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*Research Article*

# Preparation and Characterization of Coaxial Electrospinning rhBMP2-Loaded Nanofiber Membranes

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DEX and rhBMP2-loaded core-shell nanofiber membranes were synthesized by electrospinning method in one step. Zein/PLLA, Zein-DEX/PLLA, Zein/PLLA-rhBMP2, and Zein-DEX/PLLA-rhBMP2 were fabricated; and morphology, hydrophilicity, mechanics properties, *in vitro* drug release behavior, cell proliferation, and osteogenic differentiation were investigated. The results showed that the dual-release system containing rhBMP2 and DEX prepared by electrospinning had rough surface, constant drug release behavior, and could also significantly promote cell proliferation and osteogenic differentiation of RMSCs, indicating that the scaffolds we fabricated might be suitable for bone tissue engineering.

## 1. Introduction

Bone healing is still a challenge in today's clinical routine, and biomaterials provide a promising tool to treat bone defects [1]. It is found that porous materials with 3D structure is an ideal material in the field of bone tissue engineering [2]. Electrospinning technology is one of the most effective and simplest methods to prepare nanofibers, and the prepared materials have the characteristics of large specific surface area and strong size controllability [3–6]. Moreover, the electrospinning-prepared fiber membranes have 3D network structure, which is conducive to improving cell adhesion, growth, and proliferation [7]. Core-shell nanofibers which are prepared by coaxial electrospinning can effectively combine different system materials into the same fiber [8]. As a drug delivery system, electrospun coaxial fibers can protect the activity of drugs or proteins, and thus have potential application value [9]. Compared with other dosage forms, electrospun nanofiber membranes have many advantages, including fine adjustment of drug release by adjusting the morphology, porosity, and composition of nanofiber membranes [10, 11]. Besides, small diameter nanofiber membranes with high specific surface area are conducive to centralized drug delivery and effective drug release [12]. Man et al. developed a rhTGF-beta 1- and E7-loaded electrospun scaffolds which could synchronously improve all three of the basic components required for cartilage tissue engineering in vitro [13]. Kang et al. fabricated mesoporous bioactive nanocarriers in hollow fiber composites to sequentially deliver dual bone growth factors by electrospun [14].

There are lots of factors that could promote the growth of osteoblasts, such as extracellular vesicles (EVs), which can promote osteogenic function through the regulation of osteoblast differentiation and expression of osteogenic genes in vitro [15]; BMP-2 can induce bone marrow mesenchymal stem cells to differentiate into osteoblasts and promote the proliferation of osteoblasts. Mikami et al. proved that BMP-2 and DEX can support the osteogenic differentiation of bone marrow mesenchymal stem cells in vitro and jointly adding BMP-2 and DEX (a synthetic glucocorticoid [16]) to biomaterials may enhance their osteogenic differentiation efficacy in clinical bone tissue engineering [17]. It has also been proved that it is very important in bone tissue engineering to maintain their concentration of action, prolong their effectiveness, reduce the systemic risk of high burst release,

Table 1: The ingredients of each sample for test.

| Sample | Inner layer | Outer layer | Abbreviations |
|---|---|---|---|
| 1 | PLLA/HFIP 6 wt.% | Zein/HFIP (14 wt.%) | Zein/PLLA |
| 2 | PLLA/HFIP 6 wt.% | Zein/HFIP (14 wt.%)+DEX | Zein-DEX/PLLA |
| 3 | PLLA/HFIP 6 wt.% + 12 $\mu$g rhBMP2 | Zein/HFIP (14 wt.%) | Zein/PLLA-rhBMP2 (12 $\mu$g) |
| 4 | PLLA/HFIP 6 wt.% + 30 $\mu$g rhBMP2 | Zein/HFIP (14 wt.%) | Zein/PLLA-rhBMP2 (30 $\mu$g) |
| 5 | PLLA/HFIP 6 wt.% + 48 $\mu$g rhBMP2 | Zein/HFIP (14 wt.%) | Zein/PLLA-rhBMP2 (48 $\mu$g) |
| 6 | PLLA/HFIP 6 wt.% + 12 $\mu$g rhBMP2 | Zein/HFIP (14 wt.%)+DEX | Zein-DEX/PLLA-rhBMP2 (12 $\mu$g) |
| 7 | PLLA/HFIP 6 wt.%+30 $\mu$g rhBMP2 | Zein/HFIP (14 wt.%)+DEX | Zein-DEX/PLLA-rhBMP2 (30 $\mu$g) |
| 8 | PLLA/HFIP 6 wt.%+48 $\mu$g rhBMP2 | Zein/HFIP (14 wt.%)+DEX | Zein-DEX/PLLA-rhBMP2 (48 $\mu$g) |

and control the release of growth factors from biomaterial scaffolds [18].

In this work, the bovine serum albumin (BSA) was added to mechanically protect BMP-2 [19]. We prepared a kind of scaffold material for bone tissue engineering. Zein/PLLA, Zein-DEX/PLLA, Zein/PLLA-rhBMP2, Zein-DEX/PLLA-rhBMP2, and Zein-DEX/PLLA-rhBMP2 were synthesized. Scanning electron microscopy (SEM), transmission scanning electron microscopy (TEM), tensile fracture mechanical properties, and drug release behavior were tested. The culture of rat bone marrow mesenchymal stem cells (RMSCs) on fibrous membrane was also analyzed. The results showed that the fiber membranes had good mechanical properties and promoted the growth, proliferation, and osteogenic differentiation of RMSCs.

## 2. Materials and Methods

*2.1. Materials.* Zein (Sigma Co. Ltd., USA), PLLA ($M_w$ > 180,000, Daigang biotechnology Co. Ltd., Jinan, China), DEX (Bomei Biotechnology Co., Ltd., Hefei, China), recombine human bone morphogenetic protein-2 (rhBMP2; Zhongke Source Biotechnology Co., Ltd., Beijing, China), HFIP (Siensi Biochemical Technology Co., Ltd., Tianjin, China), phosphate-buffered solution (PBS; pH = 7.4, Dingguochangsheng Biotechnology Co., Ltd., Beijing, China), and bovine serum albumin (BSA; Sigma Co. Ltd., USA) were used to synthesize the membranes. ELISA test kit (Bode Biological Engineering Co., Ltd., Wuhan, China) was used to test the concentration of Zein and rhBMP2. The ALP staining kit (GOMORI, KGA353) was purchased from KeyGen Biotech Co., Ltd. (Nanjing, China), and the ARS solutions were purchased from Sigma-Aldrich Co., Ltd. (Shanghai, China). Rat mesenchymal stem cells (RMSCs) used in the cell culture were donated by Professor Zhang Yan of Sun Yat-sen University, Genetic Engineering Laboratory.

*2.2. Preparation of Fiber Membranes with Different Drugs.* One percent BSA solution was prepared: 100 mg BSA was dissolved in 10 mL sterile distilled water; then, 0.2 $\mu$m disposable filter membrane was used to filter bacterium, and sterile BSA solution with 1% mass volume fraction was prepared and stored in −20°C for reserve.

rhBMP2 was separated by BSA solution: 100 $\mu$g rhBMP2 ampoules were centrifuged at a speed of 5000 rpm for 5 min, and rhBMP2 was dissolved in 1% BSA solution using a pipette gun. The rhBMP2 was divided into 12, 30, and 48 $\mu$g/100 $\mu$L, respectively, and stored in the refrigerator at −80°C for reserve which should not be repeatedly frozen and thawed.

*2.3. rhBMP2 Labeled with 5-FITC.* 8.5 g NaCl, 0.2 g KCl, 2.85 g $Na_2HPO_4{\cdot}12H_2O$, and 0.27 g $KH_2PO_4$ were weighed, respectively, and 900 mL sterile water was added to dissolve sufficiently; the pH value was adjusted to 7.2, and then the sterile water was added to 1000 mL to prepare the PBS solution. 2 mg 5-FITC was weighed and 0.2 mL DMSO was added to dissolve the fluorescein solution. And each bottle of 100 $\mu$g rhBMP2 was dissolved in 0.2 mL sterile water and washed twice with 0.2 mL sterile water. Then, the mixed solution was placed in 10 mL round bottom flask, 8 $\mu$L of 5-FITC solution was added, stirred by a magnetic stirrer, and reacted at 4°C for 24 hours. The reactant was removed and filled with the intercepted molecular weight of 300-1000 D against 1 LPBS dialysis at 4°C to remove the unbounded fluorescein. After 5 dialysates were replaced (once every 6-8 hours), the coupling products were removed after total dialysis (dialysate is transparent and colorless solution). The rhBMP2-FITC conjugate was placed in a cillin bottle and freeze-dried in a freeze dryer to avoid light and preserved in the dark at −20°C.

*2.4. Preparation of Coaxial Fiber Membranes.* Eight groups of nanofiber membrane with different ingredients were synthesized as shown in Table 1. The synthetic method of sample 8 was as follows: a certain amount of Zein and PLLA was weighed and dissolved in 1 mL solvent hexafluoroisopropanol (HFIP), respectively, and stirred by magnetic stirrer for 4 hours at a set speed of 500 rpm, after which the homogeneous and stable spinning solutions with mass percentages of 14 wt.% and 6 wt.% were prepared. Zein solution was used as a shell spinning solution, and the PLLA solution was used as the core solution. And DEX which weighed 1% of Zein was added to the outer solution. In order to guarantee the activity of proteins, rhBMP2 aqueous solution of 48 $\mu$g/100 $\mu$L was put into inner solution. The shell and core solutions were injected into 1 mL sterile plastic syringe, respectively, and connected to coaxial spinning nozzle and then began to spinning. The optimum spinning parameters were obtained by

our experiment and some relevant literature. Therefore, the spinning conditions were set as follows: high voltage was 15 kV, spinning distance between coaxial nozzle and receiving device was 12 cm, outer and inner spinning speeds were 0.008 mL/min and 0.006 mL/min, respectively [20] (the receiving device was aluminium foil). Then, sample 8 was dried in a vacuum drying chamber at 37°C for one week. And the other samples were all synthesized in the similar way.

### 2.5. Characterization of Nanofiber Membranes

*2.5.1. Observation of Nanofiber Membrane Morphology by Environmental Scanning Electron Microscope (ESEM).* The morphological characteristics of nanofiber membranes were observed by XL-30 scanning electron microscopy of FEI Company. The prepared nanofibers were sprayed with gold for 1.5 min before testing. The acceleration voltage was 10 kV. The ESEM scanning electron microscopy diagrams obtained were measured and counted by Nano Measure Software. 100 nanofibers were measured by each group of ESEM diagrams, and the average and standard deviation were calculated. Finally, the diameter distribution of the prepared nanofibers was obtained.

*2.5.2. TEM Structural Observation.* The core-shell structure of the prepared fibrous membranes was observed by transmission electron microscopy (TEM; H-7650, Hitachi, Japan). Nanofiber samples for TEM observation were received by 400 mesh copper mesh. The acceleration voltage of TEM was 100 kV.

*2.5.3. Laser Confocal Scanning Electron Microscope (LSCM) Observation.* rhBMP2 labeled with 5-FITC in the inner layer of fibrous membrane was observed by a laser confocal scanning microscope (Fluo View FV 1000, Olympus Company, Japan). The nanofiber membrane used for LSCM observation was spun on the cover slide, and the excitation wavelength of LSCM was set at 450 nm.

*2.5.4. Hydrophilicity.* The water contact angle of the nanofiber membrane was measured by the contact angle tester (XG-CAM; Xuanyi Creative Analysis Industrial Manufacturing Co., Ltd, Shanghai). At room temperature, eight groups of samples were placed on the test bench flatly, then dripped with deionized water. At the same time, the contact angle change of the water droplets contacting the samples within 30 seconds were recorded.

*2.5.5. Tension Fracture Mechanics.* Each group of fiber membranes were tested with an electronic single fiber strength tester (LLY-06ED, Laizhou Electronic Instrument Co., Ltd.). The maximum load of the tester was 200 cN and the sensitivity was 0.01 cN. Before the tensile test, the thickness of the fibrous membrane was measured by spiral micrometer (103-256, Shanghai Shoufeng). Eight groups of fibrous membrane samples were repeated for more than five times, and their average values were obtained. Eight groups of fiber membranes were cut into $\mathbf{50 \times 5}$ mm$^2$ specimens, and the testing speed was 5 mm/min at room temperature.

*2.5.6. In Vitro Drug Release Behavior*

*(1) In Vitro. Drug Sustained Release Test of DEX.* Zein-DEX/PLLA and Zein-DEX/PLLA-rhBMP2 nanofiber membranes were weighed and put into 10 mL centrifugal tubes, and 2 mL PBS (pH 7.4) was added to the centrifugal tubes. Then, the centrifugal tubes were placed in a constant temperature water bath oscillator for 21 days. The temperature was set to 37°C, and the speed was 100 rpm. At each preset time interval, the supernatant was stored in a refrigerator at −20°C for DEX concentration test, and an equal amount of fresh PBS buffer was added. DEX concentration was determined by high-performance liquid chromatography (LC-2010AHT; Shimadzu Corp.) with a wavelength of 240 nm. The preset time interval is 1 h, 3 h, 6 h, 12 h, 1 d, 3 d, 5 d, 7 d, 14 d, and 21 d. The experiment was repeated three times to get the average value.

Sample preparation and test steps for analysis by high-performance liquid chromatography is as follows: 50 $\mu$L release solution was extracted with 1000 $\mu$L solution (dichloromethane : hexane : methyl tert-butyl ether ratio is =1 : 1 : 1, *v*/*v*/*v*). The extraction steps were repeated once [21]. After drying, the separated organic fractions were diluted and shaken with 1 mL mobile phase (acetonitrile : water = 30 : 70) and then separated into high-performance liquid chromatography (HPLC). The peak area of the drug was detected at 240 nm by ultraviolet absorption detector, and the drug content was calculated. A large number of literatures and pre-experiments were consulted to select the optimum conditions: C18 column (4.6 mm × 250 mm × 5 $\mu$m, Shimadzu-GL) was selected for the test, the mobile phase was acetonitrile : water = 30 : 70, the flow rate was 1 mL/min, the injection volume was 20 $\mu$L, and the test time was 30 min. The formula for calculating DEX concentration by high-performance liquid chromatography is as follows:

$$\text{Concentration} = \frac{\text{reference sample dilution multiple} \times \text{peak area of reference sample} \times \text{control quality}}{\text{reference sample peak area} \times \text{sample quality} \times \text{sample dilution multiple}}. \quad (1)$$

*(2) In Vitro. Drug Sustained Release Test of rhBMP2.* Zein/PLLA-rhBMP2 and Zein-DEX/PLLA-rhBMP2 nanofiber membranes were weighed in 10 mL centrifugal tubes, and 2 mL PBS (pH 7.4) was added to the centrifugal tubes. The centrifugal tubes were placed in a constant temperature water bath oscillator for 21 days. The temperature was set at 37°C, and the speed was 100 rpm. At each preset time interval, the supernatant was stored in a refrigerator at −20°C for

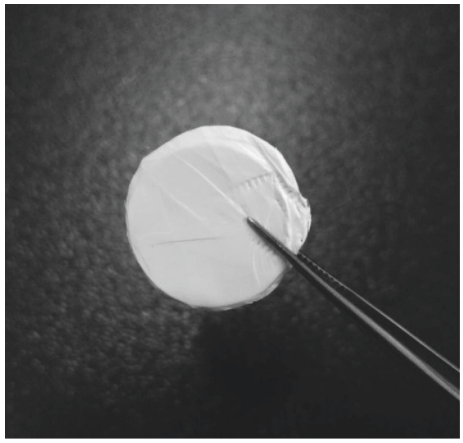


Figure 1: Image of the glass slide used for cultured cells.

rhBMP2 concentration test, and an equal amount of fresh PBS buffer was added. The collected supernatant was tested by rhBMP2 ELISA kit. The data were read by a microplate reader (Bio-Rad iMark 14071) at 450 nm. The preset time interval is 1 h, 3 h, 6, 12 h, 1 d, 3 d, 5 d, 7 d, 14 d, and 21 d. The experiment was repeated three times to get the average value.

### 2.6. Cell Experiments

*2.6.1. Culture of Rat Bone Marrow Mesenchymal Stem Cells.* The frozen cells in a liquid nitrogen tank was recovered: a frozen tube was taken out of the liquid nitrogen tank and transferred quickly to a constant temperature water bath at 37°C. After the sealing film fell off, the freezing tube was shaken in the water bath pan until it was completely thawed. Then, the water outside the tube was drained and wiped with 75% alcohol and opened on the laminar flow super clean table. Then, the cell suspension in the tube was transferred into 15 mL culture tube, 10 mL 10% fetal bovine serum (FBS) medium was added, centrifuged at 1000 rpm for 6 min, and the supernatant of cryoprotectant was removed to obtain the complete culture medium. The 10% FBS complete medium was evenly added to the petri dish in the size of 1 mL and incubated in an incubator at 37°C and 5% $CO_2$.

Electrospun fiber membrane for cell culture was prepared as follows: in the process of electrospinning, after the fiber had received a certain thickness of fiber membrane on the aluminium foil, the sterilized cover glass with a diameter of 13 mm was fixed on the fiber membrane, and electrospinning was continued until the effective dosage of drugs was reached, the receiving aluminium foil was removed, and the cover glass was clamped. The fiber membrane was cut into a circular shape with a diameter of 14 mm (Figure 1) and dried in a vacuum drying chamber at 37°C for one week, so that the residual solvents in the fiber membrane could be volatilized thoroughly. It is also noticed that before spinning, the required experimental articles were sterilized by high-pressure steam. During the electrospinning process, the prepared fibrous membranes were kept sterile as much as possible. Before cell culture, the prepared fibrous membranes were irradiated under ultraviolet lamp for 2 hours in each side to sterilize.

*2.6.2. Detection of Cell Proliferation by Cell Counting Method.* Cell proliferation was measured by cell counting method. A large number of preliminary experiments showed that the nanofibrous membrane loaded with 48 $\mu$g rhBMP2 played a better role in cell growth and proliferation. Therefore, the fibrous membrane loaded with 48 $\mu$g rhBMP2 was selected for cell proliferation experiment. RMSCs were inoculated in 24-well plate at a density of $2.5 \times 10^4$ on the nanofibers membranes of each group. Then, 1 mL cell culture medium was added into each hole for 9 days at 37°C and 5% $CO_2$. On the 3rd, 6th, and 9th day, respectively, the cell culture medium in the culture plate was removed and washed with PBS. 200 $\mu$L trypsin was added to each culture plate, digested at 37°C for 2 min, and then 10 $\mu$L cell suspension was added. Cells were counted by hematology counter plate, and then the cell proliferation diagram was obtained and analyzed. All experiments were repeated three times.

*2.6.3. Alkaline Phosphatase (ALP) Activity and Mineralization of Osteogenic Cells (ARS).* ALP activity was evaluated by Gömöri staining. After 20 days of cell inoculation, each nanofiber group was fixed with 70% ethanol for 10 min; then, the Gömöri reagent was added at room temperature. ARS staining was used for calcium mineralization induced by different scaffolds. After 21 days of cell culture, the samples were fixed with 4% paraformaldehyde at 4°C for 30 min and soaked in 1% ARS solution at room temperature for 45 min. The mineralization process was observed by an optical microscope.

*2.7. Statistical Analyses.* All quantitative results were presented as mean ± SD. Statistical analyses were performed using SPSS 13.0 software. Differences between groups were considered to be significant if $p < 0.05$.

## 3. Results and Discussion

### 3.1. The Morphology of Nanofiber Membrane

*3.1.1. Surface Morphology of Nanofiber Membrane.* Scanning electron microscopy (SEM) of coaxial nanofiber membranes (Figure 2) showed that the nanofibers had rough surfaces and no bead defects. The surface of Zein/PLLA membranes was relatively smooth, and the diameter distribution and size of the scaffolds could be visualized by the diameter distribution chart. After analysis and comparison, it could be concluded that the diameter and distribution of the nanofibers did not change significantly when different kinds and quality of drugs were added. The average diameter of the fibers was about 300 nm. The solution viscosity decreases after adding DEX with small molecular weight in Zein/PLLA fiber membrane, so the diameter became larger and the diameter distribution range was wider. The surface was roughened by the addition of drugs, which strongly supports cell adhesion and growth, and it could be also confirmed by sectional view of no-drug-loaded nanofiber mats and drug-loaded nanofiber mats as shown in Figure 3.

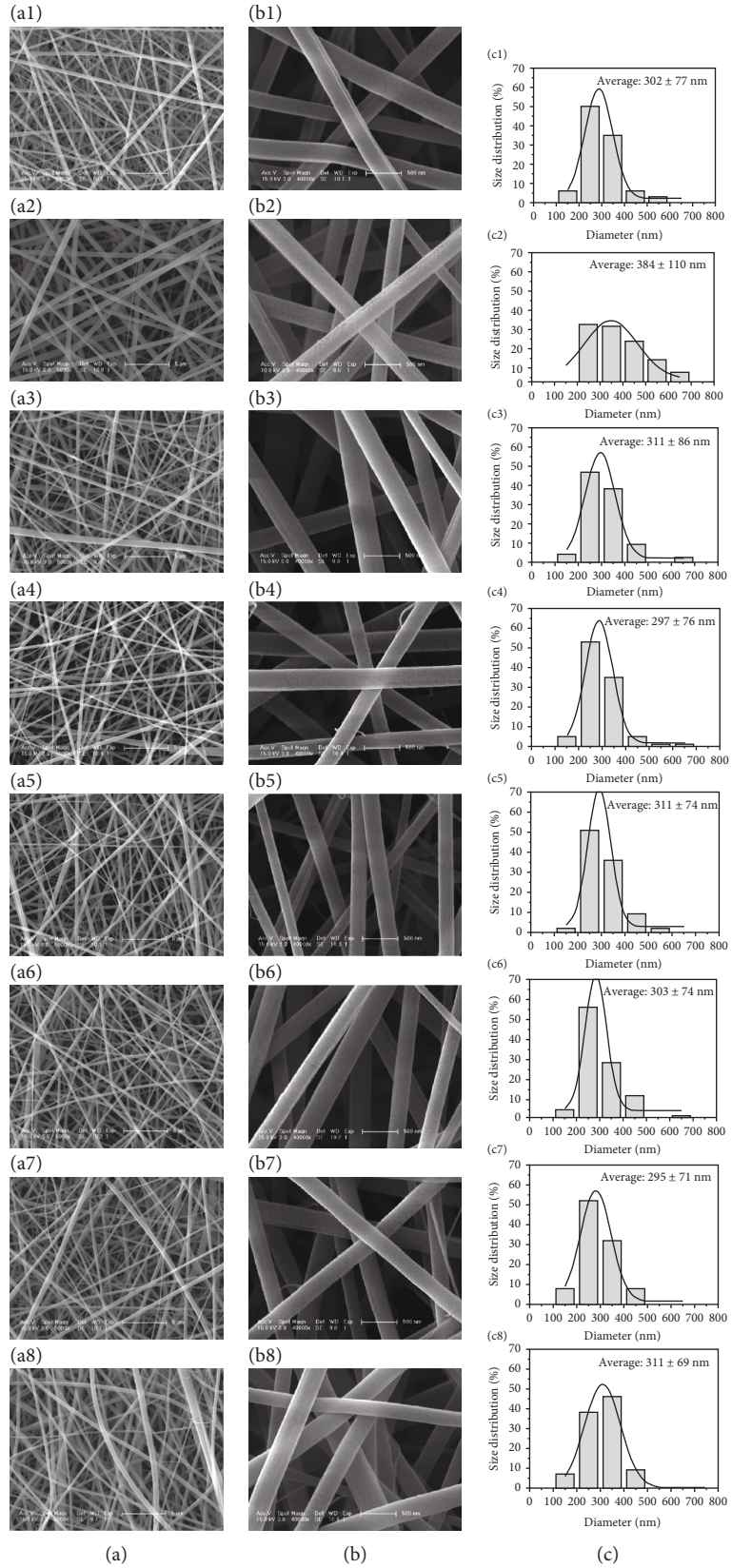
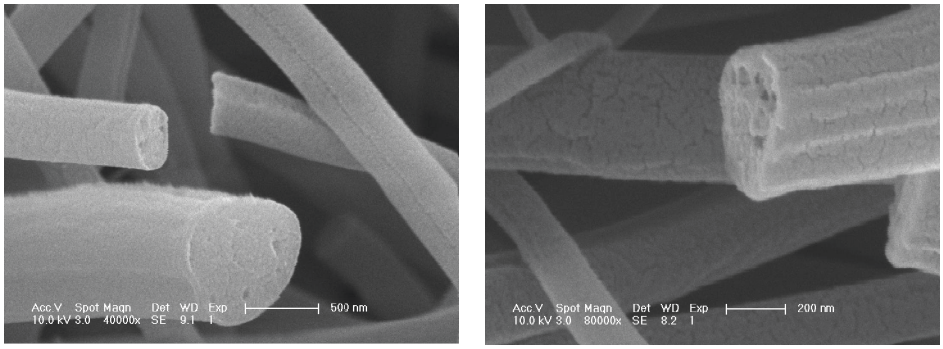
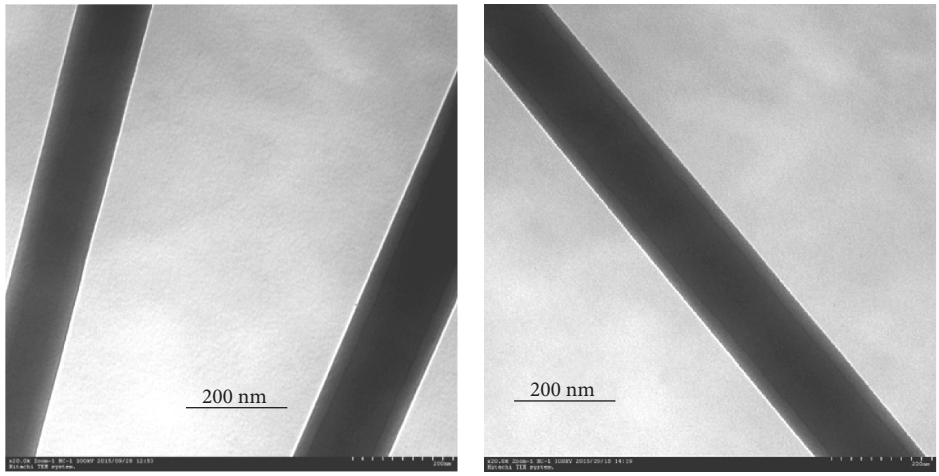



Figure 2: SEM images of coaxial nanofiber mats: ((a), a1–a8) Zein/PLLA, Zein-DEX/PLLA, Zein/PLLA-rhBMP2 (12 $\mu$g), Zein/PLLA-rhBMP2 (30 $\mu$g), Zein/PLLA-rhBMP2 (48 $\mu$g), Zein-DEX/PLLA-rhBMP2 (12 $\mu$g), Zein-DEX/PLLA-rhBMP2 (30 $\mu$g), and Zein-DEX/PLLA-rhBMP2 (48 $\mu$g) nanofiber mats, respectively. Scale bar = 5 $\mu$m. ((b), b1–b8) Scale bar = 500 nm. ((c), c1–c8) Size distribution image of the above groups.




(a) (b)

Figure 3: SEM images of the sectional view of (a) Zein/PLLA nanofiber mats and (b) Zein-DEX/PLLA-rhBMP2 (48 $\mu$g) nanofiber mats.




(a) (b)

Figure 4: TEM image of (a) Zein/PLLA nanofibers and (b) Zein-DEX/PLLA-rhBMP2 (48 $\mu$g) nanofibers.

*3.1.2. TEM and LSCM of Nanofiber Membrane.* The structure of coaxial electrospun Zein/PLLA and Zein-DEX/PLLA-rhBMP2 nanofibers were observed by TEM. Figure 4 shows that the core-shell structure of coaxial fibers had obvious difference in material contrast. Combining with the spinning process parameters, it was proved that the coaxial nanofibers prepared in the experiment had core-shell structure both before and after the drug loading with the shell structure of Zein and the core structure of PLLA.

Combining with Figure 5, rhBMP2 labeled with 5-FITC fluorescence in the core layer of nanofibers showed green fluorescence in the core structure, which proved that rhBMP2 was indeed loaded in this process condition.

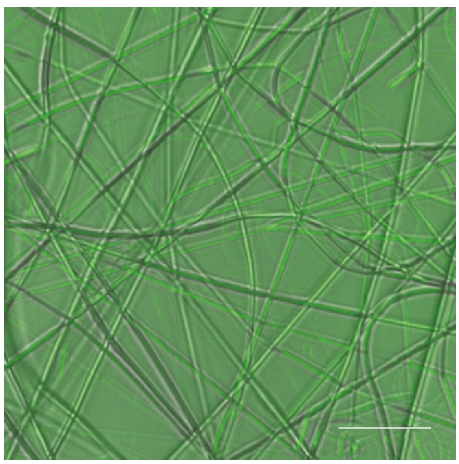


Figure 5: LSCM image of coaxial nanofibers. Scale bar = 1500 nm.

*3.2. Mechanical Properties.* The mechanical properties of the eight groups of fiber membranes are shown in Figure 6 and Table 2. The results showed that Zein/PLLA nanofiber membranes (A) had the highest tensile strength and modulus of elasticity, and the elongation at break was the largest, followed by DEX-loaded fiber membranes (B), while rhBMP2-loaded fiber membranes (C–E) had lower tensile strength, and fiber membranes which contain two drugs (F–H) were the lowest. It can be concluded that mechanical properties of the fibrous membranes would be influenced by the addition of drugs. Especially, with the addition of rhBMP2, the mechanical properties of nanofiber membrane decreased. This may be because in the core

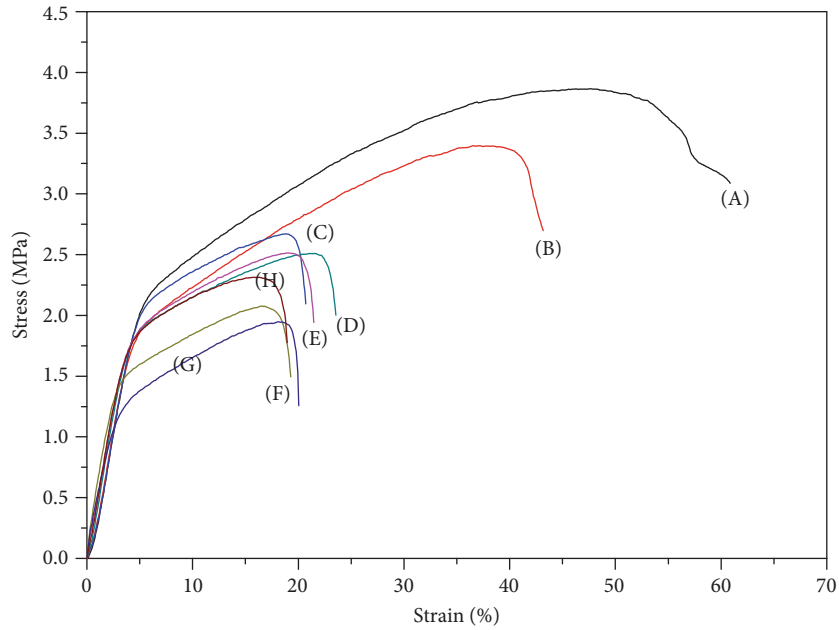



Figure 6: Tensile stress-stain curves of coaxial electrospun nanofibers: (A) Zein/PLLA nanofibers, (B) Zein-DEX/PLLA nanofibers, (C–E) Zein/PLLA-rhBMP2 nanofibers, and (F–H) Zein-DEX/PLLA-rhBMP2 nanofibers (C–E, F–H, the quality of rhBMP2 are 12, 30, 48 $\mu$g, respectively).

Table 2: Mechanical properties of Zein/PLLA electrospun nanofibers.

| Sample | Tensile strength (MPa) | Young's modulus (MPa) | Elongation at break (%) |
|---|---|---|---|
| Zein/PLLA nanofibers (A) | 4.15 ± 0.32 | 54.91 | 49.48 ± 4.27 |
| Zein-DEX/PLLA nanofibers (B) | 3.45 ± 0.29 | 42.71 | 35.17 ± 8.42 |
| Zein/PLLA-rhBMP2 (12 $\mu$g) nanofibers (C) | 2.69 ± 0.09 | 42.55 | 21.43 ± 1.91 |
| Zein/PLLA-rhBMP2 (30 $\mu$g) nanofibers (D) | 2.59 ± 0.09 | 44.41 | 19.94 ± 1.85 |
| Zein/PLLA-rhBMP2 (48 $\mu$g) nanofibers (E) | 2.53 ± 0.10 | 49.60 | 18.52 ± 2.85 |
| Zein-DEX/PLLA-rhBMP2 (12 $\mu$g) nanofibers (F) | 2.66 ± 0.14 | 50.29 | 17.37 ± 3.37 |
| Zein-DEX/PLLA-rhBMP2 (30 $\mu$g) nanofibers (G) | 2.62 ± 0.15 | 63.69 | 17.00 ± 2.37 |
| Zein-DEX/PLLA-rhBMP2 (48 $\mu$g) nanofibers (H) | 2.46 ± 0.24 | 49.25 | 15.63 ± 1.57 |

layer of the coaxial nanofiber, the mixing of rhBMP2 and PLLA leads to the destruction of the crystal growth structure of PLLA, resulting in a decline in the mechanical properties of PLLA as the mechanical support of nanofibers and a decline in the overall mechanical properties of nanofiber membrane. However, there was no significant difference between different concentrations of rhBMP2.

*3.3. Hydrophilicity.* The change of water contact angle of nanofiber membrane in 30 s is shown in Figure 7. It can be observed intuitively that Zein/PLLA and Zein-DEX/PLLA fiber membranes remain hydrophobic for 30 s. When deionized water first contacted the fiber membranes, the contact angle decreased slightly, and then remained basically unchanged. It was shown that PLLA, as the core layer structure of the fiber membranes, supported the surface stability and impeded the water droplets passing through the Zein structure of the fiber shell, which made the fiber membrane hydrophobic as a whole. The results of Figures 7(c)–7(e) and 7(f)–7(h) showed that with the increase of rhBMP2 concentration in the inner layer, the effect of PLLA on the surface stability of the fibrous membrane became weaker. When $t = 30$ s, the water contact angle of Figures 7(g) and 7(h) fiber membranes decreased to below 90°, which changed from hydrophobicity to hydrophilicity.

*3.4. In Vitro Drug Release Behavior of Nanofiber Membranes.* As shown in Figure 8, it can be observed that the release rate of DEX from single DEX-loaded nanofiber was slightly faster than that from dual-drug-loaded nanofiber membranes. The release rate of DEX from 12 $\mu$g rhBMP2-loaded fiber membranes was faster than that from other concentration and single DEX-loaded fiber membranes, and the rate of initial burst release was the highest. The release rate of rhBMP2 from

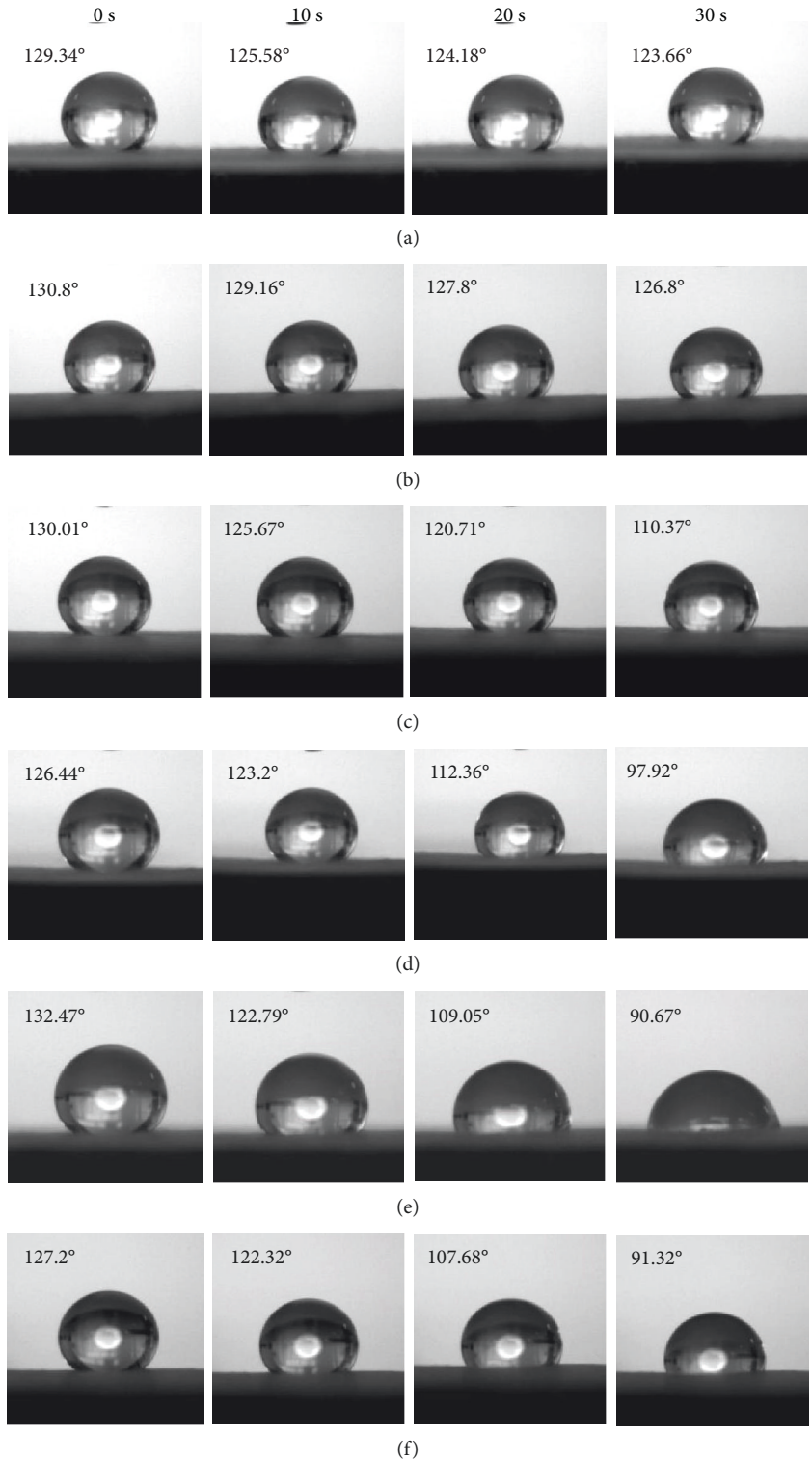



Figure 7: Continued.

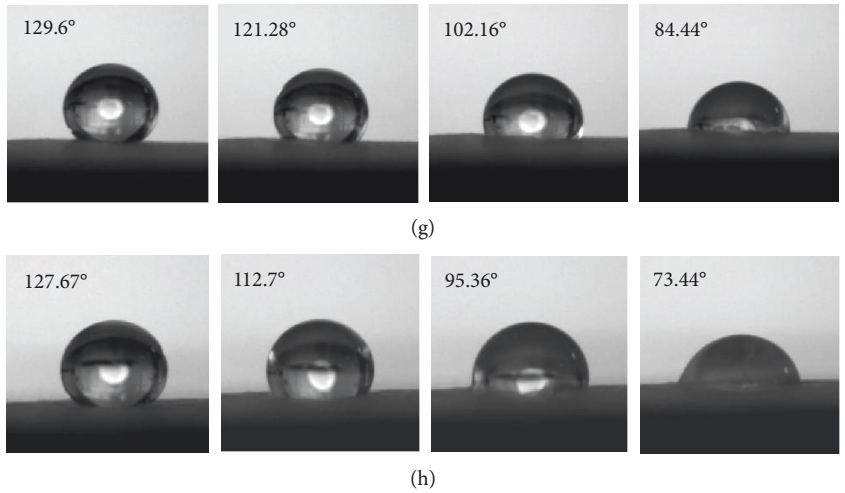



Figure 7: Water contact angle images of nanofiber mats in 30 s. (a) Zein/PLLA, (b) Zein-DEX/PLLA, (c) Zein/PLLA-rhBMP2 (12 $\mu$g), (d) Zein/PLLA-rhBMP2 (30 $\mu$g), (e) Zein/PLLA-rhBMP2 (48 $\mu$g), (f) Zein-DEX/PLLA-rhBMP2 (12 $\mu$g), (g) Zein-DEX/PLLA-rhBMP2 (30 $\mu$g), and (h) Zein-DEX/PLLA-rhBMP2 (48 $\mu$g).

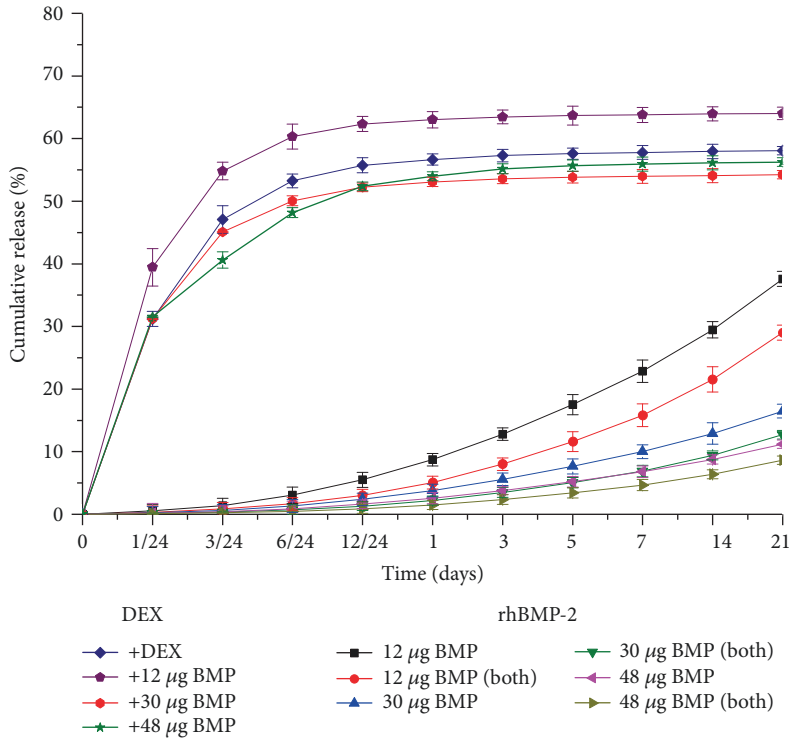



Figure 8: Cumulative release curve of double drugs from different nanofiber mats *in vitro*.

single-drug-loaded membrane was faster than that from dual-drug-loaded membrane; and the higher the concentration of rhBMP2 was, the slower the release rate was.

It was also noticed that the release of DEX was sudden within the initial 12 h, and it was because that DEX was encapsulated in Zein, the shell structure of the fibrous membrane, which could dissolve and diffuse directly into the PBS buffer. On the contrast, because PLLA, the inner layer structure, was hydrophobic, which prevented water molecules from entering the core layer structure, and rhBMP2 release process underwent the process of diffusion from internal fibers to external fibers, and then from external to solution. Due to the protection of multiple structures, the release of rhBMP2 showed a relatively slow and sustained release, which proved that the delivery fiber membrane of rhBMP2 performed better than the single structure electrospinning

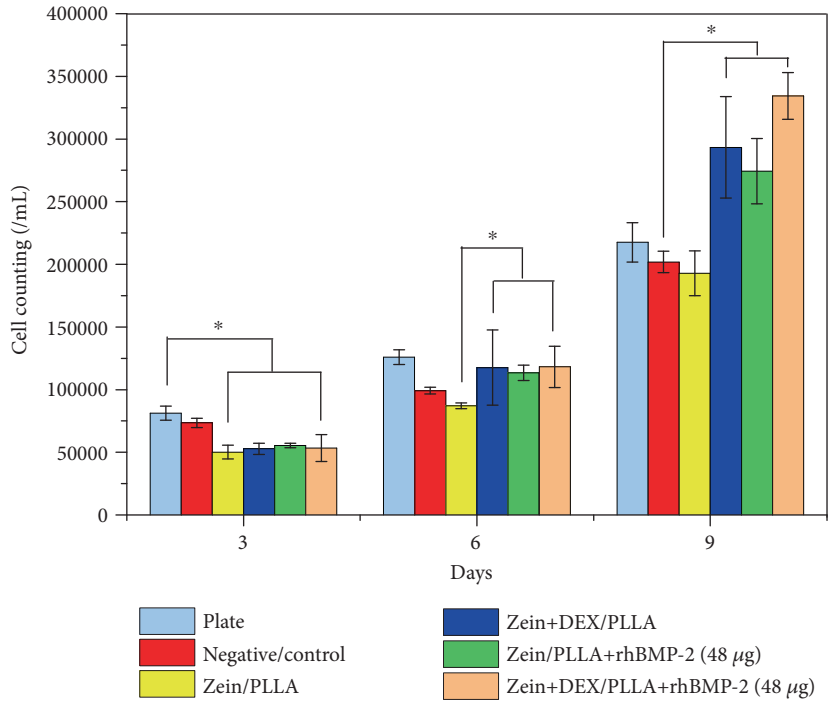



Figure 9: Proliferation of RMSCs grown on nanofiber mats after 3, 6, and 9 days of incubation ($*p < 0.05$).

nanofiber membrane in obtaining the sustained release of BMP2 [22]. The results showed that the drug release lasted 21 days, and the cumulative release rate of DEX and rhBMP2 were 55-65% and 5-35%, respectively, which indicated that the two drugs might be released for a longer time. The dual-release system containing rhBMP2 and DEX prepared by electrospinning was controllable and might be suitable for bone tissue engineering.

### 3.5. Cell Behavior

*3.5.1. Cell Proliferation.* As shown in Figure 1, in order to better achieve the effect of cell experiment, the design of cell climbing tablets was designed as a "double coating form" in this study. In cell experiments, the fiber membrane is directly spinned on the cover slide, and the thickness of the spinning depends on the amount of drug loading. If the drug loading is large, the spinning time will need to be prolonged, and the thickness of the membrane will also increase. However, when the thickness is increased to a certain extent, the nanofiber membrane will sometimes be separated from the glass surface in the cell culture medium, thus affecting the effect of cell culture and experimental results. Therefore, in this study, nanofiber membrane was used to coat the glass climber on double layer, so that the stability of the glass surface would not be affected by the thickness of the fiber membrane, and the drug could also be loaded on with positive and negative layers of the glass cover, and then the drug could also be released effectively in the cell culture medium. This design can not only reduce the thickness of the monolayer on the cover glass but also achieve the ideal drug concentration.

Figure 9 shows the proliferation of rat bone marrow mesenchymal stem cells cultured on Zein/PLLA, Zein-DEX/PLLA, Zein/PLLA-rhBMP2 (48 $\mu$g), and Zein-DEX/PLLA-rhBMP2 (48 $\mu$g) fibrous membranes for 9 days. Drug carriers were designed to minimize toxicity and side effects while maximizing the efficacy of drugs [23]. The results of cell proliferation and differentiation in 9 days by cell counting were as follows: as shown in Figure 9, SPSS statistical analysis showed that the data were significant: $*p < 0.05$ meant that there was statistical significance. Figure 9 shows intuitively that comparing with materials without drug loading, the addition of DEX and rhBMP2 could promote the proliferation of RMSCs within 9 days. On the 6th day, the proliferation of Zein-DEX/PLLA fibrous membranes was more significant than that of the other two groups, which confirmed that the release of DEX played an important role in the proliferation of RMSCs in the early stage. At the 9th day, Zein-DEX/PLLA-rhBMP2 fibrous membrane promoted cell proliferation significantly higher than Zein-DEX/PLLA and Zein/PLLA-rhBMP2 fibrous membrane, indicating that rhBMP2 and DEX together promoted cell proliferation at the later stage. Therefore, the effect of fibrous membrane with DEX in the shell layer on cell proliferation was more significant than that of single rhBMP2 in the core layer, and both of them could promote cell proliferation. The fibrous membrane loaded with DEX and rhBMP2 had the most significant effect on cell growth and proliferation in this work.

*3.5.2. Osteogenic Differentiation Analysis.* Alkaline phosphatase (ALP) activity is a marker of osteogenic differentiation and plays an important role in the formation of mineral deposits. Calcium deposition and calcium nodules were further characterized through ARS staining. Figure 10 exhibits ALP staining of RMSCs after 20 days inoculation

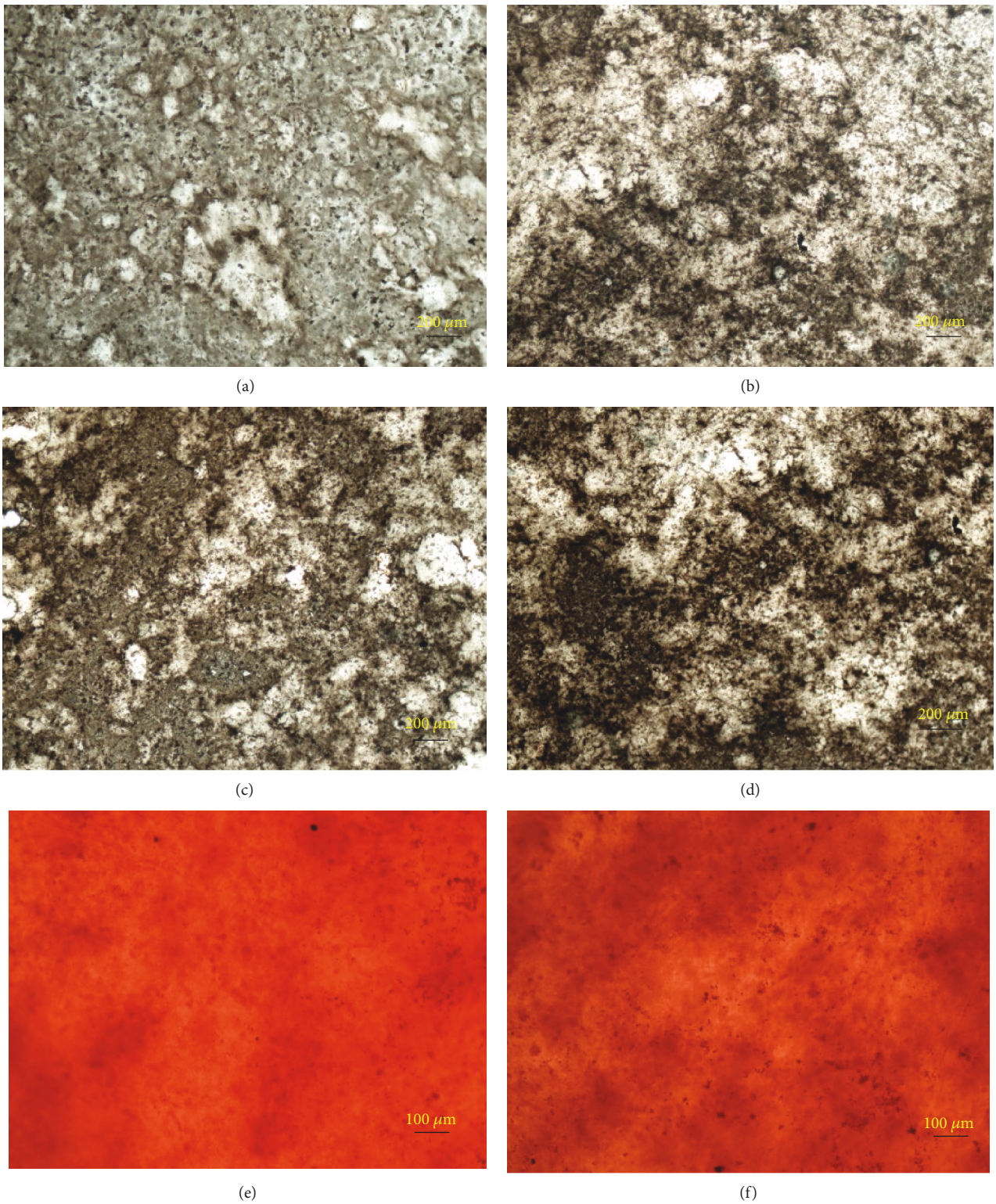
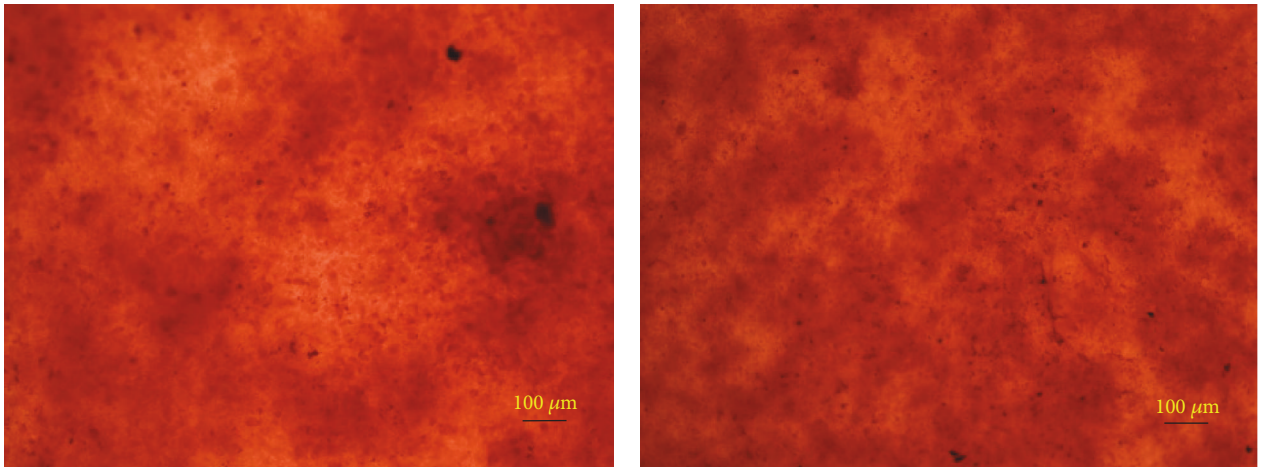



Figure 10: Continued.




(g) (h)

Figure 10: Optical microscopy images: (a–d) representative staining of ALP on day 20. Scale bar = 200 μm. (e–h) Representative staining of ARS on day 21. Scale bar = 100 μm. (a, e) Zein/PLLA, (b, f) Zein-DEX/PLLA, (c, g) Zein/PLLA-rhBMP2 (48 μg), and (d, h) Zein-DEX/PLLA-rhBMP2 (48 μg).

and the results of ARS staining in each group after 21 days of inoculation.

A significantly increased ALP activity can be showed in Zein-DEX/PLLA, Zein/PLLA-rhBMP2 (48 μg), and Zein-DEX/PLLA-rhBMP2 (48 μg) compared with in Zein/PLLA. The increased ALP activity was mainly due to the continuous release of rhBMP2 from the nanofibers, which resulted in increased concentration of osteogenic differentiation factor in the medium. More importantly, large and dense nodules can be observed on the Zein-DEX/PLLA-rhBMP2 scaffold (Figure 10(d)), indicating that combined application of rhBMP2 and DEX can significantly improve the ALP activity.

As shown in Figures 10(e)–10(h), each nanofiber group demonstrated clear positive staining. The most positive staining in Zein-DEX/PLLA-rhBMP2 (48 μg) (Figure 10(h)) showed significant dark red calcium deposits, which indicated that the synergistic effect of two drugs combined with the intrinsic ability of nanofiber surface morphology could induce osteogenic differentiation of RMSCs effectively.

## 4. Conclusion

In this work, Zein-DEX/PLLA-rhBMP2 dual-drug-loaded nanofiber membranes was synthesized by using electrospinning method in one step. Comparing with the single-drug-loaded or no-drug-loaded membranes, we found that it had rougher surface, more controllable *in vitro* drug release behavior, and more significant effect on the growth, proliferation, and osteogenic differentiation of RMSCs, which indicates that this kind of material may possess a potential use in bone tissue engineering.

## Data Availability

No data were used to support this study.

## Conflicts of Interest

There are no conflicts to declare.

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