ASSAY METHOD FOR POLYMER-CONTROLLED ANTIBIOTIC RELEASE FROM ALLOGRAFT BONE TO TARGET ORTHOPAEDIC INFECTIONS

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ABSTRACT

To mitigate and circumvent orthopaedic-associated infection, systematic oral and parenteral antibiotic therapy is often used; however, efficacy is limited due to dosing, systemic side-effects, patient compliance, effective delivery, treatment length, and resistant bacteria. A more effective method may be sustained local drug delivery of antibiotics at the wound site, using delivery vehicles that control release rates. In the case of bone for example, this could be clinically familiar bone graft. Unfortunately, without a rate-control strategy, local antibiotic delivery from allograft displays a prominent burst release: a large amount of drug payload is released as a bolus within 72 hours and depleted. Although this offers effective immediate killing, persitor bacteria remain an infection risk. Notably, drug resistance is a problem at reduced antibiotic levels. To allow better local dosing modulation, a degradable polycaprolactone (PCL) polymer allograft coating is used to modulate local delivery of the antibiotic, tobramycin. This polymer/antibiotic hybrid coats the porous structure of the cancellous bone graft, providing a substantial drug reservoir and allowing controlled release of antibiotic over extended time. PCL/tobramycin-coated bone fragments of different PCL molecular weights and variable drug loads are assayed in vitro for drug release. Tobramycin concentration is determined based on derivatization of its 5 primary amine groups with a fluorescent reagent, phthaldialdehyde (OPA). Tobramycin concentrations in release media can be calculated based on a standard curve with a reasonable accuracy and dynamic range.

Keywords: controlled drug release, orthopaedic infection, allograft bone, polycaprolactone, tobramycin, antibiotic

INTRODUCTION

Each year in the United States alone over 1 million orthopaedic surgical procedures, including over 500,000 grafting procedures, treat trauma or disease-induced boney defects [3]. One to two percent of these patients develop infection [4]. In an attempt to mitigate and circumvent infection, systemic antibiotic therapy is clinically routine. However, orthopaedic centered infections present a clinically complex and frustrating therapeutic challenge due to poor penetration of antibiotic, with drug to bone ratios ranging from 0.1 to 1.3 mg/kg, depending on the antibiotic used and type of bone [5]. Importantly, the extensive porous structure of cancellous bone allows for increased infiltration into the bone when compared to cortical bone [3]. Opportunities exist to endow bone grafting materials with new antimicrobial properties to eliminate infection locally at surgical wound sites.

The increasingly popular FDA-regulated cancellous allograft material (cadaver-harvested bone) can be used as an antibiotic depot to pack infectious, traumatic, or surgically-induced defects, providing structural support and effective local antibiotic delivery [6]. Unfortunately, as with many other

Presented at Rocky Mountain Bioengineering Symposium &
International ISA Biomedical Sciences Instrumentation Symposium
9-11 April 2010 Laramie Wyoming www.isa.org

antibiotic delivery techniques, local antibiotic delivery from allograft bone is also plagued by 1) limited loading due to cytotoxicity, 2) poor durability, and 3) bolus antibiotic jettison, effectively decreasing the longevity of antimicrobial effects [7]. Although release from allograft material offers effective immediate killing up to 72 hours after application, persistent bacteria can remain quiescent and undetected in soft tissue promoting infection and drug resistance. Antibiotic release from allograft material that is controlled using a customizable antibiotic-loaded coating of clinically familiar biodegradable polycaprolactone (PCL) polymer may prove effective as a strategy to address or circumvent these common issues.

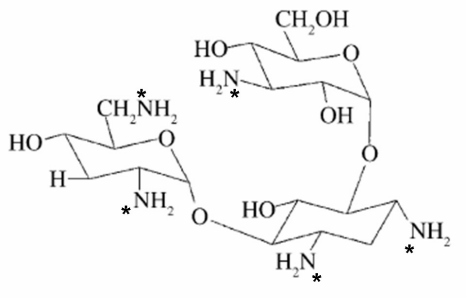


Figure 1. Chemical structure of tobramycin. Primary amines are indicated with a (*) symbol.

The kinetics of the allograft antibiotic release system are derived not only from the physicochemical properties of material carrier and the antibiotic but also from the controlled-release coating. To mitigate cytotoxic effects and limit bolus release, consequently improving longevity, antibiotic release can be retarded with the polymer, polycaprolactone (PCL), as a bone allograft coating. The polymer/antibiotic hybrid is coated over the entire cancellous allograft bone porous structure, providing a substantial drug reservoir and allowing controlled release of antibiotic over extended time periods. The variable PCL molecular weight offers versatile options to control loading and release. Differences in PCL molecular weight equate to variation in the rate of polymer hydrolysis and degradation [8]; thereby, altering release kinetics of encapsulated tobramycin.

Allograft bone can adsorb a number of antibiotics, such as vancomycin, clindamycin, gentamicin, and tobramycin [6]; however, due to the limitations of drug-bone physisorption, the released dose, as well as the kinetics of antibiotic release, critical to therapeutic efficacy, are limited. Although tobramycin is clinically relevant for treating orthopeadic trauma [9], reliably detecting and quantifying the amount released provides a major obstacle to the development of a tobramycin-containing controlled release system. Tobramycin (Figure 1) has few strong fluorescence or absorbance features; thus, detection relies either on mass spectrometry or assays often involving derivatization. Several techniques can be used to detect tobramycin: 1) derivatization with a fluorescent marker followed by high performance liquid chromatography (HPLC), 2) mass spectrometry, or 3) fluorescence polarization immunoassay (FPIA). Each technique has advantages and disadvantages. While providing excellent results, mass spectrometry during coating development is not cost effective. FPIA, based on the attachment and subsequent fluorescent polarization of fluorescein and competitive antibody binding, provides a great deal of flexibility to detect multiple antibiotics and drugs; however, based on the limited linear range, large dilutions are necessary for high concentration samples amplifying inaccuracies and invalidating the results (data not shown). Additionally, FPIA assays performed in the presence of serum are often unacceptable due to background interference and cross reactivity of the antibody; mass spectrometry from serum samples is also inconvenient. Perhaps the most cost-effective method is derivatization of tobramycin with a fluorescent reagent such as naphthyl isothiocyanate (NITC) [10], FITC, or o-phthaldialdehyde (OPA). However, with NITC and

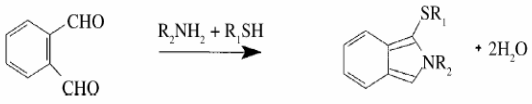


Figure 2. Reaction of OPA with primary amines in the presence of a sulfhydryl compound (β-mercaptoethanol). Notice one molecule of OPA reacts with each primary amine. Tobramycin has 5 primary amines.

FITC, excess reagent must first be excluded via HPLC prior to assay. Additionally, results can be obscured by high background and unintended solvent reactions. OPA, which has no fluorescence in the absence of primary amines, reacts (Figure 2) to quantify the number of primary amines in the presence of excess sulfhydryl moieties.

To facilitate the drug detection and formulation development, a sensitive, robust 96-well o-phthaldialdehyde (OPA)-based fluorescence drug assay was developed. Importantly, release rates *in vivo* should be similar to those measured *in vitro* [7].

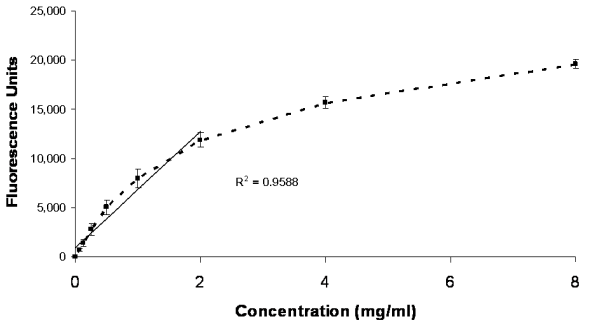
METHODS

Sample Fabrication: Cancellous allograft bone croutons (Miami Tissue Bank) were weighed and like-size fragments were selected for each cohort. PCL (Sigma CAS 24980-41-4, St. Louis, USA) (100mg/ml) was dissolved in acetone at 45˚C. Tobramycin (MP Biomedicals Cat # 199696, Solon, USA) was suspended in the PCL acetone solution at 10% weight/volume. Cohorts were dip-coated in PCL/tobramycin solution (room temperature, 30 seconds to 1 minute). After vacuum drying (5-10 minutes), each crouton was weighed again to determine the amount of drug and polymer applied. Approximately 20 mg of polymer and 2 mg of tobramycin were applied per crouton.

Tobramycin Drug Relase: Coated croutons were individually submerged in phosphate buffered saline (PBS) and incubated at 37˚C. PBS was collected at 24 hours, 72 hours, one, two, three, and four weeks.

HPLC electrospray mass spectrometry: Tobramycin was analyzed using a YMC ODS-Aq 2.1x100 mm column with 5-micron particle size (Waters) on an HPLC coupled to positive ion electrospray (Agilent 1100 LC-MSD) mass spectrometer (mobile phase 80% A (0.2% PFPA in water) + 20% B (acetonitrile) at 0.25 mL/min and 35˚C. Benzoylecgonine-d3 was used as an internal standard (m/z 293).

Tobramycin Detection/OPA Assay: Stock OPA reagent was prepared as described [11] . Briefly, 50 mg of OPA powder (Sigma P-0657) was dissolved in 4 ml of methanol, 0.5 ml of potassium borate (0.5 M boric acid adjusted to pH 10.4 with potassium hydroxide), and 50 µl 2-mercaptoethanol (Sigma M-6250) was added, and the solution was kept at 4˚C in the dark. Working reagent was prepared fresh each day by adding 50ml of OPA stock solution to 1 ml of 0.5M potassium borate buffer. Each detection reaction included 100 µl of release sample in PBS, 100 µl of isopropanol (Mallinckrodt Baker, Phillipsburg, USA #3032-22), and 200 µl of OPA reagent (Sigma P-0657). Each reaction was assembled in a 1.5 ml microcentrifuge tube, vortexed, and incubated at room temperature in the dark for 30 minutes. Tobramycin standards as internal controls were made in PBS and placed in each 96-well UV black-wall assay plate (Costar #3631) with samples. Fluorescence for each derivatization reaction (300 µl) was detected



Concentration (mg/ml)	0	0.0625	0.125	0.25	0.50	1	2	4	8
Average	28.8	721.22	1427.44	2781.00	5047.44	7945.11	11889.56	15724.67	19608.56
standard deviation	2.73	174.15	357.92	604.37	718.42	942.67	755.35	628.13	465.13
Coefficient of variation	9.48	24.15	25.07	21.73	14.23	11.86	6.35	3.99	2.37

Figure 3. OPA fluorescence signals for tobramycin standards in PBS averaged over multiple runs (n=9, ±SEM). Notice the low standard error. Limit of detection (3 times signal: noise) = 0.0625 mg/ml.[1, 2] The solid line indicates a linear regression from 0 to 2 mg/ml. The R^2 value for this regression is shown. A linear regression between 0 and 8 mg/ml gives an R^2 of 0.852 (not shown).

(excitation 360nm and emission at 460nm) in a Biotek spectrophotometer and GenePix5 software (BioTek, Winooski, USA).

Data Analysis: Fluorescence readings for the standard curves were fit with linear regression and used to calculate concentrations of tobramycin released in each sample. Amounts of tobramycin were calculated based on the weight added to each crouton and percent of tobramycin added to the coating formulation. Percent drug release was calculated by dividing the amount of tobramycin released by the amount of tobramycin in each crouton multiplied by 100. Pairwise one-way ANOVAs were used to identify significant differences.

RESULTS

Facile detection of tobramycin is often complicated by the derivatization protocol used. OPA provides a sensitive reliable reagent to derivatize the primary amines of tobramycin (Figure 2). Reliability of the 96-well assay format was assessed by averaging fluorescence signal from known tobramycin concentrations over multiple runs, and determining the error for each standard (Figure 3). OPA was reacted with the 8mg/ml tobramycin standard in isopropanol for 30 minutes at which time the reaction was serially diluted in PBS to provide 8 standards from 8mg/ml to 0 (blank). Standards from 9 assays performed on different days were used for all calculations. Standard errors ranged between 314 and 1 fluorescence units (FU) with the largest errors seen at the upper limits of the linear range (as determined by the high R^2 value) of the assay at 2 mg/ml (252 FU), 1 mg/ml (314 FU), and 0.5mg/ml (239 FU). Notably, PCL does elicit a fluorescence response (~ 500 FU) limiting the lower detection limit of the assay to approximately 125 µg/ml.

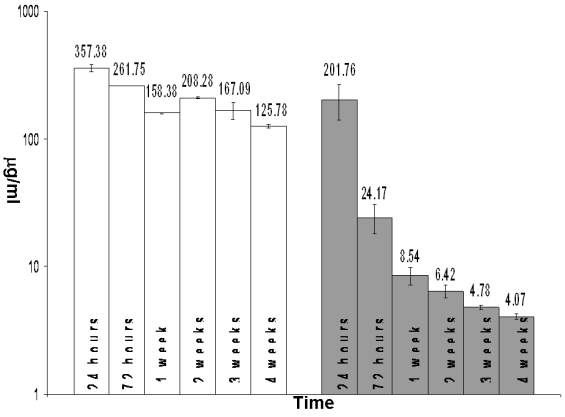


Figure 4. Comparison of the OPA fluorescence assay (white bars) to mass spectrometry (grey bars) from the same samples.

To validate the newly developed OPA-based tobramycin assay, two samples from one cohort (200kD PCL/drug with an unloaded 10kD PCL overcoat) compared using the OPA assay and mass spectrometry methods (Figure 4). Tobramycin concentrations measured based on the OPA assay were calculated using the tobramycin standard curves (all regressions had R^2 values ≥ 0.92). Drug amounts measured in PBS via mass spectrometry were significantly lower than amounts determined by the OPA assay with the exception of the 24-hour time point in which there was no significant difference (α=0.05).

To determine the utility of the OPA fluorescence assay for the PCL-drug loaded coating release, cohorts of PCL-controlled, tobramycin-loaded coated allograft fragments were assayed over time and drug release kinetics were calculated. Solutions of varying molecular weight PCL (10 kD, 80 kD, and 200kD) and 10% w/v drug concentration was used to coat cancellous allograft fragments in different cohorts. An additional cohort used a final 10kD PCL blank coat without tobramycin as an "unloaded overcoat" over a 200kD tobramycin-containing coating. Figure 5 shows drug release kinetics determined via the 96-well OPA fluorescence assay, demonstrating the ability of the assay to illuminate differences in release kinetics from varying PCL coating molecular weight. As expected in the first 24 hours, each cohort, exhibited a burst release with the largest release emanating from the smallest molecular weight PCL coating (Figure 5A and 5B). However, there were significant differences (10kD

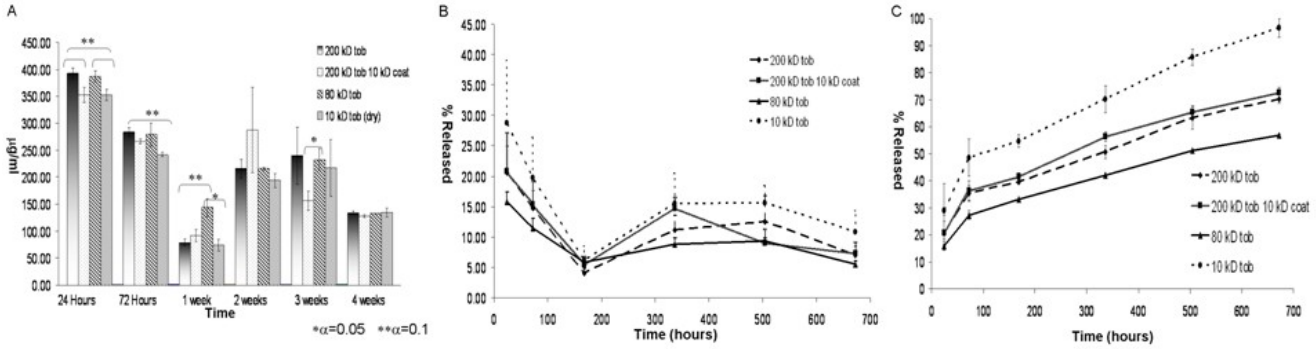


Figure 5. Comparison of tobramycin release from the PCL coating cohorts using the OPA fluorescence assay over a period of 4 weeks. A) average concentration of tobramycin released was determined at 6 time points. Significant differences ($\alpha \leq 0.1$) were determined by pair wise one-way ANOVAs and grouped and indicated by an astrix or symbol above the bar. B) average percent of tobramycin released calculated based on the measured amount of tobramycin added. C) cumulative percent of tobramycin released was calculated by adding the amounts released at each specific time point.

and 200kD (α=0.1, $p<0.05$), 10kD and 80kD (α=0.1, $p<0.08$), 200kD and 200kD with a 10kD unloaded overcoat (α=0.1, $p<0.09$) in the amount of tobramycin bursting from each coating. After the initial bolus, amounts of tobramycin released decreased to a minimal amount at week 1 (significant differences between 200kD and 80kD (α=0.1, $p<0.025$), 80kD and 10kD (α=0.05, $p<0.025$)) rebounded to approximately half of its 24 hour levels and leveled off through week 4. Interestingly, there was still a significant difference between the 80kD coating and the 200kD coating with an unloaded 10kD overcoat at 3 weeks (α=0.05, $p<0.05$), but all coatings released approximately the same amount of tobramycin at 4 weeks. By 5 weeks the amount released was negligible. When coated with 10kD PCL 100% of the tobramycin payload was released within 4 weeks (Figure 5C). At each time point, with the exception of one week for certain cohorts, the amount of tobramycin released was above the measured minimal inhibitory concentration against *E. coli.* (~1μg/ml) (Figure 5A)[12].

DISCUSSION

Methods to detect the release of tobramycin from a PCL allograft coating have proved difficult, time consuming and expensive. Results from mass spectrometry, although high quality and reliable, are expensive for routine development of tobramycin-releasing formulations. A facile, fast, cost-effective assay to determine drug release as a function of the specific coating applied would be useful. OPA, which derivatizes primary amines (Figure 2) such as those on tobramycin (Figure 1), represents such an assay. OPA fluorescence has previously been used to quantify primary amines using HPLC [10]; however, this is the first report of this method being successfully used in a 96-well format. Here we describe and validate (Figure 3 and 4) this assay for release of tobramycin into PBS. Based on the accepted interassay variation (CV$\leq$ 20%) the functional sensitivity of the OPA assay is 0.5 mg/ml [1, 2]. This corresponds with the mass spectrometry validation of this assay which revealed that OPA fluorescence is not able to accurately quantify the amount of tobramycin release at low drug concentrations (Figure 4), but does offer a simple, cost-effective screening method to rapidly produce reliable results for higher drug concentrations ($\leq$ 8 mg/ml) to compare different formulations during the early development of a new drug releasing system. It is possible that the OPA assay cannot detect low concentrations due to interference from PCL as well as additional potential interference from the allograft bone crouton.

Assay accuracy is good (Figure 3), but errors can arise from a variety of sources. Weights of allograft fragments while closely matched within each cohort, have inherent porosity differences within the allograft fragments: the PCL coating consistency may have impacted the rates of tobramycin release. Rates of drug burst release were likely also influenced by tobramycin dispersion in PCL solution and its intrinsically poor miscibility. Drug loading can influence both its uniform particulate dispersion within the polymer matrix, its homogeneity upon coating drying and its release based on the formulation's percolation threshold and drug-PBS accessibility. By overlaying an unloaded 10kD PCL blank coat onto a tobramycin-loaded 200kD PCL surface coat, surface migration of drug during coating drying was studied. This treatment significantly mitigated the amount of tobramycin released through 72 hours and again at 3 weeks. Improvements in an allograft-based controlled drug releasing system such as those described, can quickly be monitored and assessed for release kinetics using the OPA fluorescence assay.

CONCLUSIONS

The OPA-based 96-well drug release assay is useful for initial screening for drug-releasing formulations. Its utility will only be truly realized after validation using 1) drug release into physiological media, and 2) application to other amine-containing drugs released from PCL-coated allograft bone.

ACKNOWLEDGMENTS

S. Davidoff and B. Call are thanked for help fabricating the coated samples. This work was funded by the University of Utah Technology Commercialization Office.

REFERENCES