Chitosan Films

A Potential Local Drug Delivery System for Antibiotics

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Abstract Local antibiotic delivery is an emerging area of study designed to provide alternative methods of treatment to clinicians for compromised wound sites where avascular zones can prevent the delivery of antibiotics to the infected tissue. Antibiotic-loaded bone cement is the gold standard for drug-eluting local delivery devices but is not ideal because it requires a removal surgery. Chitosan is a biocompatible, biodegradable polymer that has been used in several different drug delivery applications. We evaluated chitosan as a potential localized drug delivery device. We specifically determined if chitosan could elute antibiotics in an active form that would be efficacious in inhibiting *S. aureus* growth. Elution of amikacin was $24.67 \pm 2.35 \mu\text{g/mL}$ (85.68%) after 1 hour with a final cumulative release of $27.31 \pm 2.86 \mu\text{g/mL}$ (96.23%) after 72 hours. Elution of daptomycin was $10.17 \pm 3.83 \mu\text{g/mL}$ after 1 hour (31.61% release) and $28.72 \pm 6.80 \mu\text{g/mL}$ after 72 hours (88.55%). The data from the elution study suggested effective release of amikacin and daptomycin. The activity studies indicated the eluants inhibited the growth of *S. aureus*. Incorporating antibiotics in chitosan could provide alternative methods of treating musculoskeletal infections.

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Introduction

Local antibiotic delivery is an emerging area of study designed to provide alternative methods of treatment to clinicians and patients. In compromised wound sites, avascular zones can prevent the delivery of antibiotics to the infected tissue. Whole-body dosing can also lead to systemic toxicity. Local drug delivery systems can achieve high local concentrations of drug while lowering the overall serum concentrations. According to Hanssen, the ideal local antibiotic system “would provide a more efficient delivery of higher levels of antibiotics to the site of infection and yet minimize the risks of systemic toxicity associated with traditional methods of intravenous antibiotics” [16].

Although the ideal local drug delivery system has not been discovered, several promising materials are present in modern research. Antibiotic-loaded bone cement is the current gold standard for drug-eluting local delivery devices [16, 25]. However, bone cement requires removal surgery and has the potential risk of biofilm attachment, which makes this system less than ideal [3, 18, 23, 27, 29]. Biodegradable systems offer the ability to provide extended release of therapeutic agents while being resorbable. Synthetic polymers such as polylactic acid (PLA) and poly(lactic-co-glycolic) acid as well as the mineral hydroxyapatite have been used as drug delivery matrices both alone and in composite systems [2, 4, 5–7, 11, 14, 33]. Another well-studied material used in the localized delivery of antibiotics is calcium sulfate [9, 10, 12, 13, 15, 26, 28, 30, 31, 34, 37]. Calcium sulfate is commonly used in combination with tobramycin in a commercially available kit from Wright Medical Technology (Arlington, TN). Fast dissolution and adverse reactions to wound drainage are problems associated with calcium sulfate as a localized drug delivery vehicle [32].

Chitosan is a naturally occurring biopolymer that has been used in several drug delivery systems both alone and in combination with other materials. Chitosan is biodegradable and biocompatible and is becoming more prevalent in the drug delivery arena [1, 7, 17, 19, 25, 40, 43]. Chitosan has been used as a material incorporated into implantable scaffolds in several studies [39, 41–43]. Further studies demonstrate chitosan is bacteriostatic and enhances wound healing rates [8, 19, 25, 35, 38]. Additionally, chitosan has hemostatic properties [20]. The US military currently uses a chitosan patch to control bleeding in wounds sustained by soldiers in Iraq and Afghanistan.

The aminoglycoside amikacin can be used to treat Gram-negative bacterial infections caused by bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter*. Thousands of *Staphylococcus* infections are acquired each year from American hospitals with the majority of these infections caused by *Staphylococcus aureus*. Methicillin-resistant *S. aureus* (MRSA) rates rose in hospitals from 35.9% in 1992 to 64.4% in 2003 [21]. Methicillin-resistant *S. aureus* is a Gram-positive bacterium that is normally resistant to conventional antibiotic treatment. Daptomycin is an antibiotic that offers the ability to treat multidrug-resistant Gram-positive bacteria [31]. Incorporation of either amikacin or daptomycin into chitosan can possibly lead to a desirable local drug delivery system that uses the inherent properties of chitosan. The need for improved delivery of antibiotics to infected wound sites remains a challenge that faces clinicians abroad [16, 23, 27].

Based on this need, we asked the following questions: Can chitosan serve as a carrier for amikacin and daptomycin? If so, how efficacious is chitosan at eluting these drugs? Are the released antibiotics active against *S. aureus*?

Materials and Methods

We investigated the in vitro elution of two commonly used antibiotics from a resorbable polymer matrix, chitosan. The chitosan films used in this study were preloaded with 5% (w/w) amikacin or daptomycin. Films were prepared and subjected to both elution tests to determine release profiles of the drugs. We also performed activity tests to test the inhibition efficacy of the drugs when incorporated into the chitosan matrix.

We prepared chitosan films by dissolving 2.0 g chitosan (AgraTech International, Inc, Goose Creek, SC), 80% to 81% deacetylation, into 98.0 mL of 1% (v/v) weak acid solvent to make a 2.0% (w/v) film. One hundred milligrams of antibiotic was predissolved into the acidic solvent before adding the chitosan powder. We mixed the chitosan solution for several hours and allowed it to sit overnight to

degas. The chitosan solution was then cast into flat-bottomed glass dishes and placed into a 37°C convection oven for 12 hours. After drying, we removed the films and subjected them to elution and activity tests. Small discs (5/8-inch diameter) were punched from the dried film and weighed to determine a theoretical amount of antibiotic present. We then submerged the discs into 50 mL of 1x phosphate-buffered saline (PBS) in standard 50-mL polypropylene tubes. These tubes were placed into a 37°C agitated water bath for the duration of the study. We extracted 1-mL aliquots at 1, 3, 6, 24, and 72 hours and placed them into a typical laboratory freezer until testing occurred. Aliquots were evaluated for amikacin concentration with a fluorescence polarization immunoassay technique using a TDxFLx instrument (Abbott Laboratories, Abbott Park, IL). We measured daptomycin concentration using a high-pressure liquid chromatography (HPLC) protocol previously reported in the literature [22, 31]. A Varian (Walnut Creek, CA) Prostar HPLC system was used in conjunction with a Varian Microsorb C8 column and Galaxie software. This methodology uses 38% pure acetonitrile with 62% aqueous solution containing 0.45% ammonium dihydrogen phosphate as the mobile phase [31]. Calibration curves were generated by making serial dilutions of daptomycin in the mobile phase. We used these curves to determine unknown eluate concentrations. Concentration values for both drugs are reported in microliters per milliliter and as total percentage release based on theoretical initial load.

We tested eluates and films for inhibition of *S. aureus* through activity studies. A turbidity assay was used to test the inhibition percentage of the sample eluates versus positive and negative controls. These assays allowed for a quantitative measurement of activity of the released drug. We added 200 μ L of each eluate to tubes containing 1.8 mL of Mueller-Hinton II broth with a 50 μ g/mL CaCl_2 supplement. Tubes were inoculated with 20 μ L of *S. aureus* and placed in a 37°C incubator for 24 hours. We performed and recorded absorbance measurements after incubation at a wavelength of 530 nm (A_{530}) on a spectrophotometer. Blanks were used to zero the spectrophotometer (1.8 mL of Mueller-Hinton II broth and 200 μ L of PBS) and a percent inhibition determination was made when comparing eluate absorbance measurements with positive control absorbance measurements. We also performed zone of inhibition (ZOI) tests as a method for determining the activity of loaded chitosan films against *S. aureus*. Chitosan discs were placed on an agar plate containing *S. aureus*. We tested loaded and nonloaded films.

We determined differences within groups compared within time. We considered variables different when $p < 0.05$; statistically different data was determined using a two-tailed Student t-test.

Results

The elution studies indicated release of antibiotic occurred after the first hour of testing with near complete recovery of drug after 72 hours. Amikacin release was $24.67 \pm 2.35 \mu\text{g/mL}$ after 1 hour. This amount was equal to 85.68% of the initial amikacin present. The cumulative release of amikacin increased to $27.31 \pm 2.86 \mu\text{g/mL}$ by the final timepoint of 72 hours (Table 1) (Fig. 1). The total recovery percentage of amikacin was 96.23% (Fig. 2). The

Fig. 1 Cumulative release concentrations of amikacin and daptomycin from chitosan films are shown. Substantial release of both drugs is seen after one hour through the duration of the study. The concentration values are reported in micrograms per milliliter over the duration of the 72-hour study ($n = 3$).

Fig. 2 Cumulative release percentages of amikacin and daptomycin from chitosan films are shown. Near complete recovery of both amikacin and daptomycin was achieved. The percentage values are reported over the duration of the 72-hour study ($n = 3$).

release percentages were similar ($p = 0.07$) between hour 1 and 72. Daptomycin followed a similar release pattern with an eluate concentration level of $10.17 \pm 3.83 \mu\text{g/mL}$ after 1 hour (31.61% release). The cumulative concentration level rose to $28.72 \pm 6.80 \mu\text{g/mL}$ after 72 hours (Table 1) (Fig. 1). Daptomycin release increased between hour 1 and hours 24 ($p = 0.03$) and hour 72 ($p = 0.008$). A total recovery percentage of 88.55% (Fig. 2) was determined through calculations.

The turbidity assay and ZOI studies showed the delivery system inhibited *S. aureus* growth. Eluates containing amikacin inhibited $85.13\% \pm 3.00\%$ of *S. aureus* growth after 1 hour (Table 2) (Fig. 3). With increasing release of amikacin, the inhibition percentage rose accordingly. The final eluates containing amikacin inhibited $96.91\% \pm 1.39\%$ of bacterial growth (Table 2) (Fig. 3). Bacterial growth inhibition was significantly different between hour 1 and hour 3 but was not significantly different at all subsequent timepoints. Daptomycin release inhibited bacterial growth similarly to amikacin. After 1 hour, eluates containing daptomycin had inhibition percentages of $99.32\% \pm 0.31\%$ (Table 2) (Fig. 3). The increased release of daptomycin only slightly increased the inhibition percentage. After 72 hours, the eluates containing daptomycin inhibited *S. aureus* growth $99.43\% \pm 0.10\%$ (Table 2) (Fig. 3). However, the percentage inhibition was similar after 3 hours. The ZOI studies further indicated inhibited bacterial growth. The plain chitosan films did not inhibit bacterial growth and had no visible ZOI. Both the amikacin-loaded and daptomycin-loaded films had consistent and substantial ZOIs (Fig. 4).

Discussion

The need for the localized delivery of antibiotics to infected wound sites remains a challenge for clinicians abroad. We asked whether chitosan could serve as a carrier for amikacin and daptomycin and if the eluted drugs were inhibitory to the growth of *S. aureus*.

The turbidity assay only quantifies bacterial growth inhibition of an eluate. A bactericidal assay would likely reveal the ability of an eluted drug to eradicate bacteria in a contaminated wound site. We provided no calcium supplement to the agar that the daptomycin films were tested

Table 1. Elution data for amikacin and daptomycin from chitosan films

Antibiotic	1 Hour	3 Hours	6 Hours	24 Hours	72 Hours
Amikacin ($\mu\text{g/mL}$)	24.67 ± 2.35	26.31 ± 2.70	24.86 ± 3.64	26.04 ± 3.79	27.31 ± 2.86
Daptomycin ($\mu\text{g/mL}$)	10.17 ± 3.83	14.73 ± 8.69	18.01 ± 7.16	18.11 ± 4.17	28.72 ± 6.80

Values are mean $\pm$ standard deviation ($n = 3$).

Table 2. Inhibition percentage data for eluted amikacin and daptomycin

Antibiotic	1 Hour	3 Hours	6 Hours	24 Hours	72 Hours
Amikacin	85.13 ± 3.00	96.02 ± 0.57	96.75 ± 1.24	96.65 ± 1.89	96.91 ± 1.39
Daptomycin	99.32 ± 0.31	99.63 ± 0.26	99.10 ± 0.93	99.32 ± 0.36	99.43 ± 0.10

Values are mean ± standard deviation; *inhibition percentages indicate the inhibition of *Staphylococcus aureus* growth when compared with positive and negative controls (n = 3).

Fig. 3 *Staphylococcus aureus* growth inhibition percentages of the eluates containing daptomycin and amikacin are shown. Both eluted drugs were efficacious in inhibiting the growth of *S. aureus*. Sampling time corresponds to data in Figs. 1 and 2 (n = 3).

on. The lack of calcium could potentially alter the results; however, daptomycin inhibited *S. aureus* growth in this test without the added calcium. Finally, we had no literature against which to compare data in terms of amikacin and daptomycin release from chitosan films.

We demonstrated the potential to incorporate commonly used antibiotics into a chitosan matrix. Like with other degradable delivery systems, there was considerable release of drug during the first few hours after testing [1, 2, 5, 7, 31, 37]. Amikacin recovery was much higher than that of daptomycin until the 72-hour timepoint. We found elution of antibiotic from chitosan much more rapid than reported for PMMA [3, 23, 27, 29]. PMMA is the gold standard in long-term antibiotic release from an implantable drug

delivery system; however, the retrieval surgery necessary makes PMMA less than ideal. Our delivery system offers rapid release of drug in a biodegradable matrix. The elution of amikacin and daptomycin is similar to that in studies with loaded PLA drug delivery systems and systems that incorporate antibiotics into calcium sulfate. A study performed by Richelsoph et al. [31] showed higher than minimum inhibitory concentration (MIC) levels of tobramycin and daptomycin were released out to 28 days. The release of antibiotics in this study did not achieve the extended release rates seen with the Richelsoph study. The lack of extended release could be attributed to the binding nature of drug with chitosan or the initial loading amount. Further investigation should evaluate methods to extend elution time. Cross-linking the chitosan film might aid in extending release of the incorporated antibiotic. Studies have been performed using different crosslinking agents in combination with chitosan [17, 24, 36]. Genipin is a naturally occurring crosslinking agent that is several thousand times less cytotoxic than glutaraldehyde [36]. Evaluation of crosslinking antibiotic-loaded chitosan films are required to monitor changes in release rates of drug as well as determine the activity of eluted drugs.

Drug activity of the eluates seemed unaffected by the weak acid solvent required to dissolve chitosan. Both drugs displayed excellent inhibition of bacterial growth at each timepoint. Previous studies demonstrate chitosan is bacteriostatic [8, 19, 25, 35, 38]; however, our study did not find plain chitosan bacteriostatic. The acidic solvent used could potentially be the reason for this discrepancy. The data acquired from the elution study in combination with the

Fig. 4A–C Photographs of zone of inhibition study are shown. (A) This is a plain chitosan film. No inhibition is seen with this film. (B) This representative picture of an amikacin-loaded chitosan film

clearly shows a large uniform zone of inhibition. (C) Another representative picture of a daptomycin-loaded chitosan film is shown. Again, a large uniform zone of inhibition is seen.

turbidity study results indicated a lower MIC of *S. aureus* when daptomycin is used. MIC ranges for both drugs can be found by expanding the turbidity study to include serial dilutions of known concentrations of each drug. Further testing of the eluates should include assays that can quantitatively measure the bactericidal activity of the eluted drug.

These preliminary data offer insight to the possibility of delivering amikacin and daptomycin in a resorbable polymer, chitosan. Based on the biocompatible and biodegradable nature of chitosan as well as hemostatic and wound healing properties, it exhibits potential as an ideal localized carrier for antibiotics. The large variances in the concentration readings of daptomycin eluates should be investigated further. However, the inhibition of *S. aureus* growth by both amikacin-loaded films and daptomycin-loaded films is promising. Near complete inhibition of bacterial growth was seen with the eluates and films. Studies are ongoing to examine the effects of sterilization on both antibiotic release and activity. Additional studies are needed to further characterize the delivery system such as cytotoxicity tests, degradation tests, and in vivo evaluation. This study focused strictly on a 5% preload of antibiotic from 2% (w/v) chitosan films. Examination of different loading concentrations as well as different acidic solvents can possibly lead to a more optimal formulation for potential clinical therapy in treating musculoskeletal infections.

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