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RESEARCH ARTICLE

Polymer-controlled release of tobramycin from bone graft void filler

Amanda E. Brooks · Benjamin D. Brooks · Sherry N. Davidoff · Paul C. Hogrebe · Mark A. Fisher · David W. Grainger

Abstract Despite clinical, material, and pharmaceutical advances, infection remains a major obstacle in total joint revision surgery. Successful solutions must extend beyond bulk biomaterial and device modifications, integrating locally delivered pharmaceuticals and physiological cues at the implant site, or within large bone defects with prominent avascular spaces. One approach involves coating clinically familiar allograft bone with an antibiotic-releasing rate-controlling polymer membrane for use as a matrix for local drug release in bone. The kinetics of drug release from this system can be tailored via alterations in the substrate or the polymeric coating. Drug-loaded polycaprolactone coating releases bioactive tobramycin from both cadaveric-sourced cancellous allograft fragments and synthetic hybrid coralline ceramic bone graft fragments with similar kinetics over a clinically relevant 6-week timeframe. However, micron-sized allograft particulate provides extended bioactive tobramycin release. Addition of porogen polyethylene glycol to the polymer coating formulation changes tobramycin release kinetics without significant impact on released antibiotic bioactivity. Incorporation of oil-microencapsulated tobramycin into the polymer coating did not significantly modify tobramycin release kinetics. In addition to releasing inhibitory concentrations of tobramycin, antibiotic-loaded allograft bone provides recognized beneficial osteoconductive potential, attractive for decreasing orthopedic surgical infections with improved filling of dead space and new bone formation.

Keywords Allograft bone · Tobramycin · Controlled release · Polycaprolactone · Implant infection

Introduction

Despite significant multidisciplinary clinical innovations combined with biomaterial and pharmaceutical approaches, including standard systemic antibiotic surgical prophylaxis and new bone graft materials, infection remains a major complication in total joint revision surgery, with rates ranging from 8 to 15 % and relapsing infection a significant threat (20–30 %) to wound healing [1, 2]. This represents a considerable healthcare cost burden as the demand for total joint replacements continues to rise with the aging population. The number of post-arthroplasty infectious complications is projected to increase from current levels of 17,000 cases to an overwhelming 266,000 cases annually by 2030 [3]. Unfortunately, arthroplasty represents just a fraction of the orthopedic conditions with bone infection risks. General musculoskeletal diseases account for approximately 9 % (3.4 million) of all hospital stays in the USA [4] and collectively represent the most prevalent form of chronic disability globally. Orthopedic surgeries performed to address such conditions amidst a growing aged and total population are projected to exceed six million per year by 2030 [5] generating increased global demands for effective prophylactic material platforms. Global demands for orthopedic infection prophylaxis and orthopedic

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products are steadily growing in direct correlation to an aging population and the escalating need for orthopedic procedures. Defect-filling bone graft constituents comprise ~$1.3 billion of the over $29 billion total orthopedic biomaterials market [6, 7].

Autograft bone, or patient-harvested bone, is the gold standard for bone grafting, providing a highly compatible, bioactive, structural matrix as the basis for bone healing. However, cellular death during transplantation, inadequate sourcing due to other pathologies, harvest site morbidity, pain, and cosmetic disfigurement culminate in a substantial 8.5–20 % complication risk, including acute and chronic or recurring infection [8–10]. Thus, allograft or cadaveric-sourced bone tissue has become an increasingly popular defect and wound packing material, increasing 15-fold over the past decade to now account for almost a third of the over 500,000 orthopedic graft procedures performed annually in the USA to treat traumatic or other boney defects [10, 11]. Importantly, allograft bone is processed to remove all cellular and proteinaceous components, leaving only the osteoconductive, and to a more limited extent, osteoinductive mineral component of the graft as a structural template for orthopedic repair, and promote integration and turnover by the patient's natural osteoclast and osteoblast populations. Synthetic bone fillers (i.e., calcium phosphate granules and hybrid calcium carbonate/calcium phosphate coralline ceramic bone graft) [12] and bioactive bone-based technologies (i.e., osteoinductive growth factors and drug carriers) provide new surgical options with versatile orthopedic uses for trauma, revision surgeries, and major repairs [10, 13].

Regardless of the type of implant, clinical success of bone graft void fillers relies on their ability to properly pack the orthopedic defect and facilitate adequate revascularization for graft integration. Importantly, the intrinsic low vascularity of bone and presence of susceptible avascular spaces provides a favorable niche for acute and chronic bacterial infection. These infectious events, particularly those that lead to biofilm formation, can further inhibit graft revascularization and proper cortical blood supply, leading not only to osteonecrosis (sequestra) but also to additional avascular spaces [14]. Consequently, cancellous allograft fragments and morselized, micron-sized allograft bone particulate material are often used not only for their wound packing efficiency, but also for their high surface area that provides a cellular environment conducive to tissue integration and bone remodeling [15]. Additionally, the porosity and resulting high surface area enables cancellous allograft bone fragments or morselized allograft bone as well as their synthetic surrogates [16–18] to be exploited as a drug delivery vehicle to prevent or treat osteomyelitis, with the degree of porosity directly correlating to antibiotic loading efficiency [15, 19, 20].

Based on previous studies, cancellous autogenic bone grafts impregrated with antibiotic prior to implantation show a reduction in infection with no clinical contraindications, further supporting the concept that endowing clinically familiar cancellous allograft bone tissue with controlled release antibiotic capabilities may better treat osteomyelitis [16, 19, 21–41]. However, simple antibiotic adsorption often used off-label with clinically familiar bone graft materials produces rapid bolus drug release and limited therapeutic duration—a few days maximum [5, 19, 35, 42–45]. Improved solutions to implant-centered infection might best integrate local, rate-controlled extended duration drug delivery with bone defect filler materials, particularly for implants and large bone defects with prominent avascular spaces or where penetration from systemic antibiotic administration is compromised [2, 35–39].

Treating bone infections systemically is intrinsically complicated by poor drug bioavailability and drug pharmacokinetics in bone that limit efficacy of systemically administered antibiotic therapy. Bone vascular physiology is also a niche for diverse types of opportunistic pathogens introduced at the time of injury, intraoperatively, or later by hematogenous sourcing, producing difficult-to-treat infections. Antibiotic penetration into the bone as well as the limited vasculature of the affected bone must be considered when designing a clinical treatment strategy [2, 46]. Although systemic intravenous antibiotics are often sufficient in combating these opportunistic pathogens, the negative impact of a standard 4–6-week course of antibiotics cannot be neglected. Inappropriate use of antibiotic therapies, such as poor selection, inadequate dosing, broad-spectrum antibiotic overuse, and poor patient therapy follow-through, have all accelerated pressure towards multidrug-resistant microbes. The Centers for Disease Control and Prevention reports an alarming rise in the antibiotic resistance of the major pathogen, *Staphylococcus aureus*, to at least one of the most common antibiotics from 2 % in 1972 to 63 % by 2004 (MRSA vs. MSSA) [47]. Furthermore, some systemically administered antibiotics may not achieve therapeutic levels in bone, inadvertently supporting the development of resistance. Therefore, options for local sustained antimicrobial therapies are increasingly attractive.

Local drug delivery to bone defects overcomes systemic bioavailability issues, limits development of systemic antibiotic resistance while delivering sustained amounts of drug sufficient to both resist and eliminate microbial infection locally beyond an acute time course [48]. Local delivery of antibiotics offers effective killing using higher doses (up to 1,000-fold greater than systemically delivered) [14, 42] precisely at the site of infection while avoiding systemic toxicity associated with high doses [42]. Unfortunately, many approaches to achieve local antibiotic release from bone grafts with desirable therapeutic kinetics—either actively or passively—are often characterized by an early bolus release and subsequent slow leaching of antibiotic at subtherapeutic levels that may also promote antibiotic resistance [19, 42]. Thus, improved control over local drug release is likely a necessity for efficacious long-term delivery and antimicrobial efficacy.

Drug delivery directly to bone in general and also to avascular traumatized or infected bone presents both a pharmaceutical and pharmacokinetic challenge. Currently, bone grafts are used for musculoskeletal mechanical support as well as space filling and osteoconductive foundation for new bone deposition and healing. Incorporating a space-filling material with the controlled degradation of a synthetic polymer may provide features appropriate for prophylactic controlled drug delivery. Importantly, most synthetic polymers alone are inappropriate as bulk materials for orthopedic needs that may require mechanical integrity for up to a year and bone regeneration/healing induction. Both polycaprolactone (PCL) and its copolymers exhibit requisite enhanced structural functionality for bone implant use while also providing appropriate characteristics for rate-controlled drug delivery and degradability [49–51]. With PCL's precedent in bone implants, the polymer is appropriate to endow clinically familiar bone graft filler materials with an antibiotic releasing, rate controlling programmed resorbable barrier for extended drug delivery. Hydrolytic degradation of PCL [52, 53] offers extended release kinetics and resorption. However, polymer and copolymer blends could be more versatile barrier/controlled release options [54]. Thus, antibiotic loading and subsequent release kinetics might be adjusted and tailored via the rate-controlling polymer coat formulation [55]. In addition to polymer barrier coating characteristics, other factors can be exploited in this modular approach. Graft surface area (micron-scale morselized bone may have a higher surface area for release than cancellous crouton fragments), diverse differential implant packing (i.e., mixing of large allograft cortical croutons with morselized allograft cancellous granules), and antibiotic microencapsulation prior to dose loading all provide a range of customizable release options appropriate for mitigating infectious risks in orthopedic and connective tissue surgical implant and repair sites. While directly soaking allograft bone filler materials in antibiotic has been studied extensively [33, 34, 41, 45, 56–60], the idea of endowing this matrix with a true local-controlled release strategy is only now beginning to be explored.

Synthetic bone graft materials have a lengthy clinical history. However, no Food and Drugs Administration (FDA)-approved allograft bone therapies incorporate an integrated antibiotic release scheme as a combination device [61]. The allograft bone matrix–antibiotic–polymer extended release combination device described here (shown schematically in Fig. 1) permits precise, uniform tobramycin drug loading (via the polymer overcoat) to retain the drug release depot at the surgical site controlled by polymer (PCL ± polyethylene glycol (PEG)) coating swelling and hydrolysis. This technology exhibits long-term antibiotic release and maintenance of therapeutic antimicrobial drug concentrations beyond 6 weeks.

Materials and methods

Fabrication of polymer-coated allograft fragments

Cancellous allograft bone fragments (Miami Tissue Bank) or ProOsteon 500R (BioMet, Warsaw, USA) were weighed and like size and mass fragments were selected for each cohort (n= 3). Alternatively, micron-size allograft bone particulate matter (Miami Tissue Bank) was partitioned into 100 mg aliquots for polymer–drug coating. PCL (10 kDa, Sigma Aldrich, St. Louis, USA; 60 mg/ml) was dissolved in acetone (Thermo Fisher Scientific, Waltham, USA) at 45 °C. Tobramycin (MP Biomedicals, Solon, USA) was suspended as "free" (i.e., unencapsulated) drug in PCL acetone solutions at 10 % weight/weight. Alternatively, certain PCL coating formulations included tobramycin commercially microencapsulated in vegetable triglycerides (70 w/w % tobramycin, lot# TM150-70-30, Maxx Performance Inc., Chester, USA). Importantly, the amount of encapsultated tobramycin incorporated was increased to account of the 70 % encapsulation efficiency. Formulations and cohorts are detailed in Table 1. An unloaded polymer bone control was included in all analyses and demonstrated no bioactivity when released into PBS (data not shown). Dip-coated cohorts were prepared by placing allograft bone into the PCL/free tobramycin solution at room temperature. Fragments were removed after soaking in polymer solution for 30–60 s. After vacuum drying (5–10 min at ambient temperature), each fragment was weighed again to determine amounts of drug and polymer applied. Allograft particulate cohorts of identical mass were coated in individual aluminum trays with 2 ml of polymer/drug solution, mixed twice, and then subsequently allowed to flash off, leaving coated particulate. The particulate-containing polymer film was ground using a weighing spatula prior to placing it in a 50-ml conical vial for release into PBS. To alter drug release kinetics, 45 % PEG (20 kDa, Sigma Aldrich) and/or microencapsulated tobramycin were either mixed directly with PCL solutions or coated in alternating layers with it. Importantly, a less concentrated PEG solution (35 %) was also used in certain formulations without significant effect, thus all formulations reported used a 45 % PEG solution to maximize the solubility of tobramycin and clearly distinguish it from PCL only formulations (Electronic supplementary material (ESM) Fig. 1). Allograft particulate was coated in individual aluminum trays with a total of 2 ml of polymer/drug solution (500 μl PCL with free tobramycin, 1 ml PEG with microencapsulated tobramycin, and 500 μl PCL with free tobramycin) in a layer-by-layer fashion with alternating layers of PCL and PEG. To create a polymer/drug layer, bone graft particulate was mixed twice in each polymer/drug solution and the solvent was allowed to flash off, leaving coated particulate. The dried particulate-containing polymer film was ground to granules again using a weighing spatula and the next layer was applied according to the same protocol.

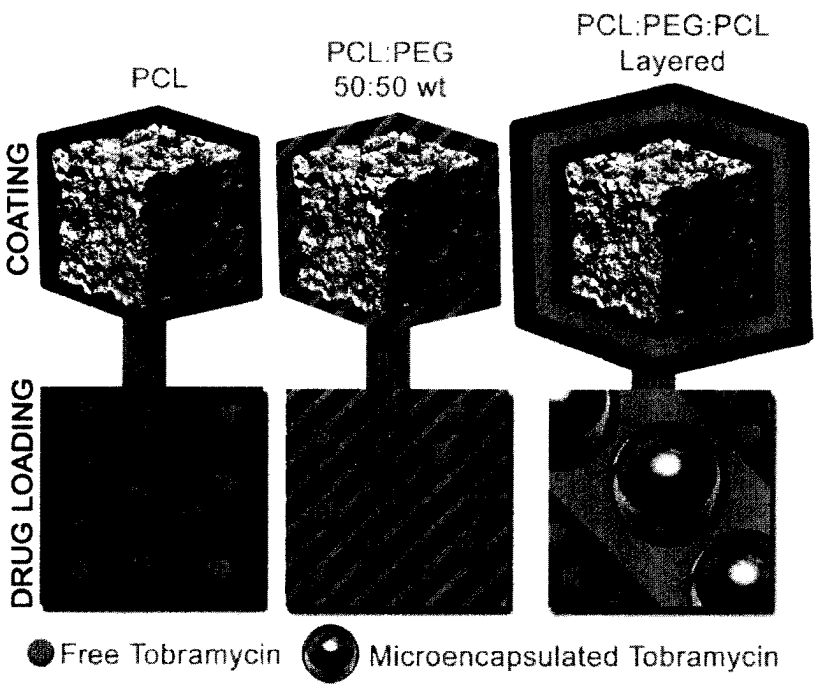

Fig. 1 Schematic describing the concept of a bone graft-based drug delivery vehicle encased within a rate-controlling degradable or porous polymer membrane. The graft porosity provides a high-surface area reservoir to load drug within the pore-filling polymer, and the polymer coating formulation and coating alternatives provide a versatile and tailorable local antibiotic releasing device. Combinations of free drug and microencapsulated drug within the graft pores and in the polymer coating allow drug loading and controlled release kinetics versatility

Drug release

Each coated allograft bone sample (~150 mg) was released into 3 ml of phosphate buffered saline pH 7.4 (PBS; Fisher Scientific). The complete release volume was drawn off and replaced at 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 72 h, and each week for up to 6 weeks to produce sink conditions. Kinetics of release from each formulation were assessed with a previously reported 96-well fluorescent assay [55] based on *o*-phthaldehyde (OPA, Sigma Aldrich) labeling of tobramycin primary amines after drug release. Briefly, 75 μl of each release sample was added to 75 μl of isopropanol in wells within black-masked 96-well plate (Fisher Scientific, Pittsburgh, USA). OPA working solution (50 μl of OPA stock solution in 1 ml of 0.5 M potassium borate buffer pH 10.5) was added to each well and incubated for 30 min prior to assessing the fluorescence of the tobramycin/OPA derivative (Biotek spectrophotometer, ex=360 nm, em=460 nm) using Gen5 1.09 software (BioTek, Winooski, USA). Each cohort contained a certain number of reference samples (n=3, 6, or 9) from which tobramycin was not released over time but instead the entire coating was dissolved in 1 ml of chloroform (Thermo Fisher Scientific) for approximately 5 min and 1 ml of water was used to phase extract tobramycin from the polymer solution by vortexing for 30 s and then centrifuging at 15,000 rpm for 2 min and 30 s. These samples were considered 100 % release samples and all amounts of tobramycin released over time from coated grafts were normalized to their cohort-matched 100 % release value as well as to the unloaded polymer bone control, and reported as a percent to facilitate direct comparison of release from different polymer formulations. Tobramycin from fragments coated with a PCL water nonsolvent mixed system was phase extracted after 8 weeks of release into PBS using chloroform and water to verify the mass balance of the system (data not shown).

Table 1 Allograft bone drug-loaded cohorts used in this work

Cohort	PCL (10 kDa)	PEG (w/v)	Tobramycin (w/v)		Application technique	Graft substrate	Graft form
	mg/ml	%	Unencapsulated (%)	Encapsulated (%)			
1	60	0	10	0	Dip-coat	Allograft	Fragment
2	60	0	10	0	Dip-coat	ProOsteon 500R®	Fragment
3	60	0	10	0	Solvent evaporation	Allograft	Particulate
4	60	45	10	0	Solvent evaporation	Allograft	Particulate
5	60	45	0	10	Solvent evaporation	Allograft	Particulate
6	60	45	5	5	Layer-by-layer	Allograft	Particulate

High-performance liquid chromatography

Standard concentrations of tobramycin were resuspended in acetonitrile–water (52:48). All samples were analyzed in triplicate using high-performance liquid chromatography (HPLC), with a precolumn OPA derivatization [55]. Data was collected from both a fluorescence detector (ex= 350 nm, em=450 nm) as well as UV–vis detector (340 nm). Samples were analyzed using a Hypersil GOLD HPLC column (100×4.6 mm; Thermo Fisher Scientific) and ChromQuest 5.0 (Thermo Fisher Scientific) software on a Finnigan Surveyor (Thermo Fisher Scientific) system. Each sample (10 μl) was injected using a 2 ml/min flow rate. The mobile phase was mixed 0.02 M phosphate (pH 6.5)/acetonitrile (52:48). The area under the tobramycin peak was plotted against standard concentration and data were fit by linear regression as a standard curve, used to calculate the concentration of unknown drug release samples.

Microbiology

Release samples (500 μl per experiment) for all microbiology studies were concentrated in a vacuum centrifuge (Labconoco Centrivap, Kansas City, USA) overnight at ambient temperature and prepared in low-bind, nontissue culture-treated 96-well microtiter plates according to their subsequent experimental use (i.e., minimal inhibitory concentration (MIC), round bottom; zone of inhibition (ZOI), flat bottom). All samples were stored dry at 4 °C until use. Preservation of antimicrobial activity after overnight drying and concentration as well as storage at −20 °C was confirmed with control conditions.

Bacteriostatic assay

Luria–Bertani (LB) broth (100 μl, Becton Dickinson, Franklin Lakes, USA) was added to each well of the round bottom 96-well plate to reconstitute the dried drug release samples. Each well was inoculated with 10^5 CFU in 200 μl of a liquid culture of *Escherichia coli* (ATCC 25922). Liquid bacterial cultures were prepared using a sterile swab to select one to three isolated colonies from a blood agar plate (Remel, Lenexa, USA). Inoculated plates were incubated overnight at 37 °C. Released drug activity, assessed by bacterial growth inhibition, was visually determined by comparing known standard tobramycin concentrations. Growth inhibition was positive if the visual turbidity of bacterial growth media differed from the positive control by 80 %. Negative growth was designated when the well was free of a visible bacterial pellet.

Zone of inhibition

For ZOI experiments, release samples were dried onto 6 mm Whatman 1 filter paper disks. Muller Hinton agar plates (Fisher Scientific, Waltham, USA) were prepared by streaking *E. coli* (ATCC 25922) to create a confluent lawn of bacterial growth (turbidity adjusted to a 0.5 McFarland standard using a nephelometer (Phoenix Spec, BD Diagnostic Systems, Franklin Lakes, USA)). Disks containing the dried-down drug from release samples were then placed with a minimum distance of 24 mm between each disk and the side of the plate. Plates were incubated overnight at 37 °C. Calipers were used to measure the diameter of the zone of inhibition around each disk. The zone of inhibition is reported as an averages calculated based on measured zones for replicate samples.

Data analysis

The amount of tobramycin released in each sample was calculated based on the linear regression of the fluorescent units for each standard. Percent drug release was calculated by dividing the amount of tobramycin released by the amount of tobramycin detected in the dissolved coating (100 % release) multiplied by 100. All formulations were tested in triplicate (biological and technical replicates) and Excel was used to calculate the propagating standard deviation. Pairwise one-way ANOVAs were used to identify significant differences ($p \leq 0.05$ for significance). Particular comparisons to be tested were selected in advance and were reported individually rather than as a group and therefore a multiple comparison correction was not necessary [62].

Results and discussion

Sample fabrication and drug release assay

Tobramycin is a clinical drug of choice used to treat orthopedic infections; however, due to associated nephro- and ototoxicity [63], maintaining adequate drug concentration to combat opportunistic microbes in bone using traditional delivery mechanisms (intravenous) may be unachievable. Therefore, a local, polymer-controlled delivery of tobramycin directly to the bone using a bone graft delivery vehicle was investigated. Importantly, no local tissue toxicity is recognized at concentrations ≤200 μg [64, 65]. Samples were fabricated according to Table 1 with all allograft fragments (~6×5× 4.5 mm) and ProOsteon 500R® fragments (~10×8×7 mm) being dip-coated to add approximately 6 mg of drug-releasing coating to the fragment's initial weight. Importantly, cohorts of bone graft fragments were weight-matched and an equivalent amount of polymer coating was applied to limit their variability. However, it is noted that dip-coating procedures are particularly susceptible to coating inconsistencies and cracking. This affect was minimized via the addition of a vacuum-drying step as previously reported [66]. Alternatively, micron-size allograft particulate (cohorts 3–6) was coated in

individual aluminum trays via a solvent evaporation procedure. The particulate-containing polymer film was carefully morselized with a spatula prior to release. Theoretical amounts of tobramycin applied to each sample were calculated based on the weight of coating applied to the allograft bone material and the percent of tobramycin included in the formulation. Tobramycin was released from the polymer coating on each cohort into PBS. PBS was sampled at designated time points and replaced to simulate sink conditions. Importantly, there is often no relationship between in vitro and in vivo pharmacokinetic studies; however, in literature, reports of local drug release into bone in vivo, the bone was sectioned and ground and the drug extracted. The maximum distance of effective drug release was 5 mm from each end of the graft [67]. Assuming the average radius of the radial bone for a rabbit, which is our animal model of choice for in vivo examination of ostemyelitis, is 3 mm, the volume of bone affected by the drug release is approximately 0.3 ml. The volume we are releasing into is 10 times this amount to provide assurance of sink conditions. Furthermore, Fickian diffusion may only be questionable within the first 24 h during the highest drug release; however, based on the almost limitless solubility of tobramycin in an aqueous solution, sink conditions should have been maintained throughout the experiment. Importantly, sink conditions most likely do not exist in vivo; however, they were simulated in vitro to provide a more robust release system. Tobramycin content in the PBS "release media" was assessed at each time point via a 96-well fluorescent assay (see Figs. 2, 3, 4, 5, and 6) [55].

Release kinetics

Tobramycin is very water-soluble and thermostable during formulation as evidenced by no loss in bioactivity after PCL formulation (data not shown) [68]. Furthermore, at neutral pH in an aqueous environment, tobramycin has proven to be very stable and resistant to hydrolysis [69]. Thus, it is not believed that the bioactivity of the drug is affected by degradation of the drug but instead is a product only of the slow degradation of PCL in the formulation, phase separation between the drug and the polymer, and perhaps a limited effect of the loaded drug's percolation threshold, particularly in the presence of PEG porogen in the coating formulation. However, detection of this small molecule aminoglycoside antimicrobial in a sample is hampered by lack of a unique optical signature. Therefore, released tobramycin was derivatized with OPA [55], yielding a chromophore by chemically coupling with primary amines on the drug, producing fluorescence signals with a dynamic range from 0 to 8 mg/ml and a limit of detection of 62.5 μg ml [55]. The OPA derivatization reaction was verified via HPLC detection of tobramycin in the presence or absence of OPA (data not shown). In the absence of OPA, tobramycin does not elicit any absorbance or fluorescence signal. Furthermore, inherent OPA fluorescence was not detected in the absence of tobramycin. Thus, release of tobramycin from a variety of polymer formulations was compared using an OPA derivatization in a 96-well assay format as previously reported and validated by mass spectrometry [55].

To facilitate comparisons, the measured amount of drug released at each time point was normalized, in a cohort specific manner, by the average (n=3–9) of the detected tobramycin after complete dissolution of the polymer coating and phase extraction (100 % release). Importantly, the phase extraction procedure was controlled by determining the percent recovery of known concentrations of both free drug (approximately 100 % recovery) and also microencapsulated (approximately 82 % recovery) tobramycin. Moreover, the validated coating-dissolution, phase extraction method was applied to time course release samples, allowing determination of the mass balance. After 8 weeks of release into PBS, between 97 and 100 % of the drug was recovered from a PCL-tobramycin coating (data not shown). The combination of fluorescent detection and mathematical validation provided accurate

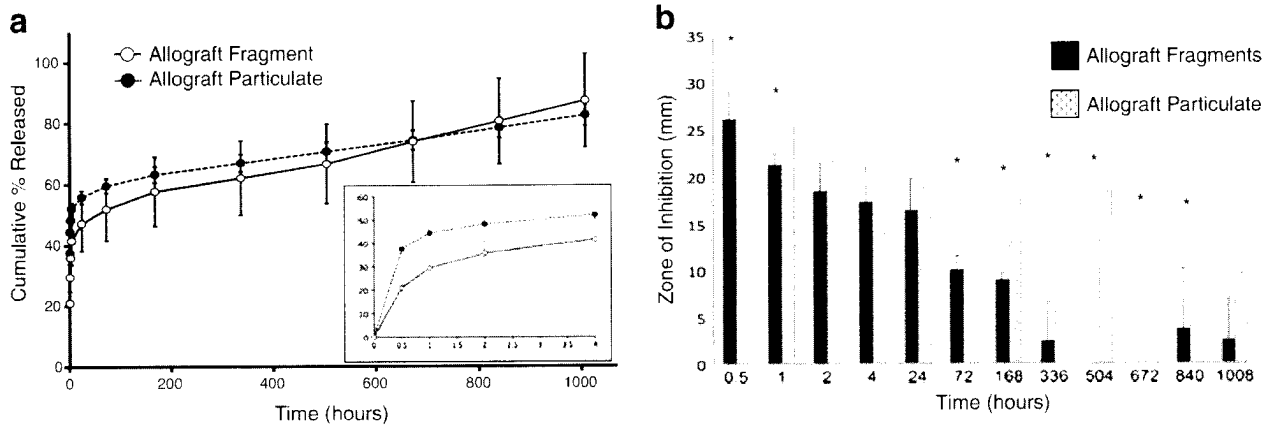

Fig. 2 Comparison of tobramycin released from allograft crouton fragments (cohort 1) and micron-sized allograft particulate matter (cohort 3). **a** Kinetics of drug release and **b** zone of inhibition in vitro against *E. coli* cultures. Note that the *inset* in **a** shows elution kinetics up to 4 h for clarity

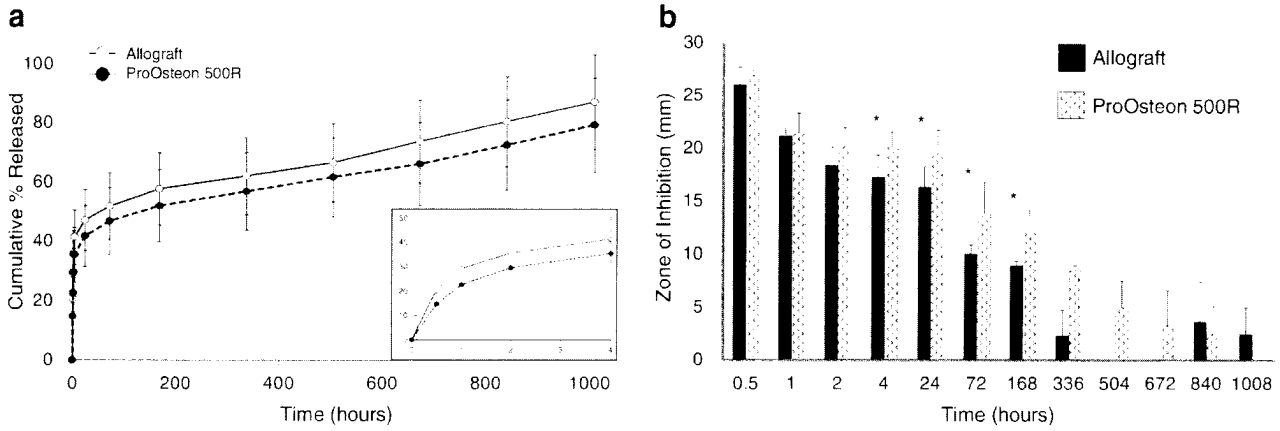

Fig. 3 Comparison of drug released from coated allograft (cohort 1) versus synthetic ProOsteon 500R® (cohort 2) substrates. **a** Kinetics of drug release and **b** zone of inhibition in vitro against *E. coli* cultures. Note that the *inset* in **a** shows elution kinetics up to 4 h for clarity

release kinetics and revealed differences among the cohorts (e.g., Figs. 2a, 3a, 4a, 5a, and 6a) as detailed below.

Impact of bone graft carrier on drug release kinetics

Drug loading and coating consistency relies on the polymer formulation, application technique, and the nature of the underlying bone graft substrate as indicated previously by scanning electron microscopy [66, 70]. Importantly, although the burst release initially appeared to not extend beyond the first week, the small amount of drug eluted at each time point still exceeded the MIC (see insets in figures). To investigate the influence of allograft substrate form (crouton or microsize particulate), cancellous allograft fragments were weighed and coated with PCL (60 mg/ml in acetone) containing tobramycin (10 % *w/w*) via dip coating with vacuum drying (cohort 1). Approximately 20 mg of tobramycin-containing polymer coating was added to each fragment. Alternatively, 100 mg of micron-size particulate was weighed and coated with the same PCL tobramycin formulation via solvent evaporation in individual aluminum trays (cohort 3). Allograft particulate-containing, tobramycin-releasing PCL films were remorselized prior to release into PBS. Micron-size allograft particulate displayed a similar initial burst release when compared to drug-releasing, polymer-coated cancellous allograft fragments, but a slightly lower cumulative percent released (Fig. 2a). Many drug formulations suffer from an early burst release of their drug payload, which, although necessary, may result in local tissue toxicity; however, previous reports using the formulation described here demonstrate not only the versatility of the system in controlling drug release kinetics, but also the ability to attenuate early burst release with an unloaded polymer overcoat, although this technique was not employed in this study [55]. The antibiotic burst from coated allograft particulate may be an unintended consequence of including additional drug-releasing polymer not adhered to the allograft particulate, polymer–drug phase separation, or of the process used to remorselize the particulate after coating. Thickness of the coating was not assessed in this manuscript but has been previously assessed [70]. To minimize the impact of the remorselization

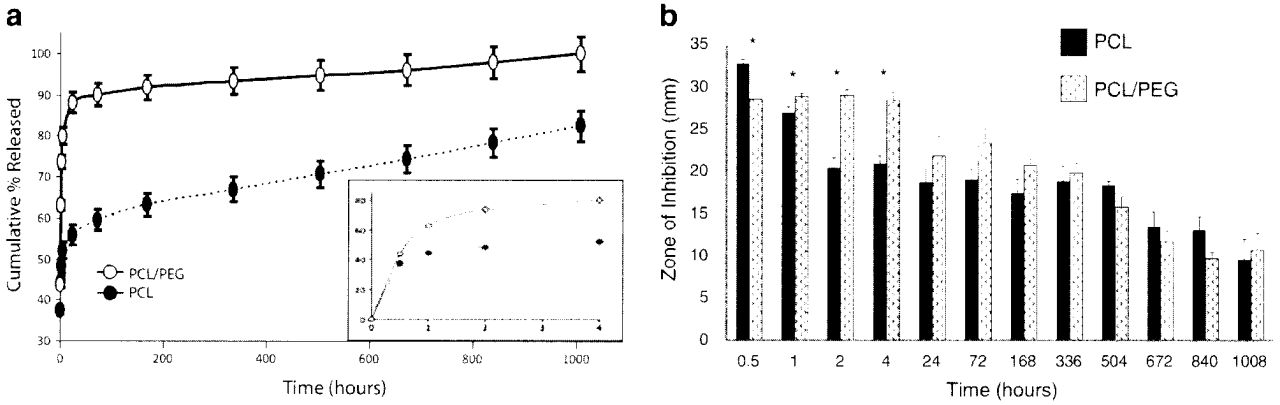

Fig. 4 Comparison of tobramycin release from cohort 3 (PCL) and cohort 4 (PCL_P/PEG), where *F*=free tobramycin. **a** Kinetics of drug release determined via a 96-well fluorescent assay and **b** zone of inhibition in vitro against *E. coli* cultures. Note that the *inset* in **a** shows elution kinetics up to 4 h for clarity

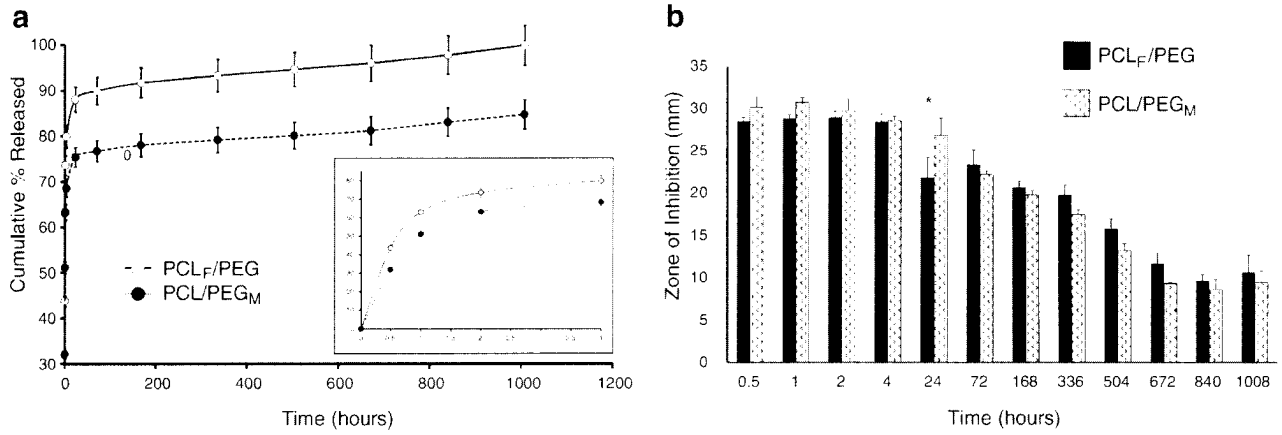

Fig. 5 Comparison of release of free (PCL_F/PEG—cohort 4) versus microencapsulated (PCL/PEG_M—cohort 5) tobramycin from coated allograft. **a** Kinetics of drug release and **b** zone of inhibition in vitro against *E. coli* cultures. Only approximately 80 % of the tobramycin was released within the 6-week time course; thus, the effective therapeutic release of microencapsulated tobramycin may be extended beyond 6 weeks. Note that the *inset* in **a** shows elution kinetics up to 4 h for clarity

process, coated micron-sized allograft particulate was not ground, but instead crushed with a weighing spatula to minimize coating damage. Differences may also be attributed to discrepancies in specific surface areas of the different allograft materials with their different porosities. Cancellous allograft fragments are highly porous, but the porosity is not consistent from fragment to fragment, leading to differences not only in drug load but also in coating integrity, culminating in larger standard deviations that preclude identification of significant differences in tobramycin release kinetics arising from allograft fragments or micron size particulate, particularly at later experimental time points. Allograft particulate also has an extremely high surface area available for coating whereas the internal porous structure of larger allograft bone may not be accessible to the coating.

The lower standard deviations associated with coated allograft particulate and increased longevity of bioactivity led to its use in all subsequent experiments with the notable exception of the synthetic bone graft filler, ProOsteon 500R® particulate, which were not micron-sized and had a porous structure reported similar to cancellous bone (pore size, ~280 to 770 μm with 55 % porosity [71, 72]; cancellous allograft pore size reported to average between 400 and 500 μm with porosity ranging between 60 and 77 % [73]). Tobramycin release for ProOsteon 500R® fragments dip-coated and vacuum dried analogously to the allograft fragments (cohort 2) did not differ significantly in its kinetics compared to coated allograft crouton fragments (Fig. 3a), demonstrating the versatility of the drug-releasing coating system for graft materials with similar microstructural features.

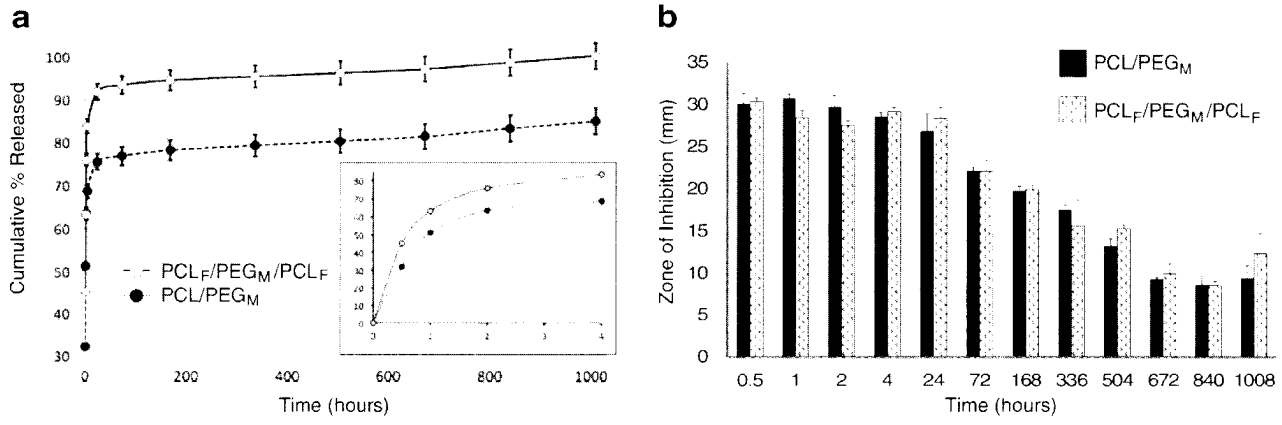

Fig. 6 Comparison of PCL coating application techniques on tobramycin release from allograft particulate. While a slight difference in release kinetics (**a**) for solvent cast (PCL/PEG_M—cohort 5) versus layer-by-layer solvent cast ($PCL_F/PEG_M/PCL_F$—cohort 6) are observed, there are no statistical differences in the resulting antimicrobial activity (**b**) as both formulations provide antimicrobial activity against *E. coli* in vitro out to 6 weeks. Note that the *inset* in **a** shows elution kinetics up to 4 h for clarity

Impact of coating formulation on tobramycin release kinetics

In solution-based drug formulating and polymer vehicle coating, component compatibility issues in drug–solvent–polymer solubility, mutual miscibility, and controlled solution stability are important design criteria [74]. Known thermodynamic predictors of these properties (i.e., matching appropriate Hildebrand solubility parameters of the drug, polymer, and solvent(s), such as with PCL (δ=20.2) [75] in acetone and tobramycin) were used to attempt to avoid compatibility issues and phase separation [74, 76]. Unfortunately, tobramycin is highly soluble in water and marginally soluble in alcohols while PCL is insoluble in water, highly soluble in chloroform, and soluble in acetone at elevated temperature. The dissimilarity in component solubility (i.e., powdered tobramycin added to acetone-solvated PCL forms a suspension) and resulting solution heterogeneity and possible phase separation as a drug delivery system impact drug release kinetics. In an attempt to limit tobramycin burst release in the first 24 h, attributed to phase separation between PCL and tobramycin, some coating formulations were modified to include a 45 % *w/v* aqueous polyethylene glycol (PEG δ=22.9 [74]; 20 kDa) feed solution (cohort 4), to help retain tobramycin in the aqueous phase and to create a more homogeneous polymer–drug coating formulation (Fig. 4). Interactions of PCL and PEG are well-studied, indicating that upon molten mixing, the structure of the blend is dictated by a balance between liquid-liquid phase separation and polymer blend crystallization [77–79]. However, in solution, miscibility can be hampered by mismatched component Hildebrand values (dispersion, polar, and hydrogen bonding) [74–76, 76, 80]; furthermore, the solvents for each polymer are different (PCL–acetone and PEG–water). Additionally, inclusion of PEG in the blended polymer formulation may have approached the percolation threshold; however, this was not considered significant since lower PEG amounts (ESM Fig. 1) had a similar effect on tobramycin release; thus, 45 % was chosen for all formulations to maximize the poragen effect of PEG on the slowly degrading PCL as well as its tobramycin-carrying capacity. Interestingly, this formulation modified tobramycin release kinetics, increasing both amounts of tobramycin released in the first 24 h (burst), as well as over the entire time course. Nevertheless, the duration of bacterial inhibitory activity of released antibiotic shows insignificant differences between the two formulations (Fig. 4b). Additionally, Fig. 4a shows that dramatic alterations in the coating solvent system improved the antibiotic solubility in the polymer formulation to allow greater than 95 % of the drug to be recovered over a 6-week release time. Previous reports have indicated that PEG inclusion in a PCL polymer formulation accelerates drug release [81]. Here, inclusion of PEG in the formulation was also used to improve drug miscibility and increase the amount of drug release from PCL using polymer blend crystallization [82]. This provides an important performance distinction over other commercially available antibiotic-releasing implantable bone delivery systems such as polymethylmethacrylate (PMMA) cements that suffer early burst releases followed by low level, and incomplete antibiotic leaching inadequate to provide longer-term efficacious antimicrobial protection [19, 42, 51]. Importantly, unlike native or resorbable synthetic bone grafts capable of both osteoclast remodeling and osteoinductivity to form osteocytes, PMMA acts as a permanent foreign body, providing a substrate for bacterial adhesion and biofilm development after antibiotic release exhaustion, promoting both secondary infections, and resistant bacteria [42, 51, 83].

Drug release kinetics were further modified using commercially oil-microencapsulated tobramycin to further slow and extend drug release by decreasing the drug burst in the first 24 h. Microencapsulated tobramycin in 45 % *w/v* aqueous PEG feed solution was mixed into a PCL–acetone–tobramycin solution prior to coating (cohort 5). The theoretical amount of tobramycin in the final formulation was equivalent to that formulated with other 45 % *w/v* aqueous PEG solutions mixed into PCL–acetone (1:1; i.e., cohort 4). Drug release kinetics from the two formulations mirror each other, with those produced from the microencapsulated formulation (cohort 5) being delayed, releasing approximately 10 % less tobramycin compared to free tobramycin (cohort 4) (Fig. 5a). Other studies (data not shown) demonstrate a minimal attenuation of the burst release (~3 %) when microencapsulated tobramycin was mixed into a PCL-only formulation.

Impact of the coating application technique on tobramycin release kinetics

Since addition of microencapsulated tobramycin retarded its release kinetics, the influence of the coating application technique was also investigated (Fig. 6). Microencapsulated tobramycin was suspended in a 45 % aqueous PEG solution while free tobramycin was suspended in the PCL–acetone solution. These two suspensions were treated as independent formulations (A and B, respectively) and applied to allograft particulate material as layers, allowing each layer to dry at ambient temperatures overnight and remorselizing the solid film prior to applying the next layer. The entire multilayer drug-releasing polymer film encasing the allograft particulate was morselized and suspended in PBS as a release medium. Notably, solvent removal was deemed to be complete once the weight of the encapsulated allograft bone filler was stable. Based on the immiscibility of formulations A and B, their alternate layering should not have dissolved each previous underlying layer. Interestingly, release kinetics from this layer-by-layer coating (cohort 6) and its analogous composition directly mixed but unlayered formulation (cohort 5) are

all dominated by the free tobramycin fraction (Fig. 6a) compared to the PCL/PEG unencapsulated formulation (cohort 4; Fig. 5a), with very little differences in release kinetics propagated over the 6-week time course beyond that initially seen in the first 30 min burst.

Antimicrobial bioactivity

Regardless of coating formulation, antimicrobial activity was confirmed via in vitro bacteriostatic assays based on modification of standard techniques for determining antibiotic MIC (data not shown), as well as classic ZOI or radial diffusion assays (see Figs. 2b, 3b, 4b, 5b, and 6b). Importantly, differences in the (tobramycin) MIC for *E. coli* (used in this study) and *S. aureus* (the most prevalent and relevant pathogenic species in osteomyelitis) are reported to be insignificant [84]. This was experimentally verified within the sensitivity of the assay (*E. coli* in LB=6.25 μg/ml, *S. aureus* in LB= 3.125 μg/ml; data not shown); thus, the more convenient, biosafety level 1 species, *E. coli*, was used for all bacteriostatic assays reported. Maintenance of local antibiotic concentration exceeding the MIC is critical over the course of both acute and chronic therapy to rapidly kill viable pathogens and prevent selection of resistant bacteria, particularly in compromised, infection-susceptible bone with limited perfusion and antibiotic penetration. Notably, to exceed the MIC for the conditions tested, more than 0.01 % of the drug must be released into liquid at each time point. This condition was met for all cohorts. Disparate sensitivity of the ZOI assays and the fluorescent tobramycin detection assay are evident. This difference in sensitivity means that even if zero-order release kinetics were observed via a kinetic assay, a zone of inhibition may still be detected as the fractional release exceeded the MIC. It is important to note that MIC assays were performed in an aqueous environment; whereas, ZOI assays are more representative of diffusion in tissue. Furthermore, the kinetic assay and ZOI assay address different questions. Thus, direct comparisons of ZOI-based antimicrobial activity with drug release kinetics are obscured, and only general trends can be compared. All coating cohorts investigated provided antimicrobial activity throughout the clinically relevant duration of the assay (6 weeks), although the crouton allograft fragment-based cohorts transiently failed to exhibit antimicrobial activity in the middle of this period as indicated by the absence of a clear ZOI (Figs. 2 and 3b).

Tobramycin release was affected by the allograft material morphology (larger porous crouton fragments or micron-sized porous particulate, Fig. 2b), showing significantly greater antimicrobial activity from coated microsized porous particulate. Although the precise amount of tobramycin released cannot be linearly related to the diameter of the ZOI studies do reveal significant differences at several time points (0.5, 1, and 72–840 h). The cancellous allograft fragment cohort displayed larger standard deviations, particularly at the later time points where only one or two samples of the cohort were still exhibiting antimicrobial activity, falling short of the desired therapeutic window (Fig. 2b). Despite being weight-matched and normalized, large standard deviations also plagued direct comparison of allograft fragments with corraline HAP synthetic fragments, with significant differences identified only between 4 h and 1 week (Fig. 3b). Conversely, consistent antimicrobial activity out to 6 weeks (1,008 h) was observed for coated allograft particulate substrates (Fig. 2b), indicating that sufficient drug load may still remain for microbial killing even past the 6-week experimental time frame, although it is important to note that there is often a disconnect between in vitro and in vivo results, and the results reported here remain to be recapitulated in vivo. This unprecedented duration may be indirectly attributed to the significant differences in coating application technique (dip-coating versus solvent casting on the two allograft materials) dependent on the substrate physical form. Nevertheless, micron-sized allograft particulate materials may provide more efficient packing into avascular dead spaces often prevalent in injured and surgically repaired bone defects with higher graft packing density to provide enhanced duration of antibacterial efficacy in vivo when compared to larger coated allograft or synthetic bone graft fragments [85].

In addition to drug release kinetic dependence on the morphology of the graft material, release rates can be altered via the polymer formulation. Disparate release kinetics measured with addition of PEG aqueous solutions (cohort 4) to the PCL–acetone base polymer formulation (Fig. 4a) should affect antimicrobial activity. However, ZOI measurements demonstrated significant differences only between 30 min and 4 h, despite relatively small standard deviations (Fig. 4b). Both formulations released tobramycin amounts sufficient to produce ZOIs throughout the entire 6-week time course. Inclusion of microencapsulated tobramycin in the coating formulation did not produce significant differences in amounts of active drug released (Fig. 5b), despite slightly slowing antibiotic release (Fig. 5a). Moreover, differences in tobramycin release kinetics from analogous formulation cohorts (i.e., cohorts 5 and 6) prepared using different application techniques were dominated by the outermost PCL layer containing free tobramycin, as indicated by a lack of significant differences (compare Figs. 5 and 6a). As such, antimicrobial activity was predicted to also be very similar (Fig. 6b) and was robust for the 6-week duration, with no statistical differences between the ZOI obtained from cohorts 5 and 6, virtually indistinguishable from that obtained from cohort 4. This may be an indication of coating inconsistencies that may be masking differences between formulations; nevertheless, maintenance of tobramycin drug concentrations throughout the 6-week experimental duration is distinct from other FDA-approved available polymer-

controlled, locally antibiotic-releasing orthopedic void-filling implants. Most of these (i.e., PMMA) not only quickly become pharmaceutically silent due to inadequate drug release kinetics but also act as a foreign bodies, facilitating infection, and ultimately promoting antibiotic resistance [42, 83].

Conclusions

The resorbable polymer-controlled, antibiotic-releasing bone graft system described here successfully delivers tobramycin antibiotic in vitro over 6 weeks, a distinguishing trait from current antibiotic releasing technologies for bone. Furthermore, the broad implications of polymer-mediated control over local drug release kinetics with some degree of versatility presents an attractive alternative technique for improved local delivery of different classes of bioactive molecules from tissue implants, particularly in diffusion-limited tissue such as bone. A facile, convenient drug fluorescence assay was used to evaluate drug release kinetics from a variety of tobramycin-loaded PCL-coated bone graft fillers. ZOI assays confirmed the antimicrobial activity of tobramycin after coating formulation and release, with inhibitory activity out to 6 weeks, independent of the underlying graft substrate or coating method (Fig. 3). Importantly, elution profiles, MIC, and ZOI assays cannot be directly compared due to different assay sensitivities and diffusion medias.

Regardless of the polymer barrier matrix controlling drug release from the formulation, micron-sized allograft bone particulate provided the most desirable bioactivity profile of tobramcyin. Due to its small size and high porosity, micronized allograft may also provide a more efficacious wound packing material to prevent formation of inadvertent avascular dead spaces, as opposed to larger porous fragments (Fig. 2). In fact, several allograft particulate-based cohort formulations yielded distinct ZOIs throughout the 6-week study duration, indicating a potentially longer window of therapeutic drug release mediated by polymer degradation, as opposed to drug leaching from coating defects and barrier inconsistencies. Surprisingly, incorporation of oil-microencapsulated tobramycin did not significantly alter the rate of tobramycin release or the resulting drug antimicrobial activity, and exhibited only a limited capacity to slow the amount of tobramycin released over the 6-week study duration (Fig. 5); however, use of an unloaded polymer overcoat did slow drug burst release in previous studies [55]. Elution kinetic assays and bioactivity assays are designed to answer different questions; thus, differences between drug release and drug bioactivities over time courses may not only be attributed to the different sensitivities of the respective assays used but also to the cumulative nature of the elution kinetics and the snapshot approach of the ZOI technique. Nonetheless, this alteration in tobramycin release kinetics provides some indication of the versatile extended drug release modulation possible with this coated bone graft resorbable implant system. Blending PEG with PCL in barrier coatings may modify coating morphology and drug compatibility to increase amounts of drug in the coating, its dissolution and subsequent rates of tobramycin release from the allograft bone.

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References

1. Conterno LO, da Silva Filho CR. Antibiotics for treating chronic osteomyelitis in adults. Cochrane Database Syst Rev. 2009;3, CD004439.
2. Landersdorfer CB, Bulitta JB, Kinzig M, Holzgrabe U, Sorgel F. Penetration of antibacterials into bone: pharmacokinetic, pharmacodynamic and bioanalytical considerations. Clin Pharmacokinet. 2009;48(2):89–124.
3. Bernthal NM, Stavrakis AI, Billi F, Cho JS, Kremen TJ, Simon SI, et al. A mouse model of post-arthroplasty *Staphylococcus aureus* joint infection to evaluate in vivo the efficacy of antimicrobial implant coatings. PLoS One. 2010;5(9):e12580.
4. Merrill C, Elixhauser A. Hospital stays involving musculoskeletal procedures, 1997–2005. HCUP Statistical Brief #34; 2007.
5. Jiranek WA, Hanssen AD, Greenwald AS. Antibiotic-loaded bone cement for infection prophylaxis in total joint replacement. J Bone Joint Surg Am. 2006;88(11):2487–500.
6. Kalorama Information Market Intelligence Report. Orthopedic biomaterials: World Market, September 2007.
7. Vasilev K, Cook J, Griesser HJ. Antibacterial surfaces for biomedical devices. Expert Rev Med Devices. 2009;6(5):553–67.
8. Reichert JC, Saifzadeh S, Wullschleger ME, Epari DR, Schutz MA, Duda GN, et al. The challenge of establishing preclinical models for segmental bone defect research. Biomaterials. 2009;30(12):2149–63.
9. Oest ME, Dupont KM, Kong HJ, Mooney DJ, Guldberg RE. Quantitative assessment of scaffold and growth factor-mediated repair of critically sized bone defects. J Orthop Res. 2007;25(7):941–50.
10. Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: an update. Injury. 2005;36 Suppl 3:S20–7.
11. Bostrom MP, Seigerman DA. The clinical use of allografts, demineralized bone matrices, synthetic bone graft substitutes and osteoinductive growth factors: a survey study. HSS J. 2005;1(1):9–18.
12. Parikh SN. Bone graft substitutes: past, present, future. J Postgrad Med. 2002;48(2):142–8.
13. McLaren AC. Alternative materials to acrylic bone cement for delivery of depot antibiotics in orthopaedic infections. Clin Orthop Relat Res. 2004;427:101–6.
14. Costerton J. The etiology and persistence of cryptic bacterial infection: a hypothesis. Rev Infect Dis. 1984;6:608–16.
15. Nandi SK, Roy S, Mukherjee P, Kundu B, De DK, Basu D. Orthopaedic applications of bone graft & graft substitutes: a review. Indian J Med Res. 2010;132:15–30.

16. McKee MD, Wild LM, Schemitsch EH, Waddell JP. The use of an antibiotic-impregnated, osteoconductive, bioabsorbable bone substitute in the treatment of infected long bone defects: early results of a prospective trial. J Orthop Trauma. 2002;16(9):622–7.
17. Koort JK, Makinen TJ, Suokas E, Veiranto M, Jalava J, Tormala P, et al. Sustained release of ciprofloxacin from an osteoconductive poly(DL)-lactide implant. Acta Orthop. 2008;79(2):295–301.
18. Koort JK, Suokas E, Veiranto M, Makinen TJ, Jalava J, Tormala P, et al. In vitro and in vivo testing of bioabsorbable antibiotic containing bone filler for osteomyelitis treatment. J Biomed Mater Res A. 2006;78(3):532–40.
19. Kanellakopoulou K, Giamarellos-Bourboulis EJ. Carrier systems for the local delivery of antibiotics in bone infections. Drugs. 2000;59(6):1223–32.
20. Winkler H, Stoiber A, Kaudela K, Winter F, Menschik F. One stage uncemented revision of infected total hip replacement using cancellous allograft bone impregnated with antibiotics. J Bone Joint Surg Br. 2008;90(12):1580–4.
21. Beaman FD, Bancroft LW, Peterson JJ, Kransdorf MJ, Menke DM, DeOrio JK. Imaging characteristics of bone graft materials. Radiographics. 2006;26(2):373–88.
22. Borkhuu B, Borowski A, Shah SA, Littleton AG, Dabney KW, Miller F. Antibiotic-loaded allograft decreases the rate of acute deep wound infection after spinal fusion in cerebral palsy. Spine (Phila Pa 1976). 2008;33(21):2300–4.
23. Buttaro M, Comba F, Piccaluga F. Vancomycin-supplemented cancellous bone allografts in hip revision surgery. Clin Orthop Relat Res. 2007;461:74–80.
24. Buttaro MA, Gonzalez Della Valle AM, Pineiro L, Mocetti E, Morandi AA, Piccaluga F. Incorporation of vancomycin-supplemented bone incorporation of vancomycin-supplemented bone allografts: radiographical, histopathological and immunohistochemical study in pigs. Acta Orthop Scand. 2003;74(5):505–13.
25. Buttaro MA, Morandi A, Rivello HG, Piccaluga F. Histology of vancomycin-supplemented impacted bone allografts in revision total hip arthroplasty. J Bone Joint Surg Br. 2005;87(12):1684–7.
26. Day RE, Megson S, Wood D. Iontophoresis as a means of delivering antibiotics into allograft bone. J Bone Joint Surg Br. 2005;87(11):1568–74.
27. Finley JM. Antibiotic supplementation of bone allografts: a review. J West Soc Periodontol Periodontal Abstr. 2001;49(1):5–9.
28. Harris RJ. A clinical evaluation of an allograft combined with a bioabsorbable membrane versus an alloplast/allograft composite graft combined with a bioabsorbable membrane. 100 consecutively treated cases. J Periodontol. 1998;69(5):536–46.
29. Kanellakopoulou K, Sahinides T, Tsaganos T, Galanakis N, Giamarellou H, Giamarellos-Bourboulis EJ. In vitro release of fusidic acid and teicoplanin from cancellous bone allografts. J Chemother. 2008;20(5):645–7.
30. Petri 3rd WH, Wilson TM. Clinical evaluation of antibiotic-supplemented bone allograft. J Oral Maxillofac Surg. 1993;51(9):982–5. discussion 6.
31. Whiteside LA. Treatment of infected total knee arthroplasty. Clin Orthop Relat Res. 1994;299:169–72.
32. Winkler H, Kaudela K, Stoiber A, Menschik F. Bone grafts impregnated with antibiotics as a tool for treating infected implants in orthopedic surgery—one stage revision results. Cell Tissue Bank. 2006;7(4):319–23.
33. Witso E, Persen L, Benum P, Bergh K. Cortical allograft as a vehicle for antibiotic delivery. Acta Orthop. 2005;76(4):481–6.
34. Witso E, Persen L, Loseth K, Bergh K. Adsorption and release of antibiotics from morselized cancellous bone. In vitro studies of 8 antibiotics. Acta Orthop Scand. 1999;70(3):298–304.
35. Ketonis C, Adams CS, Barr S, Aiyer A, Shapiro IM, Parvizi J, et al. Antibiotic modification of native grafts: improving upon nature's scaffolds. Tissue Eng Part A. 2010;16(6):2041–9.
36. Ketonis C, Barr S, Adams CS, Hickok NJ, Parvizi J. Bacterial colonization of bone allografts: establishment and effects of antibiotics. Clin Orthop Relat Res. 2010;468(8):2113–21.
37. Ketonis C, Barr S, Adams CS, Shapiro IM, Parvizi J, Hickok NJ. Vancomycin bonded to bone grafts prevents bacterial colonization. Antimicrob Agents Chemother. 2011;55(2):487–94.
38. Ketonis C, Barr S, Shapiro IM, Parvizi J, Adams CS, Hickok NJ. Antibacterial activity of bone allografts: comparison of a new vancomycin-tethered allograft with allograft loaded with adsorbed vancomycin. Bone. 2011;48(3):631–8.
39. Mathijssen NM, Petit PL, Pilot P, Schreurs BW, Buma P, Bloem RM. Impregnation of bone chips with antibiotics and storage of antibiotics at different temperatures: an in vitro study. BMC Musculoskelet Disord. 2010;11:96.
40. Witso E, Persen L, Benum P, Bergh K. Release of netilmicin and vancomycin from cancellous bone. Acta Orthop Scand. 2002;73(2):199–205.
41. Witso E, Persen L, Loseth K, Benum P, Bergh K. Cancellous bone as an antibiotic carrier. Acta Orthop Scand. 2000;71(1):80–4.
42. Diefenbeck M, Muckley T, Hofmann GO. Prophylaxis and treatment of implant-related infections by local application of antibiotics. Injury. 2006;37 Suppl 2:S95–S104.
43. Ciampolini J, Harding KG. Pathophysiology of chronic bacterial osteomyelitis. Why do antibiotics fail so often? Postgrad Med J. 2000;76(898):479–83.
44. Levin PD. The effectiveness of various antibiotics in methyl methacrylate. J Bone Joint Surg Br. 1975;57(2):234–7.
45. Miclau T, Dahners LE, Lindsey RW. In vitro pharmacokinetics of antibiotic release from locally implantable materials. J Orthop Res. 1993;11(5):627–32.
46. Chen CE, Ko JY, Pan CC. Results of vancomycin-impregnated cancellous bone grafting for infected tibial nonunion. Arch Orthop Trauma Surg. 2005;125(6):369–75.
47. Communication OotADf. CDC urges hospitals and healthcare facilities to increase efforts to reduce drug-resistant infections. 2006. http://www.cdc.gov/media/pressrel/r061019.htm?s_cid=mediarel_r061019_x. Accessed 23 June 2011.
48. Patzakis MJ, Zalavras CG. Chronic posttraumatic osteomyelitis and infected nonunion of the tibia: current management concepts. J Am Acad Orthop Surg. 2005;13(6):417–27.
49. Lowry KJ, Hamson KR, Bear L, Peng YB, Calaluce R, Evans ML, et al. Polycaprolactone/glass bioabsorbable implant in a rabbit humerus fracture model. J Biomed Mater Res. 1997;36(4):536–41.
50. Coombes AG, Rizzi SC, Williamson M, Barralet JE, Downes S, Wallace WA. Precipitation casting of polycaprolactone for applications in tissue engineering and drug delivery. Biomaterials. 2004;25(2):315–25.
51. Chang HI, Perrie Y, Coombes AG. Delivery of the antibiotic gentamicin sulphate from precipitation cast matrices of polycaprolactone. J Control Release. 2006;110(2):414–21.
52. Lam CX, Savalani MM, Teoh SH, Hutmacher DW. Dynamics of in vitro polymer degradation of polycaprolactone-based scaffolds: accelerated versus simulated physiological conditions. Biomed Mater. 2008;3(3):034108.
53. Hutmacher DW, Schantz JT, Lam CX, Tan KC, Lim TC. State of the art and future directions of scaffold-based bone engineering from a biomaterials perspective. J Tissue Eng Regen Med. 2007;1(4):245–60.
54. Wei X, Gong C, Gou M, Fu S, Guo Q, Shi S, et al. Biodegradable poly(epsilon-caprolactone)-poly(ethylene glycol) copolymers as drug delivery system. Int J Pharm. 2009;381(1):1–18.
55. Sevy JO, Slawson MH, Grainger DW, Brooks AE. Assay method for polymer-controlled antibiotic release from allograft bone to target orthopaedic infections. Biomed Sci Instrum. 2010;46:136–41.
56. Darley ES, MacGowan AP. Antibiotic treatment of gram-positive bone and joint infections. J Antimicrob Chemother. 2004;53(6):928–35.

57. Rhyu KH, Jung MH, Yoo JJ, Lee MC, Seong SC, Kim HJ. In vitro release of vancomycin from vancomycin-loaded blood coated demineralised bone. Int Orthop. 2003;27(1):53–5.
58. Winkler H, Janata O, Berger C, Wein W, Georgopoulos A. In vitro release of vancomycin and tobramycin from impregnated human and bovine bone grafts. J Antimicrob Chemother. 2000;46(3):423–8.
59. Witso E, Persen L, Benum P, Aamodt A, Husby OS, Bergh K. High local concentrations without systemic adverse effects after impaction of netilmicin-impregnated bone. Acta Orthop Scand. 2004;75(3):339–46.
60. Lindsey RW, Probe R, Miclau T, Alexander JW, Perren SM. The effects of antibiotic-impregnated autogeneic cancellous bone graft on bone healing. Clin Orthop Relat Res. 1993;291:303–12.
61. Wu P, Grainger DW. Drug/device combinations for local drug therapies and infection prophylaxis. Biomaterials. 2006;27(11):2450–67.
62. Dunnett C, Goldsmith C. When and how to do multiple comparisons. In: Buncher CR, Tsay J-Y, editors. Statistics in the pharmaceutical industry. 3rd ed. New York: Chapman and Hall; 2006. p. 421–52.
63. Begg EJ, Barclay ML. Aminoglycosides—50 years on. Br J Clin Pharmacol. 1995;39(6):597–603.
64. Gogia JS, Meehan JP, Di Cesare PE, Jamali AA. Local antibiotic therapy in osteomyelitis. Semin Plast Surg. 2009;23(2):100–7.
65. Antoci Jr V, Adams CS, Hickok NJ, Shapiro IM, Parvizi J. Antibiotics for local delivery systems cause skeletal cell toxicity in vitro. Clin Orthop Relat Res. 2007;462:200–6.
66. Davidoff SN, Call BP, Hogrebe PC, Grainger DW, Brooks AE. A robust method to coat allograft bone with a drug-releasing polymer shell. Biomed Sci Instrum. 2010;46:184–9.
67. Koort JK, Makinen TJ, Suokas E, Veiranto M, Jalava J, Knuuti J, et al. Efficacy of ciprofloxacin-releasing bioabsorbable osteoconductive bone defect filler for treatment of experimental osteomyelitis due to *Staphylococcus aureus*. Antimicrob Agents Chemother. 2005;49(4):1502–8.
68. Mousset B, Benoit MA, Delloye C, Bouillet R, Gillard J. Biodegradable implants for potential use in bone infection. An in vitro study of antibiotic-loaded calcium sulphate. Int Orthop. 1995;19(3):157–61.
69. Brandl M, Gu L. Degradation of tobramycin in aqueous solution. Drug Development and Industrial Pharmacy. 1992;18(13):1423–36.
70. Davidoff SN, Sevy JO, Brooks BD, Grainger DW, Brooks AE. Evaluating antibiotic release profiles as a function of polymer coating formulation. Biomed Sci Instrum. 2011;47:46–51.
71. Shors EC. http://alfa-med.net/Pdfs/urun45.pdf.
72. Orthovita. The packing of vitoss synthetic cancellous bone void filler into bone defects 2000. http://www.ossano.se/Bibliotek/Vitoss.5606-0057(EU).pdf. Accessed 10 Nov 11.
73. Bloebaum RD, Bachus KN, Mitchell W, Hoffman G, Hofmann AA. Analysis of the bone surface area in resected tibia. Implications in tibial component subsidence and fixation. Clin Orthop Relat Res. 1994;309:2–10.
74. Liu J, Xiao Y, Allen C. Polymer–drug compatibility: a guide to the development of delivery systems for the anticancer agent, ellipticine. J Pharm Sci. 2004;93(1):132–43.
75. Bordes C, Freville V, Ruffin E, Marote P, Gauvrit JY, Briancon S, et al. Determination of poly(epsilon-caprolactone) solubility parameters: application to solvent substitution in a microencapsulation process. Int J Pharm. 2010;383(1–2):236–43.
76. Huang J, Lin K, Deanin R. Three-dimensional solubility parameters of poly(e-caprolactone). J Appl Polym Sci. 2006;100(3):2002–9.
77. Chuang W, Jeng U, Sheu H, Hong P. Competition between phase separation and crystallization in a PCL/PEG polymer blend captured by synchronized SAXS, WAXS, and DSC. Macromol Res. 2006;14(1):45–51.
78. Chuang W, Shih K, Hong P. Kinetics of phase separation in poly(ε-caprolactone)/poly(ethylene glycol) blends. Journal of Polymer Research. 2005;12(3).
79. Liu KL, Widjaja E, Huang Y, Ng XW, Loo SCJ, Boey FYC, et al. A new insight for an old system: protein-PEG colocalization in relation to protein release from PCL/PEG blends. Molecular Pharmaceutics. 2011;8(6):2173–82.
80. Wu PL, Beerbower A, Martin A. Extended Hansen approach: calculating partial solubility parameters of solid solutes. J Pharm Sci. 1982;71(11):1285–7.
81. Wan Y, Wu H, Yu A, Wen D. Biodegradable polylactide/chitosan blend membranes. Biomacromolecules. 2006;7(4):1362–72.
82. Bramfeldt H, Sarazin P, Vermette P. Blends as a strategy towards tailored hydrolytic degradation of poly(ε-caprolactone-co-D, L-lactide)–poly(ethylene glycol)–poly(ε-caprolactone-co-D, L-lactide) co-polymers. Polym Degrad Stab. 2008;93(4):877–82.
83. Neut D, van de Belt H, Stokroos I, van Horn JR, van der Mei HC, Busscher HJ. Biomaterial-associated infection of gentamicin-loaded PMMA beads in orthopaedic revision surgery. J Antimicrob Chemother. 2001;47(6):885–91.
84. Levison ME, Knight R, Kaye D. In vitro evaluation of tobramycin, a new aminoglycoside antibiotic. Antimicrob Agents Chemother. 1972;1(5):381–4.
85. Malinin TI, Carpenter EM, Temple HT. Particulate bone allograft incorporation in regeneration of osseous defects; importance of particle sizes. Open Orthop J. 2007;1:19–24.