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Sustained release of vancomycin from polyurethane scaffolds inhibits infection of bone wounds in a rat femoral segmental defect model

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ABSTRACT

Infection is a common complication in open fractures that compromises the healing of bone and can result in loss of limb or life. Currently, the clinical standard of care for treating contaminated open fractures comprises a staged approach, wherein the wound is first treated with non biodegradable antibiotic laden poly(methyl methacrylate) (PMMA) beads to control the infection followed by bone grafting. Considering that tissue regeneration is associated with new blood vessel formation, which takes up to 6 weeks in segmental defects, a biodegradable bone graft with sustained release of an antibiotic is desired to prevent the implant from becoming infected, thus allowing the processes of both vascularization and new bone formation to occur unimpeded. In the present study, we utilized biodegradable porous polyurethane (PUR) scaffolds as the delivery vehicle for vancomycin. Hydrophobic vancomycin free base (V FB) was obtained by precipitating the hydrophilic vancomycin hydrochloride (V HCl) at pH 8. The decreased solubility of V FB resulted in an extended vancomycin release profile *in vitro*, as evidenced by the fact that active vancomycin was released for up to 8 weeks at concentrations well above both the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). Using PUR prepared from lysine triisocyanate (LTI) (PUR(LTI)), the extended *in vitro* release profile observed for V FB translated to improved infection control *in vivo* compared to V HCl in a contaminated critical sized fat femoral segmental defect. The performance of PUR (LTI)/V FB was comparable to PMMA/V HCl beads *in vivo*. However, compared with PMMA, PUR is a biodegradable system which does not require the extra surgical removal step in clinical use. These results suggest that PUR scaffolds incorporating V FB could be a potential clinical therapy for treatment of infected bone defects.

1. Introduction

Infection is a significant factor that compromises the healing of bone fractures [1,2]. A recent retrospective study found that all open type III fractures among combat casualties were infected. The presence of infection had a dramatic effect on patient outcomes, as evidenced by 37% of the patients experiencing delayed union with 14% resulting in amputation [3]. Currently, the clinical standard of care for treating contaminated open fractures comprises two steps, wherein the wound is first treated with antibiotics followed by bone grafting. In practice, PMMA beads have been used as the local antibiotic delivery platform, and have been shown to decrease infections in clinical studies [4 6]. Compared with systemic parenteral treatment, local antibiotics can achieve substantially higher concentrations at the contaminated site, resulting in more effective treatment of the infection, as well as reduced systemic toxicity concerns [7 9]. In a prospective study of patients with infected non unions, treatment with gentamycin impregnated PMMA beads resulted in similar rates of infection control and healing of non unions compared to long term systemic parenteral delivery of genta mycin [10]. Furthermore, a clinical study comparing the efficacy of local and parenteral delivery of antibiotics in patients with infected total hip and knee arthroplasty found that 30% of patients treated with systemic parental gentamycin exhibited recurring infection compared to 15% of patients treated with PMMA gentamycin beads, although the difference was not statistically significant [8]. However, since PMMA does not biodegrade, it must be removed during an additional surgical step and cannot be allowed to remain in the wound bed during definitive closure. Furthermore, the antibiotic release efficiency is typically low (e.g., <25% cumulative release), the burst release is high (>60% of the released antibiotic is within the first day), and the antibiotics are below therapeutic levels within a week or two [11 14]. Resorbable calcium phosphates and sulfates have also been investigated for local antibiotic delivery. Calcium sulfate pellets impregnated with antibiotic (Osteoset T, Wright Medical) have been shown to be effective in treating osteomyelitis in animals and humans [15 17]. These biomaterials are

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biodegradable and osteoconductive, but calcium sulfate has been associated with seromas and drainage problems [18]. Additionally, the release of antibiotic is very fast, occurring within the first 2 3 days and calcium sulfate dissolves quickly; therefore, it does not possess favorable osteoconductive properties.

While treatment with antibiotic laden PMMA beads or calcium sulfate pellets effectively controls infection in the wound bed prior to bone grafting, there is a need for a bone graft material that not only enhances bone healing, but also releases an antibiotic that protects the wound from infection during the healing process. Although generally effective at promoting bone growth, the bone graft is essentially a foreign material placed into an avascular and often contaminated defect and could thus possibly function as a nidus for infection. Additionally, blood vessel formation is a prerequisite for bone healing [19,20], and angiogenesis is known to be essential for bone regeneration in segmental defects [21,22]. It has been shown that vascularization of the fracture callus in sheep progressed during the first three weeks of healing [20], and another study has suggested that complete vascularization of implanted scaffolds may require up to six weeks [22]. Therefore, in contaminated bone wounds, it is desirable to maintain a therapeutic level of antibiotic for at least 3 6 weeks to control the infection until substantial vascularization occurs in the wound bed.

Biodegradable porous PUR scaffolds have been investigated as supportive scaffolds for cellular infiltration and new bone formation [23 25]. Polyurethanes have also been investigated as drug delivery systems. Controlled release of antibiotics has been investigated to develop infection resistant biomedical PUR implants [26 29], and delivery of biologically active growth factors from PUR scaffolds has been demonstrated [23,30,31]. In particular, a recent study has shown that controlled release of rhBMP 2 from PUR scaffolds promoted new bone tissue formation in rat femoral plug defects [23]. Biodegradable PUR scaffolds have also been shown to release biologically active tobramycin *in vitro* [26]. However, the burst release of tobramycin was high (e.g., >50%), and the tobramycin release kinetics was relatively independent of material properties.

Vancomycin, a tricyclic glycopeptide antibiotic, is an effective therapy for treating serious infections caused by gram positive bacteria such as *Staphylococcus aureus* [32,33]. Vancomycin has less negative effects on osteoblasts and skeletal cells than other commonly used antibiotics *in vitro* [34,35], and it does not impede bone growth in fractures *in vivo* [36]. Different strategies have been developed for the delivery of vancomycin from polymers, such as poly(trimethylene carbonate) based surface eroding delivery systems [37] and nano particles presenting vancomycin on the surface [38]. Ideally, the delivery system should deliver vancomycin well above the effective antibacterial concentration (i.e., the minimum inhibitory concentra tion, MIC) in a sustainable manner [2]. For treatment of osteomyelitis, it has been suggested that release of antibiotic at concentrations exceeding the MIC for 6 8 weeks is desirable [39]. Thus one objective of the present study was to synthesize PUR scaffolds incorporating vancomycin with tunable release kinetics, where the release of biologically active vancomycin was extended to 6 8 weeks so that the processes of angiogenesis and new bone formation could proceed unimpeded by infection. PUR scaffolds incorporating vancomycin with both fast and slow release kinetics were evaluated in an infected segmental defect in a rat femur to demonstrate the feasibility of the approach and identify the most effective release strategy.

2. Materials and methods

2.1. Materials

Glycolide and D,L lactide were obtained from Polysciences (Warrington, PA). The tertiary amine catalyst TEGOAMIN33, which com prised a 33 wt.% solution of triethylene diamine (TEDA) in dipropylene glycol, was received from Goldschmidt (Hopewell, VA) as a gift. Hexamethylene diisocyanate trimer (HDIt, Desmodur N3300A) was received as a gift from Bayer Material Science LLC (Pittsburgh, PA). Lysine triisocyanate (LTI) was purchased from Kyowa Hakko (New York, NY). Stannous octoate catalyst was received from Nusil technology (Overland Park, KS). Vancomycin hydrochloride (V HCl) was purchased from ACROS organics. Fluorescence labeled vancomycin (BODIPY@FL conjugate) was purchased from Invitrogen. All other reagents were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Vancomycin hydrochloride (V HCl) and vancomycin free base (V FB)

V HCl was dissolved in water at a concentration of 70 mg/ml, followed by increasing the pH to 8 (where vancomycin has no net charge and the lowest solubility [40]) by adding an appropriate amount of NaOH solution (3 N). After incubating for 30 min, the precipitated vancomycin free base (V FB) was filtered, washed with 70% ethanol and methanol separately, dispersed in water, frozen, and lyophilized overnight. The concentration of V HCl used in the conversion was selected as 70 mg/ml, which is well below the water solubility of 200 mg/ml, in order to avoid V HCl precipitates in the final V FB product. For confocal microscopy, V HCl was mixed with vancomycin BODIPY@FL conjugate at a ratio of 3725:1 in solution, and then lyophilized to obtain fluorescence generating V HCl (FL V HCl) or converted to fluorescence generating V FB (FL V FB) as described above.

2.3. Fabrication and characterization of PUR scaffolds

The polyester macrotriol (900 Da) was synthesized from a glycerol starter as published previously [41], with the backbone comprising 70% ε caprolactone, 20% glycolide, and 10% D,L lactide for T7C2G1L900 and 60% ε caprolactone, 30% glycolide, and 10% D,L lactide for T6C3G1L900. Porous PUR scaffolds were then fabricated using a one shot two component reaction between the triisocyanate and the hardener comprising polyester triol, water, TEGOAMIN33 tertiary amine catalyst, sulfated castor oil stabilizer, and calcium stearate pore opener as described previously [41]. V HCl or V FB was incorporated into the scaffolds by mixing with the hardener component prior to reacting with the triisocyanate. The targeted index (the ratio of NCO to OH equivalents × 100) was 115 for all scaffolds. Two PUR formulations were investigated: (a) PUR(HDIt), a slow degrading scaffold prepared from HDIt and T6C3G1L900; and (b) PUR(LTI), a fast degrading scaffold synthesized from LTI and T7C2G1L900. The antibiotic (V HCl or V FB) loading was selected as the maximum amount of drug that could be incorporated in the scaffolds without compromising stability and pore morphology, which corresponded to 7 wt.% for the PUR(HDIt) materials and 8 wt.% for the PUR(LTI) materials. For PUR(LTI) materials, there was no significant difference in V FB release at 7 and 8 wt.% loadings, while the release of V HCl was ~5% higher at a loading of 8 wt.% compared to 7 wt.% for days 1 15 (data not shown). The amount of water was adjusted for the antibiotic containing PUR(LTI) scaffolds in order to achieve porosities similar to blank scaffolds. The porosities of the scaffolds were calculated based on the volume and weight [23,30], and the distribution of FL V HCl or FL V FB within the PUR(LTI) scaffolds was analyzed by confocal microscopy with an excitation wavelength of 488 nm, performed on a Zeiss LSM510 confocal microscope in VUMC Cell Imaging Shared Resource [23].

2.4. Fabrication of PMMA/vancomycin beads

As described previously [26], the PMMA beads (3 mm in size) were hand made using an Osteoset® kit (Wright Medical Technology, Arlington, TN). Two grams V HCl were added to one packet (40 g powder poly(methyl acrylate, methyl methacrylate), zirconium dioxide, hydrous benzoyl peroxide) and mixed with 20 ml monomer cement (methyl methacrylate, N,N dimethyl ptoluidine). The resulting PMMA

beads contained 3.33 wt.% vancomycin for the *in vitro* release and *in vivo* animal studies, and 0.90 wt.% vancomycin for the KB tests of the scaffolds.

2.5. In vitro release of vancomycin

Three replicate PUR scaffold samples (~40 mg, 0.4 ml) or PMMA beads (~60 mg, 0.15 ml) were immersed in 1 ml PBS in polypropylene vials sealed by O rings. The volume of release medium (1 ml) was selected on the basis that the scaffold was completely immersed in the release medium, and that even if 100% of the vancomycin were released instantaneously, the concentration of the drug in solution would be at least a factor of 6 times less than its solubility. The observation that no significant difference in release profile was observed when the medium volume was increased to 10 ml further suggests that the release was performed under sink conditions. At days 1, 2, 4, 8, 14, 21, 28, 35, 42, 49, and 56, the medium was recovered by squeezing the scaffolds, followed by the addition of 1 ml fresh medium [26]. The concentration of vancomycin released at each time point was determined by measuring the absorbance at 280 nm, from which the concentration was calculated using a standard calibration curve [42,43]. The actual mass of drug released was calculated based on the measured concentration and actual collected sample volumes. The cumulative percentage release was calculated as the ratio of the mass released at each time point to the total amount of vancomycin embedded in the scaffolds. The cumulative release data were fitted to a logarithmic function, which was differentiated with respect to time in order to calculate the daily percentage release [23]. The mass of drug released per day per unit volume of scaffold was calculated from the daily percentage release and the loading of the drug in the scaffold. The Weibull function [44 46] was used to fit the cumulative release curves in order to elucidate the drug release mechanism.

2.6. Antibiotic activity assay

The Kirby Bauer assay was applied to evaluate the biological activity of: (a) the PUR scaffolds and PMMA beads, and (b) the *in vitro* releasates from the PUR scaffolds and PMMA beads. The objective of the Kirby Bauer test was to validate that the vancomycin was still active after incorporation in the PUR scaffolds. Methicillin susceptible *S. aureus* from the American Type Culture Collection (ATCC 25923) was chosen for the assay [26]. The bacteria were suspended in trypticase soy broth at a turbidity that matched a 0.5 MacFarland standard and then spread onto Mueller Hinton agar plates. PMMA impregnated with V HCl (0.9 wt.%) and PUR scaffolds with V HCl (8 wt.%) or V FB (8 wt.%) were cut into discs (6 mm diameter by 1 mm thick) and placed on the agar plates. All the three treatment groups incorporated the same mass (320 μg) of vancomycin. BBL SensiDiscs incorporating 30 μg vancomycin (BD, Franklin Lakes, NJ, USA) were used as a laboratory positive control. Blank PUR was used as negative control. After incubating the agar plates at 37 °C for 24 h, the zones of inhibition (ZI) were measured for each disc. In order to evaluate the bioactivity of vancomycin released from scaffolds and PMMA beads, the releasates from day 1 and weeks 2, 4, 6, and 8 were lyophilized and reconstituted in water to a concentration of 1.5 mg/ml. A mass of 15 μg (dissolved in a 10 μl solution) released vancomycin was subsequently loaded onto blank SensiDiscs. The positive control were SensiDiscs with 15 μg fresh vancomycin. The discs were placed on agar plates, and ZIs were measured for each disc after incubating the agar plates at 37 °C for 24 h. One way ANOVA with Bonferroni correction ($p<0.05$) was used for evaluation of statistical significance.

2.7. In vivo animal study

The animal study was approved by the Animal Care and Use Committee at the US Army Institute of Surgical Research. A previously described contaminated critical size defect (CSD) was created in the rat (Sprague Dawley) femurs [47]. Briefly, a 6 mm segmental defect was created and stabilized under aseptic conditions in 40 rats. Using aseptic technique, a longitudinal incision was made over the left anterolateral femur, and the entire femoral shaft was exposed using blunt dissection. A polyacetyl plate (length 25 mm, width 4 mm and height 4 mm) was fixed to the surface of the femur using 6 threaded K wires. A 6 mm mid diaphyseal full thickness defect was created with a small reciprocating saw blade (MicroAire 1025, MicroAire, Charlottesville, VA) under continuous irrigation with sterile saline. The defects in all animals were implanted with 30 mg of type I bovine collagen (Stryker Biotech, Hopkinton, MA, USA) that was ethanol sterilized and subsequently wetted with 10^5 of colony forming units (CFUs) of *S. aureus* lux (Xenogen 36, Caliper Life Science, Hopkinton, MA) suspended in 0.1 ml of sterile normal saline. This isolate was transgenically modified to emit photons and was sensitive to vancomycin. The contaminated collagen was packed into the defect, and the wound closed in a layered fashion. A high resolution radiograph of each femur with stabilized defect was obtained using a Faxitron X ray system (Faxitron X ray Corporation, Wheeling, Illinois (Model: MX 20, Image settings Time: 10 s, KV: 35, Window Level: 3380/1250)) at initial surgery to confirm appropriate place ment of the implant and adequate creation of the defect.

2.7.1. Treatment

Six hours after contamination, the wounds were debrided and irrigated with 60 ml of saline [47]. This time period was chosen because it is clinically relevant [48]. Model development work demonstrated that a 6 h delay was the ideal time for treatment with local antibiotics [49]. The wounds in the control group were re closed with no further treatment. For the experimental groups, PUR scaffolds (PUR(LTI)/V HCl or PUR(LTI)/V FB containing 8 wt.% vancomycin, (340 μg vancomycin) or four 3 mm PMMA/V HCl beads (clinical control containing 3.33 wt.% vancomycin, or 2800 μg vancomycin per defect) were then packed into the defects ($n=10$) prior to wound closure. To be consistent with clinical practice, two beads were placed in the bony defect and two were placed in the soft tissue. In the present study, we aimed to test our hypothesis that the PUR + vancomycin scaffolds would perform comparably to, or better than, PMMA + vancomycin beads. The cement beads were selected as the clinical control on the basis that they are an established, clinically effective treatment [4,5]. The vancomycin dosage in the PUR scaffolds was selected on the basis that it was the maximum concentration of vancomycin that could be incorporated in the PUR scaffolds while maintaining porosity and dimensional stability. The animals were allowed full activity in their cages postoperatively, and were monitored daily for signs of pain and systemic infection. Following 4 weeks of recovery, the rats were euthanized by Fatal Plus.

2.7.2. Photon counts

Immediately after euthanasia, the wound site was exposed to allow bacteria quantification using a photon counting camera (Xenogen IVIS® Imaging System 100 Series using the Living Image 2.6.2. (Software Igor Pro 4.09A)). The disarticulated extremities were placed in the Xenogen machine, and a black and white photograph was first taken of the wound followed by a luminescent image with a 2.5 s exposure time. The Region of Interest (ROI) was determined with elliptical ROIs positioned over the wound to include the entire femur and polyacetyl plate. This method assessed the amount of surface bacteria within the entire wound.

2.7.3. Quantitative cultures

The femur with defect was harvested from the animals and used for quantitative assessment [47]. The plate, K wires, and all soft tissues were removed. Each femur was weighed, snap frozen in liquid nitrogen, and ground to a fine powder under sterile conditions. The resulting bone powder was serially diluted in normal saline. Aliquots

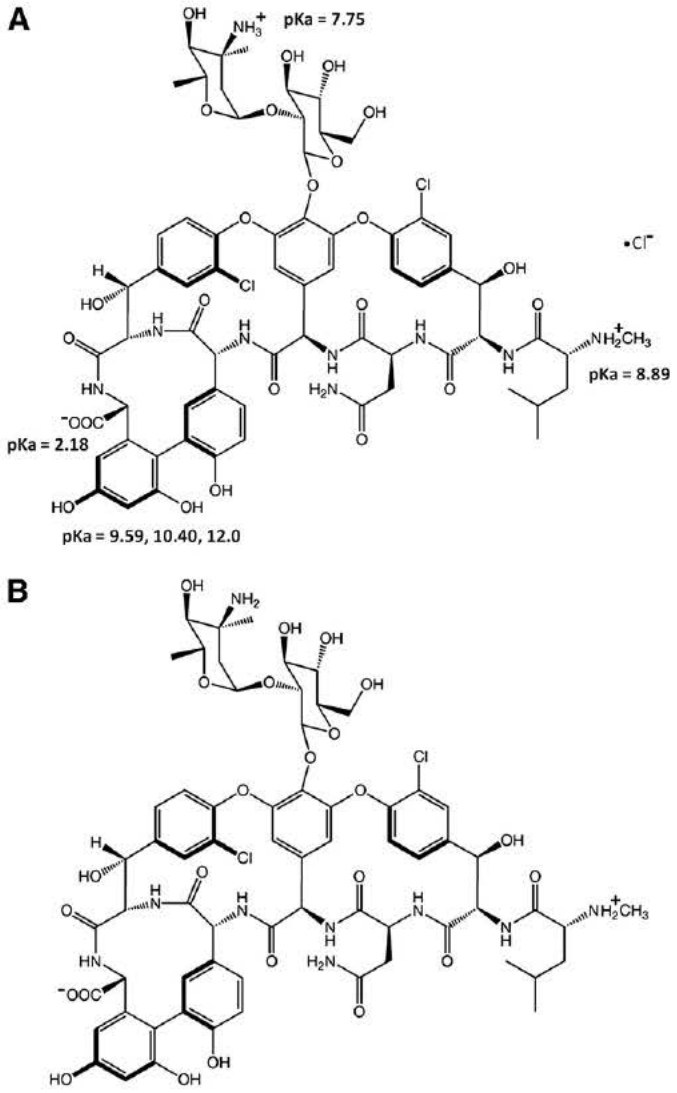

Fig. 1. The molecular structure of vancomycin hydrochloride (V-HCl, A) and free base (V-FB, B) with pKa values and charges indicated.

(100 µl) of each dilution were plated onto the surface of tryptic soy agar, and incubated at 37 °C for 48 h. The plates were examined for purity and colony morphology. Four to eight dilutions were typically required to obtain a minimum dilution level where the CFUs of bacteria were countable on the culture plate; the actual numbers of recovered CFUs of bacteria were obtained by correcting for the magnitude of the dilution used to obtain them and reported in CFUs per gram of bone tissue.

Table 1
Treatment groups.

Treatment group	V form	V loading		Porosity
		(wt.%)	(µg/cm^3)	
PUR(LTI)/V-HCl	V-HCl	8	7.90×10^3	91.87%
PUR(LTI)/V-FB	V-FB	8	7.40×10^3	92.40%
PUR(LTI)	N/A	N/A	N/A	91.45%
PUR(HDIt)/V-HCl	V-HCl	7	8.93×10^3	89.46%
PUR(HDIt)/V-FB	V-FB	7	9.10×10^3	89.26%
PUR(HDIt)	N/A	N/A	N/A	92.9% [23]
PMMA/V-HCl	V-HCl	3.33	2.91×10^4	24.06%

V-HCl denotes the hydrochloride form of vancomycin, while V-FB denotes the free base form of vancomycin. PUR(LTI) scaffolds were synthesized from LTI and T7C2G1L900 polyester triols, while PUR(HDIt) scaffolds were synthesized HDIt and T6C3G1L900 polyester triols. Vancomycin loading is specified in both wt.% and µg/cm^3 scaffold.

3. Results

3.1. Properties of vancomycin (V HCl and V FB)

Vancomycin contains 6 functional groups which exhibit dissociable hydrogen atoms at specific pH values (Fig. 1), including a carboxyl group with a pK_a of 2.18, a primary amino group with a pK_a of 7.75, a secondary amino group with a pK_a of 8.89, and three phenol groups with pK_a values of 9.59, 10.40, and 12.0 respectively [40]. Therefore, V HCl has one net positive charge in solution (Fig. 1A), and has solubility in water equal to 200 mg/ml. V FB was obtained through precipitation of vancomycin at pH 8. V FB contains no net charge (Fig. 1B) and has a water solubility of less than 20 mg/ml, which is substantially lower than that of V HCl.

3.2. Fabrication and activity of PUR scaffolds with vancomycin

Two types of PUR scaffolds were fabricated: PUR(LTI), a fast degrading material (100% mass loss after 36 weeks *in vitro*) synthesized from LTI and T7C2G1L900, and PUR(HDIt), a slow degrading material (32% mass loss after 36 weeks *in vitro*, unpublished data) synthesized from HDIt and T6C3G1L900 [26]. Two forms of vancomycin were separately incorporated into different PUR scaffolds by mixing with the polyester triol prior to reacting with the triisocyanate. The loadings of antibiotic within the PUR(LTI) and PUR(HDIt) scaffolds were 8 wt.% and 7 wt.%, respectively. As shown in Table 1, all PUR scaffolds exhibited porosity of ~89 93%, while PMMA beads were only 24% porous.

In order to investigate the distribution of vancomycin within the scaffolds, vancomycin BODIPY@FL conjugate was mixed with V HCl in order to obtain fluorescence labeled V HCl (FL V HCl). A portion of FL V HCL was then converted to FL V FB using the same method of converting V HCl to V FB. When incorporated in PUR scaffolds, as evidenced by confocal fluorescence microscopy images (Fig. 2), both V HCl and V FB were distributed uniformly throughout the PUR scaffold walls. The V HCl particles appeared to have a more needle like (e.g., high aspect ratio) morphology relative to the V FB particles.

The bioactivity of both PUR(LTI) and PUR(HDIt) scaffolds incorporating vancomycin (6 mm diameter by 1 mm thick discs) was measured using the Kirby Bauer diffusion test. The positive laboratory control, comprising BBL SensiDiscs used in clinical pathology laboratories containing 30 µg vancomycin, showed a ZI of 19.5 mm, and the negative control, blank PUR scaffolds, showed a ZI of 0 mm (data not shown). These controls demonstrate that the KB test was performing properly. As shown in Fig. 3, PUR(LTI) discs incorporating V HCl (PUR(LTI)/V HCl) demonstrated ZIs comparable to PMMA/V HCl at the same volume

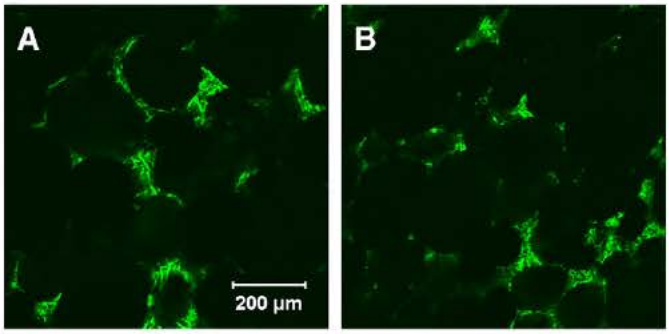

Fig. 2. Distribution of FL-V-HCl (A) and FL-V-FB (B) in PUR(LTI). Vancomycin BODIPY@FL conjugate was mixed with V-HCl to obtain FL-V-HCl and then converted to FL-V-FB. The loading of either type of FL-V in PUR(LTI) was 8 wt.%. The fluorescence images were taken using a confocal microscope with an excitation wavelength of 488 nm.

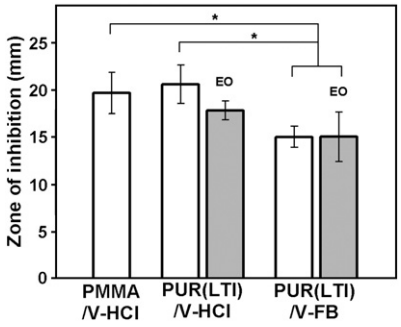

Fig. 3. Zones of inhibition measured for PUR(LTI)/V-HCl, PUR(LTI)/V-FB, and PMMA/V-HCl materials. *Staphylococcus aureus* (ATCC 25923) were spread onto Mueller–Hinton agar plates. PUR scaffolds and PMMA beads (6 mm × 1 mm discs) were placed on the agar plates. BBL SensiDiscs incorporating 30 ug V-HCl were used as a laboratory control. After incubating the agar plates at 37 °C for 24 h, the zones of inhibition (ZI) were measured for each disc. One way ANOVA with bonferroni correction ($p<0.05$) was used for evaluation of statistical significance, and the significance between groups was depicted as * where existed.

concentration, while PUR(LTI) discs loaded with vancomycin free base (PUR(LTI)/V FB) showed 25% lower activity than the PUR(LTI)/V HCl material. It is also interesting to note that sterilization by ethylene oxide (EO) resulted in no significant loss in biological activity of either V HCl or V FB. As shown in Fig. 4, similar to PUR(LTI) materials, the PUR(HDIt) discs incorporating V HCl (PUR(HDIt)/V HCl) showed comparable ZIs PMMA/V HCl, while PUR(HDIt) discs loaded with V FB (PUR(HDIt)/V FB) showed decreased (statistically significant) bioactivity com pared with both PUR(HDIt/V HCl). The lower ZIs observed for PUR discs incorporating V FB are conjectured to result from the lower water solubility of V FB relative to V HCl, resulting in slower release of the drug into the agar gel.

3.3. In vitro release of vancomycin from PUR scaffolds and PMMA beads

The *in vitro* release kinetics of vancomycin from PUR(LTI)/V HCl, PUR(LTI)/V FB, and PMMA/V HCl (3.23 wt.% V HCl, the clinical control) were measured in PBS. The PUR(LTI)/V HCl materials showed a burst release of 42% and 28% on days 1 and 2, respectively, and the cumulative release was 90% at day 8. After day 8, the release was slow (i.e., 0.80%/day at day 8 and 0.09%/day release at day 56) (Fig. 5A and B). When vancomycin was transformed from V HCl to V FB and embedded in PUR(LTI), the lower solubility was expected to slow the release of vancomycin. This hypothesis is supported by the observation that the release on day 1 of PUR(LTI)/V FB was only about 11%, compared to 42%

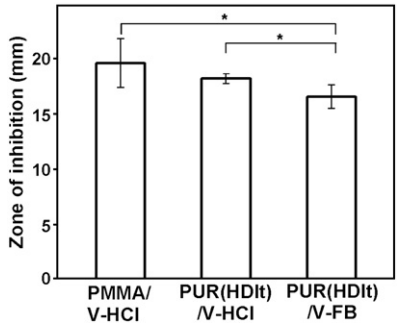

Fig. 4. Zones of inhibition measured for PUR(HDIt)/V-HCl and PUR(HDIt)/V-FB. *Staphylococcus aureus* (ATCC 25923) were spread onto Mueller–Hinton agar plates. PUR scaffolds and PMMA beads (6 mm × 1 mm discs) were placed on the agar plates. BBL SensiDiscs incorporating 30 ug V-HCl were used as a laboratory control. After incubating the agar plates at 37 °C for 24 h, the zones of inhibition (ZI) were measured for each disc. One way ANOVA with bonferroni correction ($p<0.05$) was used for evaluation of statistical significance, and the significance between groups was depicted as * where existed.

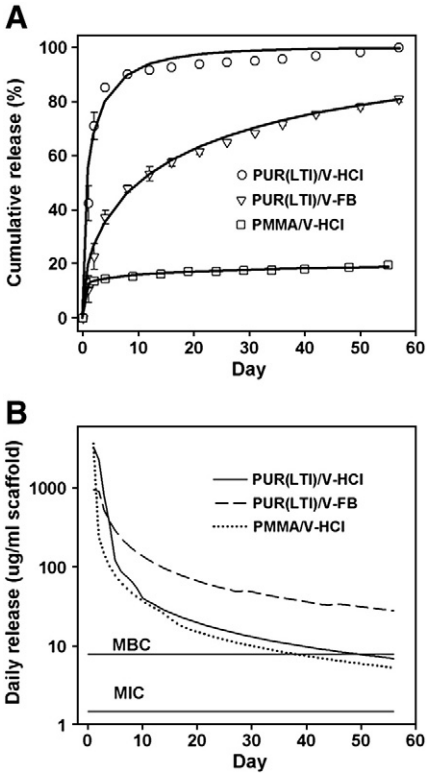

Fig. 5. Vancomycin release kinetics from PUR(LTI)/V-HCl, PUR(LTI)/V-FB, and PMMA/V-HCl materials. (A) Cumulative release (%, symbols) and simulation by empirical Weibull model (solid lines), and (B) daily release (μg/cm^3 scaffold). PUR scaffolds (~40 mg, $n=3$) or PMMA beads (~60 mg, $n=3$) were immersed in 1 ml PBS in polypropylene vials sealed by O-rings. The medium was refreshed as indicated in the methods. The amount of vancomycin released at each time point was determined by measuring the absorbance at 280 nm.

observed for PUR(LTI)/V HCl. Furthermore, as shown in Fig. 5B, the V FB exhibited a more sustained release profile from day 8 to day 56 (i.e., 2.42%/day at day 8 and 0.36%/day at day 56) which is 3 fold greater than the corresponding release rates for the V HCl formulation. Due to the lower burst and more sustained release of V FB, the concentration of vancomycin remained well above the minimum inhibitory concentra tion (MIC, 0.75~2 μg/ml) and minimum bactericidal concentration (MBC, 8 μg/ml) [50,51] for at least 8 weeks (Fig. 5B). In contrast, PMMA beads exhibited a burst release of 10% on day 1 followed by a small sustained release on days 8 56 (i.e., 0.16%/day at day 8 and 0.02%/day at day 56). The sustained release from PMMA beads is less than that observed for the PUR treatment groups, although the loading of vancomycin per volume PMMA is 4 fold that of PUR scaffolds as shown in Table 1.

The release profiles of vancomycin from PUR(HDIt) scaffolds were similar to those observed for PUR(LTI) scaffolds. PUR(HDIt)/V HCl materials exhibited a burst release for days 1 4 followed by a period of slower release from days 8 56 (Fig. 6). The PUR(HDIt)/V FB materials showed a minimal burst release followed by a sustained release for a period of 6 weeks. The cumulative release of vancomycin was generally less from HDIt scaffolds compared to LTI scaffolds. For example, on day 42, the cumulative release of V HCl was 95% for the LTI materials compared to 85% for the HDIt materials. Similarly, on day 42, the cumulative release of V FB was 70% for the LTI materials compared to 52% for the HDIt materials. The PUR(HDIt)/V (HCl + FB) materials, which were loaded with 3.5 wt.% V HCl and 3.5 wt.% V FB, resulted in a release profile intermediate to that of the PUR(HDIt)/V HCl and PUR(HDIt)/V FB materials, suggesting the possibility of tuning the release profile of vancomycin for different applications by blending the V HCl and V FB forms.

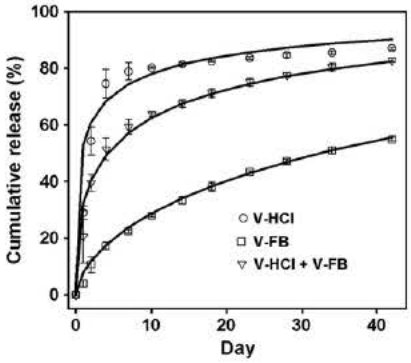

Fig. 6. Vancomycin release kinetics from PUR(HDIt)/V-HCl, PUR(HDIt)/V-FB, and PUR(HDIt)/V-FB + V-HCl scaffolds: cumulative release (%, symbols) and simulation by empirical Weibull model (solid lines). PUR scaffolds (~40 mg, $n=3$) were immersed in 1 ml PBS in polypropylene vials sealed by O-rings. The medium was refreshed as indicated in the methods. The amount of vancomycin released at each time point was determined by measuring the absorbance at 280 nm.

3.4. In vitro vancomycin release mechanism

There are several empirical models available for simulating drug release from polymer scaffolds. Although the power law model has been extensively used, it is most accurate for short time periods or <60% cumulative release of the drug [46]. In the present study, the release of vancomycin was measured for up to 56 days and nearly 100% cumulative release for some of the treatment groups. While the power law model was observed to fit the first 4 7 days of release, the quality of the fit was poor at longer time points where the cumulative release exceeded ~50%. Therefore, the vancomycin release data from PUR scaffolds and PMMA beads were fit to the Weibull function, another empirical equation which has been used to model drug release for more extended periods of time [44,46]:

$$\frac{M_t}{M_\infty} = 1 - \exp\left(-a \cdot t^b\right) \quad (1)$$

where M_t = the mass of drug released at time t, M_∞ = the mass of drug released at infinite time (assumed equal to the amount of drug added), and a and b are constants [45]. A value of $b<0.75$ suggests a Fickian diffusion release mechanism [44,46]. The fitting parameters deter mined from the vancomycin release data are listed in Table 2. The values of b calculated for PUR(LTI) scaffolds were found to be approximately 0.50 for both V HCl and V FB. Similarly, the values of b determined for PUR(HDIt) materials were found to be 0.30, 0.40, and 0.61 for V HCl, V HCl + V FB, and V FB respectively. For all treatment groups, $b<0.75$ which suggests that the release of either type of vancomycin from PUR scaffolds is diffusion limited. For PUR(LTI), the values of a were found to be 0.81 and 0.22 for V HCl and V FB, respectively. Similar trends were observed for the PUR(HDIt) materials, with values of a equal to 0.76, 0.39, and 0.08 for V HCl, V HCl + V FB, and V FB respectively, thus suggesting a faster release rate for V HCl as a result of its higher water solubility relative to V FB. The parameter $\tau = a^{-b}$, defined as the time at which 63.2% of the drug has been delivered [45], was calculated from the Weibull fitting parameters. The values of τ were calculated to be 1.5 and 20.8 days for V HCl and V FB released from PUR(LTI) scaffolds; and 2.5, 10.5, and 58.8 days for V HCl, V HCl + V FB, and V FB released from PUR(HDIt) scaffolds. These values of τ are consistent with the experimentally observed times required for 63.2% cumulative release of vancomycin (Figs. 5A and 6).

The a and b values for V HCl release from PMMA were 0.14 and 0.11 respectively, which are significantly lower than those from PUR(LTI) scaffolds. The differences in the Weibull model parameters are attributed to the fact that the majority (e.g., <80%) of the antibiotic is sequestered in the PMMA beads, with only the drug trapped near the surface being released [52,53].

Table 2
Simulation of *in vitro* release to the empirical Weibull model.

$\frac{M_t}{M_\infty} = 1 \quad \exp(\quad a\ t^b)$		a	b	R^2
PUR(LTI)	V-HCl	0.81	0.50	0.978
	V-FB	0.22	0.50	0.990
PUR(HDIt)	V-HCl	0.76	0.30	0.923
	V-HCl + V-FB	0.39	0.40	0.982
	V-FB	0.08	0.61	0.997
PMMA	V-HCl	0.14	0.11	0.996

M_t/M_0, t, and R^2 represent cumulative percentage released (%), time (day), and the coefficient of determination respectively. Both a and b are constants.

3.5. Bioactivity of released vancomycin

The vancomycin released from PUR(LTI)/V HCl, PUR(LTI)/V FB, and PMMA/V HCl materials up to specified time points were lyophilized and reconstituted to the same concentration (1.5 mg/ml) to evaluate the bioactivity of the released vancomycin at the same concentration of antibiotic. Using fresh vancomycin as a positive control, 15 µg of released vancomycin was loaded onto SensiDiscs and the zones of inhibition measured using the Kirby Bauer test. As shown in Fig. 7, the released vancomycin from each treatment group at each time period was verified to be bioactive, and the bioactivity decreased with time. For PMMA/V HCl, the bioactivity of released vancomycin decreased as early as week 2, while the bioactivity of released vancomycin from the PUR(LTI)/V HCl materials decreased at week 4. The bioactivity of released antibiotic was highest for the PUR(LTI)/V FB materials, where the bioactivity did not decrease until week 6. Due to the more sustained release of vancomycin from these materials, biologically active antibiotic was released up to week 8 with a significant but modest (~15%) reduction in bioactivity.

3.6. Infection inhibition by PUR scaffolds in vivo

The PUR(LTI) materials were sterilized by EO treatment at room temperature overnight, which was shown to have a negligible effect on the biological activity of the scaffolds (Fig. 3). PUR(LTI)/V HCl, PUR(LTI)/V FB, and PMMA/V HCl beads were implanted in the con taminated rat femoral segmental defect and the femurs harvested after 4 weeks. The spatial distribution of the bacteria within the wound, detected by photon imaging, shows that all the vancomycin

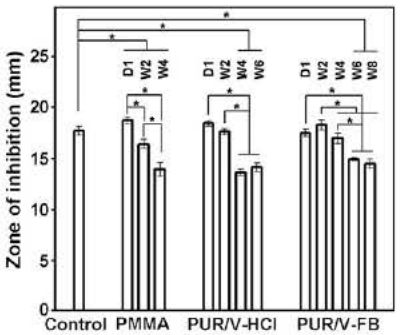

Fig. 7. Zones of inhibition measured for vancomycin released from PUR(LTI)/V-HCl, PUR(LTI)/V-FB, and PMMA/V-HCl materials for up to 8 weeks. Releasates from day 1 and weeks 2, 4, 6, and 8 were lyophilized and reconstituted to a concentration of 1.5 mg/mL A mass of 15 µg (dissolved in a 10 µl solution) released vancomycin was subsequently loaded onto blank SensiDiscs. The positive control was SensiDiscs with 15 µg fresh vancomycin. The discs were placed on agar plates, and ZIs were measured for each disc after incubating the agar plates at 37 °C for 24h. One way ANOVA with bonferroni correction ($p<0.05$) was used for evaluation of statistical significance, and the significance between treatment groups and the positive control or different time points within each treatment group was depicted as * where existed.

treatment groups inhibited bacteria growth in the soft tissue significantly compared with the untreated negative control (Fig. 8). No significant differences were observed between any vancomycin treatment groups for the soft tissue bacteria counts. The femurs were then frozen, ground to a fine powder, and cultured for counting of CFUs per unit weight of bone. The CFU data (Fig. 9) indicate that all the vancomycin treatment groups inhibited bacteria growth in bone tissue, but only PUR(LTI)/V FB and PMMA/V HCl showed significant inhibition effects compared with the untreated control. No significant differences existed between the performance of PMMA/V HCl beads and the PUR(LTI) treatment groups. Consis tent with the definition of a CSD, no significant new bone formation was observed when PUR scaffolds were implanted in rat femoral CSDs in the absence of an osteoinductive factor such as rhBMP2 (unpublished data).

4. Discussion

Vancomycin is an important antibiotic due to its potential in preventing infections caused by gram positive bacteria such as methicillin resistant *S. aureus* (MRSA) [1,32]. In previous studies, delivery of vancomycin hydrochloride resulted in low release efficiency when delivered using non biodegradable materials such as PMMA [11 14]. Furthermore, a high burst with minimal sustained release resulted due to the high water solubility of V HCl in PMMA and other materials [37,42,43]. In the present study, PMMA beads impregnated with V HCl resulted in maximum daily release on day 1 followed by slow release thereafter, with only 20% cumulative release of V HCl. A similar release profile has been reported for tobramycin incorporated within PMMA [26]. When incorporated in a porous biodegradable PUR, the release efficiency was significantly enhanced as evidenced by the nearly 100% cumulative release of V HCl from PUR(LTI) scaffolds at week 8. Additionally, the sustained release is extended to longer time periods relative to PMMA (Fig. 4). By incorporating the more hydrophobic, less soluble free base form of vancomycin (V FB), the burst release was decreased and the sustained release increased. Release profiles of V HCl and V FB were fit to the Weibull equation, resulting in value of the exponent *b* ranging from 0.4 0.6, which suggests that the release of vancomycin was diffusion controlled. The model calculations also showed that by decreasing the water solubility of the drug, the diffusion rate from PUR scaffolds was decreased, which resulted in a more sustained release profile for the hydrophobic V FB relative to hydrophilic V HCl. The daily release concentration of V FB released from PUR(LTI)/V FB was well above the MIC and MBC up to week 8, and the released V FB was verified to

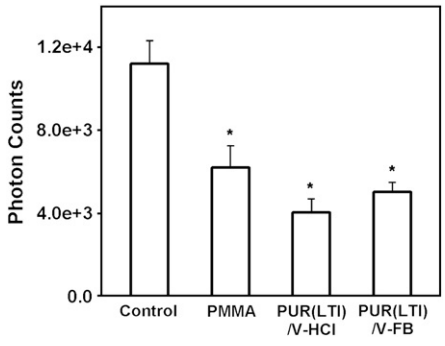

Fig. 8. Photon counts measured for PUR(LTI)/V-HCl, PUR(LTI)/V-FB, and PMMA/V-HCl materials after 4 weeks implantation time. At 4 weeks, the rats were euthanized, tissue around and within the defects was harvested, and quantitative cultures performed. A photon-counting camera was used to capture the quantitative and spatial distribution of the bacteria within the wound following euthanasia and harvesting of the femur. One way ANOVA with bonferroni correction ($p<0.05$) was used for evaluation of statistical significance, and the significance between vancomycin treatment groups and the negative control was depicted as * where existed.

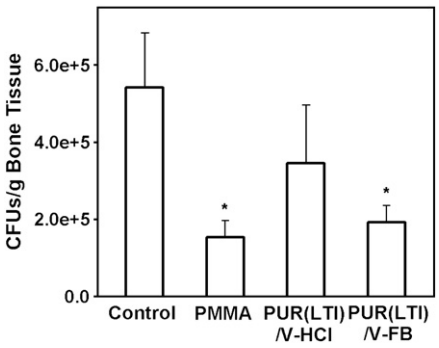

Fig. 9. CFU counts measured for PUR(LTI)/V-HCl, PUR(LTI)/V-FB, and PMMA/V-HCl materials after 4 weeks implantation time. Each femur was weighed, snap-frozen in liquid nitrogen, and ground to a fine powder under sterile conditions. The resulting bone powder was serially diluted in normal saline. Aliquots (100 µl) of each dilution were plated onto the surface of tryptic soy agar, and incubated at 37 °C for 48 h in 5% CO_2. All CFU counts were normalized to counts/g bone tissue. One way ANOVA with bonferroni correction ($p<0.05$) was used for evaluation of statistical significance, and the significance between vancomycin treatment groups and the negative control was depicted as * where existed.

be bioactive in the Kirby Bauer bacteria inhibition test. Furthermore, confocal microscopy images revealed that the vancomycin was distributed uniformly throughout the pore walls, which is consistent with our previous results reported for bovine serum albumin [23]. PUR(LTI) scaffolds exhibit interconnected pores and undergo hydro lytic degradation with resorption times varying from 26 36 weeks *in vitro* [54]. In a previous study, we reported that ~75% of the tobramycin incorporated in PUR scaffolds was released after 1 week incubation time *in vitro*, resulting in a 20 30% reduction in compressive strength [26]. This reduction in strength is attributed to the additional channels and pores created within the scaffold resulting from diffusion of the drug from the polymer into the solution [55].

PUR(LTI) scaffolds incorporating vancomycin were evaluated in an infected rat femoral segmental defect to verify the activity of the released antibiotic *in vivo*, as well as identify the preferred formulation. At week 4, compared with the untreated group, both PUR(LTI)/V HCl and PUR(LTI)/V FB treatment groups reduced the number of bacteria counts in soft tissue significantly, and PUR(LTI)/ V FB performed similarly to PMMA/V HCl in reducing the number of bacteria counts significantly within bone tissue. Taken together, these observations suggest that the PUR(LTI)/V FB implants exhibited superior infection control relative to the PUR(LTI)/V HCl implants at 4 weeks. Furthermore, the *in vivo* data show the PUR(LTI)/V FB implants performed comparably to the PMMA/V HCl clinical control. Due to processing constraints associated with the PUR scaffolds, the maximum achievable vancomycin loading was 8 wt.%. Due to the high porosity of the PUR scaffolds (~90%), the density of the scaffolds was much lower than that of the PMMA beads; thus the loading of vancomycin in the defects (3 mm × 6 mm) was substantially less for animals treated with PUR scaffolds (340 µg) compared to those treated with PMMA beads (2800 µg). Since the efficiency of release of vancomycin was considerably higher for PUR scaffolds, the daily re lease (in units of $\mu g \cdot cm^{-3}$ implant) was comparable for the PUR(LTI)/ V HCl and PMMA/V HCl treatment groups at all time points (Fig. 5). However, it is important to note that four beads were placed in the femoral CSDs, two in the bone defect and two in soft tissue, which is consistent with clinical practice. Therefore the volume of the PMMA implants was approximately twice that of the PUR scaffolds (which were only placed in the bone defect), so that the total mass of antibiotic released from the PMMA implants is estimated to be twice that released from the PUR scaffolds based on the *in vitro* release data (Fig. 5). The observation that the PUR(LTI)/V HCl implants inhibited the infection in bone to a lesser extent than the PMMA/V HCl control at 4 weeks is thus

attributed to the larger mass of V HCl released from the PMMA beads compared to the PUR(LTI)/V HCl scaffolds. The data in Fig. 5B indicated that at 28 days the daily release exceeded the MIC for all treatment groups, although after day 2 the daily release from the PUR(LTI)/V FB scaffolds exceeded that from the other treatment groups. Thus the observation that PUR(LTI)/V FB scaffolds performed comparably to PMMA beads is conjectured to result from the more sustained release achieved with the PUR(LTI)/V FB scaffolds. While the PUR(LTI)/V FB implant performed comparably to the PMMA/V HCl control at 4 weeks, it is anticipated that the more sustained release profile achieved with the PUR(LTI)/V FB scaffolds would significantly reduce the infection burden relative to PMMA/V HCl at longer time points (e.g., 8 weeks) where the release from PUR(LTI)/V FB continued to exceed the MIC.

The objective of the present study was to compare the perfor mance of biodegradable PUR + vancomycin implants to that of PMMA beads incorporating vancomycin which is the current clinical standard of care for local delivery of antibiotics. Thus no direct comparisons can be made between local delivery of vancomycin from PUR(LTI)/V FB scaffolds and systemic delivery due to the lack of an additional clinical control comprising an empty scaffold with systemic parenteral delivery of vancomycin. However, previous studies have shown that local delivery of antibiotics can achieve higher concentra tions at the contaminated site compared to systemic parenteral treatment [7 9], resulting in potentially more effective management of the infection and reduced systemic toxicity risks. Local delivery of gentamycin from PMMA beads performed comparably to long term systemic parenteral delivery of gentamycin in the treatment of infected non unions [10]. Additionally, local delivery of antibiotics was reported to reduce the risk of infection for total hip and knee arthoplasties relative to systemic delivery, although the difference was not statistically significant [8]. Taken together, these previous studies suggest that local delivery of antibiotics using PMMA beads is at least as effective at controlling infections as systemic delivery. Considering that the PUR(LTI)/V FB therapy performed comparably to the PMMA/V HCl beads in the infected rat segmental defect model at 4 weeks, it is anticipated that the PUR(LTI)/V FB therapy would also perform at least comparably to the systemic delivery approach.

Beads or spacers made from antibiotic laden bone cements have been clinically adopted for treating infection associated with hip anthroplasty [56,57]. Simplex P, a PMMA bone cement with tobramycin, has been approved by FDA for second stage revision of total joint replacement, where the antibiotic is claimed to protect the PMMA from infection. PMMA bone cements incorporating antibiotics have also been used to treat chronic osteomyelitis [58 60], and the two stage procedure comprising treatment with antibiotics to control the infection followed by grafting to heal the defect is the current clinical standard of care for treating contaminated bone wounds [56,61 63]. Nevertheless, cases of secondary infectious complications have been reported [64], which have been attributed to insufficient sustained release of the antibiotic. Furthermore, too low a concentra tion of antibiotic may induce antibiotic resistance of bacteria [65]. Local sustained delivery of antibiotics from the bone graft implanted after the stabilization of the wound bed is anticipated to protect the graft from infection and reduce the frequency of secondary complica tions. Bone wound healing is dependent on angiogenesis and vascularization [19 22], and previous reports have suggested that a period of up to 6 weeks may be required for full vascularization of scaffolds implanted in segmental defects to occur [22]. Another study has shown that at least 6 weeks of effective antibiotic treatment are required in order to control infection for total hip and knee arthroplasties [66]. Therefore, sustained release of antibiotic exceed ing the MIC for at least 6 weeks post implantation is anticipated to be necessary for protecting the bone graft from infection and enabling the processes of vasculogenesis and wound healing to proceed. The biodegradable (PUR(LTI)/V FB) delivery system developed in this study, which is a single stage approach, presents a potentially significant innovation in the clinical use of antibiotics. By protecting the graft from contamination, infection control and tissue regenera tion are allowed to occur simultaneously.

Recent studies have shown that sustained release of recombinant growth factors, such as rhPDGF BB [30] and rhBMP 2 [23], from PUR scaffolds accelerates cellular infiltration and ingrowth of new tissue *in vivo*. However, infection is known to inhibit new bone formation, even in the presence of an osteogenic factor [67]. While systemic delivery of antibiotics and local delivery of rhBMP 7 from a collagen carrier have been observed to enhance bone formation relative to local delivery of rhBMP 7 alone [68], the effects of local delivery of antibiotics and an osteoinductive factor on new bone formation in infected wounds have not been investigated. Therefore co delivery of V FB and rhBMP 2 from a biodegradable PUR scaffold could be a potentially promising approach for healing contaminated bone defects: the vancomycin is anticipated to control the infection so that the osteoinductive effects of the rhBMP 2 are not impeded. If successful, the dual delivery approach could accelerate healing by protecting the scaffold from bacterial contamination, which could substantially reduce the frequency of secondary infectious complications.

5. Conclusions

Porous biodegradable PUR scaffolds have been shown to support tunable, sustained release of vancomycin *in vitro*. When implanted in infected segmental defects in rats, PUR/V FB scaffolds significantly reduced the infection relative to the untreated control and performed comparably to PMMA bone cements after 4 weeks. The release profile of vancomycin from PUR was extended to at least 8 weeks when hydrophilic V HCl was converted to hydrophobic V FB. Therefore, PUR scaffolds incorporating V FB could be a potential clinical therapy for treatment of contaminated bone defects.

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