EVALUATING ANTIBIOTIC RELEASE PROFILES AS A FUNCTION OF POLYMER COATING FORMULATION

Sherry N. Davidoff[1*], Justin O. Sevy[2*], Benjamin D. Brooks[2], David W. Grainger[1, 2], Amanda E. Brooks[2]
[1]Department of Bioengineering
[2]Department of Pharmaceutics and Pharmaceutical Chemistry
University of Utah, Salt Lake City, UT 84112-5820 USA

*These authors contributed equally to this work

ABSTRACT

To address persistent 1-3% infection rates associated with orthopedic implant surgeries, the next generation of bone graft filler materials is no longer pharmacologically silent being endowed as a local drug delivery vehicle to maintain locally high levels of antibiotic. Bone allograft material, used as a structural support to fill the avascular spaces in bone defects, revision surgeries, and traumatic injury, can be used as a drug depot to provide effective antibiotic delivery over the orthopedically relevant six-to-eight week time period. Passive antibiotic coatings, applied in the surgical theater, are quickly depleted from the site, inadvertently promoting the development of drug-resistance. Alternatively, many promising controlled-delivery strategies provide an initial burst release of antibiotic within 24 to 72 hours; however this remains inadequate to combat the onslaught of ubiquitous pathogens that can persist only to reemerge once drug concentrations fall below the minimal inhibitory concentration (MIC). To improve the longevity of this strategy, a variety of coating techniques were evaluated in which clinically-accepted, FDA-recognized, degradable polycaprolactone (PCL) polymer acts as a rate-controlling membrane to retard the release of the antibiotic tobramycin from allograft bone. Using a combination of dipping and rapid drying, the drug-releasing polymer coating was applied concurrently maintaining the high surface area of the allograft bone; however, SEM imaging reveled an imperfect coating that negatively affected the release kinetics. Altering the drug-containing polymer formulation to incorporate water provided a smoother, more uniform coat and ultimately improved the drug-release profile and longevity. Drug bioactivity was assessed using both bacteriostatic and bacteriocidial assays.

Keywords: infection, allograft bone, tobramycin, polycaprolactone, controlled release, bioactivity

INTRODUCTION

Orthopedic infections often cause patients to suffer severe pain and psychological distress, and require extensive clinical pharmaceutics intervention [1]. Of the 1 million orthopedic surgeries performed annually in the United States, 1-3% result in infection [1, 2]; however, the infectious risk during orthopedic revision surgeries escalates dramatically occurring as frequently as 8-15% [3] with rates of reoccurrence reported at a staggering 20-30% [4]. To combat these grim statistics, systemic oral antibiotic therapy is often utilized. Unfortunately, efficacy of systemic therapy is often limited due to dosing, treatment length, patient compliance and poor penetration of antibiotics into the avascular "dead" spaces surrounding the orthopedic wound and surgical site [5]. A more promising method may be to provide local drug delivery directly to the wound site using clinically familiar cadaver-harvested allograft bone as the carrier. Although, allograft bone provides a depot for drug due to its porous

structure, previous techniques involving cancellous bone as a drug delivery system have been plagued by limited antibiotic loading and ineffective antibiotic release kinetics leading to a limited duration of bactericidal protection [6]. Without a rate-controlled release strategy, drug dose releases as a bolus jettison, killing bacteria only up to 72 hours [5]. Unfortunately, persistent bacteria can survive quiescently, only to re-emerge to cause further infection after the drug depot has been depleted and released drug metabolized by the host [5]. Ultimately, this progression can inadvertently promote antibiotic-resistance, accelerating antibiotic clinical inability to combat a host of infectious events.

A more efficacious approach may be to endow allograft bone with a drug-releasing polymer to retard release of antibiotic. Polycaprolactone (PCL) may be a suitable biodegradable polymer that addresses the common problems associated with local drug delivery by allowing a long-term antibiotic release. Incorporating an antibiotic into an allograft-coating PCL shell for implantation as an orthopedic void filler will not only provide structural support for orthopedic healing but may also mitigate cytotoxic effects and prevent rapid drug loss associated with suboptimal antibiotic release kinetics. Although tobramycin is a clinically relevant antibiotic for treating some orthopedic infections [7], its insolubility in organic media is incompatible for use in a PCL-based polymer/drug hybrid coating, as PCL is insoluble in water. Variations in coating formulations, including altering drug miscibility in coating solutions using solvent mixtures, can modify drug loading, release, and the rate of polymer degradation and hydrolysis [8]; thus, altering and potentially extending the duration of active antibiotic release. This study evaluates effects of the addition of water to an antibiotic-containing PCL solution coating formulation for release of a water-soluble drug from a water insoluble rate-controlling polymer membrane coating. Water addition to an antibiotic-containing PCL/acetone solution dramatically changes the release kinetics of tobramycin from the PCL coating over the course of 6 weeks.

METHODS

Sample Fabrication: Cancellous allograft bone croutons (Miami Tissue Bank) were weighed and similar weights were selected for each cohort. 10kD or 80 kD PCL (Sigma CAS 24980-41-4, St. Louis, USA) (100mg/ml) was dissolved at 45˚C in acetone with 4% v/v deionized water. Tobramycin (MP Biomedicals Cat.# 199696, Solon, USA) was suspended in the PCL/acetone/water solution at 10% weight/volume. Cohorts were dip-coated in PCL/tobramycin solution (room temperature, 30 seconds to 1 minute). After incubating for 5 minutes at -20˚C, croutons were vacuum dried (5-10 minutes). Croutons were dipped 4-6 times. Each crouton was weighed after coating to determine the amount of drug and polymer applied to each crouton. For all comparisons, the amount of drug released was normalized to the amount of drug applied to the crouton based on the weight of applied coating as well as the percent of tobramycin in the formulation.

Tobramycin Drug Release: Coated croutons were individually submerged in 3 ml of phosphate buffered saline (PBS, cat#BP661-10, Fisher Scientific) and incubated at 37˚C. The complete release volume was collected and replaced at 24 hours, 72 hours, and each week up to six weeks. A 96-well colorimetric assay was used to compare the release kinetics for each formulation as previously reported [9].

High Performance Liquid Chromatography (HPCL): Samples were analyzed using HPLC as previously described[10]. Briefly, tobramycin was derivatized using OPA (o phthalaldehyde) reagent. The reaction was then stopped by an addition of 50µl of 1M HCl. Each sample (10 µl) was injected using a 1ml/min flow rate and data was analyzed using both a fluorescence detector (Ex_{340}, Em_{450}) and a UV-Vis (254 nm) detector. The area under a specific tobramycin peak was plotted against standard

concentration and the data were fit to a linear regression as a standard curve. Concentration of unknown release samples were calculated from the standard curve using regression analysis.

Microbiology: Release samples (500 ul) were concentrated in a heated-vacuum centrifuge (Labconoco Centrivap, Kansas City, Missouri) and prepared in low-bind, non-tissue culture treated 96-well microtiter plates (MIC- round bottom, ZOI - flat bottom). All samples were then stored dry at 4˚C until use.

Bacteriostatic Assay: LB broth (100µl, cat# 244620, Difco) was added to each well of the round bottom 96-well plate to reconstitute the lyophilized drug release samples. Each well was inoculated with 10^5 CFU (10^5 = OD_{600}~1) of a liquid culture of *E. coli* (ATCC 25922). The plates were incubated overnight at 37˚C. Plates were imaged using UV (Bio-Rad, Hercules, CA) and growth was visually determined via comparison with known standard tobramycin concentrations.

***Zone of Inhibition (ZOI)*:** For ZOI experiments, release samples were dried onto 6mm Whatman 1 filter paper disks. Muller Hinton agar plates (cat# B21800X, Fisher Scientific) were prepared by streaking *E. coli* (ATCC 25922) to create a contiguous lawn of bacterial growth. Disks containing the dried-down drug were then placed with a minimum distance of 24mm between each disk and the side of the plate. Plates were incubated overnight at 37˚C. The diameter of the zone of inhibition or bacterial clearing around each disk was measured.

Data Analysis: The amount of tobramycin released in each sample was calculated based on the linear regression of the fluorescent units (FU) for each standard. Percent drug release was calculated by dividing the amount of tobramycin released by the amount of tobramycin in each formulation multiplied by 100. All formulations were tested in triplicate (biological and technical replicates) and Excel was used to calculate the average and standard deviation. Pairwise one-way ANOVAs were used to identify significant differences ($p < 0.05$).

RESULTS

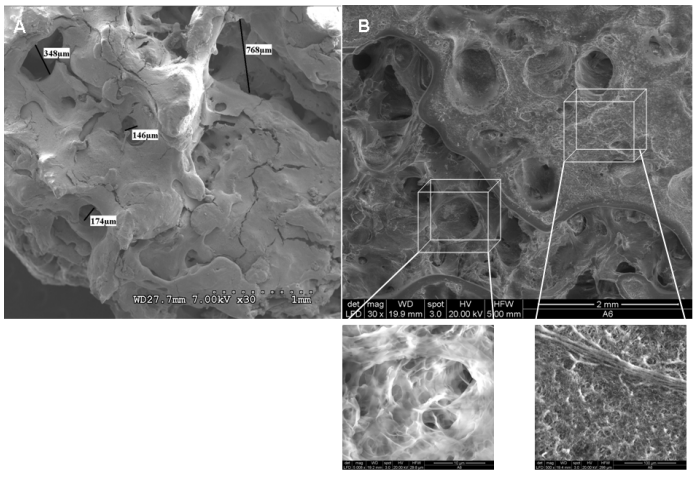


Figure 1: SEM imaging showing 2 different formulations A) acetone-dissolved polymer-coated allograft bone, and B) freeze-dried water-containing polymer formulation-coated allograft bone. Notice the increased porosity shown clearly in the insets of B and the cracked surface and occluded pores in A.

Slight variations in coating formulations of allograft bone yielded important differences in physical characteristics of the surfaces. Figure 1 portrays visual differences between two different coating techniques analyzed by SEM imaging. Differences can be explained by either variations in the coating formulation or differences in the application technique used. Coating inconsistency is evident for formulations in which the polymer and antibiotic were dissolved in acetone prior to dip-coating application. Additionally, significant cracking and occlusion of the porous cancellous structure is also apparent following air drying (Figure 1A). Alternatively, when 4% water is added to the formulation in addition to a freeze drying step, little or no cracking is observed (Figure 1B). Notably, the

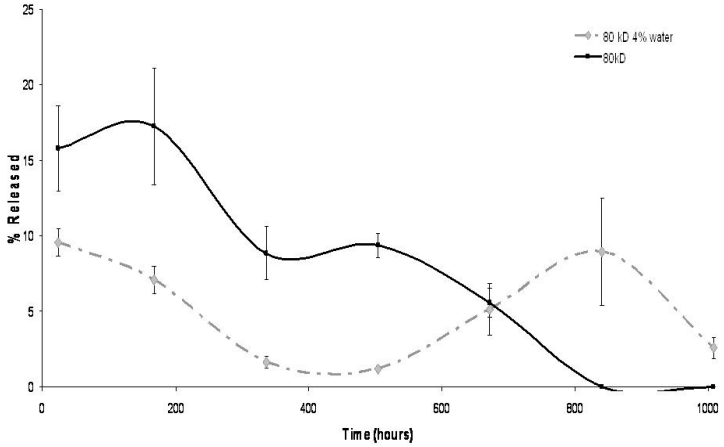
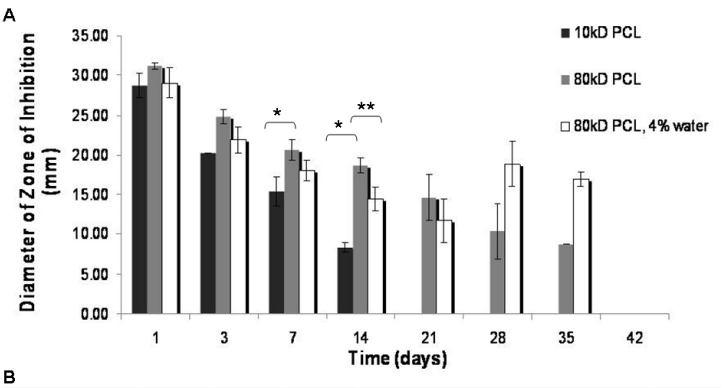


Figure 2: 96-well OPA-tobramycin detection assay comparing different polymer coating formulations. Certain formulations included 4% water that appeared to impact tobramycin release kinetics.



B

	24 hr	72 hr	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk
10kD PCL	-	-	-	-	+	+-	ND	+
80kD PCL	-	-	-	-	-	-	+	-
80kD PCL 4% water	-	ND	-	-	-	-	+-	+

Figure 3: Antimicrobial testing A) Zone of inhibition results compared for 3 different formulations (10kD PCL with 10% tobramycin, 80 kD PCL with 10% tobramycin, 80kD PCL with 4% water and 10% tobramycin) B) Minimal inhibitory concentration compared for 3 different formulations out to 42 days. (+) indicates growth at that particular time point, (-) indicates the absence of growth, (+/-) indicates potential growth, and ND indicates that the MIC was not determined at that time point.

freeze-drying procedure produces an intricate lattice structure in addition to the structural porosity of the cancellous allograft fragments (Figure 1B insets).

Tobramycin was derivatized via a chemical reaction with OPA and detected using product fluorescence. HPLC confirmed that tobramycin elicited no fluorescent or absorbance in the absence of OPA; additionally, OPA exhibited very limited background fluorescence (data not shown). Thus, the fluorescence of OPA-derivatized tobramycin was detected via a 96-well assay [9]. Polymer coating formulations were easily and cost-effectively compared using this assay to reveal differences in release kinetics (Figure 2). Drug anti-microbial activity was assessed using both bacteriostatic and zone of inhibition studies. Bacterial killing varied among three coating techniques, although points of significance were minimal (Figure 3).

DISCUSSION

Tobramycin release kinetics were dramatically altered depending on the polymer coat formulation and application technique used (Figure 2). Although the OPA-derivatization assay employed is not capable of precisely quantifying amounts of tobramycin released, it provides a valuable comparison of formulations and reveals that incorporating water into the drug-containing polymer formulation not only decreases the initial burst release but also imparts a secondary burst of antibiotic after ~5 weeks (Figure 2, Figure 3). Although tobramycin is an orthopedically relevant pharmaceutical agent for the treatment of osteomyelitis, it is inherently difficult to incorporate tobramycin in a PCL rate-controlling membrane due to significant differences in their solubility. Thus, the addition of water to the

formulation was very challenging. Nonetheless, addition of 4% water to the formulation provided superior bioactivity at 5 weeks when compared to an analogous coating without water and a significant improvement in bioactivity when compared to a 10kD polymer coating (Figure 3A). Unfortunately, statistical significance could not be determined past 4 weeks due to release sample contamination in the cohort coated in the non-water polymer formulation. The alteration in release kinetics with the addition of water cannot however be completely attributed to variations in the antibiotic-polymer coat formulation. The lattice structure produced (Figure 1B) by the freeze drying protocol may be critical to achieve these desired kinetics. Finally, the addition of water in the PCL formulation is thought to promote the miscibility of tobramycin and PCL, limiting the drug "blooming" effect to the matrix surface, and its subsequent burst. This is supported by the more limited burst release at 24 hours.

CONCLUSIONS

Engineering a secondary burst of antibiotics locally late in the clinically relevant orthopedic time frame (6-8 weeks) should provide a significant advantage over many other antibiotic controlled release systems where the antibiotic depot is either exhausted prior to 4 weeks or the drug is sequestered in the bulk substrate indefinitely, leaving the patient vulnerable to opportunistic infections particularly by drug-resistant bacteria. Addition of water in the polymer coat formulation not only provides an efficacious rate-controlling membrane to retard the release of tobramycin over the clinically relevant time frame but also endows porous allograft bone graft fragments with enhanced capabilities to ward off late assaults by quiescent pathogens. Furthermore, SEM imaging provides insight as to the mechanism of this enhanced capacity as it reveals an open pore structure with an intricate lattice polymer network. Alternatively, SEM imaging of a "standard" 80kD polymer coating applied via dip-coating with subsequent vacuum drying reveals a more limited pore structure and inconsistencies in the coating integrity. These results appear promising but additional long-term *in vitro* as well as *in vivo* studies are necessary to fully assess the potential of the technology.

ACKNOWLEDGMENTS

This research was funded by the Technology Commercialization Office at the University of Utah as well as a Technology Commercialization Grant from the Utah State Technology and Research initiative.

REFERENCES

[1] "Epsicom 2006 market report " in *https://www.espicom.com/prodcat.nsf/Product_ID_Lookup/00001955?OpenDocument*.

[2] "Complications of Orthopedic Surgery," in *http://www.ofc-oregon.com/assets_ofc/COMPLICATIONS_OF_ORTHOPEDIC_SURGERY.pdf*.

[3] C. B. Landersdorfer, J. B. Bulitta, M. Kinzig, U. Holzgrabe, and F. Sorgel, "Penetration of antibacterials into bone: pharmacokinetic, pharmacodynamic and bioanalytical considerations," *Clin Pharmacokinet*, vol. 48, pp. 89-124, 2009.

[4] L. O. Conterno and C. R. da Silva Filho, "Antibiotics for treating chronic osteomyelitis in adults," *Cochrane Database Syst Rev*, pp. CD004439, 2009.

[5] H. Winkler, O. Janata, C. Berger, W. Wein, and A. Georgopoulos, "In vitro release of vancomycin and tobramycin from impregnated human and bovine bone grafts.," *J Antimicrob Chemother*, vol. 46, pp. 423-8, 2000.

[6] S. K. Nandi, S. Roy, P. Mukherjee, B. Kundu, D. K. De, and D. Basu, "Orthopaedic applications of bone graft & graft substitutes: a review," *Indian J Med Res*, vol. 132, pp. 15-30, 2010.

[7] A. Aneja, J. Woodall, S. Wingerter, M. Tucci, and H. Benghuzzi, "Analysis of tobramycin release from beta tricalcium phosphate drug delivery system.," *Biomed Sci Instrum*, vol. 44, pp. 88-93, 2008.

[8] G. Sivalingam, S. Chattopadhyay, and G. Madras, "Enzymatic degradation of poly (-caprolactone), poly (vinyl acetate) and their blends by lipases," *Chemical Engineering Science*, vol. 58, pp. 2911-2919, 2003.

[9] J. O. Sevy, M. H. Slawson, D. W. Grainger, and A. E. Brooks, "Assay method for polymer-controlled antibiotic release from allograft bone to target orthopaedic infections - biomed 2010," *Biomed Sci Instrum*, vol. 46, pp. 136-41, 2010.

[10] A. Brooks, B. Brooks, S. Davidoff, B. Call, P. Hogrebe, M. Fisher, and D. and Grainger, "Tailored Polymer-Controlled Release of Tobramycin from Allograft Bone Void Filler," *Pharmaceutics and Pharmaceutical Chemistry, Pathology, and Bioengineering.* Salt Lake City: University of Utah, 2010.