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RESEARCH ARTICLE

# Development and evaluation of dual-controlled release antibiotic-loaded bone scaffolds

## Supplementary file

### S1. Preparation of RM-PLGA microspheres

Moxifloxacin/rifampicin—poly(lactic-co-glycolic acid) (PLGA) microspheres were prepared via the double emulsion–solvent evaporation method, with the outer phase serving as the oil phase, and the inner phase as the aqueous phase. Liquid paraffin, petroleum ether, and moxifloxacin were added to a beaker and stirred until fully saturated. Then, the solution was centrifuged, and the supernatant was collected; 0.1% Span 85 was added to the supernatant and stirred again to form the outer phase. An appropriate amount of PLGA was dissolved in an acetonitrile and isopropanol solution, vortexed, and ultrasonicated. Subsequently, moxifloxacin and rifampicin were added and vortexed again. Dichloromethane was added to the mixture and vortexed, forming the inner phase. After the outer and inner phases were prepared, an appropriate amount of the outer phase was transferred to a beaker for shearing and reserved. The inner phase was added to the outer phase and emulsified using a high-speed disperser, after which the remaining outer phase was added and mixed on a vortexer. During vortexing, small pink particle microspheres were observed in the solution. After centrifugation and discarding the supernatant, the microspheres were washed with detergent, vortexed, and centrifuged. The resulting microspheres were then fully dried on a culture dish to obtain moxifloxacin/rifampicin–PLGA microspheres.

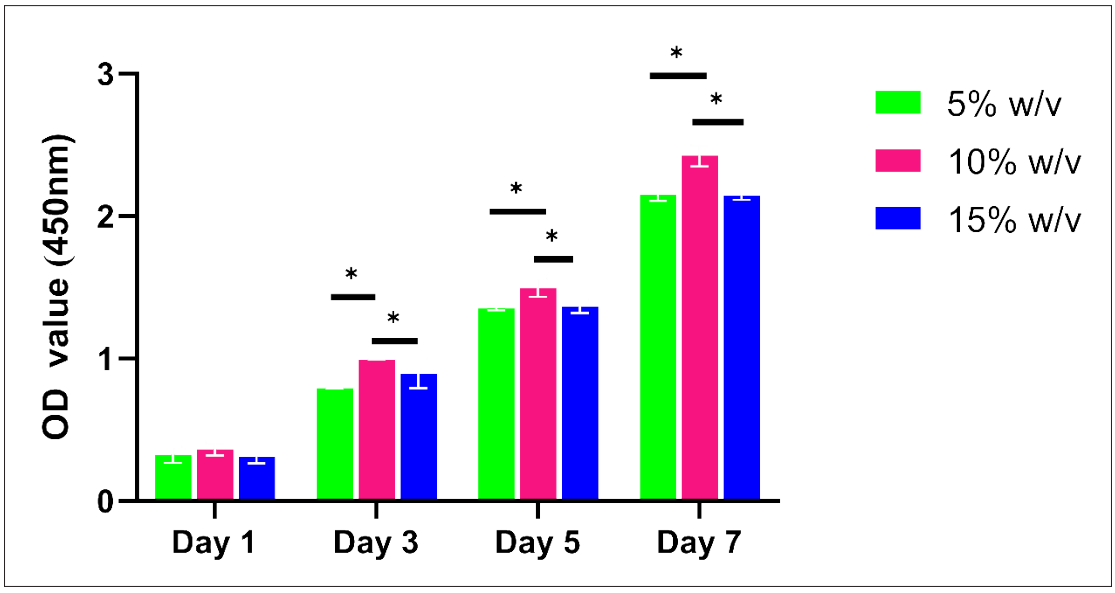


**Figure S1.** MC3T3 cells were co-cultured with 5%, 10%, and 15% (w/v) different concentrations of hydrogel for 24 h. Then, a CCK8 cell proliferation assay was conducted, and the absorbance value at 450 nm wavelength was detected using a microplate reader.

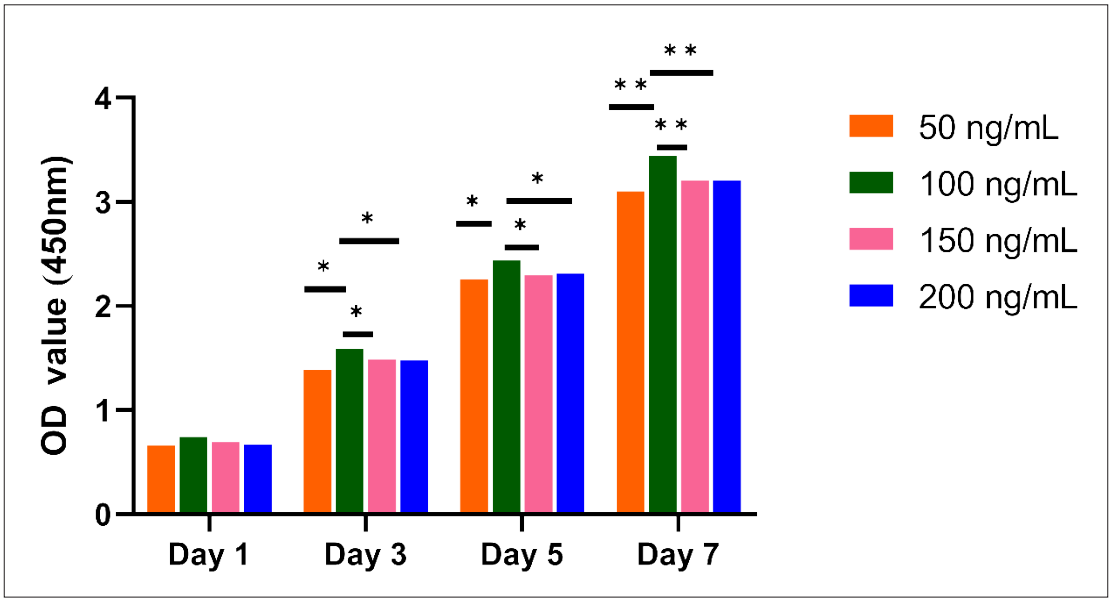


**Figure S2**. MC3T3 cells with 50, 100, 150, and 200 ng/mL bFGF for 24 h, a CCK8 cell proliferation assay was conducted. The absorbance value at a wavelength of 450 nm was measured using an enzyme reader.

## S2. Sustained-release testing of drugs

The support was placed in a dialysis bag with a relative molecular weight of 3500. The two ends of the dialysis bag were sealed, and 1 mL of phosphate-buffered saline (PBS) was added. The bag was transferred into a polyethylene (PE) tube containing 5 mL of PBS, the PE tube was sealed, and incubation was carried out in a constant-temperature water bath at 37°C. Samples were collected at 3, 6, 12, and 24 h, as well as on days 2, 3, 5, 7, 10, 14, 21, and 27. At each time point, 5 mL of the sample was collected and stored in a sealed container at −20°C for analysis. Subsequently, 5 mL of fresh PBS was added to the PE tube to maintain constant volume. The content of moxifloxacin in the release solution was determined using an ultraviolet spectrophotometer. The release amount was calculated as:

$$\text{Release amount (\%)} = \frac{m1}{m2} \times 100\% \quad \text{(I)}$$

where $m1$ is the amount of moxifloxacin and rifampicin released by the microspheres into the buffer, and $m2$ is the total amount of moxifloxacin and rifampicin in the microspheres.

## S3. Slow-release detection of fibroblast growth factor-basic

The composite scaffold was placed in 1 mL of buffer solution and incubated at 37°C. At the predetermined time points (days 1, 3, 5, 7, 14, 21, and 28), the supernatant was collected and replaced with an equal volume of fresh buffer. The release amount of Fibroblast Growth Factor-basic (bFGF) in the buffer was determined using a bFGF ELISA kit. Briefly, 100 μL of the diluted standard solution was added to the standard wells, 100 μL of standard solution and sample diluent to the blank wells, and 100 μL of sample to the remaining wells. The microplate was covered and incubated at 37°C for 90 min. Subsequently, 100 μL of the working solution of biotinylated antibody was added to each well, the plate was covered, and incubation was continued at 37°C for 1 h. Each well was then washed three times with 350 μL of washing solution, with soaking for 1 min per wash. Next, 100 μL of the working solution of Horseradish peroxidase-conjugated enzyme was added to each well, followed by incubation at 37°C for 30 min. The wells were then washed five times, after which 90 μL of 3,3′,5,5′-Tetramethylbenzidine substrate solution was added to each well. The plate was incubated at 37°C in the dark for about 15 min. Finally, 50 μL of stop solution was added to each well to terminate the reaction, and the optical density of each well was immediately measured at 450 nm using a microplate reader.

## S4. Live imaging detection of small animals

All Sprague-Dawley rats inoculated with fluorescent enzyme bacteria were subjected to real-time in vivo fluorescence analysis at 0, 7, and 14 days after surgery. At specific time points, the animals were first anesthetized

with isopentane gas for 5 min. Once adequate anesthesia was achieved, the animals were placed in the small-animal in vivo imaging system. The relevant imaging parameters were adjusted, and after 5 min of exposure, bioluminescence images were acquired. Based on the areas of concentrated bacterial luminescence, circular regions of interest (ROIs) were defined using the built-in processing software (Living Image) to obtain and compare the average fluorescence intensity (unit: p/s/cm$^2$/sr) within each ROI. The in vivo imaging data presented in this study were randomly selected from three animals per group.

## S5. Western blot assay for osteogenic markers

Soft tissue samples were collected from the modeling area of Sprague-Dawley rats (around the implanted stent) and washed with pre-cooled PBS to remove surface blood and impurities. The samples were cut into pieces using scissors, and RIPA lysis buffer containing protease and phosphatase inhibitors was added. The lysed samples were centrifuged to remove cell debris and insoluble substances, and the supernatant was carefully transferred to a new centrifuge tube. The extracted protein samples were then used for Western blot analysis of osteogenic markers (RUNX2 and OPN).

## S6. Reverse transcription-quantitative polymerase chain reaction for detecting osteogenic differentiation-related genes

Soft tissue samples were collected from the modeling area of Sprague-Dawley rats (around the implanted stent) and washed with pre-cooled PBS to remove surface blood and impurities. The samples were cut into pieces using scissors, and RNA was extracted and reverse transcribed. Quantitative polymerase chain reaction was used to detect osteogenic differentiation genes (*Runx2* and *Opn*).

**Table S1. Primers used in RT-qPCR for detecting osteogenic differentiation genes**

| Gene | Primer sequence (5'–3') | |
|---|---|---|
| | Forward | Reverse |
| *Runx2* | ACTCCAAGACCCTAAGAAACCGAT | TGGCTCCTCCCTTCTCAACCTC |
| *Opn* | CTCAGAATTCAGCCAGGAGAAC | CCAAACAGGCAAAAGCAAAT |
| *Actb* | ACATCCGTAAAGACCTCTATGCC | TACTCCTGCTTGCTGATCCAC |

Abbreviation: RT-qPCR, reverse transcription quantitative polymerase chain reaction.