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Formulation Development and Evaluation of Gelatin Scaffolds by Lyophilization

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ABSTRACT

The scaffold is the central component that is used to deliver cells, drugs, and genes into the body. Ciprofloxacin HCl is a fluoroquinolone derivative, widely used as an antibiotic in osteomyelitis because of its favorable penetration and antibacterial effect. The aim of present study is to prepare gelatin scaffolds containing antibiotic by lyophilization. Different trial batches (T1-T6) of gelatin scaffolds were prepared by using the different amount of gelatin and glutaraldehyde as a crosslinking agent. Prepared scaffolds were evaluated for porosity using liquid displacement method, drug content, in vitro drug release and differential scanning calorimetry. The porosity was found in the range of 6.3% to 8.21%. Drug content was found in the range of 93.7% to 95.00%. The batch B6 was showed 96.46 % CDR in 6 hrs. The optimized batch was formulated by using 3% gelatin and 30 µl glutaraldehyde (5 %v/v) and 2% ciprofloxacin HCl which showed 95.00% CDR in 13hrs. All characterization parameter satisfy the scaffold requirement for treatment of bone disease such as osteomyelitis.

INTRODUCTION

"Scaffold is the central component that is used to deliver cells, drugs, and genes into the body" [1]. Tissue engineering aims to replace or facilitate the regrowth of damaged or diseased tissue by applying a combination of biomaterials, cells and bioactive molecules. Every day thousands of clinical procedures are performed to replace or repair tissues in the human body that have been damaged by disease or trauma. The damaged tissue is replaced by using donor graft tissues (autografts, allografts, or xenografts), but the main problems associated with these are a shortage of donors or donor sites, transmission of disease, rejection of grafts, donor site pain, and morbidity, the volume of donor tissue that can be safely harvested, and the possibility of harmful immune responses. Compared with replacing damaged tissues with grafts, tissue engineering, or regenerative medicine, there are aims to regenerate damaged tissues by developing biological substitutes that restore, maintain, or improve tissue function. Biodegradable polymeric scaffolds for tissue engineering have received much attention because they provide a temporal and spatial environment for cellular growth and tissue in-growth. The scaffold is the central component that is used to deliver cells, drugs, and genes into the body [1, 2]. Different biomaterials can be used for the preparation of scaffolds [2].Natural polymers include alginate, proteins, collagens, gelatin, fibrin, albumin, elsinan, pectin (pectinic acid), galactan, curdlan, gellan, levan, emulsan, dextran, pullulan, gluten, elastin, fibroin, hyaluronic acid, cellulose, starch, chitosan (chitin), scleroglucan can be used as biomaterial for delivery of drug/gen/cells [2,4]. Synthetic polymers are largely divided into two categories, biodegradable and nonbiodegradable. Biodegradable polymers are polyglycolide, polylactide and its copolymer poly (lactide-*co*-glycolide), polyphosphazene, polyanhydride, poly (propylene fumarate), polycyanoacrylate, polycaprolactone, polydioxanone, and polyurethanes. Nonbiodegradable polymers include polyvinyl alcohol, polyhydroxyethylmethacrylate, and poly (N-isopropylacrylamide) [4]. Melting inorganic raw materials to create an amorphous or crystalline solid body is known as *bioceramics*, and these porous final products are used mainly for scaffolds. Alumina, zirconia, and silicon nitride, glass ceramics such as dense hydroxyapatites [9CaOCa $(OH)_2 \cdot 3P_2O_5$] are the examples of bioceramics [3].

"Lyophilization or freeze drying is a process in which water is frozen, followed by its removal from the sample, initially by sublimation (primary drying) and then by desorption (secondary drying)" [5].The main principle involved in freeze drying is a phenomenon called

sublimation, where water passes directly from solid state (ice) to the vapor state without passing through the liquid state. Sublimation of water can take place at pressures and temperature below triple point i.e. 0.00603 mm of Hg and 0.01 degree Celsius [5].

"Osteomyelitis is an infection of the bone, a rare but serious condition" [6]. The majority of infections of bone or joint are caused by the spread of bacteria through the bloodstream or occasionally by the entry of organisms through an open wound, by puncture or by extension of infection from adjacent tissue. The most common causative organism is *Staphylococcus aureus* but many other organisms may be responsible for a bone or joint infection. The treatment of osteomyelitis is based on antibiotic therapy in combination with surgical drainage if pus or infected tissue is present [6, 7].

MATERIALS AND METHODS

Ciprofloxacin HCl was obtained as a gift sample from Wockhardt Pvt Ltd (Aurangabad, India). Gelatin was purchased from Loba Chemicals (Mumbai, India). Glutaraldehyde (5% v/v) was purchased from Loba chemicals (Mumbai, India). Lysozyme (50 mg/ml) was purchased from Malegaon Pathological Laboratory (Malegaon, India). All other reagents and solvents used were of analytical grade.

Preparation of placebo gelatin scaffolds

Placebo gelatin scaffolds were prepared as accurately weighed the quantity of gelatin (200 mg) was taken in distilled water (20 ml) and kept at 60ºC until it was completely swollen, then it was stirred at speed of 350 rpm to form homogenous gelatin solution. Then glutaraldehyde (5%v/v, 10µl) as a crosslinking agent was added to the gelatin solution and allowed to age for 2 hrs. Another placebo was prepared by using 200mg gelatin and 20µl glutaraldehyde. Finally, the solution was transferred to Petri plate. This solution was lyophilized under the conditions shown in table No.1.

Table No. 1 Lyophilization conditions

Segments	Temperature (°C)	Ramp (°C/min)	Vacuum (mbar)	Time (h)
Prefreezing	-70	-	-	6
1	-30	0.25	0.040	6
2	-15	0.25	0.040	6
3	-5	0.50	0.040	4
4	5	0.50	0.040	4
5	15	0.50	0.040	4

Evaluation of placebo scaffolds

Scaffolds were subjected for following Evaluation Parameters.

Swelling study

Scaffolds of equal weight in triplicate were immersed in 30 ml of phosphate buffer pH 7.4 solution at 37ºC. The scaffolds were retrieved at 1 h time interval and excess phosphate buffer pH 7.4 was removed using filter paper. The swollen scaffold weight was recorded using electronic balance (D455003609, Shimadzu, Japan). The swelling ratio was calculated as

$$S = \frac{Ws - Wd}{Wd} \times 100$$

Where W_s and W_d correspond to the swollen and dry weights of the scaffolds [8].

Porosity determination

Liquid displacement method was used to measure the porosity of scaffolds. Scaffolds of dry weight (0.047 gm and 1cm diameter) were immersed in 15 ml ethanol for 5 mints, taken out, and its weight was recorded. The porosity of scaffolds was evaluated using

$$\epsilon = \frac{Ww - Wd}{\rho \times \pi \times (\frac{D}{2})^2 \times H} \times 100$$

Where *Ww* and *Wd* are the wet and dry weights of scaffolds, ρ the density of ethanol (0.789/cm^3), π, the pi-constant (3.14), D, the diameter and H, a thickness of scaffolds [9].

Preliminary trails batches for drug-loaded scaffolds

Trail batches of scaffolds were prepared by using different concentrations of gelatin and glutaraldehyde as shown in Table 2. The Same procedure and Lyophilization conditions were used as described in placebo preparation.

Table 2 Composition of different trial batches of scaffolds

Trail batches Code	Gelatin (mg)	Glutaraldehyde (µl)	Distilled water (ml)	Drug (mg)
T1	600	5	20	400
T2	800	5	20	400
T3	600	10	20	400
T4	600	20	20	400
T5	600	30	20	400
T6	600	40	20	400

Evaluation of drug loaded scaffolds:

Swelling study

The same procedure was used as described for placebo sample [8].

Porosity determination

The same procedure was used as described for placebo sample [9].

Drug content determination:

Scaffolds (10 mg) were dissolved in 100 ml of distilled water and 0.1 ml of sample was taken out and diluted with 10 ml of water. The absorbance of this solution was measured at 271 nm (λmax) using distilled water as a blank, using UV visible spectrophotometer [8].

In vitro drug release of scaffolds in simulated body fluid:

Preliminary trial batches of scaffolds (T1 to T6) were subjected for *in vitro* drug release study using USP dissolution apparatus I. The equal weight of scaffolds in triplicate was immersed in the 900 ml of simulated body fluid (pH7.4) and samples were taken out after one hour time intervals by maintaining the sink condition. The drug release was determined by taking absorbance using UV spectrophotometer [10].

Drug release models

To describe the kinetics of the drug release from the scaffolds, the release data were evaluated with the help of mathematical models such as Zero-order, First-order, Higuchi, Hixon-Crowell, and Koresmeyer-Peppas[11].

Selection of optimized Batch:

Based on the outcome of above evaluation obtained, batch T5 showed satisfactory results in the term of the swelling index, porosity, drug content and *in vitro* drug release as compared to other formulation. *In vitro* drug release indicated that T5 showed the better-sustained release effect over a period of 12 hrs. Hence, batch T5 was selected as optimized formulation on basis of percent drug release and other evaluation parameters. Optimized batch was subjected for further evaluation parameters.

Fig. 1 Image of optimized batch scaffolds prepared by lyophilization

Differential scanning calorimetry of scaffold

Differential scanning calorimetry equipped with an intra-cooler and a refrigerated cooling system was used to analyze the thermal behavior of gelatin scaffolds in the range of 35 to 300^0C. Indium standard was used to calibrate the DSC temperature. Nitrogen was purged at 50 ml/min and 100 ml/min through cooling unit. The DSC graph of the drug-loaded was compared with that of the scaffolds without the drug to check for any possible changes [8].

Scanning electron microscopy:

SEM was performed at Sophisticated Analytical Instruments Facilities, Cochin, India. The porosity of gelatin scaffolds contains drug and gelatin scaffolds without the drug was observed after SEM analysis.

Enzymatic degradation:

The *In vitro* degradation of developed gelatin scaffolds under physiological condition was determined. Previously weighed scaffolds was immersed in 10 ml of phosphate buffer solution (pH 7.4) containing 1 ml of lysozyme solution (50 mg/ml) and incubated at 37 °C as well as scaffolds was also immersed only in the phosphate buffer solution (pH 7.4) which does not contained lysozyme and it was used as control. At the specific time interval, the scaffolds were removed from the solution, washed with deionized water and dried. The percentage of degradation was calculated using the formula [10].

$$\% \text{ degredation} = \frac{Initial\ weight - dry\ weight}{initial\ weight} \times 100$$

Sterility testing of scaffolds

Prepared scaffolds were subjected to the sterilization by ethanol sterilization and the sterility was tested by direct inoculation method using thioglycolate media [12]. A dry scaffold (0.050 gm and 1 cm diameter) was immersed in the 96 % of ethanol for 30 mints. Scaffolds were retrieved and aseptically transferred in Petri plate containing 20 ml of thioglycolate media. The sample was incubated at 37ºC for the 24 hrs and observed for any growth of the microorganism.

Stability studies of optimized formulation

To assess the drug and formation stability, stability studies were done according to ICH and WHO guidelines (ICH guideline Q1A (R2). The optimized formulations sealed in aluminum packaging and kept in the humidity chamber maintained at 40 ± 2ºC and 75% ± 5 RH for three months. At the end of each interval, samples were analyzed for the physical observation, drug content and *in vitro* release profile.

RESULT AND DISCUSSION

Swelling studies for placebo gelatin scaffolds were carried out and it was found that the placebo (200 mg gelatin and 10 µl glutaraldehyde) shows the 100 % swelling around the 5 hrs. and placebo (200 mg gelatin and 20 µl glutaraldehyde) shows the 100% swelling in 12 h. The change in the concentration of glutaraldehyde (crosslinking agent) the swelling index of the prepared gelatin scaffolds was changed. The porosity of different placebo gelatin scaffolds was determined by using liquid displacement method results are shown in Table 3. Due to change in the concentration of crosslinking agent (glutaraldehyde) the porosity of prepared gelatin scaffolds was changed.

Table 3 Porosity of placebo gelatin scaffolds

Placebo Batch code	Porosity (%)
P1	95.12
P2	88.31

Swelling studies of different batches of scaffolds were carried out and the results are shown in Table 4. It was found that T5 shows the 100% swelling in 12 h. T6 shows the 100% swelling in 15 h. For the treatment of bone infection (osteomyelitis) the requirement of drug release is 12 h. so the batch T5 shows the complete swelling in 12 h i.e. the drug will be released till the scaffolds complete swell.

Table 4 Swelling study of scaffolds

Time (h)	Swelling index (%)					
	T1	T2	T3	T4	T5	T6
1	45.51	48.81	35.0	28.56	18.50	15.0
2	88.62	85.34	42.23	36.31	26.32	21.0
3	93.41	96.12	51.78	48.57	34.0	29.34
4	98.0	99.0	64.0	55.45	42.0	35.46
5	-	-	78.32	62.0	49.12	41.87
6	-	-	90.61	69.87	61.34	47.21
7	-	-	98.34	80.67	68.45	50.0
8	-	-	-	88.78	75.29	55.65
9	-	-	-	95.89	83.78	62.34
10	-	-	-	100	89.23	70.35
11	-	-	-	-	96.56	76.78
12	-	-	-	-	100	83.89
13	-	-	-	-	-	92.45
14	-	-	-	-	-	98.19
15	-	-	-	-	-	100

Porosity determination

The porosity of drug loaded scaffolds was determined by the liquid displacement method. It was observed that the prepared gelatin scaffolds show different porosity due to the change in concentration of cross-linking agent as well as gelatin as shown in Table 5.

Table 5 Porosity of scaffolds

Trail batches Code	Porosity (%)
T1	84.0
T2	86.14
T3	80.10
T4	77.0
T5	75.32
T6	59.41

Drug content determination:

The drug content of the scaffolds was determined. The gelatin scaffolds show the drug content in percentage shown in Table 6.

Table 6 Percent drug content of scaffolds

Trail batches Code	Percent drug content
T1	60.12%
T2	81.41%
T3	88.53%
T4	90.0%
T5	98.0%
T6	65.10%

The percent drug content was found in the range of 60 to 98 %. Batch T5 shows the 98 % drug content so it was the highest percent drug content. Figure 2 shows the release profile of all formulations. Batch T5 shows the 98.0% drug release in 12 h and it is required for the treatment of osteomyelitis [11].

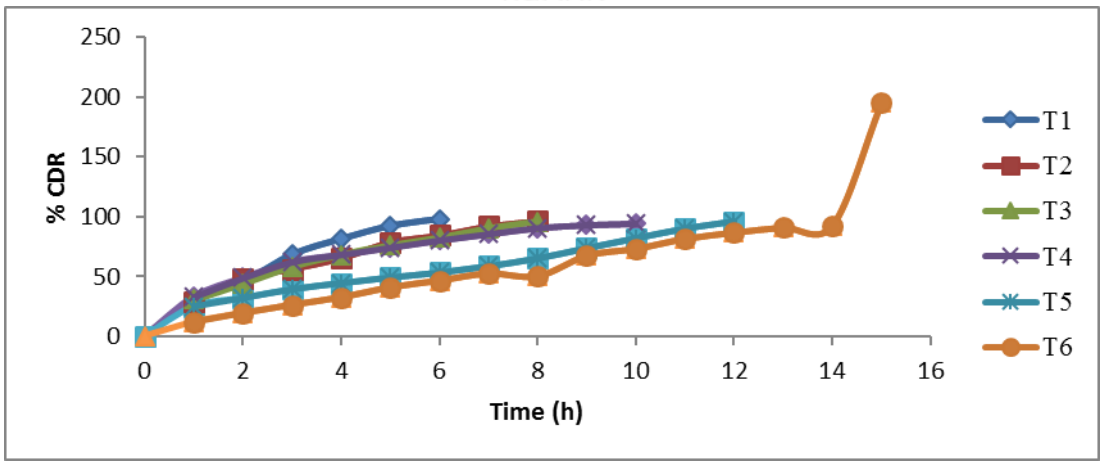

Fig. 2 Release profiles of all formulations T1 to T6

Drug release kinetics

The regression coefficient (R^2) values are shown in Table 7. Release profiles of all formulations were treated with Korsemeyer Peppas equation. Value of n (Release exponent)

was 0.597 displaying non-Fickian diffusion mechanism or anomalous transport responsible for release of drug [11]

Table 7. Drug release kinetic study

Trail batches Code	Zero Order	First Order	Higuchi	Hixon Crowel	Korsmeyer Peppas
T1	0.9860	0.8583	0.9672	0.893	0.9961
T2	0.9752	0.9451	0.9654	0.904	0.9963
T3	0.9561	0.9340	0.9833	0.895	0.9931
T4	0.9403	0.9529	0.9859	0.9874	0.9921
T5	0.9183	0.9743	0.9668	0.9718	0.9940
T6	0.9331	0.9256	0.9134	0.9351	0.9939

Evaluation of optimized batch

All the above batches T1 to T6 were analyzed for all the parameters and the results were compared with available standards. On the basis of the swelling index, porosity, drug content and *In vitro* drug release the batch T5 is selected as optimized batch and subjected for further studies.

Differential scanning calorimetry:

Differential scanning calorimetry of scaffolds containing drug and without drug (placebo) was performed. The DSC thermogram of scaffolds containing ciprofloxacin HCl is shown in Fig 3 and it was compared with DSC thermogram of placebo scaffolds as shown in Fig 4. It was observed that onset temperature (149.44ºC) and peak temperature (150.45ºC) of scaffolds containing drug is similar to placebo scaffolds. So that no any change occurred after loading of drug into the scaffolds.

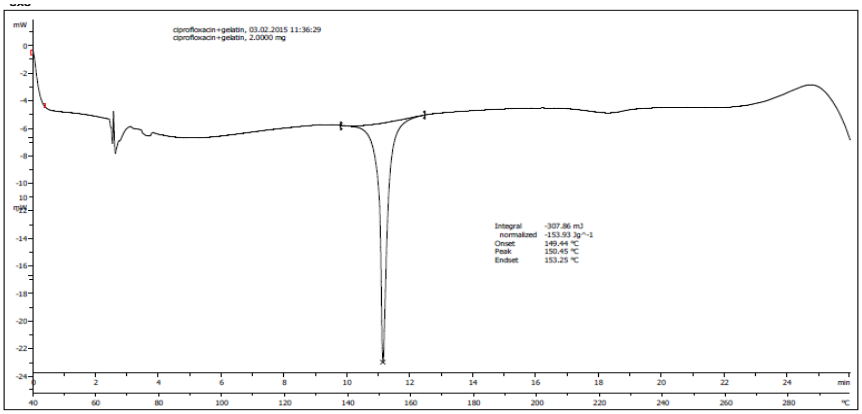

Fig. 3 DSC spectra of gelatin scaffolds with ciprofloxacin HCl

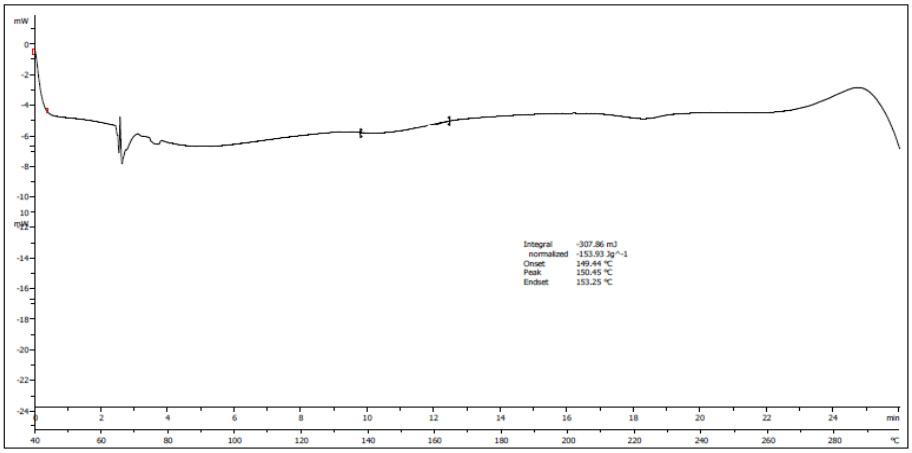

Fig. 4 DSC spectra of gelatin scaffolds (placebo)

Scanning electron microscopy:

The pore size of the scaffolds was determined by scanning electron microscope. Images of both drug containing scaffolds (Fig.5) as well as without drug (placebo 6) was obtained. It was observed that the scaffolds containing dug shows the pore size 100 µm and placebo (gelatin scaffolds without the drug) shows the pore size 100 µm i.e. after addition of drug no any changes of pore size was observed. So that the prepared scaffolds can be used for drug or cell loading as well as treatment of bone infection such as osteomyelitis.

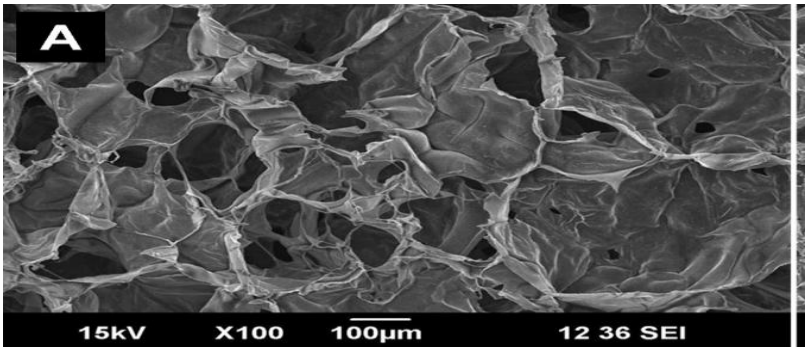

Fig. 5 SEM image of gelatin scaffolds without drug

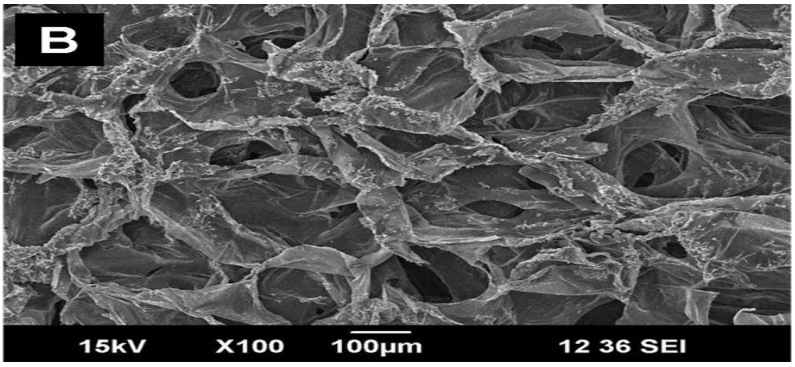

Fig. 6 SEM image of gelatin containing ciprofloxacin HCl

Enzymatic degradation:

Enzymatic degradation of scaffolds was carried out in phosphate buffer pH 7.4 using lysozyme solution and results are shown in Table 8. It was observed that scaffolds show the complete degradation in 16hrs. So it can be used for the treatment because release will be in 12h and scaffolds degrade in 16 hrs.

Table 8 Percentage degradation of scaffolds

Time (h)	% Degradation
1	6.70
2	15.45
3	22.56
4	30.14
5	35.67
6	42.53
7	49.76
8	56.83
9	62.0
10	66.13
11	70.0
12	75.34
13	82.91
14	90.56
15	94.89
16	100

Sterility testing:

Sterilized scaffolds were transferred aseptically to the fluid thioglycolate media and incubated at 37ºC for 24hrs. After incubation for 24hrs, no any growth of microorganisms and no changes were observed on the scaffolds. So the scaffolds were sterilized by ethanol treatment and it can be used for the treatment of bone infection.

Stability studies of optimized formulation:

The stability study of optimized formulation revealed that no significant changes in the physical parameters when stored at temperature and humidity conditions at 40 ± 2^{0}C and 75±5% RH. No significant variation *in vitro* drug release over a period of three months. The stability data for the optimized formulation is shown in Table 9.

Table 9 Results of optimized formulation during stability studies

Parameter/ Time	Temperature 40 ºC / 75% RH			
	Initial	1st month	2nd month	3rd month
Physical appearance	Solid	No Change	No Change	No Change
In-vitro drug released (%) 12 h.	96.16	95.92	96.00	96.10
Drug content	98.0%	98.10%	97.90%	98.0%

CONCLUSION

The aim of the present study was to formulate and evaluate a gelatin scaffolds containing ciprofloxacin HCl for the treatment of bone infection such as osteomyelitis. The formulation process was carried out by freeze drying method using Triad™ lyophilizer (Labconco, USA). All the production characteristics and performance characteristics are confirmed and these give optimized and stable gelatin scaffolds of ciprofloxacin HCl for treatment of bone infection such as osteomyelitis. It was concluded that the gelatin scaffolds containing ciprofloxacin HCl prepared by lyophilization method can be used for the treatment of bone infection such as osteomyelitis and also used migration, proliferation, and new tissue formation.

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