**Size and composition of synthetic calcium sulfate beads influence dissolution and elution rates in vitro**

Randy Roberts, Stephen J. McConoughey, Jason H. Calhoun  
Department of Orthopaedics, The Ohio State University Wexner Medical Center, Columbus, Ohio 43210

Received 22 April 2013; revised 15 August 2013; accepted 10 September 2013  
Published online 00 Month 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.33045

**Abstract:** Treatments of osteomyelitis lag behind bacterial resistance to antibiotics. We tested different-sized calcium sulfate beads and their ability to elute multiple antibiotics in vitro as a possible method to improve the therapeutic delivery in patients. Two sizes of calcium sulfate beads (4.8 and 3.0 mm diameter) that contained vancomycin, tobramycin, or both were dissolved in phosphate-buffered saline, and the rate of dissolution by weight and antibiotic elution by the disc diffusion assay and high-pressure liquid chromatography were measured. The 4.8 mm beads showed significantly higher dissolution rates relative to the 3.0 mm beads (2.3 mg/day vs. 1.3 mg/day). While the vancomycin-loaded 4.8 mm beads eluted for a longer time relative to the 3.0 mm beads (20 days vs. 10 days), the smaller beads had threefold higher elution for the first 2 days, before dropping to near zero elution by day 4. The presence of tobramycin extended the elution of the vancomycin to day 40, which closely matches the recommended 6 weeks to treat orthopedic staphylococcus infections. These data suggest that size and content of the bead are variables that could affect their clinical success, and both could be exploited to tailor treatments of specific infections and injuries. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2013.

**Key Words:** calcium sulfate, antibiotics, elution

---

**INTRODUCTION**

Orthopedic infections are becoming more difficult to treat in the clinic. Bacteria responsible for these infections continue to evolve resistance to commonly used antibiotics (i.e., methicillin), and infection clearance in patients can be difficult to achieve due to the presence of multiple forms of bacteria. Because of these complications, multiple surgical debridements followed by long-term parenteral antibiotics are often required for effective therapy. The resulting space created by the debridement is often filled with antibiotic-impregnated bone cement. Initially, poly(methyl methacrylate) (PMMA) beads were used for localized antibiotic delivery; however, due to low antibiotic elution rates and the requirement of a secondary surgery PMMA is beginning to be replaced with other biodegradable bone graft materials.

Biodegradable antibiotic carriers have been shown to be able to carry multiple antibiotics, and they have also been effective against experimental osteomyelitis. Calcium sulfate is a biodegradable carrier that is replaced by new bone tissue. Because of this complete resorption these beads show greater antibiotic release compared to PMMA beads. Calcium sulfate has been a significant improvement in the treatment of orthopedic infection. Currently, surgeons use 4.8 mm, 3.0 mm, or a combination of both sizes of beads to fill the space following debridement; however, the effect of bead size on the rate of dissolution or antibiotic elution has not been directly tested. Furthermore, the size of the bead and addition of multiple antibiotics could influence the ability to construct solid beads. Numerous laboratories have reported that loading calcium sulfate beads with antibiotics change the solidification and dissolution characteristics of the beads.

The purpose of this study was to investigate (1) whether the size of the beads significantly influences the rate of antibiotic elution or (2) dissolution, (3) whether tobramycin, vancomycin, or a combination of tobramycin and vancomycin could be eluted from calcium sulfate beads in vitro, and (4) whether the presence of a second antibiotic would affect the elution rate of the first.

**MATERIALS AND METHODS**

We tested the elution of antibiotic(s) from two different sizes of beads in vitro (Figure 1). To determine the concentration of each antibiotic in the elutant from all conditions (beads loaded with tobramycin or vancomycin alone or tobramycin combined with vancomycin), the elution samples were analyzed by the disc diffusion assay and high-pressure
liquid chromatography (HPLC). The disc diffusion assay can be used to determine the concentration of antibiotics when compared to a standard curve. More importantly, this assay measures the active concentration, or the concentration that is still able to inhibit bacterial growth. HPLC can measure less than 1 μg/mL of vancomycin in solution, which is well below the lower limit of detection for the disc diffusion assay (25 μg/mL), but HPLC cannot differentiate between active and nonactive antibiotic. The use of both assays allowed us to determine an accurate concentration (HPLC) and whether the antibiotic was still active (disc diffusion).

For control beads, 5 cm³ (9.5 g) of powder Stimulan® (provided by Biocomposites, Keele, UK) were mixed with 3 mL of sterile water as directed by the manufacturer. To test for the elution of only tobramycin, 3 mL of clinical grade tobramycin dissolved in H₂O (40 mg/mL, APP Pharmaceuticals, Schaumburg, IL) were added in place of the 3 mL of water. For the testing of vancomycin elution, 0.5 g of vancomycin powder (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the 5 cm³ of Stimulan® and the powders were mixed until uniform. Then the 3 mL of sterile water were added. To test the elution of tobramycin and vancomycin from the same bead, the Stimulan® and vancomycin powders were mixed until uniform, and then 3 mL of tobramycin (40 mg/mL) were added. Each of the four resulting mixtures (no antibiotic, tobramycin, vancomycin, or tobramycin and vancomycin) was then smoothed onto molds to form the 4.8 mm diameter and 3.0 mm diameter beads. The smaller beads had an approximate weight of 35 mg, whereas the larger beads weighed approximately 100 mg. By weight tobramycin made up approximately 0.9% of each bead ([0.120 g tobramycin/13 g total mixture) × 100] and vancomycin was approximately 4% ([0.5 g vancomycin/13 g total mixture] × 100).

The beads solidified on the bench top at room temperature for 2 h before removal from the mold. One bead from each of the eight conditions [4.8 mm: control, tobramycin, vancomycin, and combined (T-V) beads] and 3.0 mm beads: control, tobramycin, vancomycin, and combined (T-V) beads] was placed in a sterile microcentrifuge tube containing 1 mL or 0.5 mL of phosphate-buffered saline (PBS) for 4.8 mm and 3.0 mm beads, respectively. The microcentrifuge tubes containing both the PBS and the bead were incubated in a water bath at 37°C. The eluent was removed each day and replaced with an equal amount of fresh PBS. The eluent was divided equally and placed into two microcentrifuge tubes (one for disc diffusion measurements and one for HPLC analysis). The eluents were stored at −80°C until analysis.

Antibiotic standards of tobramycin and vancomycin were prepared using twofold serial dilutions to yield a concentration series ranging from 800 mg/L to 12.5 mg/L to generate standard curves for the disc diffusion bioassay described subsequently.

To measure bead dissolution, each day the beads were removed from the PBS, gently dried on a paper towel and weighed. After they were weighed, the beads returned to the same microcentrifuge tube and fresh PBS was added.

Twenty microliters of each sample were added to one of the three sterile blank, 6 mm diameter Bacto Concentration discs (Difco, Detroit, MI). These discs were placed on Escherichia coli (ATCC25922) seeded defibrinated sheep blood Trypticase soy agar plates (to measure elution from the tobramycin beads) or Enterococcus faecalis (ATCC29212) seeded plates (to measure elution from the vancomycin and TV beads). The plates were incubated at 37°C for 18–24 h. The diameter of the zone of growth inhibition around each disc was measured twice with a caliper along orthogonal axes to the closest 0.1 mm. A standard curve of growth inhibition was generated for each antibiotic individually (tobramycin against E. coli and vancomycin against E. faecalis), as well as a standard curve for the mixture of both antibiotics tested against E. coli and E. faecalis individually.

Least-squares linear regression was performed for disc diffusion standard curves [plots of log₁₀ antibiotic concentration (μg/mL) vs diameter (mm) of the zone of growth inhibition], and the values for each antibiotic concentration in the experimental samples were determined. The equation used to calculate percent of antibiotic eluted was (micrograms of antibiotic eluted each day/total micrograms of antibiotic in each bead) × 100.

HPLC was run in parallel to the disc diffusion assay to confirm the disc diffusion data and to measure the concentration of individual antibiotics below 25 μg/mL. Briefly, HPLC was performed on samples from days 1–5, 10, 20, 30, and 40, for detection of vancomycin concentrations. The HPLC system used was comprised of a Waters Alliance (Milford, MA) 2695 HPLC with Photodiode Array. Data processing utilized Waters Empower 3 software Data System. Mobile phase was HPLC-grade methanol and water (6:94, v/v) solution including 0.2 M trifluoroacetate (Sigma-Aldrich, St. Louis, MO). A Phenomenex (Torrance, CA) Kinetex 5µm XB-C18 100A 50 mm × 210 mm column was used to separate vancomycin at a flow rate of 0.8 mL/min. These settings were verified with standards of vancomycin before running our elution samples.
For experimental testing, every sample was run in triplicate and each experiment was repeated at an independent time. Two-tailed $t$ tests were run to compare the dissolution and elution rates.

RESULTS

We saw no inhibition of bacterial growth in our control groups (beads containing no antibiotic).

Both 3.0 mm and 4.8 mm beads were able to function as antibiotic carriers in our study; however, the larger beads showed a significantly greater rate of dissolution as measured by weight of the bead [2.3 mg/day vs. 1.3 mg/day, $p < 0.05$; Figure 2(A)]. The smaller beads were completely dissolved within 30 days, and the larger beads took approximately 48 days to dissolve [Figure 2(A)]. Interestingly, the small beads had a more rapid early elution on day 1 that quickly dropped to near zero by day 4 as measured with the disc diffusion assay (Figures 3 and 4). This differed from the larger beads, which had slower elution rates that were sustained for at least 5 days (Figures 3 and 4) as measured by percent (Figure 3) and concentration (microgram of antibiotic/mL of solution/grams of bead) eluted (Figure 4). Antibiotic could be measured in the elutant from the 4.8 mm tobramycin beads for 10 days using the disc diffusion assay, as opposed to 5 days from 4.8 mm vancomycin or T-V beads (Figures 3 and 4).

HPLC confirmed the disc diffusion data (percent and concentration of eluted vancomycin) through the first 5 days of the beads dissolving in vitro. The lower detection limit of HPLC was under 1 µg/mL, which allowed measurement of vancomycin in the large beads with vancomycin alone until day 20 (14.97 µg/mL/g) and T-V large beads until day 40 (180.53 µg/mL/g). These data suggest the presence of tobramycin significantly slowed the elution vancomycin when they were loaded in the same bead (Table I, $p < 0.05$). The concentration of vancomycin after day 5 was below the lower detection limit of the disc diffusion assay as determined by HPLC, suggesting why it was not possible to measure vancomycin using this assay past day 5. It should also be noted that the increase in concentration (µg/mL/g) observed at day 40 of the large T-V beads (Table I) was likely due to the diminished size of the bead rather than a true increase in elution. For comparison of the HPLC data, we normalized the data to the volume of elution and the daily weight of the bead, giving a new unit of microgram of vancomycin/mL of PBS/g of bead as previously reported. $^{24}$ Unfortunately, due to technical limitations we were unable to measure tobramycin concentrations using the HPLC.

To ensure equilibrium was not reached on the dissolution or elution rates of our samples, we tested multiple volumes for elution and dissolution of the 4.8 mm beads. There was no difference in elution rate when the bead was placed in 0.5, 1.0, or 1.5 mL (Figure 5). In addition to the elution rate, we found no significant difference in the dissolution rate over the first 5 days for the 4.8 mm beads between any of the elution volumes (data not shown). This finding suggests that the increased elution from the 3.0 mm beads is not a result of the

DISCUSSION

Though calcium sulfate has not received Food and Drug Administration approval as a means to deliver drugs
clinically, there is a growing body of literature that suggests this function of calcium sulfate could benefit infection patients.\textsuperscript{26–32} Loaded calcium sulfate beads used to treat osteomyelitis by providing antibiotics to the previously infected debridement site can eradicate any remaining infection and lower the probably of a recurring infection. Although these beads are currently used off-label clinically, there has been a lack of studies to investigate the effect(s) bead size and multiple antibiotics have on the dissolution and elution profile. These results could advance the use of these beads in the clinic; not only for osteomyelitis, but in all infections that use antibiotic-loaded cement (joint replacements, etc.). Our comparative elution data provide
ample information to select candidate therapies for clinical efficacy studies.

The limitations of this study result from the in vitro model that was used. Specifically, the beads were dissolved in PBS, which could differ significantly from the in vivo environment. Additionally, we used one bead in a closed environment, whereas the patient will have multiple beads implanted following debridement. Obviously, it is difficult to model the complexities of the body in a microcentrifuge tube, but we argue that the most important factor in this model is the ratio of volume of PBS = volume of calcium sulfate bead. Next, although the beads were dissolving, there was no shaking or disruption of the bead, but in the human body these beads will be exposed to numerous forces (pressure, erosion from fluids, etc.). Another possible source of error could have been in the drying of beads before weighing them. It is possible antibiotic could have leached from the beads during this step; however, our results suggest we achieved close to 100% elution. Furthermore, we exchanged the solvent every day, which could enhance the rate of elution by increasing the concentration gradient. Our future work will continue to test ways in which this model could be improved.

The results of this study raise several interesting points on the role of antibiotic-loaded beads against infection. First, there was a significant difference between the rate of dissolution and the elution of the 4.8 mm beads compared to the 3.0 mm beads. This result suggests that beads of varying size could have unique advantages in the clinic. In situations that require shorter, higher elution the smaller beads might provide a better option, but in times that necessitate longer, lower levels of elution, the larger beads would be more beneficial. Another possible treatment option would be loading the two sizes of beads with different compounds to better control the environment in the healing bone. Additionally, loading these beads with antibiotics did not alter their dissolution curve, suggesting beads containing both tobramycin and vancomycin could be a viable option for use in patients. Though previous reports have noted issues in bead dissolution after addition of an antibiotic, it should be noted that the antibiotic thought to account for this change in dissolution (daptomycin) was not used in the current study.

Consistent with PMMA beads loaded with multiple antibiotics, calcium sulfate beads loaded with a second antibiotic (tobramycin) had elevated elution of vancomycin on days 10, 20, 30, and 40 when compared to beads only containing vancomycin (p < 0.05, Table I). These T-V beads also eluted for a longer time (40 days vs. 20 days for

| TABLE I. Concentrations (µg/mL/g) of Vancomycin Detected by High-pressure Liquid Chromatography (HPLC) in Elutes up to Day 40 in T-V 4.8 mm Beads With Standard Deviations in Parentheses |
|---|---|---|---|---|
| 4.8 mm Beads | 3.0 mm Beads |
| Vancomycin | Tobramycin + Vancomycin | Vancomycin | Tobramycin + Vancomycin |
| Day 1 | 18413.3 ± (4099.0) | 17094.7 ± (531.1) | 52203.5 ± (947.1) | 48154.7 ± (6081.9) |
| Day 2 | 10277.0 ± (905.4) | 7580.4 ± (1039.0) | 22029.3 ± (5066.6) | 24009.5 ± (4995.9) |
| Day 3 | 5715.8 ± (920.1) | 4617.6 ± (612.7) | 2512.9 ± (1512.5) | 10576.8 ± (1032.9) |
| Day 4 | 3550.9 ± (590.7) | 3121.9 ± (294.8) | 355.9 ± (168.3) | 2298.3 ± (1175.9) |
| Day 5 | 707.0 ± (268.0) | 2102.1 ± (336.6) | 160.8 ± (16.6) | 371.6 ± (301.4) |
| Day 10 | 14.7 ± (3.2) | 33.9 ± (6.0) | 69.9 ± (6.6) | 87.2 ± (12.1) |
| Day 20 | 15.0 ± (1.6) | 40.6 ± (3.9) | 0 | 204.9 ± (9.24) |
| Day 30 | 0 | 62.0 ± (12.6) | 0 | 0 |
| Day 40 | 0 | 180.5 ± (53.9) | 0 | 0 |

Shaded concentrations are below the minimum inhibitory concentration required to inhibit growth of 90% of organisms (MIC90) for vancomycin against *E. faecalis*. N = 6.
vancomycin alone beads). This elevated elution of vancomycin may be due to the tobramycin making the Stimulan® more porous, which in turn increased the availability for its elution; however, this finding requires further evaluation.

It should be noted that after day 5 we did not observe elution concentrations (µg/mL) that were above the minimum inhibitory concentration required to inhibit growth of 90% of organisms (MIC90) for vancomycin against *E. faecalis* (≥4 µg/mL).33 This is consistent with the fact that we could measure vancomycin using HPLC from days 5 to 40, but saw no bacterial growth inhibition as measured by the disc diffusion assay. While independently these beads are not eluting vancomycin at a concentration that is above the MIC90 for *E. faecalis*, in patients these beads will be supplementing the intravenously delivered antibiotic, and therefore could help to maintain antibiotic concentration above the MIC90. This raises the question of whether the presence of long-term, sublethal antibiotics from the calcium sulfate beads would be beneficial, or if it might actually enhance the formation of antibiotic-resistant bacteria. This is a critical question, as pathogen growth in the presence of sublethal antibiotic concentrations has been one of the major mechanisms often reported for creating antibiotic-resistant strains of bacteria.34 Further testing will be required to determine the concentration of eluted vancomycin from highly purified calcium sulfate beads in vivo to answer whether the concentration of vancomycin at or above the MIC90 can be maintained from the 4.8 mm beads. Interestingly, though the 3.0 mm beads dissolved at a slower rate (as measured by weight), they did not maintain this sublethal concentration for as long (Table I), and therefore they may be the better option clinically.

Though groups have shown mixing antibiotics with the calcium sulfate can affect the rate of bead dissolution, we could only find one previous article measuring how the size of PMMA bead affects elution,35 and this article was using beads 10–100 times larger than those in our study (approximately 1–5.65 g). Our findings suggest that 4.8 mm and 3.0 mm beads could be used to obtain different elution patterns within the clinic; however, further testing in vivo is required to validate this.

Calcium sulfate beads loaded with tobramycin or vancomycin alone as well as tobramycin and vancomycin in combination have the advantage of averting the need for surgical bead removal, function as a carrier for multiple antibiotics, and completely dissolve within 40 days. The rapid drop in elution of antibiotic is consistent with previous reports that examined the individual elution of tobramycin or vancomycin from calcium sulfate beads,24,36–38 Further characterization of antibiotic-loaded beads will improve the potential role of these beads in the prevention and management of musculoskeletal infections.

REFERENCES


21. Nelson CL, McLearon SG, Skinner RA, Smeltzer MS, Thomas JR, Olsen KM. The treatment of experimental osteomyelitis by...