

# Rifampicin liposomes loaded on calcium phosphate cement scaffold constructed using 3D printing technology for sustained drug release

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Rifampicin (RFP) is used for the treatment of chronic bone tuberculosis owing to its powerful and wide spectrum antibacterial activities. However, effective concentrations of RFP required to treat bone tuberculosis are maintained for a short time in vivo, and adverse reactions and bone defects may occur after surgery. Therefore, the construction of a new drug delivery system to overcome these problems is required. In this study, we designed, constructed, and demonstrated the applicability of a calcium phosphate cement scaffold loaded with RFP liposomes for the treatment of bone tuberculosis. RFP liposomes were prepared using a film dispersion method. The preparation method was optimized using the encapsulation rate as an indicator and the morphology, mean particle size, zeta potential, encapsulation rate, and drug loading of RFP liposomes were characterized. Calcium phosphate cement scaffolds were constructed using 3D printing technology and used as RFP liposome carriers for sustained-release drug delivery. Finally, some of the properties were verified in vivo through experiments in rabbits. The results indicated that composite scaffolds can provide sustained drug release and are a promising treatment option for bone tuberculosis.

**Keywords:** Rifampicin. Liposomes. 3D printing technology. Calcium phosphate cement. Bone tuberculosis.

## INTRODUCTION

Tuberculosis (TB) is among the deadliest infectious diseases (Fernandes *et al.*, 2022). As the floating population grows, so does the prevalence of immunodeficiency diseases such as acquired immunodeficiency syndrome, as well as other chronic conditions such as diabetes and hepatitis B. This increase increases the risk of infection with *Mycobacterium tuberculosis* (Furin, Cox, Pai, 2019; Drobnik *et al.*, 2017). China has the world's second-largest TB epidemic after India. About 10–15% of all patients with TB have extrapulmonary TB, with the leading type being that of the bones and joints (Chakaya *et al.*, 2021; Sharma, Mohan, Kohli, 2021; Chen *et al.*, 2015). Some studies have found that delayed diagnosis or treatment of multidrug-resistant *Mycobacterium tuberculosis* infection

increases the risk of spinal and bone joint damage and even death (Hua *et al.*, 2021). Therefore, patients with bone TB often undergo surgery combined with anti-TB drug treatment. However, because the bone TB lesion tissue is surrounded by several layers of connective tissues and does not have a blood supply, anti-TB drugs mostly fail to penetrate these tissues and achieve a therapeutic effect (Zhu *et al.*, 2015). In addition, most drugs cannot effectively kill TB bacilli even in non-sclerosing necrotic and caseous lesions owing to environmental factors, such as insufficient drug concentration or hypoxia, which contribute to bone TB recurrence (Liang *et al.*, 2019). Therefore, the direct administration of anti-TB drugs into lesions would maintain an effective bactericidal concentration and effectively treat bone TB.

In recent years, several materials have been developed for repairing bone TB lesions (Fang *et al.*, 2022; Dong *et al.*, 2014; Zhao *et al.*, 2015). However, these materials exhibit several disadvantages. Initially, these materials are limited to mending bone defects without any anti-

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TB properties. Furthermore, individuals with bone TB experience prolonged recovery periods compared to those with other bone conditions, because the absorption and degradation rates of these substances fall short of the human body's needs for bone regeneration (Wang, Gao, Hao, 2020). Therefore, in this study, we propose a novel bone reconstruction material comprising a drug-loaded cement scaffold for the treatment of TB bone defects. This novel material can be used to fill the lesions, eradicate the TB bacilli via sustained drug release, and induce bone formation.

Rifampicin (RFP) is a powerful broad-spectrum antibacterial drug that is often used to treat TB in clinical practice (Hua, Qian, Lei, 2022; Zumla, Nahid, Cole, 2013). However, high oral doses of RFP are required for wide distribution throughout the body, resulting in several adverse reactions. Furthermore, the effective treatment concentration is maintained for only a short time, allowing the re-growth of *Mycobacterium tuberculosis* (Khadka *et al.*, 2021). Therefore, to overcome these problems, we prepared RFP liposomes using thin-film dispersion to prolong the retention time and maintain the drug at high concentrations in the lesion.

Bone cement implantation is commonly used to repair bone defects and restore its structure after tuberculous lesion resection. Calcium phosphate bone cement has good biocompatibility (Xu *et al.*, 2023), and being similar to the mineral components of bone, it can bind well with bone at the defective site, preventing fibrous tissue formation during the bone repair process. Furthermore, it has good biodegradability, mechanical strength and is suitable for bone grafting (Parasaram *et al.*, 2019). Currently, calcium phosphate bone cement is used as a carrier for many drugs, such as antimicrobial, antitumor, and anti-osteoporosis agents, to achieve the desired therapeutic effects (Costa, Grenha, 2013). Presently, polymethyl methacrylate, calcium phosphate, and silicate are commonly used to prepare bone cement for clinical use. Among these, calcium phosphate is the most widely used for clinical applications due to its excellent biocompatibility, biodegradability, bone conductivity,

and mechanical properties, including strong plasticity (Gong, Zhang, Yan, 2022).

3D printing, also known as rapid prototyping technology, can be applied to medical imaging data to accurately construct a physical model of a specific anatomical structure of a patient. It can convert virtual images into three-dimensional physical objects, providing advantages such as personalization and high accuracy. This technology has been used to develop personalized treatment, particularly in the field of orthopedics (Suwanprateeb, Sanngam, Panyathanmaporn, 2010). Due to the biocompatibility, degradation, and excellent photothermal effects of polypyrrole, 3D-printed bioceramic scaffolds with uniform self-assembled calcium-phosphorus/polypyrrole surface nanolayers can be utilized to achieve anti-tumor effects and osteogenic regeneration (Liu *et al.*, 2023). Thus, 3D printing technology holds wide applicability for the effective treatment of extensive bone defects arising from bone surgeries, demonstrating appreciable therapeutic outcomes. In this study, the anti-TB agent RFP was loaded on liposomes using the film dispersion method to construct a sustained-release system. A calcium phosphate bone cement scaffold was fabricated using 3D inkjet printing technology based on tissue engineering principles. Finally, the prepared liposomes were combined on the calcium phosphate scaffold to prepare a functional composite material with bone repair and anti-TB therapeutic effects. The RFP liposome-loaded calcium phosphate cement scaffold ensured sustained drug release that maintains an effective bactericidal concentration in the bone.

## MATERIAL AND METHODS

### Material

Rifampicin standard was purchased from the National Institutes for Food and Drug Control (Beijing, China), soybean lecithin from Lipoid GmbH (Baden-Wuerttemberg, Germany), cholesterol from Meilun Bio (Suzhou, China), chloroform from Xilong Scientific Co. Ltd. (Guangdong, China), and nano- $\beta$ -tricalcium phosphate from Sigma (Missouri, USA).

Preparation of RFP liposomes

RFP liposomes were prepared using the film-dispersion method. Briefly, soybean lecithin and cholesterol were dissolved in chloroform at various ratios. The chloroform solvent was evaporated using a rotating evaporator for 30 min at an appropriate temperature. Once the lipid film was produced, RFP dissolved in PBS (pH 7.4) was added to obtain an emulsion, which was magnetically stirred for 10 min and sonicated for 20 min until the mixture became translucent. Then, an RFP liposome nanoemulsion was obtained by filtration through a 0.45 µm microporous membrane.

Optimization of RFP liposome preparation

Based on the results of the single-factor investigation, the RFP-to-soybean lecithin mass ratio, hydration temperature, and cholesterol-to-soybean lecithin mass ratio were selected as factors that strongly influenced the physicochemical properties of RFP liposomes, such as encapsulation rate, drug loading, and particle size. Furthermore, an orthogonal test with three factors and three levels was used to screen for the optimal formulation and processing of RFP liposomes, with encapsulation efficiency as the investigation index. These factors and their levels are listed in Table I.

TABLE I - Design factors and levels

| Level | Factor                              |                                |                                             |
|-------|-------------------------------------|--------------------------------|---------------------------------------------|
|       | A<br>RFP: Soybean lecithin<br>(m/m) | B<br>Hydration temperature, °C | C<br>Cholesterol: Soybean lecithin<br>(m/m) |
| 1     | 1:6                                 | 25                             | 1:3                                         |
| 2     | 1:8                                 | 30                             | 1:4                                         |
| 3     | 1:10                                | 35                             | 1:5                                         |

Encapsulation efficiency and drug loading ability

RFP liposomes were centrifuged for 20 min (18000 rpm/min). The amount of RFP in the supernatant after centrifugation (W1), representing the non-encapsulated drug, was determined by high-performance liquid chromatography (HPLC). An equal amount of RFP liposomes was destroyed by sonication with methanol, and

the total amount of RFP (W0) was similarly determined using HPLC. The drug-loading efficiency (DL) and encapsulation efficiency (EE) of RFP liposomes were calculated using formulas (1) and (2), respectively, where W2 is the total weight of the RFP liposomes.

$$EE(\%) = \frac{W_0 - W_1}{W_0} \times 100 \tag{1}$$

$$DL(\%) = \frac{W_0 - W_1}{W_2} \times 100 \tag{2}$$

Characterizations of RFP liposomes

Several methods were employed to investigate the morphology, particle size, and zeta potential of RFP

liposomes. The appearance and morphology of liposomes were observed and photographed using transmission electron microscopy. A sample from the prepared

RFP liposome solution was dropped on the copper net followed by one drop of 2% phosphotungstic acid, left to dry naturally for 10 min, and then observed and photographed. The particle size distribution, zeta potential, and polydispersity index (PDI) of RFP liposomes were measured using a Malvern laser particle size analyzer.

### **Preparation of RFP liposome-loaded calcium phosphate cement scaffold**

The preparation begins with the formulation of the premix solution, mixing acrylic resin monomer, dispersant BYK111 (1 wt%, relative to the ceramic powder mass), organic additive tributyl citrate (3 wt%, relative to the ceramic powder mass), and photoinitiator TPO (1 wt%, relative to the mass of acrylic acid). The premix requires stirring for 15–30 min until uniform. Calcium phosphate ceramic powder is then gradually added to the aforementioned premix, and through ball milling for 6 h, a ceramic slurry with low viscosity and high solid content is prepared, wherein the solid content mass fraction is 75%. A 3D porous scaffold model was designed using computer-aided design software, the data of which were imported into the control software of the ceramic laser 3D printer, and the ceramic paste was injected into the tray. The laser 3D printing parameters were adjusted to create a ceramic biscuit. The printed ceramic pigment biscuit was degreased and sintered in an electric furnace (1150 °C) to obtain the final scaffold.

The prepared calcium phosphate cement scaffold was cut according to the size of the bone wounds. RFP liposomes were added to centrifuge tubes and mixed thoroughly. The liposomes were then centrifuged at 4000 rpm/min for 15 min to fill all the pores of the calcium phosphate bone cement scaffold. The supernatant was discarded, and the calcium phosphate bone-cement scaffold loaded with RFP liposomes was obtained and placed in a vacuum freeze-dryer for 24 h to maintain the quality. The RFP scaffolds were prepared similarly.

### **Determination of loading capacity of calcium phosphate cement scaffold for RFP liposomes**

Calcium phosphate cement scaffolds loaded with RFP liposomes or RFP were crushed ( $n = 6$ ), 2 mL of methanol were added, and the supernatant was centrifuged and analyzed by HPLC to calculate the RFP content.

### **Effect of RFP liposome-loaded scaffolds on drug delivery and bone repair in rabbit models**

Chinchilla rabbits (6 months old) were supplied by the Laboratory Animal Center of Zhejiang University and divided into the RFP liposome and RFP groups ( $n = 9$ ). A rabbit femoral defect model was established and the rabbits in the two groups were implanted with scaffolds loaded with RFP liposomes or RFP. Each rabbit was uniformly created with a femoral defect model approximately  $10.5\text{mm} \times 10.5\text{mm} \times 5.5\text{mm}$  in size. To avoid contact contamination and reduce mechanical stress, we used laser cutting to achieve the required size and shape of the scaffolds. This method effectively preserved the integrity of the scaffold structure, with no instances of cutting fractures or damage observed in this experiment. The final scaffolds loaded were about  $10\text{mm} \times 10\text{mm} \times 5\text{mm}$  in size to maintain the consistency of the baseline dosing by controlling for the same size. Three rabbits from each group were sacrificed each week after the operation and the scaffolds were removed. The RFP content was determined to evaluate the rate of drug release.

## **RESULTS**

As shown in Table II, orthogonal experiments were conducted to optimize the formulation. According to the range (R) visual analysis, the influence of each factor on the encapsulation rate of liposomes was in the order  $B > A > C$ . Based on these results, the optimal formulation was determined to be A2B3C2 as follows: a RFP to soybean lecithin mass ratio of 1:8, a hydration temperature of 35 °C, and a cholesterol to soybean lecithin mass ratio of 1:4.

**TABLE II** - Results of orthogonal experiment

| Serial number | A     | C     | EE (%) |
|---------------|-------|-------|--------|
| 1             | 1     | 1     | 29.32  |
| 2             | 1     | 2     | 21.93  |
| 3             | 1     | 3     | 42.73  |
| 4             | 2     | 2     | 47.06  |
| 5             | 2     | 3     | 59.45  |
| 6             | 2     | 1     | 33.65  |
| 7             | 3     | 3     | 49.55  |
| 8             | 3     | 1     | 37.68  |
| 9             | 3     | 2     | 31.56  |
| K1            | 31.33 | 33.55 |        |
| K2            | 46.70 | 33.52 |        |
| K3            | 39.60 | 50.56 |        |
| R             | 15.38 | 17.04 |        |

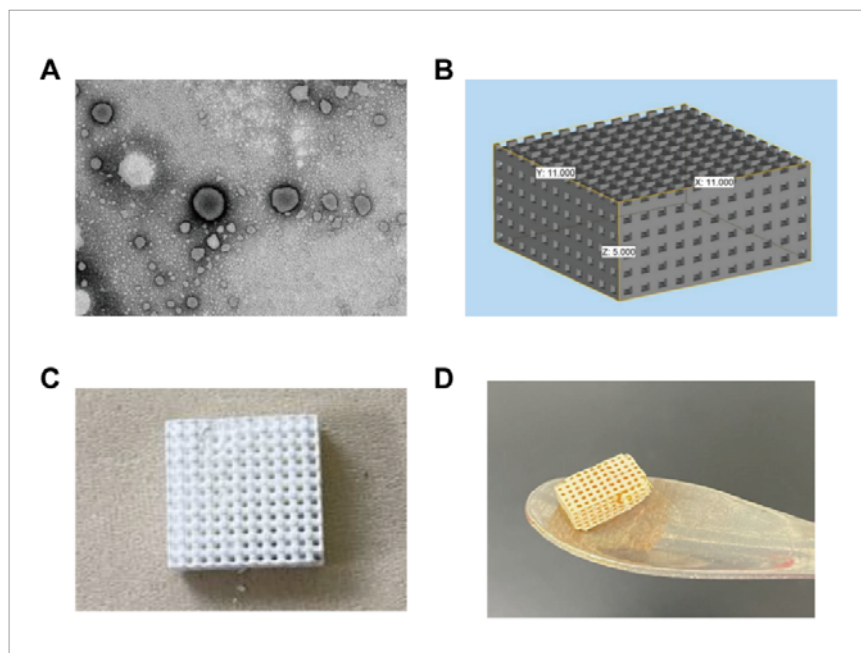
K1, K2, and K3: mean values of the three levels, R: range.

Three batches of RFP liposomes were prepared according to the optimal formulation determined by orthogonal experiments. The encapsulation efficiencies of the RFP liposomes were 49.56%, 49.95%, and 50.31%, with an average value of 49.94%. The drug loadings of the liposomes were 21.50%, 19.40%, and 20.00% with an average value of 20.30%. We considered the optimal formulation prepared according to the orthogonal experiments to be stable and feasible because the encapsulation efficiency of the liposomes exceeded 49%.

The size distribution of RFP liposomes prepared by the optimal method was  $402.9 \pm 10.87$  nm, and the zeta potential was  $-17.0 \pm 1.10$  mV. According to the TEM results, as shown in Figure 1A, the RFP-loaded liposomes were regular spheroids with a uniform particle size distribution, indicating good morphology.

A 3D model of the porous scaffold was obtained by computer-based 3D modeling (Figure 1B). Based on

the design scheme, 3D printing was used to prepare a scaffold prototype. The printed ceramic pigment biscuit was degreased and sintered in an electric furnace to obtain the final scaffold (Figure 1C). Subsequently, a RFP liposome-loaded calcium phosphate cement scaffold was obtained by centrifugal drying, as shown in Figure 1D. The scaffold primarily comprises calcium phosphate ceramic powder, known for its excellent osteointegration capacity. The manufacturing process utilizes ball milling to ensure a thorough mix of the ceramic powder with the premix solution, achieving a high ratio of solid ceramic powder. According to previous research, calcium phosphate ceramic scaffolds, when designed with a porous structure, have been proven capable of withstanding performance tests under conditions such as centrifugation and drying. These elements fundamentally ensure the scaffold's robust performance.

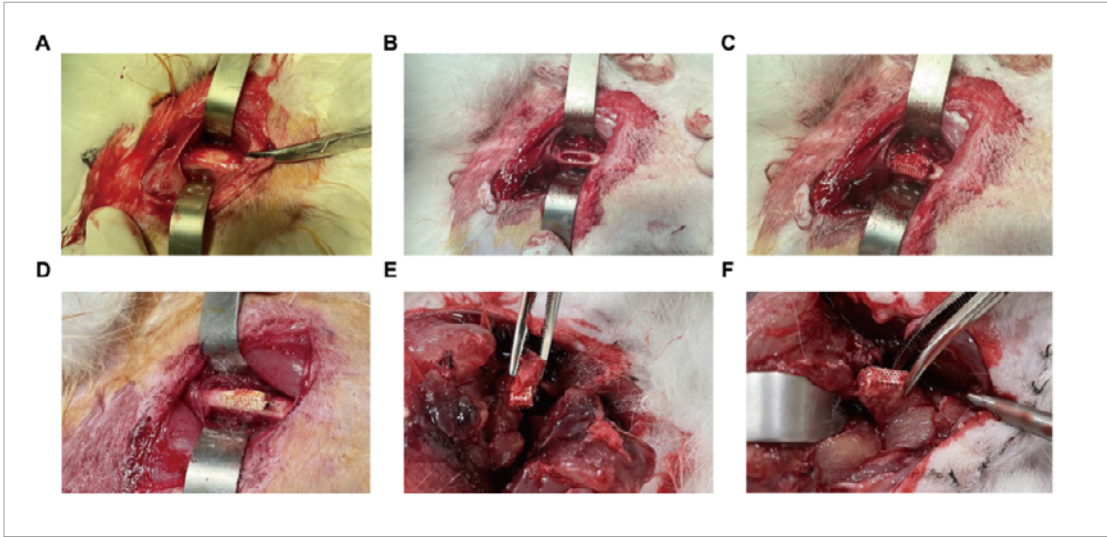


**FIGURE 1** - (A) TEM of RFP liposomes. (B) 3D print model. (C) Final scaffold. (D) RFP liposome-loaded calcium phosphate cement.

The drug content of the bone cement scaffold affected the speed and efficacy of drug release to some extent. To ensure the stability of drug loading within the materials and to determine the amount of drug carried by each scaffold, we randomly sampled scaffolds loaded with the drug. The scaffolds, varying in weight, were randomly crushed and placed into conical centrifuge tubes for measurement via HPLC. The average drug content of the RFP-loaded calcium phosphate cement scaffold was 0.657 mg/g, whereas that of the RFP liposome-loaded calcium phosphate cement scaffold was 0.894 mg/g. The results indicate that the liposomal formulation of RFP could improve drug-loading efficiency and drug release at the lesion site. This implies that as long as the size and weight of the scaffolds are controlled, the total amount of drug loaded fluctuates within a relatively consistent range under the same processing conditions, providing a comparative basis for subsequent studies.

The rabbits were anesthetized and secured on an experimental table. The hair on the lower middle section of the left thigh was shaved to expose the skin. The surgical area was routinely disinfected with iodine alcohol. An

approximately 2 cm horizontal median incision was made on the skin of the lower lateral portion of the left thigh. The incision entered the femoral condyle through blunt dissection between the lateral femoral muscles, separating the periosteum and exposing the lateral femoral condyle and the middle-lower section of the femur (Figure 2A). A bone drill was used to create a lesion above the surface of the lateral femoral condyle joint and a bone knife was used to remove the lateral cortex and internal cancellous bone on the same side. The contralateral cortex remained intact, forming a  $10 \times 10 \times 5$  mm bone lesion (Figure 2B). As shown in Figures 2C and D, after the scaffold was implanted, all rabbits showed varying degrees of swelling but remained alive. All rabbits showed good wound healing and no complications, such as infection, abscesses, or fluid build-up, were observed. Food and water intake was normal, but most rabbits had poor postoperative mental status. The femurs of rabbits in both groups were well-wrapped with muscle tissue, demonstrating excellent biocompatibility, and the outer surface of the material had abundant callus tissue (Figure 2E-F).



**FIGURE 2** - (A) Rabbit femur. (B) Bone defect was 10 × 10 × 5 mm. (C–D) Images of RFP-loaded scaffolds and RFP-loaded liposomes implanted into bone defects. (E–F) Images showing scaffold removal from the surgical site.

A batch of rabbits (n=3) was sacrificed in the first, second, and third weeks, and the scaffolds were removed. The drug content in the scaffold was calculated to determine the cumulative RFP release. As shown in Table

III, the RFP liposome-loaded calcium phosphate cement scaffold slowly released RFP in rabbits and maintained effective drug concentrations for a long time.

**TABLE III** - Cumulative release amount of LFP

|                                                        | one-week      | two-week     | three-week   |
|--------------------------------------------------------|---------------|--------------|--------------|
| Cumulative release amount of drug (%<br>LFP liposomes) | 29.81 ± 13.14 | 36.85 ± 2.81 | 44.76 ± 2.62 |
| Cumulative release amount of drug (%<br>LFP)           | 37.12 ± 5.83  | 59.79 ± 5.20 | 84.64 ± 3.58 |

**DISCUSSION**

Herein, we report the optimal formulation of RFP liposomes. The efficiency of drug encapsulation is important for the safety and effectiveness of liposomal drug delivery systems for targeted therapies. Therefore, this study aimed to screen for the formulation with optimal encapsulation efficiency of RFP liposomes. RFP liposomes were loaded onto a calcium phosphate cement scaffold

prepared using a 3D printing technique based on the physiological environment of bone tuberculosis. This composite material exhibited good biological properties, bone conductivity, osteogenesis, and histocompatibility. The RFP liposome-loaded calcium phosphate scaffold prepared in this study not only enabled the sustained release of RFP liposomes in bone lesions but also provided osteogenic repair and long-term bone graft support functions by the bone cement scaffold.

This approach can be used to treat a wide range of bone defects resulting from postoperative osteoporosis while promoting patient recovery and reducing recurrence; therefore, it provides clinicians with more effective and safer treatment options. Composite materials have great potential for the development of therapeutic systems that can be used to repair tubercular defects in loaded or unloaded areas of cancellous bones. However, this study still has limitations. Although technologies for 3D printing based on wound size already exist, being applied in fields such as interventional cardiology to achieve individualized wound treatment, they require precise wound imaging technology. Since the wounds in this study could be artificially controlled and were relatively regular, we did not adopt this approach under the premise of optimizing costs. Merely using laser cutting can achieve a support structure that matches the wound. However, in the future, as we conduct further research, we will employ more precise 3D printing technology to bridge the gap.

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