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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 mesoprotoporphyrin 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, supplanting 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.

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 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

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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 μM, 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 *versus* BSA was shown to release a greater fraction of loaded antibiotic.

Gram-positive *Staphylococcus epidermidis* is the most prevalent bacterial strain of the human skin and mucous membrane microflora, and the epitome of 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, 500 mL) 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 h 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 λmax (nm) DMF: 412, 542, 580; GaMP: UV–Vis λmax (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 8.5 cm × 4.0 cm 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 ± 0.03 mm. 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 60 s 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