Gatifloxacin-loaded PLGA and β -tricalcium phosphate composite for treating osteomyelitis

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Gatifloxacin (GFLX)-containing poly(lactide-co-glycolide) (PLGA) was introduced to the pores and surfaces of porous β -tricalcium phosphate (β TCP) granules by melt compounding whereby no toxic solvent was used. The granular composite of GFLX-loaded PLGA and β TCP released GFLX for 42 days in Hanks' balanced solution and exhibited sufficient *in vitro* bactericidal activity against *Streptococcus milleri* and *Bacteroides fragilis* for at least 21 days. For *in vivo* evaluation, the granular composite was implanted in the dead space created by the debridement of osteomyelitis lesion induced by *S. milleri* and *B. fragilis* in rabbit mandible. After a 4-week implantation, the inflammation area within the debrided area was markedly reduced accompanied with osteoconduction and vascularization in half of the rabbits, and even disappeared in one of the six rabbits without any systemic administration of antibiotics. Outside the debrided area, inflammation and sequestrum were observed but the largest of such affected areas amounted to only 0.125 times of the originally infected and debrided area. These findings showed that the granular composite was effective for the local treatment of osteomyelitis as well as an osteoconductive scaffold which supported and encouraged vascularization.

Keywords: Gatifloxacin, Poly(lactide-co-glycolide), β -tricalcium phosphate, Osteomyelitis

INTRODUCTION

Chronic osteomyelitis is an intractable infection of the bone associated with the destruction of bone tissues and vascular channels¹⁻⁴. The destruction of vascular channels leaves a portion of dead and infected bone (sequestrum) detached from the adjoining healthy bone and surrounded by avascular soft tissue. Impaired vascularity prevents antibiotics to be delivered to the lesion *via* the intravenous route. Therefore, chronic osteomyelitis cannot be eradicated without a radical surgical debridement of the sequestrum. Since adequate debridement is down to the living bone, the debridement can leave a large avascular dead space that must be managed to prevent infection recurrence before tissue reconstruction. The debridement is usually accompanied with prolonged antibiotic therapy, or antibiotic and anti-inflammatory therapy administered by the intravenous route⁵. Despite such radical treatment, the recurrence rate of chronic osteomyelitis is still high.

A promising strategy to manage the dead space after debridement is local antibiotic delivery using biodegradable polymers accompanied by intravenous antibiotic delivery. The effectiveness of local antibiotic delivery using biodegradable polymers has been substantiated by *in vitro* and *in vivo* studies. The biodegradable polymers that have been studied include poly(D,L-lactic acid)^{6,7}, polylactide-co-glycolide⁸⁻¹¹, copolymers of L-lactide and DL-lactide¹², polyanhydrides

of bis-carboxyphenoxypropane and sebacic acid¹³, polycaprolactone¹⁴⁻¹⁶, and polyhydroxyalkanoate¹⁷. These antibiotic biodegradable polymers released antibiotics for several hours to 40 weeks *in vitro* and were effective for several weeks *in vivo*^{18,19}. Biodegradation makes second surgical removal of the implant unnecessary. However, the current antibiotic biodegradable polymers are not osteoconductive.

A biodegradable polymer combined with osteoconductive ceramics would be better than a biodegradable polymer alone for use as the local antibiotic delivery carrier. Since biodegradable polymers are not osteoconductive, osteoconductive ceramics have been combined with biodegradable polymers for use as bone substitutes²⁰⁻²³. Recently, cyprofloxacin (CFLX)-loaded poly(D,L)-lactide matrix containing bioglass microspheres of 90–125 μ m diameter showed *in vivo* efficacy in controlling osteomyelitis and osteoconduction^{24,25}. In a previous study, we developed a composite that consists of porous β -tricalcium phosphate (β TCP) ceramic matrix with gatifloxacin (GFLX)-loaded poly- ϵ -caprolactone (PCL) in the pores and on the surfaces of β TCP²⁶. The composite of GFLX-loaded PCL and β TCP released GFLX for 4 weeks *in vivo* and had sustained bactericidal activity against *Streptococcus milleri* and *Bacteroides fragilis*. This composite showed efficacy in controlling infection recurrence at the dead space formed by the debridement of the osteomyelitis lesion and supported bone tissue reconstruction. Twelve and 50 weeks after the implantation, the inflammation even disappeared without intravenous antibiotic

administration. However, the composite remained almost unresorbed even after 50 weeks due to the low resorption rate of PCL. Prolonged presence of a biodegradable polymer also induces intervention of low calcified bone tissues. Therefore, the recommended follow-up strategy is to use a biodegradable polymer having a shorter resorption period while maintaining *in vivo* efficacy in sterilizing the dead space to improve bone tissue regeneration in the dead space.

The resorption rate of resorbable polymers depends on the type of polymer and molecular weight. In the present study, the antibiotic polymers used were GFLX-loaded copolymers of lactic acid and glycolic acid (PLGA) with different molecular weights and lactide/glycolide ratios. A composite was then fabricated from a selected GFLX-loaded PLGA and β TCP and characterized in terms of its *in vitro* antibiotic release efficiency and bactericidal property as well as *in vivo* effectiveness and safety.

MATERIALS AND METHODS

Preparation of GFLX-loaded PLGA

The PLGA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) used in this study had a lactic acid-to-glycolic acid molar ratio of 50:50 or 75:25 with an weight-average molecular weight of 5,000, 10,000, or 15,000 g/mol. The

copolymers were denoted using the lactic acid ratio and molecular weight as PLGA5005, PLGA5010, PLGA5015, PLGA7505, PLGA7510, and PLGA7515, as listed in Table 1.

One gram of PLGA with a molecular weight of 5,000, 10,000, or 15,000 was heated at 120, 130, or 140°C respectively in a glass tube using an aluminum block heater. For each of these molecular weights, these temperatures were correspondingly the lowest ones for heating PLGA to attain sufficiently low viscosity for mixing with GFLX by mechanical stirring (Table 1). These temperatures were 47–67°C lower than the decomposition temperature of GFLX (187°C). Molten PLGA was mixed with 0.5, 1.0, or 2.0 wt% GFLX ($C_{19}H_{22}FN_3O_4 \cdot 1.5H_2O$; LKT Laboratories, Inc., MN, USA) by stirring at 7,000 rpm for 1 minute.

Preparation of GFLX-loaded PLGA disks

Using a metal mold, GFLX-containing molten polymers were solidified into disks measuring 6.35 mm in diameter and 1.2 mm in thickness. The weight of each disk was 90 ± 11 mg.

Preparation of granular composite of GFLX-loaded PLGA5015 and porous β TCP

Commercially available porous β TCP granules (OSferion Type G2-1, Olympus Terumo Biomaterials Corp., Tokyo,

Table 1 Molar ratios of lactic and glycolic acids, molecular weights and inherent viscosities of PLGA used

Polymer	Composition (mol%)		Molecular weight	Viscosity (dL/g)
	Lactic acid (mol%)	Glycolic acid (mol%)		
PLGA5005	50	50	5,000	0.088–0.102
PLGA5010	50	50	10,000	0.122–0.143
PLGA5015	50	50	15,000	0.154–0.186
PLGA7505	75	25	5,000	0.082–0.098
PLGA7510	75	25	10,000	0.119–0.140
PLGA7515	75	25	15,000	0.152–0.185

Fig. 1 (a) Scanning electron microscope image and (b) Light microscope image of β TCP granules with a porosity of 75%, a pore diameter range of 100–400 μ m, and a granule diameter range of 1.0–3.0 mm. (c) Granular composite of GFLX-loaded PLGA and β TCP.

Japan) — with a 75% porosity, a pore diameter range of 100–400 μm , and a granule diameter range of 1.0–3.0 mm— were immersed in molten PLGA5015 containing 1.0 wt% GFLX at 140°C in a glass tube. The glass tube was deaerated to a pressure of 0.02 MPa for 1 minute to load the molten GFLX-loaded PLGA into the pores and onto the surface of the porous β TCP granules.

The thus-prepared granular composite of GFLX-loaded PLGA and β TCP was lightly ground with a zirconia ball mill (Mixer Mill MM301, Retsch GmbH, Haan, Germany) to remove polymer burrs on the granules. Granular composites of two different sizes were thereby obtained: One was 3.6 ± 0.5 mm in size and 13 ± 2 mg in weight, while the other was 2.1 ± 0.5 mm in size and 5.9 ± 2.2 mg in weight. Each initial β TCP granule and the corresponding composite granule (Fig. 1) were weighed to obtain the masses of GFLX-loaded PLGA and GFLX.

In vitro release of GFLX from GFLX-loaded PLGA disks and the granular composite

The minimum duration for antibiotic administration is 4 weeks when treating chronic osteomyelitis in the mandible. Therefore, *in vitro* release tests were performed to examine whether the release of GFLX could be sustained for 4 weeks or longer.

1. GFLX-loaded PLGA disks

For GFLX-loaded PLGA disks, their observation time points were set at 1, 2, 3, 4, 5, 6, 12 and 18 hours, and 1, 2, 3, 5, 7, 10, 14, 28, 42 and 56 days. Five samples from each group were measured at each time point. Therefore, 90 GFLX-loaded PLGA disks per group were placed into glass tubes each containing 10 mL of Hanks' balanced solution (Sigma, MO, USA), and then incubated at 37°C. It was predetermined that 10 mL of Hanks' balanced solution per 90 mg of disk would be used. Besides, the Hanks' balanced solution was not stirred to avoid any stirring-induced deformation of polymers.

Using a UV-Vis spectrophotometer (Model V-550, Jasco Co., Tokyo, Japan), GFLX concentration was determined from the absorbance at 286.2 nm with no background subtraction. Glass tubes of Hanks' balanced solution containing GFLX-free PLGA disks were used as reference solutions for absorbance measurement at the same time points. For comparison, GFLX-loaded PCL disks as per those used in our previous study²⁶⁾ were also subjected to the *in vitro* release test.

2. Granular composite of GFLX-loaded PLGA5015 and β TCP

For the granular composite of GFLX-loaded PLGA5015 and β TCP, their observation time points were set at 1, 2, 3, 4, 5, 6, 12 and 18 hours, and 1, 2, 3, 5, 7, 10, 14, 28 and 42 days. Five composite samples were measured at each time point. Therefore, 85 samples of granular composite were placed into glass tubes each containing Hanks' balanced solution (Sigma, USA), and then incubated at 37°C. The predetermined volume-to-weight ratio was 10 mL of Hanks' balanced solution per 90 mg of composite granule. Since each sample consisted of 40–50 mg of granular composite, the actual volume of Hanks'

balanced solution used was approximately 5 mL per glass tube. Released GFLX was measured, as described above, using the UV-Vis spectrophotometer at 286.2 nm absorbance with no background subtraction.

3. Statistical analysis

Data on released GFLX were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. Statistical significance was set at $p < 0.001$.

In vitro bactericidal properties of heat-processed GFLX and granular composite of GFLX-loaded PLGA5015 and β TCP

1. GFLX heated without PLGA

The bactericidal activity of GFLX heated without PLGA at 140°C for 20 minutes was examined by a microbiological assay using *Escherichia coli* Kp as the test microorganism. Heated GFLX was dissolved in calcium- and magnesium-free phosphate buffered saline solution (PBS(-)) at concentrations of 1, 5, and 25 $\mu\text{g/mL}$. Paper disks of 8 mm diameter were used to absorb the PBS(-) containing heated GFLX.

An aliquot of a suspension of *E. coli* Kp was added to molten heart infusion agar (Becton Dickinson and Company, MD, USA) at a constant concentration. The agar medium was allowed to set in plates. Paper disks loaded with heated GFLX were placed on these agar plates for 18–20 hours at 35°C. After the culture period, inhibitory-zone diameter was measured for the concentration of active GFLX. A diameter-concentration working curve was obtained using paper disks that absorbed PBS(-) solutions containing unheated GFLX with concentrations ranging between 0.05 and 100 $\mu\text{g/mL}$. Data on the bactericidal activities of heated and unheated GFLX were compared using unpaired Student's *t*-test.

2. GFLX heated with PLGA5015

The bactericidal activity of GFLX heated with PLGA5015 at 140°C was also examined using *E. coli* Kp as the test microorganism. GFLX-loaded PLGA5015 disk (90 mg) containing 1% GFLX, prepared *via* heat processing, was dissolved in 5 mL of chloroform. For the control, a GFLX-free PLGA5015 disk and 900 μg of unheated GFLX were dissolved in 5 mL of chloroform. The chloroform solution was mixed with 95 mL of PBS(-) by stirring, and then centrifuged at 3,000 rpm for 5 minutes to remove PLGA5015, as much as possible, that had resolidified in PBS(-).

More than 95% of GFLX was eluted from the PLGA5015 disk into PBS(-) (data not shown). The concentration of active GFLX in PBS(-) was measured by a microbiological assay using *E. coli* Kp as described above. Data on the bactericidal activities of heated and unheated GFLX were compared using unpaired Student's *t*-test.

3. Granular composite of GFLX-loaded PLGA5015 and β TCP

The bactericidal property of the granular composite of GFLX-loaded PLGA5015 and β TCP after eluting GFLX in the Hanks' balanced solution for 1 to 21 days was evaluated by the Kirby-Bauer method using *Streptococcus*

milleri NCTC7331 (*S. milleri*; distributed by the Public Health Laboratory Service, London, UK) and *Bacteroides fragilis* NCTC9343 (*B. fragilis*; distributed by the Public Health Laboratory Service, London, UK)²⁷. *S. milleri* was aerobically cultured at 37°C for 24 hours in heart infusion broth (Eiken Chemical Co., Ltd., Tokyo, Japan), and *B. fragilis* was anaerobically cultured at 37°C for 48 hours in GAM broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) using BBL™ GasPak™ Anaerobic System Envelopes (Becton, Dickinson and Company, MD, USA).

An aliquot of a suspension of *S. milleri* was added to molten heart infusion agar (Eiken Chemical Co., Ltd.) at a concentration of 1.0×10^8 colony forming units per mL (CFU/mL). Similarly, an aliquot of a suspension of *B. fragilis* was added to molten GAM agar (Nissui Pharmaceutical Co., Ltd.) at a concentration of 1.0×10^8 CFU/mL. These agar media were allowed to set in plates. The granular composite of GFLX-loaded PLGA5015 and β TCP after eluting GFLX in a period between 1 and 21 days was placed in a circular area with a diameter less than 6.35 mm on each agar medium. The set agar medium with *S. milleri* and the granular composite was allowed to stand aerobically at 37°C for 24 hours, while the set agar medium with *B. fragilis* and the granular composite was allowed to stand anaerobically at 37°C for 48 hours using BBL™ GasPak™ Anaerobic System Envelopes.

Data on inhibitory zone diameter changes were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test; statistical significance was set at $p < 0.001$. Data on inhibitory zone diameter against *S. milleri* were compared with the clinical criterion of sensitivity (18 mm) by Student's *t*-test; $p < 0.001$ was assumed to be significant.

Implantation of granular composite of GFLX-loaded PLGA5015 and β TCP

Using the Satoh-Heimdahl method, *S. milleri* and *B. fragilis* osteomyelitis was induced on either left or right side in the mandible of six skeletally mature male New Zealand White rabbits weighing from 2.5 to 3.0 kg^{28,29}.

Using aseptic techniques, after an intravenous injection of pentobarbital (25 mg/kg body weight) and an additional local injection of lidocaine hydrochloride, an initial incision was made on the skin covering the mandible. The skin and subcutaneous tissues were separated from the periosteum. A second incision was made in the periosteum of the mandible. The periosteum was elevated and carefully dissected from the underlying mandible. A small cavity of 2 mm diameter and 2 mm depth was made by drilling through the buccal cortex and into the underlying trabecular bone using a 2-mm-round steel bur bit. Collagen sponge (NMP Collagen Sponge, Nippon Meat Packers Inc., Osaka, Japan) containing a suspension of *S. milleri* and *B. fragilis* at 2.5×10^8 CFU was inoculated into the cavity. After inoculation, the periosteum was sutured using a single resorbable suture (4-0 Vicryl, Johnson & Johnson, NJ, USA) and skin incision was tightly sutured using a nylon suture (3-0 Ethilon, Johnson & Johnson). Infection

was allowed to develop for 4 weeks.

At 4 weeks after inoculation, the osteomyelitis lesion of each rabbit was treated by debridement and implantation of the granular composite of GFLX-loaded PLGA5015 and β TCP. Using the aseptic surgical technique as mentioned above, the osteomyelitis lesion was exposed. The infected impaired granulation and bone tissues were debrided as much as possible. The surface of the bone cavity was then further cleaned using a 2-mm round steel burr bit. After washing the bone cavity with a physiological saline, the bone cavity was filled tightly with granular composite of two different size classes. The number of granular composite packed was from 7 to 13 depending on the size of bone cavity. To prevent connective tissue invasion, an absorbable collagen membrane (BioMend, approximately 0.2 mm in thickness; Zimmer Dental Inc., NJ, USA) was placed over the bone cavity filled with the granular composite of GFLX-loaded PLGA and β TCP. The periosteum was sutured using 4-0 Vicryl®, and the wound was closed using 3-0 Ethicon®. Apart from the granular composite of GFLX-loaded PLGA and β TCP, no other antibiotics were administered during the operation and implantation period.

At 4 weeks after implantation, the animals were sacrificed with an overdose of pentobarbital sodium (1.5 mL/kg). Serum and tissue samples from the gingiva, palate, tongue, submandibular lymph nodes, parotid gland, submandibular gland, buccal mucosa, skin of the neck, and skin of the dorsum were collected and stored at -80°C for subsequent determination of transferred-GFLX concentration. Following mandibulectomy, the removed mandible was cut in the middle and subjected to roentgenographic analysis. The mandible was fixed in 10% neutral buffered formalin, decalcified, and embedded in paraffin. The embedded mandible was sectioned horizontally at the center of the implanted site, sliced to a thickness of 5 μ m, and stained with hematoxylin and eosin. All the animal experiments were carried out in accordance with the guidelines of the Ethical Committee of Nippon Dental University and the National Institutes of Health for the care and use of laboratory animals (NIH Pub. No. 85-23 Rev. 1985).

High-performance liquid chromatography (HPLC) for determination of GFLX concentration in tissues and serum

Using a zirconia ball mill at 5,000 rpm for 5 minutes, a tissue sample (0.4 g) was homogenized in 500 μ L of PBS(-) and then centrifugally filtered (Ultrafree-CL, 0.22 μ m pore size; Millipore) to obtain the supernatant. The GFLX concentration in the serum and supernatant were determined by column-switching reversed-phase HPLC with fluorescence detection as reported previously^{26,30}.

Briefly, a 20- μ L sample was directly injected into the HPLC system (LC-10, Shimazu, Kyoto, Japan) using an automatic injector. The sample was loaded onto a pretreatment column (TSK precolumn BSA-ODS, 3.5 cm $\times$ 4.6 mm diameter; Tosoh, Tokyo, Japan) for

deproteinization, followed by analysis using an analytical column (TSKgel ODS-80TM 15 cm×4.6 mm diameter; Tosoh). The excitation and emission wavelengths for fluorescence detection were 295 and 485 nm respectively. The mobile phase used for deproteinization was a 0.01 M phosphoric acid solution containing 0.02% sodium l-octane sulfonate at a flow rate of 1.0 mL/min. The mobile phase used for analysis was a mixture of 0.01 M phosphoric acid and acetonitrile (69:31, vol/vol) containing 0.02% sodium l-octane sulfonate at a flow rate of 1.0 mL/min. Differences in GFLX concentration of tissues between implanted side and un-implanted side were analyzed by paired Student's *t*-test; $p < 0.001$ was assumed to be significant.

RESULTS

In vitro release of GFLX from GFLX-loaded PLGA disks and the granular composite

1. GFLX-loaded PLGA disks

Regardless of the lactic acid-to-glycolic acid molar ratio and molecular weight of the PLGA used, the release of GFLX from GFLX-loaded PLGA disks was sustained for over 4 weeks through early slow-release stage, mid fast-release stage, and late slow-release stage. The early slow-release stage was due to the hydration of PLGA before degradation. The mid fast-release stage was due to PLGA degradation associated with a decrease in molecular weight of PLGA³¹.

During the early slow-release stage, GFLX concentration reached MIC levels for *S. milleri* (0.25

Fig. 2 Release of GFLX from GFLX-loaded disks of (a) PLGA7505; (b) PLGA5005; (c) PLGA5010; and (d) PLGA5015.

* $p < 0.001$: Comparison between PLGA+GFLX0.5% and PLGA+GFLX1%.

** $p < 0.001$: Comparison between PLGA+GFLX0.5% and PLGA+GFLX2%.

*** $p < 0.001$: Comparison between PLGA+GFLX1% and PLGA+GFLX2%.

Fig. 3 Release of GFLX from GFLX-loaded PLGA5015 disks, GFLX-loaded PCL disks, and the granular composite of GFLX-loaded PLGA5015 and β TCP.
 * $p < 0.001$: Comparison between PCL+GFLX1% and PLGA5015+GFLX1%.
 ** $p < 0.001$: Comparison between PCL+GFLX1% and PLGA5015+GFLX1%/ β TCP.
 *** $p < 0.001$: Comparison between PLGA5015+GFLX1% and PLGA5015+GFLX1%/ β TCP.

$\mu\text{g/mL}$) and *B. fragilis* (0.5 $\mu\text{g/mL}$) at 3 hours, 1 and 5 days for PLGA5005, PLGA5010, and PLGA5015 respectively. For PLGA7505 containing 1% GFLX, average value of the cumulative amount of GFLX released for 4 weeks was $112 \pm 29\%$ of the initial GFLX loading amount (Fig. 2(a)). For PLGA5005 containing 1% GFLX (Fig. 2(b)), PLGA5010 containing 1% GFLX (Fig. 2(c)), and PLGA5015 containing 1% GFLX (Fig. 2(d)), their corresponding average values of the cumulative amount of GFLX released for 6 weeks were $114.8 \pm 8.3\%$, $107 \pm 6.3\%$, and $95.6 \pm 9.7\%$ respectively. The differences among these average values were not statistically significant, and on the overall the cumulative GFLX release amount reached 100% for 4–6 weeks. Due to the existence of measurement error, some of these average values exceeded 100%. The error probably arose from an overlap in absorbance at 286.2 nm caused by factors including degraded PLGA. PLGA with 75% lactic acid acidified the Hanks' balanced solution sooner and more intensely than PLGA with 50% lactic acid. As intense acidification could trigger inflammation, PLGA with 75% lactic acid was excluded from subsequent experiments.

The kinetics of GFLX release from PLGA was different from that of GFLX release from PCL. The release curve of PCL was regressed as $(\text{GFLX concentration}) = 5.6297 \times (\text{day})^{0.41315}$, $r = 0.9947$ for the entire 6 weeks' release, while that of PLGA disk comprised three almost linear parts corresponding to the early

Fig. 4 Bactericidal properties of the granular composite of GFLX-loaded PLGA5015 and β TCP against *S. milleri* and *B. fragilis* after eluting GFLX in Hanks' balanced solution for 1–21 days.
 * $p < 0.001$: Comparison against day 1.
 ** $p < 0.001$: Comparison against the clinical criterion of sensitivity (18 mm).

slow-release stage, mid fast-release stage, and late slow-release stage (Fig. 3). At the early slow-release stage, the amount of GFLX released from PLGA was lower than that from PCL during the corresponding early period. Beyond this stage, the rate of GFLX release from PLGA accelerated and it even became higher than that of PCL. Finally, GFLX was released more completely from PLGA than from PCL after 6 weeks.

2. Granular composite of GFLX-loaded PLGA5015 and β TCP

GFLX release from the granular composite of GFLX-loaded PLGA5015 and β TCP was also sustained for 4 weeks (Fig. 3). For GFLX-loaded PCL disks, GFLX-loaded PLGA5015 disks, and the granular composite of GFLX-loaded PLGA5015 and β TCP, their average values of the cumulative amount of GFLX released for 6 weeks were $48.3 \pm 6.7\%$, $95.6 \pm 9.7\%$, and $130 \pm 14\%$ of the initial GFLX loading amount. Note again the existence of measurement error which caused some average value to exceed 100%. The error probably arose from an overlap in absorbance at 286.2 nm caused by factors including degraded PLGA and/or β TCP.

In vitro bactericidal properties of heat-processed GFLX and granular composite of GFLX-loaded PLGA5015 and β TCP

1. GFLX heated with and without PLGA

Heat-processed GFLX retained its bactericidal property. GFLX heated at 140°C without PLGA5015 for 20 minutes

Fig. 5 Histopathologic sections of: (a) osteomyelitis lesion induced by *S. milleri* and *B. fragilis* at 4 weeks after inoculation and without implantation of the granular composite of GFLX-loaded PLGA and β TCP; and (b) new bone formation after a 4-week implantation of granular composite of GFLX-loaded PLGA and β TCP. Without implantation, sequestrum (+) was observed in the expanded inflammatory area (*); with implantation, new bone formation (*) was observed on the surface and in the pores of β TCP (#).

had a bactericidal activity that corresponded to $101 \pm 4.3\%$ that of unheated GFLX. According to unpaired Student's *t*-test, there were no statistically significant differences in bactericidal activity between unheated GFLX and the heated GFLX ($p=0.770$). GFLX heated at 140°C with PLGA5015 had a bactericidal activity that corresponded to $100 \pm 1.0\%$ that of unheated GFLX. Similarly, there were no statistically significant differences in bactericidal activity ($p=0.986$) as determined by unpaired Student's *t*-test.

2. Granular composite of GFLX-loaded PLGA5015 and β TCP

The granular composite of GFLX-loaded PLGA5015 and β TCP demonstrated sustained *in vitro* bactericidal activities against *S. milleri* and *B. fragilis*. If the inhibition zone diameter is larger than 18 mm against *S. milleri*, it means that the strain is sensitive to GFLX, and thus, the loaded-GFLX has clinical efficacy for the strain. In this study, the inhibitory zone diameters of the granular composite of GFLX-loaded PLGA5015 and β TCP against *S. milleri* were statistically larger than 18 mm up to 14 days of GFLX elution in the Hanks' balanced solution (Fig. 4). The inhibitory zone diameters initially increased with increasing elution period, which would correspond to the early slow-release stage.

In vivo efficacy of granular composite of GFLX-loaded PLGA5015 and β TCP against infection and toward bone tissue reconstruction

Without implantation of the granular composite, inflammatory cells including neutrophils and lymphocytes markedly infiltrated around the separated

Fig. 6 New bone formation with vascularization was observed in the pores of β TCP (#).

and necrotized bone associated with an unhealed cortical defect (Fig.5(a)). On the other hand, 4 weeks after the implantation, inflammatory cell infiltration disappeared in half of the rabbits in the debrided area. Even though the inflammation remained in the debrided area, the inflammation area became much smaller than the debrided area. In areas outside the debrided area, $0.7\text{--}2.5\text{ mm}^2$ of inflammation area and $0.1\text{--}0.8\text{ mm}^2$ of sequestrum area were observed in all specimens. However, these areas were much smaller than the originally infected and debrided area which was

Table 2 GFLX concentrations in tissues ($\mu\text{g/g}$) and serum ($\mu\text{g/mL}$) at 4 weeks after implantation of the granular composite of GFLX-loaded PLGA5015 and βTCP

		4 weeks	serum ratio
Serum		0.025	1
Gingiva	(I)*	0.0092 $\pm$ 0.0002	3.68
	(U)*	0.0089 $\pm$ 0.06	3.56
Palate		0.0049 $\pm$ 0.08	1.96
Tongue		0.0211 $\pm$ 0.06	8.44
Submandibular lymph node	(I)	0.0442 $\pm$ 0.01	17.68
	(U)	0.0212 $\pm$ 0.02	8.48
Parotid gland	(I)	0.0231 $\pm$ 0.03	9.24
	(U)	0.0189 $\pm$ 0.06	7.56
Submandibular gland	(I)	0.0207 $\pm$ 0.005	8.28
	(U)	0.0196 $\pm$ 0.01	7.84
Buccal mucosa	(I)	0.0037 $\pm$ 0.1	1.48
	(U)	0.0022 $\pm$ 0.1	0.88
Neck Skin		<0.005	–
Dorsum Skin		0.0019 $\pm$ 0.01	0.76

* (I): Implanted side, (U): Un-implanted side

The detection limits for GFLX concentration are 0.005 $\mu\text{g/g}$ for the tissues and 0.03 $\mu\text{g/mL}$ for serum.

approximately 20–30 mm² in area. In one of the six rabbits, the cortical defect was even completely closed.

Based on the barely noticeable presence of residual βTCP in the bone tissue, it was highly likely that the granular composite of GFLX-loaded PLGA5015 and βTCP was almost completely resorbed. New bone was formed around the βTCP granules and in their pores, and was continuously connected with surrounding bone regardless of presence and absence of inflammation (Fig. 5(b)). Besides, it is shown in Fig. 6 that vascularization accompanied new bone formation. On average, the total dose of GFLX per rabbit was 635 $\pm$ 28 μg .

All these lines of evidence demonstrated that the granular composite of GFLX-loaded PLGA5015 and βTCP were capable of preventing infection recurrence, supporting bone and vascular formation, and being resorbed at the dead space formed by the debridement of the osteomyelitis lesion.

HPLC determination of GFLX concentration in tissues and serum

GFLX concentrations in tissues demonstrated sustained release of GFLX *in vivo* (Table 2). High GFLX concentrations were recorded in average in submandibular lymph node, parotid gland, and submandibular gland on the implanted side, whereas low GFLX concentrations on the un-implanted side. Among the examined tissues, only the submandibular lymph node had a statistically significant difference in GFLX concentration between the implanted and un-implanted sides. GFLX concentrations of half of neck and dorsum skin samples are under detection limit

(<0.005 $\mu\text{g/g}$).

The non-uniformity in GFLX distribution among the tissues was an evidence of the local delivery of GFLX from the implanted granular composite. Very importantly too, the serum GFLX concentration was lower than detection limit, which indicated a low risk of inducing adverse effects of GFLX.

DISCUSSION

The granular composite of GFLX-loaded PLGA5015 and βTCP was successfully fabricated by melt compounding with almost no decrease in the bactericidal property of GFLX. Like other fluoroquinolone antibiotics (with decomposition temperatures in the range of 220–310°C), GFLX has a high decomposition temperature (187°C). In this study, PLGAs were melted in the temperature range of 120–140°C for mixing with GFLX and the resulting mixture was loaded into the pores of βTCP granules. As heat processing was done 47–67°C below GFLX's decomposition temperature, the thus heat-processed GFLX retained its bactericidal property. Based on the elution test results, PLGA with a high lactic acid content (PLGA7505, PLGA7510, and PLGA7515) was excluded due to rapid and intense acidification during dissolution. PLGA5015 was finally selected as the delivery carrier of GFLX.

Typically, chronic osteomyelitis lesions consist of sequestrum surrounded by avascular inflamed soft tissues and sclerotic bone surfaces which prevent intravenous antibiotics from being delivered effectively—despite the elevation in blood drug concentration through this administration route. Therefore, an

alternative and potentially promising treatment would be a three-fold combination of: (i) surgical debridement of infected tissues; (ii) scaffold-supported bone tissue reconstruction with vascularization at the debrided site; and (iii) local antibiotic delivery using a resorbable polymer in addition to systemic antibiotic administration to prevent infection recurrence during tissue reconstruction.

In our previous study²⁶⁾, a composite of GFLX-loaded PCL and β TCP porous ceramic was developed. However, only limited bone tissue reconstruction and vascularization were observed at the debrided site even after 50 weeks of implantation²⁶⁾. In the present study, the granular composite of GFLX-loaded PLGA5015 and β TCP exhibited *in vivo* efficacy for the reconstruction of bone and vascular channels in the dead space created by the debridement of chronic osteomyelitis lesion. Scaffold-supported bone tissue reconstruction and vascularization were accomplished at 4 weeks after implantation. The vascular channels thus formed would significantly contribute to the sterilization of the debrided site *via* systemic antibiotic administration.

In general, synthetic resorbable polymers are not osteoconductive. Hence, a prolonged presence of such polymers prevents bone tissue reconstruction and vascularization. The composite of GFLX-loaded PCL and β TCP porous ceramic in our previous study²⁶⁾ remained mostly unresorbed due to the low dissolution rate of PCL. Apart from type of resorbable polymer, another factor that influences resorption is the molecular weight of resorbable polymer. Pellets of PLGA-ciprofloxacin-bioglass composite showed only minor structural changes after a 6-month implantation in the proximal rabbit tibia due to the high molecular weight of the PLGA used (6.62 dL/g in inherent viscosity)²⁵⁾. In contrast, PLGA in the present granular composite of GFLX-loaded PLGA and β TCP was mostly resorbed after a 4-week implantation due to a lower molecular weight of the PLGA used (0.15–0.19 dL/g in inherent viscosity).

As PLGA resorption progressed, the osteoconductive surface of β TCP became gradually exposed, thereby resulting in early bone tissue reconstruction with normal vascularity. During the period of bone tissue reconstruction, the granular composite of GFLX-loaded PLGA and β TCP also released a sufficient dose of GFLX (Fig. 4) to sterilize the debrided site. The localized release of GFLX was indeed confirmed by a non-uniform distribution of GFLX in tissues around the implanted site (Table 2). In addition, local acidification which arose from the degradation of PLGA could have further contributed to the sterilization of the dead space and degradation of β TCP. In the infection control and management of osteomyelitis, an early replacement of the polymer with vascular tissues is important because the polymer in the avascular infected site can become colonized with bacteria, as in the cases of antibiotic-impregnated poly(methyl methacrylate) beads and cement^{32,33)}.

Based on the amount of GFLX loaded and the weight

of the experimental rabbits (approximately 3 kg), the dose of GFLX administered was calculated to be 212 μ g of GFLX/kg body weight, which corresponded to 1/27 of a single daily dose of GFLX for adults with a body weight of 70 kg (5.71 mg of GFLX/kg body weight)³⁴⁾. Long-term oral administration of GFLX can induce adverse reactions including abnormal blood sugar level, diarrhea, gastric discomfort, anorexia, dizziness, and headaches; but such a low dose of GFLX poses a low risk for systemic toxicity. However, although a low dose of GFLX was released from the granular composite of GFLX-loaded PLGA and β TCP, it effectively caused the inflammation in the affected area to decrease and even disappear in 50% of the rabbits without systemic antibiotic administration—thereby fulfilling the purpose of infection control which is a principal concern in the treatment of chronic osteomyelitis.

CONCLUSION

Based on the experimental strategy of this study, the following findings were obtained:

1. GFLX retained its bactericidal property after heat processing below its decomposition temperature.
2. The granular composite of GFLX-loaded PLGA and β TCP released GFLX for 4 weeks in Hanks' balanced solution, and demonstrated sustained *in vitro* bactericidal activities against *S. milleri* and *B. fragilis* for at least 3 weeks.
3. In the rabbit mandible, the granular composite of GFLX-loaded PLGA and β TCP showed efficacy in controlling infection as well as supporting bone tissue reconstruction and vascularization in the dead space created by the debridement of osteomyelitis lesion induced by *S. milleri* and *B. fragilis*.

Bone tissue reconstruction and vascularization resulted from an early exposure of the osteoconductive surface of β TCP due to the resorption of PLGA. Concomitant with the resorption of PLGA is the sustained bactericidal activity. Therefore, the granular composite of GFLX-loaded PLGA and β TCP is a promising local antibiotic delivery means to control infection recurrence in dead space caused by the debridement of osteomyelitis lesions.

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