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Elution of amikacin and vancomycin from a calcium sulfate/chitosan bone scaffold

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Abstract. Treatment of polymicrobial infected musculoskeletal defects continues to be a challenge in orthopaedics. This research investigated single and dual-delivery of two antibiotics, vancomycin and amikacin, targeting different classes of microorganism from a biodegradable calcium sulfate-chitosan-nHA microsphere composite scaffold. The addition of chitosan-nHA was included to provide additional structure for cellular attachment and as a secondary drug-loading device. All scaffolds exhibited an initial burst of antibiotics, but groups containing chitosan reduced the burst for amikacin at 1hr by 50%, and vancomycin by 14-25% over the first 2 days. Extended elution was present in groups containing chitosan; amikacin was above MIC (2-4 μg/mL, Pseudomonas aeruginosa) for 7-42 days and vancomycin was above MIC (0.5-1 μg/mL Staphylococcus aureus) for 42 days. The antibiotic activity of the eluates was tested against S. aureus and P. aeruginosa. The elution from the dual-loaded scaffold was most effective against S. aureus (bacteriostatic 34 days and bactericidal 27 days), compared to vancomycin-loaded scaffolds (bacteriostatic and bactericidal 14 days). The dual- and amikacin-loaded scaffolds were effective against P. aeruginosa, but eluates exhibited very short antibacterial properties; only 24 hours bacteriostatic and 1-5 hours bactericidal activity. For all groups, vancomycin recovery was near 100% whereas the amikacin recovery was 41%. In conclusion, in the presence of chitosan-nHA microspheres, the dual-antibiotic loaded scaffold was able to sustain an extended vancomycin elution longer than individually loaded scaffolds. The composite scaffold shows promise as a dual-drug delivery system for infected orthopaedic wounds and overcomes some deficits of other dual-delivery systems by extending the antibiotic release.

Keywords: drug delivery; biodegradable scaffolds; chitosan; calcium sulfate; bone regeneration

1. Introduction

Infections in complex orthopaedic wounds can delay or inhibit healing and can be very difficult to treat (Thomas *et al*. 2005, Noel *et al*. 2010, Nair *et al*. 2011). Together, infection and severe

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tissue damage can reduce blood flow, which can prevent the necessary inflammatory and reparative cells from accumulating and acting at the wound site (Nair *et al*. 2011). Reported infection rates after surgery and trauma range from 1-55% and open fracture infections range from 10-50% depending on the severity of the wound (Patzakis and Wilkins 1989, Jain and Panchagnula 2000, Zalavras *et al*. 2005, Anderson *et al*. 2011, Wenke and Guelcher 2011, Hogan *et al*. 2013). Common pathogens responsible for infecting these injuries include both Gram-positive and Gram-negative bacteria such as Pseudomonas aeruginosa, Escherichia coli, Staphylococcus epidermidis and Staphylococcus aureus (Gentry 1997, Kobayashi *et al*. 2008, Hatzenbuehler and Pulling 2011, Hogan *et al*. 2013). Treatment can be particularly challenging in wounds containing polymicrobial bacterial species and/or resistant species such as methicillin-resistant S. aureus (MRSA). In addition, identifying the infecting organism is time-consuming and can be misleading when results are falsely negative (Brady *et al*. 2006, Kobayashi *et al*. 2008, Hogan *et al*. 2013, Parvizi *et al*. 2014).

Polymicrobial orthopaedic infections may require more than one antibiotic for sufficient treatment. Local delivery devices loaded with antibiotics have been investigated due to toxicity issues with systemic delivery and reduced vasculature in the wound (Zilberman and Elsner 2008, Noel *et al*. 2010, Rathbone *et al*. 2011, Schlickewei *et al*. 2014), but only a handful of dual delivery systems have been reported (Lodenkämper *et al*. 1982, Penner *et al*. 1996, Bucholz 2002, Phillips *et al*. 2007, Atilla *et al*. 2010, McPherson *et al*. 2013, Sakamoto *et al*. 2013, Howlin *et al*. 2015). A dual-antibiotic-loaded local delivery device made from polymethylmethacrylate (PMMA) was investigated by Penner *et al*. and Phillips *et al*. and found to have a much faster rate of elution than the single antibiotic loaded devices (Penner *et al*. 1996, Phillips *et al*. 2007). Although PMMA has been used to deliver antibiotics, it is not an ideal delivery device since it requires additional surgery for removal, can only be used with certain heat-stable antibiotics, and can elute low-level antibiotics that contribute to bacterial resistance.

The limitations of PMMA have resulted in ongoing research on degradable drug delivery systems, such as calcium sulfate, which can be loaded with a wider variety of antibiotics than PMMA (McLaren 2004, Wenke *et al*. 2006; Gogia *et al*. 2009, Thomas and Puleo 2009, McConoughey *et al*. 2015). Amikacin and vancomycin were co-delivered from a degradable calcium sulfate scaffold by Atilla *et al*. (Atilla *et al*. 2010). They reported results similar to PMMA studies; faster elution with the dual-loaded scaffold compared to single loaded scaffolds. The fast elution rates of dual-loaded scaffolds may be considered a shortcoming for local delivery systems because of a loss of long-term antibiotic and therapeutic effectiveness. The reason for the fast elution rate of dual-loaded scaffolds is not clear. Speculations from literature are that the presence of two antibiotics results in an increased porosity of the scaffold and allows for faster elution through the pores, and/or that the potential for chemical interactions between the antibiotics and the scaffold may increase scaffold degradation rates leading to faster antibiotic release (Penner *et al*. 1996, Phillips *et al*. 2007, Atilla *et al*. 2010). Additionally, high levels of antibiotic incorporation can interfere with complete conversion of calcium sulfate to the dehydrate form, possibly causing faster degradation and faster antibiotic elution (Richelsoph *et al*. 2007, Thomas and Puleo 2009, Atilla *et al*. 2010). Regardless of the reason, current research reports that dual-loaded scaffolds elute antibiotics faster than scaffolds with a single antibiotic. However, extending local antibiotic administration for 4-6 weeks is desired to completely eradicate and prevent serious infections. This time frame comes from clinical studies with antibiotic therapy and the time necessary for revascularization of bone in animal studies (Lew and Waldvogel 1997, Lazzarini *et al*. 2004, Nair *et al*. 2011). Additionally, polymicrobial infections may require more than one

antibiotic for treatment. Thus, there is a need for a scaffold system that can deliver two antibiotics with extended elution times while retaining necessary properties to support new bone growth.

To address the extended dual-delivery problem, a scaffold composed of antibiotic-loaded chitosan-nano hydroxyapatite (C-nHA) microspheres embedded into antibiotic-loaded calcium sulfate (CaS) paste was investigated. Calcium sulfate was chosen because it is a commercially used bone graft material, has the versatility to be loaded with multiple different antibiotics, can be manipulated by the surgeon at the point of care, and can easily act as a binder for other materials such as microspheres (Wichelhaus *et al*. 2001, Thomas *et al*. 2005, Richelsoph *et al*. 2007, Thomas and Puleo 2009, Parker *et al*. 2011). Drug-loaded C-nHA microspheres were selected because they have been shown to be effective for local drug delivery, and they may also be easily incorporated into other materials such as CaS paste, which can act as a temporary barrier to fluid flow from the surrounding environment and slow/extend drug release from the microspheres (Pecora *et al*. 1997, Thomas and Puleo 2009, Reves *et al*. 2009, Doty *et al*. 2014). The C-nHA microspheres may also provide a necessary framework for tissue regeneration, since a limitation of calcium sulfate is a fast resorption rate (Chesnutt *et al*. 2009, Thomas and Puleo 2009, Dash *et al*. 2011, Reves *et al*. 2012). By loading antibiotics into two individual parts of a composite scaffold, we hope to eliminate or minimize interactions between the two therapeutic agents and to provide an extended elution profile for both antibiotics.

In order to develop effective treatment of polymicrobial wounds, a co-delivery system for two antibiotics from a bone graft substitute was fabricated from a composite of CaS and C-nHA microspheres. Two antibiotics, amikacin and vancomycin, were used as model therapeutic agents since they provide coverage over both Gram-positive (vancomycin and amikacin) and Gram-negative (amikacin) pathogens. These antibiotics were also selected as they are commonly used to treat infections such as MRSA and have lower toxicity issues compared with other antibiotics (Edin *et al*. 1996, Zilberman and Elsner 2008, Jackson *et al*. 2009, Atilla *et al*. 2010, Noel *et al*. 2010, Rathbone *et al*. 2011, Wenke and Guelcher 2011). While there is a potential for antagonistic, additive, or synergistic interactions when co-delivering two antibiotics, no change in activity for vancomycin or amikacin has been reported when co-delivered (Atilla *et al*. 2010, Noel *et al*. 2010, Thomas *et al*. 2011, Sakamoto *et al*. 2013). Potential effects of the composite scaffold on antibiotic activity will be examined. It is hypothesized that this composite scaffold will be able to deliver antibiotics for an extended period of 4 weeks at biologically active levels and that the incorporation of C-nHA microspheres will aid in extending the time course of elution of therapeutic agents.

2. Materials and methods

2.1 Chitosan-HA microsphere fabrication

A previously described co-precipitation method was employed for the fabrication of the composite chitosan-nano hydroxyapatite (C-nHA) microspheres (Chesnutt *et al*. 2009). Briefly, 3.57 g 80% DDA chitosan powder (260 kDa, Primex EHF, Siglufjordur, Iceland) was dissolved in 84 mL of 2 wt% acetic acid and mixed with 10 mL 1*M* $CaCl_2$ and 6 mL 1*M* NaH_2PO_4 (Ca:P ratio=1.67). The mixture was slowly dripped into a continuously stirred solution of 20% NaOH, 30% methanol, and 50% water (pH=13) to precipitate spherical chitosan-calcium phosphate microspheres. The C-nHA microspheres were kept in the precipitating solution for 24 hours to

allow for the formation of nano-crystalline hydroxyapatite. The C-nHA microspheres were then washed with deionized (DI) water until a neutral pH was achieved (pH 7-8). The C-nHA microspheres were frozen at 20℃ for one hour and then transferred to a 2.5 liter Labconco freeze-dryer for 48 hours to create lyophilized/porous C-nHA microspheres (Reves *et al*. 2009).

2.2 Composite scaffold fabrication and antibiotic loading

Each composite scaffold was made by mixing 600 mg of calcium sulfate (CaS, α-hemihydrate, Wright Medical Technologies, Inc, Arlington, TN) and 80 mg lyophilized C-nHA microspheres with 0.24 mL of DI water. The mixture was pressed into a mold to form a pellet that yielded a CaS to microsphere ratio of 88:12. This ratio was determined empirically for the size of the beads and the amount of CaS that was necessary to hold the scaffold together. C-nHA microspheres were pre-loaded via swelling (24 hrs at room temperature) with 1.5 mL amikacin (10 mg/mL, MP Biomedical, LLC. Solon, OH) in DI water. Any remaining amikacin solution was removed and the C-nHA microspheres were washed once with 3 mL of dIH_2O in order to remove non-absorbed drug. The C-nHA microspheres were then mixed with CaS to form composite pellets. CaS was loaded with 0.24 mL vancomycin hydrochloride (75.2 mg/mL, Acros Organics, New Jersey) in DI water to yield 2 wt% of vancomycin per pellet. All pellets were dried overnight at room temperature to allow conversion to dihydrate form of CaS before use. The handling ability of the composite scaffold was tested after setting by manually pressing with a spatula and observing any crushing or breaking of the pellets. Two control groups were amikacin-loaded C-nHA microspheres (no CaS) and vancomycin-loaded CaS (no microspheres). Five test groups were evaluated: (1) Amikacin loaded C-nHA Microspheres incorporated into Vancomycin loaded Calcium sulfate (AMVC), (2) Amikacin C-nHA Microspheres alone (AM, control), (3) Vancomycin loaded Calcium sulfate without microspheres (VC, control), (4) Amikacin C-nHA Microspheres in Calcium sulfate without vancomycin (AMC) and (5) C-nHA Microspheres without amikacin in Vancomycin loaded Calcium sulfate (MVC). Vancomycin is commonly loaded in CaS and thus we loaded it into the CaS matrix components of our scaffold (Wichelhaus *et al*. 2001, Gitelis and Brebach 2002, Rauschmann *et al*. 2005, Jackson *et al*. 2009, Atilla *et al*. 2010, McPherson *et al*. 2013). We choose amikacin because it is effective against many types of bacteria such as Acinetobacter, Pseudomonas, and Enterobacter (Petersen *et al*. 2007). The C-nHA microspheres were loaded with amikacin and incorporated into calcium sulfate in order to prolong the antibiotic release.

2.3 Antibiotic elution and degradation

Specimens (n=5 per group) were placed in individual wells of 48-well plates with 1.5 mL PBS. Elution samples were taken at 1, 5, 12, 24 hrs and 2, 4, 7, 14, 20, 27, 34 and 42 days. The PBS solution was completely refreshed after each elution time point. The collected elution samples were analyzed with a fluorescent polarization immunoassay (TDxFLx; Abbott Laboratories, Abbott Park, IL) (Jackson *et al*. 2009, Noel *et al*. 2010, Doty *et al*. 2014). Percent degradation and antibiotic release were calculated with Eqs. (1) and Eqs. (2) respectively

$$\%\, Degradation = \left(\frac{Initial\ mass - Final\ mass}{Initial\ mass}\right) * 100 \quad (1)$$

$$\%\, Antibiotic\ Recovery = 100 - \left(\frac{A_L - A_C}{A_L} * 100\right) \quad (2)$$

Where A_L=Theoretical amount of loaded antibiotic and A_C=Cumulative antibiotic release over 42 days. The theoretical amount of amikacin was determined by dissolving three sets of amikacin-loaded C-nHA microspheres (as mentioned above for scaffold fabrication) in 5 mL of 1% acetic acid over night. The dissolved solution was frozen at -20C until analysis. The theoretical amount of vancomycin was calculated by the amount that was loaded into each scaffold.

2.4 Inhibition of growth and bactericidal assay

Turbidity assays using *S. aureus* (Cowan I strain, ATCC 12598, Manassas, VA) and *P. aeruginosa (*ATCC 27317, Manassas, VA) in trypticase soy broth (TSB) were used to evaluate the inhibitory effectiveness of the released antibiotics (Noel *et al*. 2010, Doty *et al*. 2014). Techniques were adapted from the Clinical and Laboratory Standard Method M07-A9 (CLSI 2012). Briefly, 200 μl composite scaffold eluates were added to sterile tubes containing 1.75 ml Trypticase soy broth (TSB) and inoculated with ~2×10^6 colony forming units (CFU) of *S. aureus* and *P. aeruginosa* respectively. Turbidity was determined spectrophotometrically (Spectronic Instruments, 20 Genesis) at OD_{530} after an overnight incubation at 37℃ and no-growth was indicated by OD<0.050. Bactericidal activity of samples was determined by plating dilutions of all samples that inhibited growth. Those samples that killed greater than 99.9% of the inoculum were considered bactericidal.

2.5 Statistical analysis

Statistical analysis was performed with a Two-Way ANOVA on the release of vancomycin and amikacin from the composite scaffolds and on the activity of the eluates between groups and over time. Composite scaffold degradation was analyzed with One-Way ANOVA. Post hoc analysis was carried out with Student-Newman-Keuls (SNK, $P<0.05$) when statistical differences were present. Turbidity and bactericidal statistical analysis performed with proportions test using contingency tables between the rate of inhibition of growth or bactericidal effect with a Fisher Exact test.

3. Results

3.1 Chitosan-nHA microsphere fabrication

Chitosan-nHA microspheres were teardrop shaped, with a textured surface and a diameter of 1.40 mm±0.23 mm (Fig. 1). The composite scaffold measured approximately 12.5 mm diameter by 6.5 mm high, with C-nHA microspheres uniformly distributed throughout. The set pellet was hard and did not crumble with manipulation of forceps.

3.2 Composite scaffold elution and degradation

Elution data showed that amikacin loaded C-nHA microspheres embedded in CaS with or without vancomycin (AMC and AMVC) exhibited a reduced burst and extended the release of amikacin compared to elution from C-nHA microspheres (AM) alone ($P<0.001$) (Fig. 2). Statistical analyses indicated a significant interaction between time and groups ($p<0.001$). Because

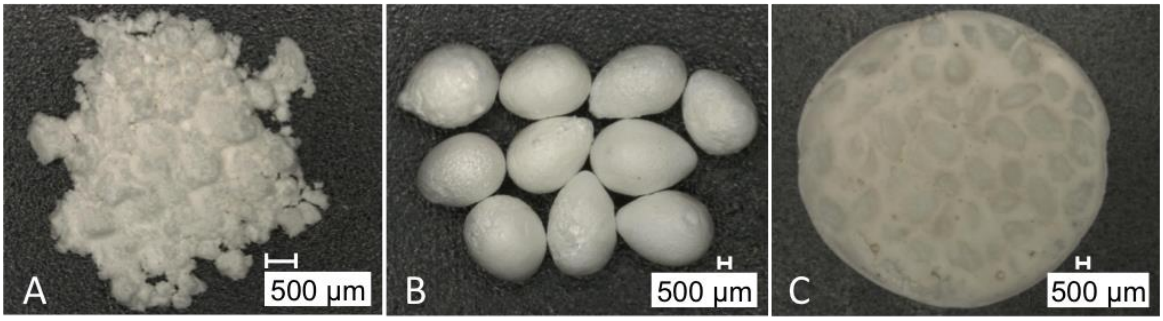

Fig. 1 Digital microscopy images (Keyence VHX-1000) of calcium sulfate powder (A), lyophilized chitosan-nHA microspheres (B), and composite pellet of chitosan-nHA microspheres incorporated into calcium sulfate (C)

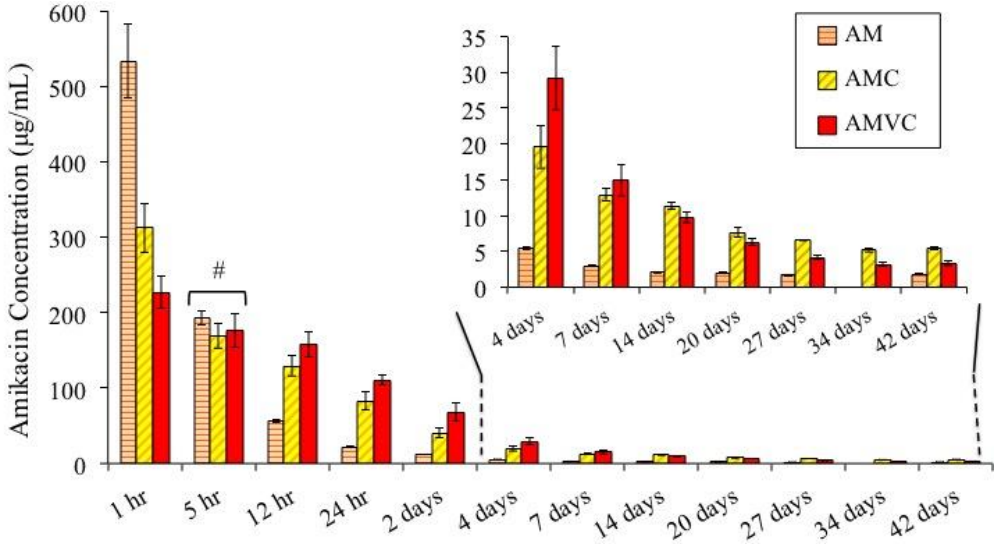

Fig. 2 Amikacin elution from scaffolds composed of chitosan-nHA microspheres embedded in calcium sulfate with or without vancomycin (AMC and AMVC) and amikacin loaded chitosan-nHA microspheres (AM) over 42 days. (#) Indicates no statistical difference

of thisinteraction, subsequent analysis evaluated groups at each time point. There were statistically significant differences in the release of amikacin from AM, AMVC and AMC at every time point ($p<0.05$ except at 5 hr, $p=0.132$). Amikacin release from AM was significantly greater than either AMC or AMVC ($p<0.05$) only at the 1hr time point. After 5hrs, the amikacin release from AM was significantly less than AMC and AMVC at every time point ($p<0.05$). This indicated a very rapid burst release from the C-nHA microspheres alone (AM) as compared to microspheres incorporated into the CaS matrix (AMVC/AMC). Initially, at 1hr, the amikacin release from AMC was higher than that of AMVC, but by 5 hr there were no differences. From 12 hr to14 days the AMVC released more amikacin than AMC but at Day 20 this shifted and AMC released more than AMVC throughout the rest of the study. Amikacin recovery for all groups, regardless of the elution profile, was 41-42% and there were no statistical differences in percent recovery between the three groups, $p=0.582$ (Table 1).

The vancomycin release profile for all three groups showed an initial burst and subsequent decay over time (Fig. 3). Significant differences in the vancomycin release over time ($p<0.05$) were detected as well as significant interactions between time and group factors ($p<0.05$) indicating that the release of vancomycin was dependent on both time and scaffold composition. Further analysis at each time point revealed that there were statistical differences in vancomycin release between groups at all time points ($p<0.05$) except for 1hr where no statistical differences were reported ($p=0.053$). The vancomycin release from VC was notably high through day 2, and then dropped rapidly exhibiting the statistically lowest levels of release for the rest of the study. Vancomycin release from MVC remained high through day 4, and then, similar to the VC, exhibited a drop in release, but the drop was less dramatic, and the levels of release remained statistically higher than VC after day 4. AMVC followed a different release pattern, with a statistically lower release compared to the other groups from 12 hr through day 2 and a statistically higher release from day 4 to 42 suggesting a reduction in initial burst with a concomitant extension of vancomycin release. Vancomycin recovery from the scaffolds was nearly complete at 98-102%, with no statistical differences, $p=0.133$ (Table 1).

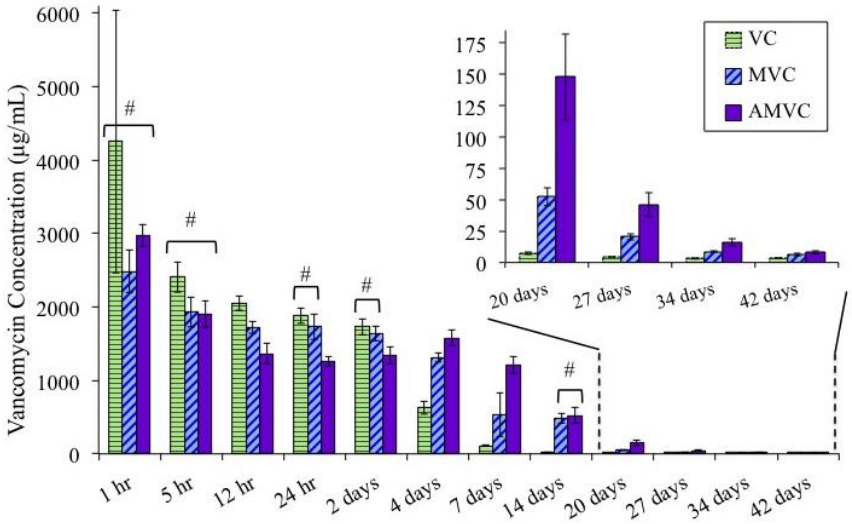

Fig. 3 Vancomycin elution from scaffolds composed of chitosan-nHA microspheres embedded in calcium sulfate with or without amikacin (MVC and AMVC) and vancomycin loaded calcium sulfate (VC) over 42 days. (#) Indicates no statistical difference

Table 1 Antibiotic recovery and change in mass of composite scaffolds over 6 weeks (n=5)

Group	% Amikacin Recovery	% Vancomycin Recovery	% Degradation
AMVC	41.35±1.91	102.42±2.33	47.8±2.3
VC	na	102.00±1.44	46.5±1.5
AMC	41.04±1.50	na	47.1±3.4
MVC	na	98.72±4.20	45.1±3.5
AM	42.45±2.94	na	nd

na - not applicable because drug not in scaffold
nd - not determined, degradation was determined for composite scaffold only

Table 2 Bacteriostatic and bactericidal activity of eluates containing vancomycin against *S. aureus*, and amikacin against *P. aeruginosa* (n=5). (-) indicates no growth, (+) indicates growth, (--) indicates reduction in the number of surviving CFU from the inoculum by 99.9% (killing), (++) indicates CFU growth >0.01% of inoculum (no killing) and (--/++) partial killing in replicate samples due to slight variations in drug release at levels near the MIC. Symbol (*) denotes a statistical difference in proportion between (- or --) inhibitory group(s) and (+ or ++) non-inhibitory group(s) at a given time point ($p<0.05$)

	S. aureus						***P. aeruginosa***					
	Bacteriostatic			Bactericidal			Bacteriostatic			Bactericidal		
	VC	MVC	AMVC	VC	MVC	AMVC	AM	AMC	AMVC	AM	AMC	AMVC
1hr	-	-	-	--	--	--	-	-	-	--	--	--
5hr	-	-	-	--	--	--	-	-	-	--	-- / ++	--/++
12hr	-	-	-	--	--	--	-	-	-	++	++	++
24hr	-	-	-	--	--	--	-	-	-	++	++	++
2d	-	-	-	--	--	--	+	+	+	++	++	++
4d	-	-	-	--	--	--	+	+	+	++	++	++
7d	-	-	-	--	--	--	+	+	+	++	++	++
14d	-	-	-	--	--	--	+	+	+	++	++	++
20d	+	- *	- *	++	-- *	-- *	+	+	+	++	++	++
27d	+	- *	- *	++	-- *	-- *	+	+	+	++	++	++
34d	+	+	- *	++	++	--/++	+	+	+	++	++	++
42d	+	+	+	++	++	++	+	+	+	++	++	++

Pellets exhibited an average change in mass of 47±3% after six weeks and no statistical differences were noted, $p=0.064$ (Table 1).

3.3 Inhibition of growth and bactericidal assay

The turbidity assays showed that the antibiotics eluted from AMVC inhibited *S. aureus* growth for 34 days and was bactericidal for 27days (Table 2). Groups VC and MVC were effective in inhibiting and killing *S. aureus* for 14 and 27 days respectively. Group AMVC abated *P. aeruginosa* for 24 hrs but was bactericidal for only 1 hr. Groups AM and AMC showed inhibition of *P. aeruginosa* through 24hrs and bacterial killing for 5 hrs.

4. Discussion

In this study we evaluated *in vitro* a composite bone graft substitute for the dual delivery of vancomycin and amikacin to address the problem of treating polymicrobial orthopedic infections. Amikacin was absorbed into the C-nHA microspheres (36 mg/g microsphere), and combined with the vancomycin-loaded CaS component (2wt% of CaS) without affecting the handling properties of the scaffold after setting, unlike some studies that report changes in crushability and setability with quinolone antibiotics or daptomycin (CLSI 2012) (Mousset *et al*. 1995, Richelsoph *et al*. 2007). These two antibiotics were used to create a broad-spectrum antibiotic effect against both *P.*

aeruginosa, and *S. aureus*, two bacteria commonly found in orthopaedic wounds (Hatzenbuehler and Pulling 2011, Hogan *et al*. 2013). This study demonstrates that the use of the C-nHA microspheres and dual-antibiotic loading reduced the *in vitro* burst release and extended the elution of both antibiotics compared to CaS or C-nHA microspheres alone. All scaffolds, regardless of composition, were less effective in inhibiting and killing *P. aeruginosa* than *S. aureus.* In bone scaffolds that incorporated C-nHA microspheres and vancomycin, the bacterial inhibition and killing of *S. aureus* was improved by 50-60% as compared to the vancomycin-loaded calcium sulfate group. In the presence of both antibiotics, the inhibition of *S. aureus* was further extended 20% due to an extended release and positive interaction between amikacin and vancomycin (Gentry 1997). The most effective antibacterial results reported for *S. aureus* were expected because the elution values of vancomycin containing scaffolds were 100-1000x above the breakpoint MIC (1 μg/mL) for *S. aureus* for 7 days, whereas the highest elution value for amikacin was 55-130x above the breakpoint MIC (4 μg/mL) for *P. aeruginosa* at 1 hr (Jackson *et al*. 2009, Noel *et al*. 2010).

Even though the vancomycin release for AMC was lower in the initial time points than the other groups, there was an extended elution that allowed the composite to remain active against *S. aureus* for the greatest amount of time and within the recommended 4-6 weeks period for antibiotic treatment of orthopaedic wounds (Lew and Waldvogel 1997, Lazzarini *et al*. 2004, Nair *et al*. 2011). After 7 days, 96% of vancomycin was recovered from eluates for the AMVC and VCM composites compared to the same recovery after only 2 days for VC scaffolds, which shows the significant elution extension in the presence of C-nHA microspheres. One possible mechanism for the extended release is that vancomycin was absorbed into and retained by the exterior surface of the porous C-nHA microspheres during preparation. The large vancomycin molecules could have become entangled in the C-nHA microspheres and further delayed the antibiotic release. The bacterial inhibition we report for our dual-loaded scaffold containing C-nHA microspheres is 33 days longer than the 18-24 hour inhibition against several types of *S. aureus* (including MRSA) and *S. intermedius* reported by Atilla *et al*. for dual-loaded amikacin and vancomycin CaS beads (Atilla *et al*. 2010). Additionally, Howlin *et al*. reported no benefit in dual-loaded vancomycin-tobramycin CaS beads over single-loaded vancomycin CaS beads in zone of inhibition (ZOI) tests against MRSA (Howlin *et al*. 2015). Even though vancomycin recovery from the dual-loaded scaffold at day 34 was 102±2%, there remained high enough concentration to inhibit *S. aureus* at this time point. This indicates that the CaS and C-nHA microspheres did not affect the activity of eluted antibiotics. The high recovery of vancomycin for all groups not only contributes to the extended antibiotic activity but also leads to less risk of bacterial resistance from long-term low-level elution.

The amikacin release also followed a burst release pattern similar to other studies that report drug release from chitosan microspheres (Sinha *et al*. 2004, Aranaz *et al*. 2009, Reves *et al*. 2009). The delayed burst effect from groups containing C-nHA embedded in CaS, may be due to the physical barrier of calcium sulfate slowing elution or possible interactions with the matrix. Therefore, it is understandable that the amikacin recovery from AM is ~40% at 2 days compared to groups with C-nHA microspheres, where the recover is ~40% after 14 days. A similar elution delay was noted in Doty et al. where a CaS matrix acted as a physical barrier and caused a one-day delay in rhBMP-2 elution (Doty *et al*. 2014). While the amikacin elution fell below the MIC on days 7 and 27 for groups AM and AMVC respectively, the release for AMC was detectable throughout the 42-day time course, suggesting that amikacin was still entrapped within the matrix and being slowly released. Although the eluted amikacin concentrations were 3-17 fold higher

than MIC levels at 24 hrs, the reduced efficacy of bacterial inhibition and killing compared to the measured concentrations may indicate that the drug is reacting with matrix components and either being inactivated or possibly forming a salt. Atilla et al. reported possible inactivation of amikacin when in lower pH environment, as may occur at the micro level with degrading CaS (Walsh *et al*. 2003, Atilla *et al*. 2010). Thus, the amikacin activity in the composite scaffolds may have been compromised and the activity reduced. Overall, our amikacin release was extended 2.6-4 times longer than what Atilla et al. reported for a dual amikacin-vancomycin-loaded plain CaS scaffold, indicating a benefit of slowed and extended release using C-nHA microspheres with CaS matrix (Atilla *et al*. 2010). Although Atilla *et al*. tested different strains of bacteria; they also reported short antimicrobial activity times of eluates, 18-24 hr for scaffolds containing both antibiotics, possibly due to amikacin inactivation in a CaS environment (Atilla *et al*. 2010). Future studies would require investigation into increasing the amikacin concentration, improving the loading efficiency and/or choosing another non-pH affected antibiotic in order to achieve a longer bacterial inhibition period.

A look into the antibiotic loading of CaS and C-nHA microspheres reveals information about the elution potential. Vancomycin was loaded at 2 wt%, which is an effective "low dose" for premixed cements (Clyburn and Cui 2007). Although we achieved ~5 times higher amikacin loading than Reves et al. (~6.5 mg/g chitosan) (Reves *et al*. 2009) our amikacin loading yielded only 0.42 wt% pellet (36.7 mg/g chitosan), a much smaller amount compared to the vancomycin loading. Thus, it is understandable that the vancomycin eluates were able to sustain a longer activity against bacteria *in vitro* than the amikacin eluates. Inefficient loading, loss of amikacin upon loading (washing step), or inactivation could explain why the amikacin recovery was only ~41%. It is possible that increasing the surface area of the microsphere would allow more antibiotic absorption, thus using chitosan nanoparticles instead of microspheres could result in higher loading and subsequent release (Masri *et al*. 1995, Phillips *et al*. 2007, Romainor *et al*. 2014). The overall benefit of C-nHA microspheres in the CaS matrix loaded with two antibiotics is a slowed initial and extended elution rate for amikacin, despite the low antibiotic levels and the possible inactivation of amikacin in CaS. It is likely that by increasing antibiotic loading levels in the C-nHA microspheres or by using an alternative antibiotic to amikacin, the issues of reduced activity and short duration of elution could be minimized.

This preliminary work is limited to *in vitro* assays and offers no direct correlation to clinical efficacy. However, it does provide valuable foundational information about the co-delivery of two antibiotics from a CaS based scaffold that can be used to further develop composite implant strategies. The antibacterial assays for vancomycin suggest that extended elution would occur *in vivo*. The interactions of antibiotics with bone scaffold materials is not well known, and although in this study a composite approach altered elution and antibiotic activity, the exact mechanisms by which these effects occurred is not well understood. Further work is needed to improve the efficiency of amikacin loading and elution to determine if residual drug is remaining in the scaffold. Evaluating the effects of combinations of antibiotics and calcium sulfate in solution as well as antibiotic salts may help to determine reasons for the lack of antibiotic activity against microorganisms at inhibitory concentrations. Additionally, more pH stable antibiotics may be studied to replace amikacin in the C-nHA microspheres, or synthetic calcium sulfates could be used as they have less acidic degradation products as well as extended elution (Parker *et al*. 2011, McPherson *et al*. 2013). A long-range goal, not encompassed in this work, would be for the slower-degrading, C-nHA microspheres to provide an osteoconductive scaffold for tissue regeneration as the CaS is resorbed.

5. Conclusions

Our goal to deliver therapeutically active antibiotics for 4 weeks from our composite scaffold was exceeded for the vancomycin release only, with the composite scaffold containing both amikacin and vancomycin. The dual antibiotic loaded calcium sulfate-chitosan-nHA microsphere composite scaffold was able to elute and sustain bacterial activity against *S. aureus* for 2.4 times longer than vancomycin loaded calcium sulfate scaffolds alone. The amikacin loaded into the chitosan-nHA microspheres did show a slightly extended elution when embedded in the calcium sulfate matrix compared to free chitosan-nHA microspheres, however, the amikacin may have been inactivated in the calcium sulfate scaffold as it was degrading. Together these results indicate that dual antibiotic delivery over an extended period in one scaffold is possible and favorable compared to single antibiotic delivery systems. Loading one antibiotic into each component of the scaffold helped to slow the elution of both agents. Modifications to improve elution of amikacin could make this dual delivery method advantageous for treatment of orthopaedic wounds with polymicrobial infection. Studies evaluating these biomaterials in preclinical models of contaminated musculoskeletal trauma should follow to confirm potential clinical efficacy.

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