP, GaPP and GaMP appear more active against Gram-positive bacteria. This result is consistent with the previous data of Ga and Zn complexes in planktonic cultures (Fig. 2). Note the intent here is to authenticate the concept of local drug delivery rather than systemic. The PEU films are not meant to kill bacteria in the liquid phase, a veritable mile away from the surface of the films. Rather, the films are to release a lethal amount of therapy in the near local region, killing only those cells in close proximity to the films. Thus, the release of therapy would not be detected systemically. Fig. 4 shows except in one case, that the net growth of bacteria exposed to the films releasing Ga- and Zn-complexes is about the same as controls.

The efficacy of the drug-releasing PEU films against bacterial adhesion and biofilm formation was also investigated (Fig. 5). After 8 hours, only ZnMP (against *S. epidermidis*, 2 log₁₀ reduction) and GaMP (against *P. aeruginosa*, 1 log₁₀ reduction) showed any significant reductions compared to the control films releasing nothing. After 24 hours, all three drug-loaded PEU films showed some reduction of adherent cells versus control films (GaMP and GaPP films against *S. epidermidis* with 1 log₁₀ reduction, ZnMP films against *S. epidermidis* 6 log₁₀ reduction and all three drug-loaded films against *P. aeruginosa* exhibited a 2 log₁₀ reduction). For adherent cells, after 24 hours, the levels of dead cells on the three drug-loaded PEU films (~90% dead *S. epidermidis* and 50% dead versus the negative control without drug (~20% dead *S. epidermidis* and 30% dead *P. aeruginosa*)

(Fig. SIV). SEM images also showed a significant reduction of total cell number adherent on PEU films with drugs compared to the control films without drugs (Fig. SV).

To evaluate the bactericidal efficacy of the PEU films after an extended period of time, film samples that had released drugs for over 4 months were used to repeat the above experiments. CFU counts of the adherent bacteria still decreased at least 90% (1 log₁₀ reduction) compared to the negative control (Data not shown).

3.4 *In vitro* cytotoxicity testing of PEU films

It is essential to evaluate the cytotoxicity of the drug containing PEU films prior to any *in-vivo* studies. BioSpan® PEU is an FDA approved biomaterial, backed by a comprehensive FDA master file with numerous biological tests demonstrating its non-cytotoxic properties when used in various medical devices [36]. However, there is no such information on the cytotoxicity of the siderophore-chelated gallium and zinc complexes at the concentrations released from the sustained drug delivery vehicles.

Consequently, we investigated the toxicity of three PEU films releasing ZnMP, GaMP and GaPP on mouse fibroblast viability *in vitro*. Mouse fibroblasts were chosen since they are instrumental in wound healing and maintaining the extracellular matrix [37]. As shown in Fig. 6, an AlamarBlue cell viability assay revealed that unloaded PEU films did not result in any greater toxicity to the fibroblasts compared to the cells cultured on TCPS significantly ($P < 0.5$). In contrast, significant differences in 3T3 fibroblasts proliferation were observed (Fig. 6) for ZnMP drug-loaded PEU film compared to the cells cultured on TCPS control surface ($P > 0.001$). ZnMP PEU film inhibited fibroblasts proliferation by approximately 20%. However, the other two drug-loaded PEU films, GaPP and GaMP films did not affect cell viability. Release of GaPP and GaMP from PEU films was less toxic than ZnMP, with a fibroblast viability of $92\% \pm 5\%$ after 24 hour exposure, which is comparable to other commonly applied antibiotics; for example, 2% kanamycin sulfate reduces cell viability to only $93 \pm 10\%$ [38],

3.5 *In vitro* endotoxin testing of PEU films

Endotoxin concentrations in the extracts prepared from the PEU drug release films were determined using the endotoxin-specific LAL test (Fig. 7). Resultant levels are significantly lower than the FDA endotoxin tolerance limit for medical device by extraction (0.5 EU/ml) [8]. The LAL assay used in this study is based on the activation of a pro-enzyme in the presence of very low levels of endotoxin (QCL-1000™ detection limit, 0.1 EU/ml).

3.6 *In vivo* efficacy of PEU/PEG films containing GaMP complex

To mimic nosocomial infection and evaluate the efficacy of PEU films releasing Ga-complexes against bacterial infection, PEU/PEG films loaded with GaMP complex were pre-inoculated in *Ps. aeruginosa* suspensions for 6 hours and then implanted into mice subcutaneously. PEU/PEG films without drug were used as the control. As shown Fig. 8A, mice in both groups had a body weight drop the second day of implantation, due most likely to the implantation surgery. All mice in the GaMP group exhibited normal activity, and gradually regained their body weight over time. In contrast, the control group mice became very weak on the second day of implantation, indicating serious infection. All mice in the control group were euthanized after consultation with the veterinarian service staff. These results suggest that the GaMP films possess a strong ability to protect the animals against bacterial infection. We further examined the blood bacterial concentration of mice in both groups. As shown in Fig. 8B, one day after implantation, the blood bacterial concentration reached $\sim 10^6$ CFU/ml in the control group using two different culture methods, while no bacteria were detected in the GaMP group. Consistent with our *in vitro* observations, this

test further proves that without the protection from GaMP, bacteria contaminated implants will cause serious systemic infection.

4. Discussion

Gallium is a FDA approved drug to treat hypercalcemia cause by solid cancer tumors [19]. PP and MP are prosthetic units of hemoglobin and other hemoproteins. We discovered that a group of these combined chemical compounds, complexes of GaPP, GaMP and ZnMP, showed strong antibacterial activities against both Gram-positive and Gram-negative bacteria. These formulated Ga- and Zn-complexes hold great promise and could offer some advantages compared to other conventional antibiotic therapies. First, the ZnMP, GaMP and GaPP drugs used in this work possess a potent and broad antibacterial efficacy against both Gram-negative and Gram-positive bacteria. Significant data has validated the efficacy of free gallium and their siderophore-chelated complexes in inhibiting suspended and eradicating biofilm bacteria of Gram-negative strains, such as *PA*. The bactericidal killing effects of $\text{Ga}(\text{NO}_3)_3$ on *P. aeruginosa* strain PA01 were reported at 99% at a concentration of 100 μM and 99.9999% at a concentration of 1000 μM in both planktonic and biofilm cultures [32]. A metallo-complex, desferrioxamine-gallium (DFO-Ga) kills planktonic *P. aeruginosa* bacteria with a MIC of 32 μM and blocks biofilm formation at a sub-inhibitory concentration 1 μM [39]. However, these antibacterial activities of free Ga ion and DFO-Ga were only investigated using *P. aeruginosa*. Unfortunately, neither Ga ion nor DFO-Ga appear to inhibit the growth of Gram-positive strains commonly found in medical device related infections, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*. Our findings show that both Gram-positive and Gram-negative strains are sensitive to ZnMP, GaMP and GaPP complexes used in this work. Table 2 summarizes the relative minimum inhibitory concentrations for the various Zn- or Ga-complexes, reported on an equivalent mass metal ion (Ga or Zn) basis. GaMP and GaPP exhibited MIC values of 0.17 and 0.14 mg-Ga/L respectively; approximately 3.5 $\times$ lower than the MIC for ciprofloxacin (0.5 mg/L), 50% lower than $\text{Ga}(\text{NO}_3)_3$ and 15 $\times$ lower than DFO-Ga or ZnMP.

Pathogenic bacteria face a particularly acute problem in acquiring sufficient iron. The host specifically limits iron availability as part of its innate defense against invading microorganisms. Mammals employ iron-binding proteins (transferrin, lactoferrin) to reduce the levels of free extracellular iron to around 10^{-18} M; levels insufficient for bacterial growth. In addition, the host produces proteins that bind haem and hemoglobin (*e.g.*, haemopexin and haptoglobin), consequently limiting the availability of haem as an iron source for pathogenic bacteria. Pathogens counter the iron restriction imposed by their hosts through the use of siderophores, receptor-mediated transport systems specific for host iron complexes, or direct degradation of haem. Siderophores are of low molecular mass (<1000 Da) and have high specificity and affinity ($K_{\text{aff}} \sim 10^{30}$) towards ferric iron. Approximately 500 siderophores of both Gram-positive and Gram-negative bacteria have been characterized. We hypothesize that the efficacy of our Ga-PP and Ga-MP complexes is due to the importation of the metal complexes via the iron siderophore uptake mechanism, replacing iron with the non-redox cycling gallium. Table 1 suggests that gallium- and zinc-complexes are released by simple, passive diffusion through interconnecting pores formed upon the dissolution of the porogen, PEG. Increasing PEG content appears to increase the pore tortuosity. It is hypothetically possible that gallium or zinc ions are released from the porphyrin siderophore complexes; either within the PEU films prior to diffusing out or immediately upon escaping the PEU. Neither scenario has any validity. Stability experiments showed that soluble complexes of either PP or MP versions of the Ga- and Zn-complexes were stable for weeks, not releasing any free metal ion (data available upon request). If GA- or Zn-ion were release from the PP or MP siderophores, then their anti-microbial efficacy (the MIC level) would be the same as that for $\text{Ga}(\text{NO}_3)_3$. Table 2 shows

that the MIC levels for Ga-MP or GA-PP, on a Ga mass concentration basis, were 3-4x lower than with Ga(NO₃)₃. These results suggest that the Ga antimicrobial efficacy is due to the importation of the metal complexes via the iron siderophore uptake mechanism.

The Ga- and Zn-siderophore complexes within the PEU polymer films proffer several advantages over conventional antibiotics for antibacterial protection based on biomaterial release. First, the very low MIC values of GaPP and GaMP are well-suited for a sustained drug delivery vehicle to prevent biofilm formation *in-vivo*. While the MIC of ZnMP (<30 μM) is high and similar to the reported level of DFO-Ga [39], the MIC values of GaPP and GaMP complexes (< 2 μM) in planktonic cultures are extremely low; which reduces the release rates required from a dispensing biomaterial. Secondly, any dispensing biomaterial will eventually reach a point where most of the loaded drug is gone and drug concentrations drop below effective levels. It has been reported that a *sub-inhibitory* concentration of Ga³⁺ and DFO-Ga of 1 μM can prevent biofilm formation without actually killing cells [32, 39]. In contrast, conventional antibiotics at sub-inhibitory concentrations do not affect planktonic growth and can *actually exacerbate* biofilm formation. Several groups, including ours, have documented that sub-inhibitory concentrations of many different acting antibiotics (β-lactams, imipenem [41]; glycopeptides, vancomycin [42]; aminoglycoside antibiotics, *e.g.*, tobramycin [43], kanamycin [44], and gentamycin [44]) can cause structural changes in the biofilm, *e.g.*, an increased biofilm volume, cell numbers, alginate expression, and biofilm thickness of >100%. Thirdly, continual exposure of bacteria to lethal or sub-lethal dosages can create a rise in drug resistant strains. Since bacteria have only one mechanism to import iron, it is unlikely they can evolve a resistance mechanism against these Ga complexes [32].

A controlled drug delivery system was developed here using Biospan® PEU as the base polymer; individually GaPP, GaMP or ZnPP were loaded as antimicrobial agents to achieve a continuous drug-release profile, where the concentration of released drug remains constant in a region near the implant. The PEU film system loaded with GaPP, GaMP and ZnPP in this study provides some advantages over above conventional implantable drug delivery devices. First, homogeneous drug distribution within smooth films was obtained without additional sieving procedures as reported previously [23]. Second, the constant antimicrobial drug release from our PEU films was persistent for 4 months. In contrast, conventional antibiotic encapsulated drug delivery films, such as ciprofloxacin in PEU or poly (2-hydroxyethyl methacrylate) (pHEMA), release almost 100% of their drug within a week [23, 45]. Third, the sustained drug concentrations released from films (Fig. 4) did not kill either *S. epidermidis* or *P. aeruginosa* species in planktonic phase, but did prevent their colonization on surfaces, even at low concentrations (Fig. 5; Fig. SIII). Finally, the base polymer PEU is a well-known non-toxic biomaterial and has been FDA approved for use in numerous device applications. The porogen, PEG, is a substance that is generally recognized as safe (GRAS list. Food and Drug Administration) [46]. There are reports that PEG itself only shows toxicity at high, parenteral doses associated with human toxicity [47]. Our study indicates that, at least within the total amount contained in the PEU films, the pore former PEG did not seem to inhibit the proliferation of fibroblast cells (Fig. 6). The toxicities of all siderophore-chelated gallium complexes drug-loaded films presented in this study are also relatively low except for the ZnMP.

Unlike Fe, Ga exposure does not induce the expression of immunosuppressive related virulent factors [32]. Endotoxin concentrations for the four PEU films are all below the recommended FDA limit of 0.5 EU/mL [8].

5. Conclusions

In this study, two Gallium complexes, Gallium protoporphyrin IX and Gallium Mesoporphyrin IX were synthesized via chelation reaction. The bacterial growth in planktonic cultures of both Gram-positive (*S. epidermidis*) and Gram-negative species (*P. aeruginosa*) were effectively inhibited by siderophore-chelated gallium and zinc complexes (ZnMP, GaMP and GaPP) at relatively low MICs (GaMP and GaPP less than 2 μ M). These complexes may represent a new family of antimicrobial drugs to treat bacterial infections caused by antibiotic resistant strains. Furthermore, incorporation of these drugs into PEU polymer films containing 0.5% Ga-complexes and 8% of PEG (MW1450) achieved a constant rate of drug release for approximately one year. ZnMP, GaMP and GaPP released from PEU films in a controlled-sustained manner inhibited bacteria growth suspended in the liquid phase, but significantly reduced live bacteria colonizing the PEU film surfaces. Mice implanted with PEU films containing GaMP survived a constant *Ps. aeruginosa* bacterial challenge versus empty PEU films releasing nothing; GaMP-releasing PEU films produced undetectable levels of *P. aeruginosa* in their blood with 100% survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by grants to JDB from NIH/NIAID (1R01EB007575-01). We thank Dr. P. Singh for supplying *Pseudomonas aeruginosa* strain and Winston Ciridon for helpful technical support.

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Fig. 1. Schematic of Protoporphyrin IX (A); Mesoporphyrin IX dihydrochloride (B) and Gallium protoporphyrin IX complex via chelation reaction (C).

Fig. 2.

Inhibition effects of Zinc and Gallium siderophore based complexes on planktonic bacterial growth in a concentration-dependent manner: (A) Gram positive strain of *Staphylococcus epidermidis* SE RP62A, and (B) Gram-negative strain of *Pseudomonas aeruginosa* PA O1. The bacteria were cultured in TSB (10g/L) at 37°C to mid-exponential phase. The isolated cells were incubated at a starting concentration of 5×10^4 cells/ml in 0.3 g/L TSB with or without antimicrobial agents at 37°C for 24 hours. The colony forming units (CFU) were counted on agar plates.

Fig. 3.

Cumulative percent release profiles of ZnMP from PEU films by varying PEG molecular weights (A); and PEG weight percentage (B). (A): each specimen contains 0.55% of ZnMP and 8% of PEG varying molecular weight from 1450 to 8000; (B) each specimen contains 0.55% of ZnMP and PEG (MW =1450) amounts varying from 2% to 40% (w/w).

Temperature = 37°C pH = 7.2. Data is taken from two separate experiments, each with n=3. Error bars are standard deviations (S.D.).

Fig. 4.

Kinetics of proliferation of the two bacteria strains grown in suspended culture in the presence of different drug-loaded PEU films: (A) Gram positive strain of *Staphylococcus epidermidis* SE RP62A, and (B) Gram-negative strain of *Pseudomonas aeruginosa* PA O1. The bacteria were cultured in TSB (10g/L) at 37°C to mid-exponential phase. The isolated cells were incubated at a starting concentration of 5×10^7 cells/ml in 0.3 g/L TSB with different drug-loaded PEU films at 37°C for 24 hours. The colony forming units (CFU) were counted on agar plates. The bars represent S.D., n=3.

Fig. 5. Adhesion of the two bacteria strains on Zinc or Gallium siderophore complexes loaded PEU films: (A) Gram positive strain of *Staphylococcus epidermidis* SE RP62A, and (B) Gram-negative strain of *Pseudomonas aeruginosa* PA O1. Asterisk, $P < 0.001$ when compared to controls. The bars represent S.D., $n=3$.

Fig. 6.

The viability of mouse NIH-3T3 fibroblasts after exposed to drugs-loaded (GaPP, GaMP and ZnMP) and non-drug loaded PEU films for 24 hours. Cell viability was quantified by Alamarblue assay through the conversion of resazurin to resorufin and normalized to fibroblasts cultured on TCPS. Error bar represent standard deviation. The bars represent S.D., n=3. Asterisk, $P < 0.001$ when compared to control.

Fig. 7.

Endotoxin concentrations in the extracts from various PEU films (drug-loaded and control without drug) were expressed as a unit of EU/ml. The maximum permissible endotoxin level for sterile water set by FDA is 0.5 EU/ml (shown as a dashed line). The bars represent S.D., n=3.

Fig. 8. GaMP drug release from PEU/PEG films prevents systemic infection in the implanted mice on (A) survival and body weight loss; and (B) bacterial concentration in blood. The bars represent S.D., n=3. Asterisk, $P < 0.001$ when compared to the control films.

Table 1

Tortuosity (τ) varied with the different PEU/PEG formulations.

PEG (% w/w)	Slope ($1 / \sqrt{day}$)	D_{eff} (cm ² /day)	Tortuosity (τ)
0	0.0329	2.1242E-06	0
4	0.0522	5.3475E-06	368
8	0.065	8.2916E-06	475
18	0.0759	1.1306E-05	740
30	0.0855	1.4346E-05	961
40	0.0983	1.8963E-05	1038

Slopes were gathered from several plots of M_{total} versus $t^{0.5}$ with different PEU/PEG formulations. D_{eff} and tortuosity (τ) were calculated from Equation (4) and (5), respectively.

Table 2

Minimum inhibitory concentration (**MIC**) of various Ga- and Zn-Complexes against planktonic bacteria.

Therapeutic	MW	MIC (μ M)	MIC (as mg/L)
Ga(NO ₃) ₃	629.7	5.0 (<i>Ps. aeruginosa</i>) [32]	0.349 (mg-Ga/L)
Ga-desferrioxamine	255.7	32.0 (<i>Ps. aeruginosa</i>) [39]	2.22 (mg-Ga/L)
ZnMP	630.1	30.0 (<i>Ps. aeruginosa</i> and <i>S. epidermidis</i>) (this study)	1.95 (mg-Zn/L)
GaMP	634.8	2.0 (<i>Ps. aeruginosa</i> and <i>S. epidermidis</i>) (this study)	0.174 (mg-Ga/L)
GaPP	630.7	2.0 (<i>Ps. aeruginosa</i> and <i>S. epidermidis</i>) (this study)	0.140 (mg-Ga/L)
Ciprofloxacin	330.1	1.50 (<i>Ps. aeruginosa</i> and <i>S. epidermidis</i>) [40]	0.5 (as mg-cipro/L)