Preliminary In Vitro Evaluation of Degradable Biopolymer Sponges for the Local Release of Amphotericin B

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Introduction: Invasive fungal infections (IFI) have recently become increasingly problematic for both military and civilian populations. Many fungal strains adhere to tissue and implanted orthopaedic hardware within wounds to form biofilms, and can easily spread to become prominent resistant infections. Invasive Candida albicans (C. albicans) infection is the third most common cause of hospital-acquired bloodstream infections.[1] These problematic, often polymicrobial, infections can result in high healthcare costs, high mortality rates, and significantly higher amputation rates than those from bacterial infections alone.[2-4] In 2010, 78% of the wounded U.S. soldiers in Afghanistan with IFI required lower extremity amputations.[4] An outbreak of cutaneous mucormycosis was also recently reported in victims from the 2011 tornado in Joplin, Missouri, where 38% of the infected patients died.[5] Amphotericin B is one of the most commonly used antifungals to treat IFI, but because systemic toxicity can be a concern with this antifungal, local wound delivery is highly desirable.[6] The objective of this study is to determine if chitosan and polyethylene glycol sponges can locally deliver antifungals as a degradable, adjunctive therapy for surgical treatment for early abatement of infection.

Methods: Chitopharm S chitosan obtained from Chitinor AS (Tromsøe, Norway) at a 250.6 ± 16.60 kDa weight-average molecular weight and 82.46 ± 1.68% degree of deacetylation was used to make 0.5% (w/v) and 1% (w/v) sponges in 1% acetic acid, as previously described.[7] Polyethylene glycol was obtained from Fisher Scientific in two different molecular weights, 6,000 and 8,000 g/mol. Combination chitosan (0.5% w/v) and polyethylene glycol (PEG) (0.5% w/v) sponges were prepared in 1% acetic acid, with varying PEG molecular weights and either 1 or 2 lyophilizations (1 Lyo or 2 Lyo). Sponges that were lyophilized twice were also neutralized between lyophilizations. In vitro sponge degradation was assessed by mass loss after two or four days (n = 3 per time point) in a 1 mg/ml lysozyme solution. The lysozyme solution was completely refreshed after two days. For antifungal elution measurements, a 1 mg/ml solution of sodium deoxycholate (Sigma-Aldrich) with varying PEG molecular weights and either 1 or 2 lyophilizations (1 Lyo or 2 Lyo) was loaded into sponges via absorbance and elution was measured over 72 hours. Sponges were incubated at 37°C in 1 x PBS (n = 3) with complete PBS refreshment at each time point. Amphotericin B concentration was measured via high pressure liquid chromatography. For biocompatibility testing, a modified ASTM F813-07 protocol was used where normal human dermal fibroblasts (NHDFs) were seeded at 3.75 x 10^4 cells/cm^2 in 12 well plates in DMEM with 10% FBS and 1 x antibiotic/antimycotic. After 24 hours, spent media was replaced with fresh media and 8 mm sponges (n = 5) were added to the wells. Polyurethane and 1% chitosan sponges were used as contact inhibition controls. After 3 days, sponges were removed and cell numbers were determined by ATP quantity normalized to standard concentrations of cells using Glo® luminescent cell viability assay. Degradation, elution, and biocompatibility data were analyzed using ANOVA with Holm-Sidak post hoc analysis.

Results: Chitosan (Chit)/PEG 8000 1 Lyo, Chit/PEG 6000 1 Lyo, and Chit/PEG 6000 2 Lyo sponges all exhibited increased degradation as compared to control chitosan sponges (Fig. 1A). Number of lyophilizations also affected degradation; sponges with 1 lyophilization showed greater degradation than sponges with 2 lyophilizations. The Chit/PEG 6000 1 Lyo sponges released the most amphotericin B after 1 and 6 hours of elution, while the Chit/PEG 8000 2 Lyo sponges eluted the most amphotericin B of all the PEG modified sponges at 3, 24, and 72 hours (Fig. 1B). Among all sponges, the 1% and 0.5% chitosan sponges released the most amphotericin B at 48, 24, and 72 hours, respectively. None of the chitosan/PEG sponges exhibited significantly lower cell numbers than the control polyurethane sponges, and all exhibited higher cell numbers than the chitosan sponges (Fig. 1C).

Discussion: Results indicate that adding polyethylene glycol to the chitosan sponges increases in vitro degradation, except for the Chit/PEG 8000 2 Lyo sponges after 4 days. All sponges released the antifungal at levels well above the amphotericin B minimum inhibitory concentration of C. albicans; however, the sponges also retained a significant portion of the originally loaded antifungal.[8] A major limitation of this preliminary in vitro study is that the elution experiment is a short term test which does not factor in sponge degradation. Based on the preliminary in vitro degradation data, an improved analysis of antifungal release might combine in vitro elution and degradation tests. The addition of PEG to the chitosan sponges did not cause any viability issues with NHDFs, and biocompatibility of the amphotericin B eluates on NHDFs will be evaluated in future studies. To ensure there is no loss of activity after release from the sponges, antifungal activity of the amphotericin B eluates will also be evaluated with C. albicans. The degradation and biocompatibility of the chitosan/PEG sponges is currently being evaluated in vivo in a rat model.
Because of toxicity concerns with antifungals, the efficacy and biocompatibility of locally delivered amphotericin B should be evaluated in vivo before clinical use. Although this is a preliminary in vitro study, these experiments serve as a first step in identifying a local antifungal delivery system, and due to the polymicrobial nature of orthopaedic wound infections, should be further evaluated with both antifungals and antibiotics.

**Significance:** The results of these studies are useful in identifying a degradable local delivery system that can be utilized as a local antifungal therapy, adjunctive to surgical treatment, for orthopaedic wounds.

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**References:**
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Figure 1. (A) Percent sponge remaining (Mean ± standard deviation) after 2 or 4 days in vitro lysosome-based degradation (n = 3), where * represents p < 0.05 versus, † indicates p < 0.05 vs. 1% and 5% chitosan, and ‡ denotes p < 0.05 vs. all except †. (B) Mean amphotericin B in µg/ml (± standard deviation) released in vitro from various sponges over 72 hours (n = 8), where * denotes p < 0.05 versus. (C) Mean cell number in each condition (± standard deviation) after 5 days of incubation.