

参赛学生姓名： 顾凌菲

中学： 上海中学国际部

省份： 上海

国家/地区： 中国

指导老师 1 姓名： 廖辉

指导老师 1 单位： 上海中学国际部

指导老师 2 姓名： 高峰青

指导老师 2 单位： 苏州大学血液和血管疾病
诊疗药物技术工程研究中心

论 文 题 目 : A Three-Layer Gradient
PLGA/Hydroxyapatite Scaffold with pH-Responsive
Curcumin Release for Combined Osteogenic Induction
and Macrophage Immunomodulation

A Three-Layer Gradient PLGA/Hydroxyapatite Scaffold with pH-Responsive Curcumin Release for Combined Osteogenic Induction and Macrophage Immunomodulation

Angelina Ling Fei Gu

Abstract

Bone repair, particularly after complex fractures, remains an enduring clinical challenge. Conventional homogeneous biodegradable scaffolds are limited by uniform pore structure, mismatched degradation kinetics, insufficient osteogenic induction, and limited immunomodulatory capacity, which hinder their performance for complex bone repair. This study proposed a novel three-layer gradient PLGA/HA composite scaffold loaded with curcumin to address the limitations of homogeneous scaffolds. Different PLGA ratios and gradient HA and curcumin contents were designed for outer, middle and inner scaffold layers. Physicochemical characterizations showed that this gradient scaffold possessed more open, irregular porous structure with higher porosity (~65 %) compared with homogeneous scaffold (~30 %), which underpinned its superior osteogenic performance, as evidenced by the significantly increased expression of osteogenic markers RUNX2 and ALP at both protein and mRNA levels in the in-vitro cell tests using MC3T3 E1 pre osteoblasts. Benefiting from varied PLGA ratio across layers, the three-layer gradient scaffold realized a tunable degradation rate. It also achieved pH responsive curcumin release: fast drug release under inflammatory pH 5.5 condition, while reduced drug leakage at physiological pH 7.4 condition. Moreover, curcumin released from the scaffold markedly suppressed pro-inflammatory M1 macrophage polarization, promoted reparative M2 macrophage polarization, and elevated anti-inflammatory IL 10 secretion. Taken together, this three layer gradient scaffold with pH responsive curcumin release exhibited favorable structural and degradative properties, enhanced osteogenic capacity, and immunomodulatory function, representing a promising candidate biomaterial for complex bone fracture repair and deserving further in-vivo validation toward clinical application.

Keywords: Bone repair; three-layer gradient scaffold; PLGA; HA; curcumin; degradation; osteogenic induction; pH-responsive release; macrophage immunomodulation

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Contents

1 Introduction	1
1.1 Background	1
1.2 Objective and Innovation	2
1.3 Research Question and Hypotheses	3
1.4 Overall Study Design and Technical Roadmap	3
2 Materials and Methodology	4
2.1 Materials	4
2.1.1 Chemicals	4
2.1.2 Cells and Reagents	5
2.1.3 Equipment	5
2.2 Experimental Methods	6
2.2.1 Fabrication of Scaffolds	6
2.2.2 Scaffold Material Characterization	7
2.2.3 Degradation Performance	8
2.2.4 pH-Responsive Curcumin Release Test	9
2.2.5 Evaluation of Osteogenic Differentiation Performance	10
2.2.6 Evaluation of Anti-inflammatory Effects	11
3 Results and discussion	13
3.1 Scaffold Morphology and Structure	13
3.2 Porosity	14
3.3 Chemical Structure (FT-IR Spectra)	15
3.4 Crystalline Structure	16
3.5 Degradation Behavior	17
3.6 pH-responsive Drug Curcumin Profile	18
3.7 Osteogenic Differentiation	19
3.8 Anti-inflammatory Effect	22
4 Future Plan	24
5 Conclusion	25
References	27
Acknowledgements	30

1 Introduction

1.1 Background

Bone fractures are one of the most common and painful traumatic injuries worldwide, affecting millions of individuals and causing long-term physical disability across global populations [1]. A fractured bone requires a long recovery period and brings continuous physical discomfort and impaired mobility throughout the healing process. Approximately 10–15% of fracture patients develop bone nonunion and eventually require revision surgery [2]. Therefore, developing advanced biomaterials that can support efficient bone regeneration and improve postoperative healing microenvironments remains highly necessary.

Traditional permanent metallic implants have been widely used for fracture fixation, but they possess two prominent clinical drawbacks: first, a second surgery is mandatory for implant removal after bone healing, adding extra surgical trauma, pain and excessive medical expenditure for patients; second, the traditional permanent metallic implants possess no therapeutic function to suppress the sustained inflammation response induced by bone fracture, and such persistent inflammation state disrupts the bone microenvironment, inhibits osteoblast growth, delays tissue repair and causes continuous swelling and pain [3].

With the rapid development of tissue engineering, biodegradable polymer scaffolds have emerged as a promising alternative to permanent metallic implants to resolve the critical drawback of mandatory secondary surgery [4]. A large number of animal studies have confirmed that polymer scaffolds can support cell growth and promote skeletal tissue regeneration [5]. These temporary porous biomaterials degrade gradually inside the human body without the need of additional surgical extraction after bone repair, significantly reducing surgical trauma, pain, and clinical expenditure for fracture patients.

Nevertheless, conventional homogeneous biodegradable scaffolds still possess prominent limitations. They lack intrinsic therapeutic capacity for anti-inflammatory and immunomodulatory effects and osteogenic induction. On the other hand, most current scaffolds adopt simple, uniform structures with fixed degradation rates. These non-gradient structures cannot adapt to the dynamic biological demands across different bone-healing stages, leading

to obvious structural and functional defects that restrict their repair performance in clinic fracture treatments [6]. These practical limitations are well demonstrated in multiple animal studies focusing on polymer-based scaffold regeneration. Polo-Corrales (2014) investigated diverse bone graft techniques for skeletal fracture repair and bone defect regeneration. Although conventional grafting modalities achieved promising reparative outcomes, autografts incur donor site morbidity while allografts carry infection and immune rejection risks. Current substitutes still fail to fabricate living constructs comparable to native bone in structural and functional properties [7]. Zadpoor (2015) highlighted that geometrical parameters such as surface curvature, pore size and pore shape directly regulate cellular response and bone regeneration rate within porous scaffolds, yet few existing additively manufactured scaffolds harness geometric gradient architectures to optimize bone repair [8]. Even structurally customized PLA/PGA scaffolds designed for TMJ cartilage repair exhibits native-like mechanical strength; however, their single-layered structure fails to recapitulate the natural bone-cartilage transitional tissues [9]. Consequently, these homogeneous polymer scaffolds are only applicable to simple single-tissue repair and remain inadequate for treating complex bone fractures requiring staged degradation, osteogenic induction, and immunomodulation.

1.2 Objective and Innovation

To address the above-mentioned drawbacks including mismatched degradation behavior, persistent postoperative inflammation, and insufficient osteogenic induction capacity, this study proposes a novel three-layer gradient Poly (lactic-co-glycolic acid) (PLGA) / hydroxyapatite (HA) scaffold loaded with curcumin. PLGA, HA and curcumin are promising biomaterials in bone fracture repair. PLGA enables control of degradation behavior through adjusting its lactic/glycolic monomer ratio (e.g. 50:50 PLGA degrades within 1–2 months, while 85:15 PLGA remains structurally stable for 6–12 months, matching the full timeline of bone healing) [10]. As the main inorganic component of natural bone, HA supports cell attachment and activates osteogenic signaling through the release of calcium and phosphate ions, effectively promoting bone reconstruction [11]. Curcumin, a natural anti-inflammatory compound, can be incorporated into the scaffold. The scaffold enables pH-responsive curcumin

release, which alleviates postoperative inflammation and modulates the local immune microenvironment [12].

Collectively, this study targets to fabricate a curcumin-loaded three-layer gradient scaffold, which integrates multiple functions: gradient-matched degradation, pH-responsive drug release, enhanced osteogenic induction, and anti-inflammatory immunomodulatory therapy, aiming to overcome the deficiencies of conventional homogeneous scaffolds.

1.3 Research Question and Hypotheses

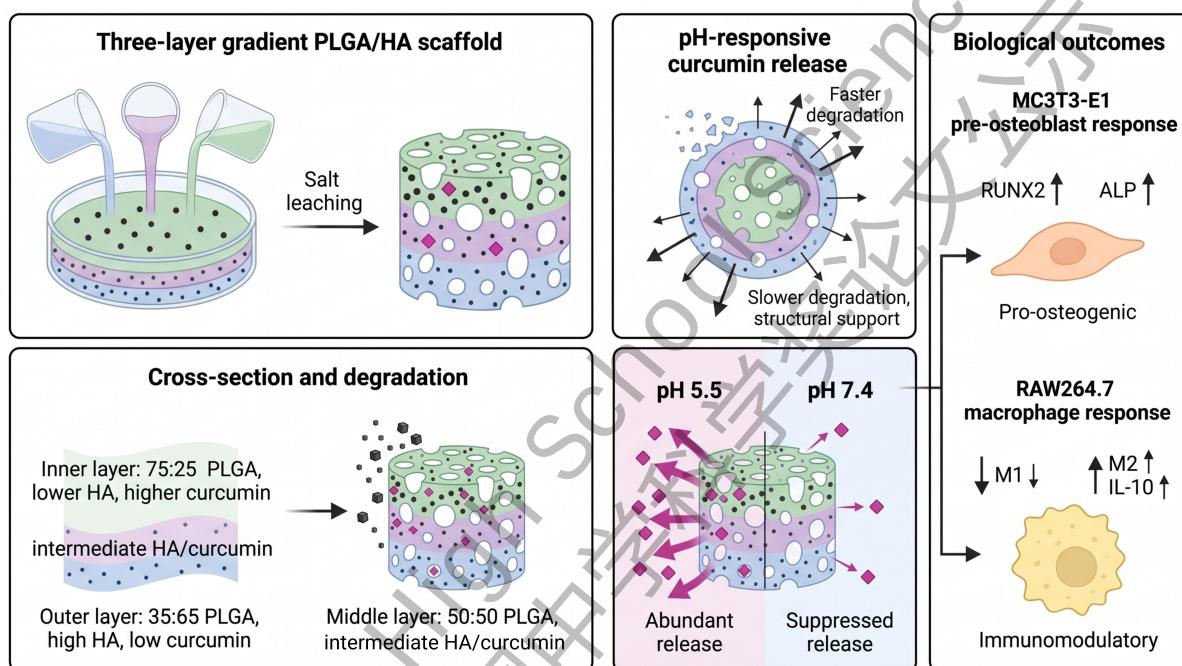
The core research question of this study is: Can a curcumin-loaded three-layer gradient PLGA/HA scaffold achieve staged scaffold degradation, pH-responsive sustained curcumin release, favorable osteogenic induction, and simultaneous inflammation suppression?

To answer the research question, three hypotheses are proposed. First, the three-layer gradient PLGA/HA scaffold will exhibit tunable staged degradation behavior compatible with the physiological bone healing cascade. Second, compared to homogeneous scaffolds, the three layer gradient scaffold will more efficiently promote osteogenic differentiation of pre osteoblasts by up regulating the expression of core osteogenic markers RUNX2 and ALP. Third, the curcumin loaded three-layer gradient scaffold can realize pH responsive curcumin release, suppress pro inflammatory M1 macrophage activation, drive anti-inflammatory M2 macrophage polarization, and boost the secretion of anti-inflammatory cytokine IL-10.

1.4 Overall Study Design and Technical Roadmap

Herein, to address the limitations of conventional homogeneous biodegradable scaffolds and to test the above hypotheses, this study designs and fabricates a curcumin-loaded three-layer gradient PLGA/HA scaffold with layer-specific PLGA ratios (outer layer 35:65, middle layer 50:50, inner layer 75:25), gradient HA contents, and curcumin loading (**Scheme 1**). The scaffold is prepared by sequential solvent casting and salt leaching, while a homogeneous 50:50 PLGA/HA/curcumin scaffold is fabricated as the control. The study then systematically evaluates the scaffold from material, drug-release, and biological perspectives: SEM, porosity measurement, FT-IR, XRD, and in vitro degradation tests are used to characterize its structure, composition, and staged degradation; curcumin release is measured under inflammatory pH

5.5 and physiological pH 7.4 conditions; MC3T3-E1 pre-osteoblasts are used to assess RUNX2 and ALP expression at both protein and mRNA levels; and LPS-stimulated RAW264.7 macrophages are used to evaluate M1/M2 polarization and IL-10 secretion. Through this integrated design, we aim to demonstrate that the three-layer gradient scaffold can achieve tunable degradation, enhanced osteogenic induction, pH-responsive curcumin release, and macrophage immunomodulation, offering a promising biomaterial strategy for complex bone fracture repair.



Scheme 1. Schematic illustration of the design, fabrication, degradation, pH-responsive curcumin release, and biological functions of the curcumin-loaded three-layer gradient PLGA/HA scaffold

2 Materials and Methodology

2.1 Materials

2.1.1 Chemicals

PLGA with three different lactic-glycolic acid copolymer ratios (35:65, 50:50, 75:25) with a molecular weight of 40-60 kDa, Potassium bromide (KBr, spectral grade, CAS No. 7758-02-3), Concentrated hydrochloric acid (HCl, analytical grade, CAS 7647-01-0), Dichloromethane (DCM, 75-09-2), Lipopolysaccharide (LPS), and absolute ethanol were purchased from Adamas-beta (Shanghai, China). Nano-hydroxyapatite (HA, CAS: 1306-06-5, Cat. No.

H906378-25 g, purity $\geq 97\%$, particle size < 100 nm (BET)), Curcumin (CAS: 458-37-7, Cat. No. C138632, purity $\geq 98\%$, HPLC grade), Sodium chloride (NaCl, analytical grade), and Sterile phosphate-buffered saline (PBS, powder, pH 7.2-7.4) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) Double-distilled water (ddH₂O) was self-prepared in the laboratory. Paraformaldehyde (PFA) fixative solution (Cat. No. G1101) was purchased from Wuhan Servicebio Technology Co., Ltd.

2.1.2 Cells and Reagents

MC3T3-E1 murine pre-osteoblast cells (CRL-2593) were obtained from ATCC. Complete osteogenic culture medium, α -MEM (12571-063), DMEM medium, Fetal Bovine Serum (FBS), ALP primary antibody (PA5 106391), and RUNX2 Recombinant Rabbit Monoclonal Antibody (SD208 0, MA5 41185) were obtained from Gibco. Penicillin-Streptomycin (P/S), Trypsin-EDTA solution (C0208-100ml), 5% Triton X-100 solution (P0096-100ml), the confocal nuclear staining reagent (C1002), Mouse IL-10 high-sensitivity ELISA detection kit (Cat. No. PI521), RNAeasy™ Animal RNA Isolation Kit with Spin Column (R0024) were obtained from Beyotime Biotechnology (Shanghai, China). RAW264.7 macrophage cells were obtained from Cell Bank of the Chinese Academy of Sciences.

2.1.3 Equipment

Scanning electron microscopy (SEM, Regulus8230, Hitachi, Japan), Fourier transform infrared spectroscopy (FT-IR, Nicolet iS20, Thermo Scientific, USA), X-ray diffraction (XRD, SmartLab SE, Rigaku, Japan), PrimeScript™ RT Master Mix (Perfect Real Time; RR036A; Takara Bio, Japan), TB Green® Premix Ex Taq™ II (Tli RNaseH Plus; RR820A/B; Takara Bio, Japan), multi-mode microplate reader (Model: CYTATION 3, BioTek, USA), confocal laser scanning microscope (FV3000, Olympus, Japan), CelMate CO₂ incubator (Model: CLM-170B-8, Esco Lifesciences, Singapore), Constant Temperature Shaker (MaxQ 420, Thermo Fisher Scientific), Microplate Reader (VICTOR TMX4, Perkin Elmer), and bench-top laminar flow cabinet (SW-CJ-2FD, Hu Jing Purification) were used in this study.

2.2 Experimental Methods

2.2.1 Fabrication of Scaffolds

The three-layer gradient scaffolds were fabricated by the solvent casting and salt leaching method using three PLGA/HA ratios (**Table 1**). Three precursor solutions with different copolymer ratios (inner layer 75:25, middle layer 50:50, outer layer 35:65) were prepared separately. In detail, 1.2 g of PLGA (75:25), 1.0 g of PLGA (50:50), and 0.6 g of PLGA (35:65) were individually dissolved in 15 mL of dichloromethane (DCM). Each solution was magnetically stirred at 500 rpm for 10 minutes at room temperature to achieve complete dissolution. Gradient hydroxyapatite (HA) powder was added correspondingly: 0.8 g HA to PLGA (75:25), 1.0 g HA to PLGA (50:50), and 1.4 g HA to PLGA (35:65). All mixtures were stirred continuously.

Then, curcumin was loaded. 300 mg of curcumin powder was dispersed in 5 mL of DCM and vortexed for 5 minutes to prepare a mixture. The curcumin mixture was added into the three composite systems with fixed gradient volumes: 1.0 mL for PLGA (75:25), 0.83 mL for PLGA (50:50), and 0.5 mL for PLGA (35:65). Each mixture was stirred for another 10 minutes to ensure uniform curcumin distribution. Following this, a total of 2.0 g of NaCl particles was added into each group as the agent in order to form pores. 10 minutes of stirring was conducted to obtain fully mixed layered solutions.

Next, the three-layer gradient scaffold was constructed by sequential casting. The inner-layer solution was first poured into a 10 cm-diameter Petri dish and oven-dried for 15 minutes for preliminary solidification. Then, the middle-layer precursor was cast on the pre-solidified outer layer and oven-dried for 15 minutes. Finally, the outer-layer solution was overlaid and cured to form an integrated three-layer scaffold (**Figure 1**).

A homogenous single-layer curcumin-loaded scaffold with 50:50 PLGA and PLGA/HA ratio and curcumin amount same as the middle layer of the gradient scaffold was fabricated to serve as the control group.

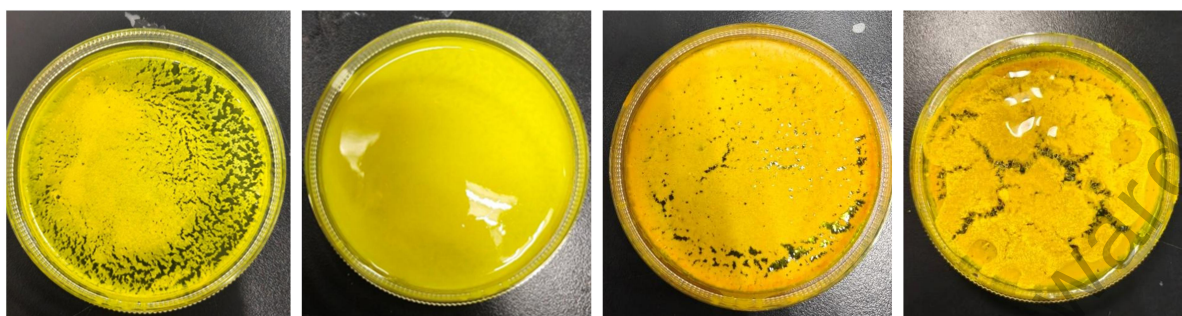


Figure 1. Process photos of scaffold synthesis with different PLGA/HA ratios and curcumin loading amounts

All scaffolds were fully dried before the removal of NaCl particles. NaCl particles were leached by continuous immersion in double-distilled water (ddH₂O). The leaching solution (ddH₂O) was replaced every 10 minutes for several repeated cycles to ensure complete removal of salt. After thorough leaching, all porous scaffolds were fully dried and stored in a dark, low-temperature environment for subsequent characterization and biological testing. All scaffold groups were prepared in triplicate for parallel experiments.

Table 1. Formulation parameters for the outer, middle, and inner layers of three-layer gradient PLGA/HA/curcumin scaffolds prepared by solvent casting and salt leaching

Scaffold Layer Group (PLGA Ratio)	PLGA Mass	DCM Volume	HA Mass	Curcumin Dispersion Volume	NaCl Mass
Inner layer (75:25)	1.2 g	15 mL	0.8 g	1.0 mL	2.0 g
Middle layer (50:50)	1.0 g	15 mL	1.0 g	0.83 mL	2.0 g
Outer layer (35:65)	0.6 g	15 mL	1.4 g	0.5 mL	2.0 g

2.2.2 Scaffold Material Characterization

SEM was used to observe the microscopic morphology and structural characteristics of the scaffolds. All fractured samples were sputter-coated with 20nm gold before imaging (**Figure 2**).



Figure 2. Process of sample preparation for SEM and microscopic morphology observation

The porosity of the scaffolds was determined by using the ethanol liquid displacement method. The initial dry weight (W_1) and the wet weight (W_2) of the scaffolds after fully immersed in absolute ethanol and degassed under vacuum for 30 minutes were recorded. The ethanol-impregnated scaffold was then submerged in ethanol with a known initial volume (V_1), and the final total volume (V_2) was recorded. The scaffold porosity was calculated according to the following formula: $[(W_2 - W_1) / \rho_{\text{ethanol}}] / [(V_2 - V_1) + (W_2 - W_1) / \rho_{\text{ethanol}}] \times 100\%$, where the density of absolute ethanol ρ_{ethanol} was set as 0.789 g/cm^3 . Five parallel samples ($n=5$) were tested for each group, and the average porosity was reported.

FT-IR was applied to analyze the chemical composition and functional group of the scaffolds. Scaffold samples were fully dried and ground into fine powder, then uniformly mixed with spectroscopic-grade KBr for spectral testing. The scanning wavenumber range was set from 4000 cm^{-1} to 400 cm^{-1} with a spectral resolution of 4 cm^{-1} and 32 scan for each sample.

XRD testing was performed to characterize the crystal structure changes of the scaffolds at predetermined incubation time points of 0 and 4 weeks. The XRD scanning range was set from $2\theta = 5^\circ$ to 80° to collect diffraction patterns of all samples.

2.2.3 Degradation Performance

The in vitro degradation performance of scaffolds was evaluated by measuring dry-weight loss. Three parallel samples were tested and averaged for each group. The initial dry mass of each scaffold was recorded. All scaffolds were fully immersed in pH 7.4 PBS and placed in a constant temperature shaker for continuous simulated physiological degradation. After 1 w, 2 w, 3 w and 4 w of incubation, all scaffold samples were retrieved and completely dried to measure residual dry weight. The mass loss curve was produced by calculating the percentage

of remaining weight.

2.2.4 pH-Responsive Curcumin Release Test

Three-layer gradient scaffolds and homogenous scaffolds both loaded with curcumin were placed separately into 12-well plates with three parallel wells for each experimental condition. The prepared pH 7.4 and pH 5.5 PBS buffer solutions were added into the corresponding wells to fully immerse the scaffold samples. To prevent photodegradation of photosensitive curcumin, all 12-well plates were tightly wrapped with aluminum foil and placed in a constant temperature shaker for continuous incubation. At scheduled time points of 0.5 h, 1 h, 2 h, 4 h, 6 h, 12h, 24h, 48h, 72h, 144h, and 168h during incubation, release supernatant was collected from each well to be detected using a multimode microplate reader at a fixed wavelength of 405 nm to measure the UV-Vis absorbance of curcumin.

The standard curve of curcumin concentration absorbance was established in advance for subsequent quantitative calculation of cumulative drug release. To construct this curve, curcumin stock solution was serially diluted to nine gradient working concentrations: 2 mg/mL, 20 $\mu\text{g/mL}$, 2 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, 0.8 $\mu\text{g/mL}$, 0.4 $\mu\text{g/mL}$, 0.2 $\mu\text{g/mL}$, 0.1 $\mu\text{g/mL}$, and 0.05 $\mu\text{g/mL}$. The absorbance of each concentration was measured by the multimode microplate reader at 405 nm wavelength to generate the curcumin standard curve. Then, the cumulative curcumin release percentage of supernatant samples was calculated according to the standard curve of curcumin concentration-absorbance (**Figure 3**).

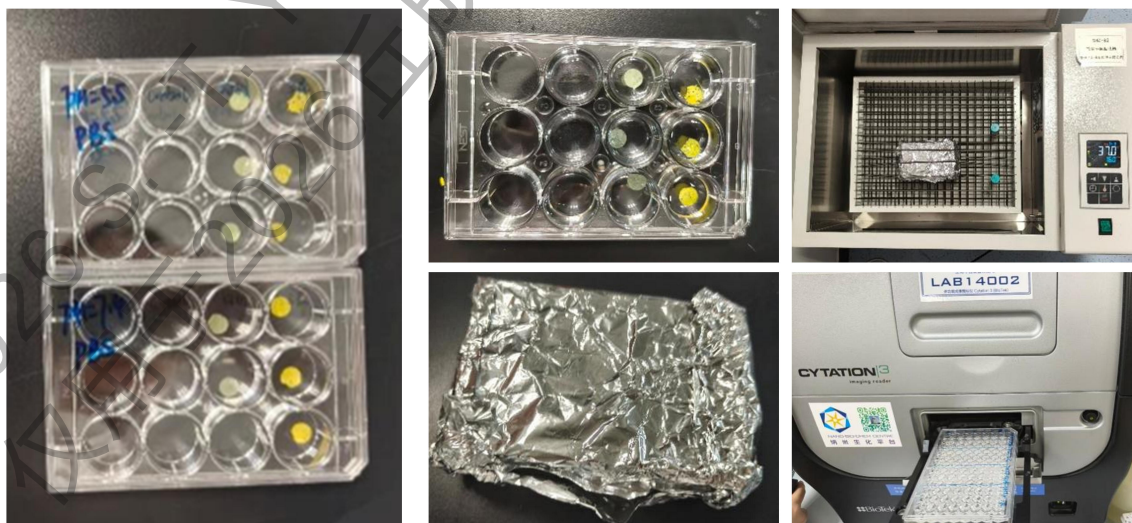


Figure 3. Photographs of the pH-responsive curcumin release experiment

2.2.5 Evaluation of Osteogenic Differentiation Performance

MC3T3-E1 Cell Culture and Seeding: Cell resuscitation was performed in a laminar flow cabinet. Frozen MC3T3-E1 mouse osteoblast precursor cells were thawed in 37 °C water bath and centrifuged at 1000 r/min for 5 minutes. Then, cell pellets were resuspended in α -MEM complete medium with 10% FBS and 1% P/S and cultured in a 37 °C incubator with 5.0% CO₂ for stable proliferation (**Figure 4**).

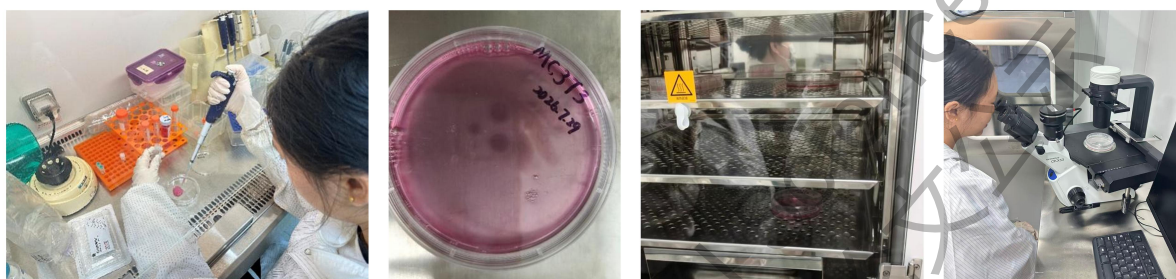


Figure 4. Resuscitation, subculture, and microscopic observation of MC3T3-E1 pre-osteoblasts in α -MEM complete medium

Immunofluorescence Staining of RUNX2 and ALP Osteogenic Proteins: Before immunofluorescence staining, the culture medium was completely removed, and each well was rinsed with PBS. 100 μ L paraformaldehyde (PFA) fixative was added to each well for 15 minutes at room temperature to preserve cell morphology and intracellular proteins. After fixation, 100 μ L of 0.5% Triton X-100 solution was added for 15 minutes to permeabilize cell membranes to facilitate antibody penetration. Then, 400 μ L of ALP primary antibody and 400 μ L of RUNX2 primary antibody were added to corresponding wells, and the plate was incubated at 37 °C for 1 hour. Unbound primary antibodies were removed by three PBS rinses. Next, 400 μ L of diluted secondary antibody working solution was added to each well. The plate was sealed and incubated overnight in a 4 °C refrigerator. On the next day, nuclear staining was completed by adding the confocal nuclear staining reagent. After 10 min, cell samples were observed and imaged under a confocal laser scanning microscope to analyze the fluorescence intensity and positive expression of osteogenic proteins RUNX2 and ALP.

qPCR Analysis: Cultured cells were lysed in the scaffolds. Total RNA was isolated using the RNAeasy™ Animal RNA Isolation Kit. The cell lysate was mixed with an equal volume of binding buffer and transferred to an RNA purification column. The column was sequentially

washed with Wash Buffers I and II, and the purified RNA was eluted in 30-50 μL of elution buffer. RNA concentration and purity were assessed by a spectrophotometer.

500ng RNA was reverse-transcribed into complementary DNA using PrimeScript™ RT Master Mix in a 10- μL reaction system. Reverse transcription was performed at 37°C for 15 min, followed by enzyme inactivation at 85°C for 5 s.

Real-time quantitative PCR was performed using TB Green® Premix Ex Taq™ II. Each 20- μL reaction contained 10 μL of 2 $\times$ TB Green Premix Ex Taq II, 0.8 μL each of forward and reverse primers (10 μM), 2 μL of the reverse-transcription product, 0.4 μL of ROX Reference Dye or ROX Reference Dye II and 6 μL of nuclease-free water. The amplification program consisted of an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 30–34 s. A melting-curve analysis was subsequently performed to verify amplification specificity.

RUNX2 and ALP expressions were normalized to that of GAPDH and calculated using the comparative Ct method. ΔCt was defined as $\text{Ct_ALP} - \text{Ct_GAPDH}$, and the mean ΔCt of the homogeneous scaffold group on day 1 was used as the calibrator for calculating $\Delta\Delta\text{Ct}$. Relative gene expression was expressed as $2^{-\Delta\Delta\text{Ct}}$. Three independent biological replicates were analyzed and averaged per group for subsequent calculations. Statistical analyses were performed using ΔCt values, whereas $2^{-\Delta\Delta\text{Ct}}$ values were used for graphical presentation.

2.2.6 Evaluation of Anti-inflammatory Effects

Macrophage Culture: RAW264.7 macrophage cells were cultured in high-glucose DMEM medium containing 10% FBS and 1% P/S at 37 °C with 5% CO_2 .

To prepare curcumin extract derived from the gradient scaffolds, 100 mg scaffolds were immersed in 10 mL of DMEM medium and incubated in a constant temperature shaker at 37 °C and 100 rpm for 24 h. The supernatant was collected, centrifuged at 3000 rpm for 10 min, and filtered through a 0.22 μm sterile filter to obtain curcumin extract.

RAW264.7 cells were seeded in 24-well plates at a density of 2×10^5 cells per well and cultured overnight for complete adherence. Cells were then treated with three different medium groups with $n=6$ biological replicates: medium with LPS, medium with LPS and free curcumin, and medium with LPS and curcumin extract from the gradient scaffold. After 24 h of

continuous inflammatory intervention, cell culture supernatants and cell pellets were collected for subsequent inflammatory detection.

Immunofluorescence Staining for Macrophage Polarization Assessment: RAW264.7 cells from each experimental group were harvested and thoroughly washed with sterile PBS. Cells were then incubated with a mixture of antibodies for immunofluorescent staining. W CD86-FITC and iNOS-PE are used as markers for M1 pro-inflammatory macrophages, and CD206-APC together with Arg-1-PerCP for M2 anti-inflammatory macrophages. Cells were first permeabilized to allow antibodies to cross the cell membrane and bind intracellular targets. The antibody incubation was performed at 4 °C in the dark for 30 minutes. After incubation, unbound antibodies were completely removed by repeated PBS washing. Prepared cell samples were analyzed and imaged under a confocal laser scanning microscope to analyze the fluorescence intensity of M1 and M2 (**Figure 5**).



Figure 5. Workflow for immunofluorescence staining and imaging analysis of macrophage polarization

Detection of Inflammatory Factor (ELISA): ELISA detection kit was used to quantitatively detect the expression level of anti-inflammatory cytokine IL-10 in cell culture supernatant. The lyophilized mouse IL-10 standard powder was fully dissolved in matched standard dilution liquid. Five-gradient: 100 pg/mL, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, 6.25 pg/mL dilution was

made to establish a standard curve for quantitative detection, with $n=3$ replicates for each gradient group.

IL-10 standard solutions and cell culture supernatants from different experiment groups were added into multi-well plate, with five replicate wells set for each group. The plate was then tightly sealed with sealing film and incubated in a constant temperature incubator to allow sufficient antigen antibody binding.

After incubation, HRP Streptavidin conjugate was added to bind the antigen antibody complex to allow the enzyme HRP to attach to it. Next, TMB chromogenic solution was added to react with HRP to produce a blue color, and the deeper the blue, the more IL 10 in the sample. When the reaction reached stability, ELISA stop solution was added to terminate the reaction. Finally, the absorbance value of each well was measured at 450 nm using a multimode microplate reader. The IL 10 concentration of each sample was calculated from the standard curve (**Figure 6**).

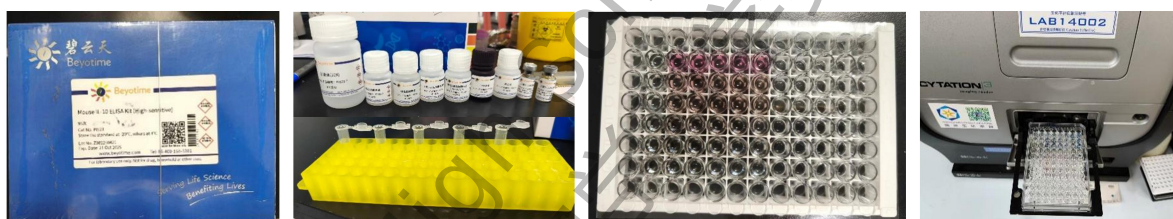


Figure 6. Experimental workflow for ELISA detection of the anti-inflammatory cytokine IL-10

3 Results and discussion

3.1 Scaffold Morphology and Structure

Scanning electron microscopy (SEM) was applied to characterize the fracture cross section and surface porous microstructure of the homogeneous and three-layer gradient scaffolds (**Figure 7**). The homogeneous scaffold exhibited a relatively smooth surface. Its surface featured small sized pores that were densely packed and evenly distributed across the whole field of view, forming a uniform single phase porous architecture. In comparison, the three layer gradient scaffold displayed a visibly rougher surface. Distinct layered structures were clearly distinguished on its fracture surface. Its surface possessed larger and more irregular pores, which matched the design of three stacked layers with varied PLGA HA blending ratios.

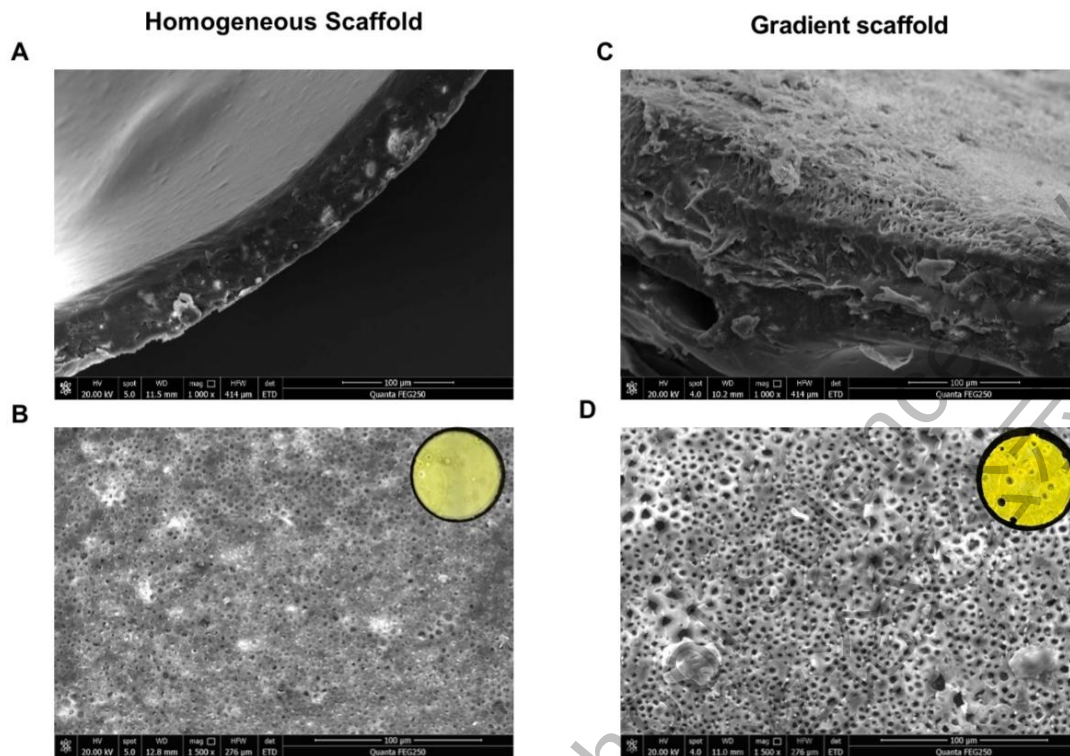


Figure 7. SEM morphological characterization of homogeneous scaffolds cross section(A) and surface (B), and three-layer gradient scaffolds cross section (C) and surface (D)

3.2 Porosity

Quantitative porosity measurement (Figure 8) revealed that the homogeneous scaffold had an average porosity of approximately 30 %. By contrast, the three-layer gradient scaffold achieved around 65 % porosity, more than doubling the value of the homogeneous group. These numerical findings corroborate the qualitative observations from SEM, where the three-layer gradient scaffold exhibited more open pore architecture.

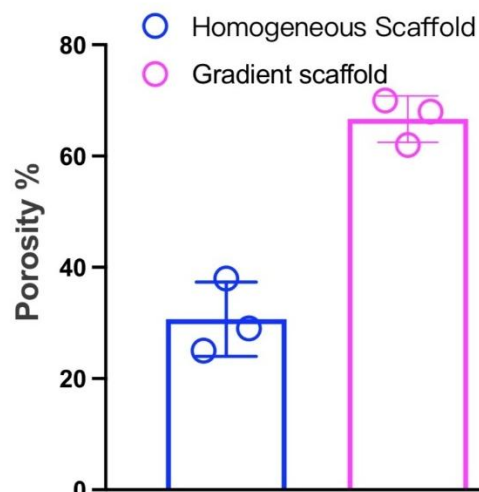


Figure 8. Porosity of homogeneous and three-layer gradient scaffolds

3.3 Chemical Structure (FT-IR Spectra)

Fourier transform-infrared spectroscopy (FT-IR) was used to assess chemical groups of the homogeneous and three-layer gradient scaffolds from 0 to 4 weeks during in-vitro degradation. Peaks in the spectrum corresponds to infrared absorption by specific chemical groups: PLGA-associated C=O stretching vibration peak near 1750 cm^{-1} , HA-associated P-O peak at $1000\text{--}1100\text{ cm}^{-1}$, and O-H peak around 3300 cm^{-1} . A stronger absorption peak indicates higher relative content of the target groups [13].

Both types of scaffolds exhibited similar overall characteristic absorption peaks of PLGA and HA, confirming successful component compounding of the scaffolds (**Figure 9**). The C=O peak of three-layer gradient scaffold became shallower at week 4, indicating the hydrolysis of PLGA. Meanwhile, a new minor absorption peak emerged beside the C=O peak at near 1710 cm^{-1} , assigned to the hydrogen-bonded carboxylic acid (-COOH) groups generated by ester hydrolysis. The three-layer gradient scaffold also showed a broad O-H peak at week 4. This could be caused by hydroxyl-containing degradation by-products and absorbed moisture.

Despite that both scaffolds showed absorption peaks for similar chemical components, the shallower C=O peak, the newly emerged -COOH peak, and stronger O-H peaks at week 4 of the three-layer gradient scaffold indicated that the three-layer gradient scaffold underwent a faster degradation.

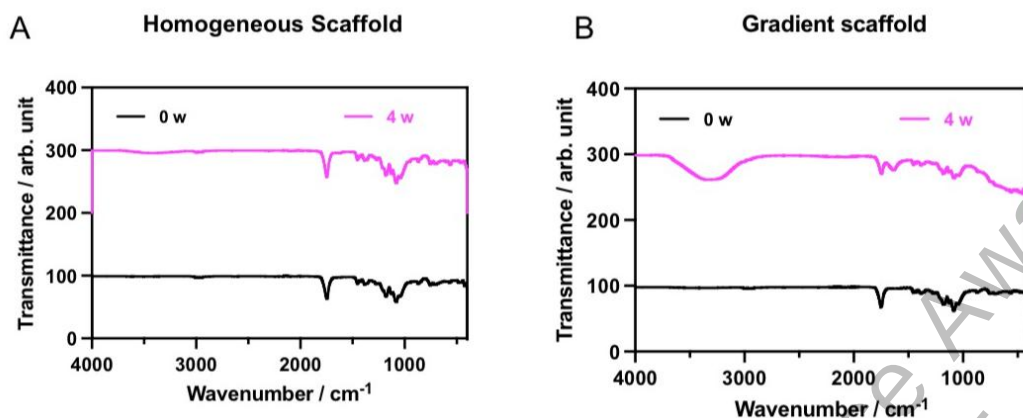


Figure 9. FT-IR spectroscopic characterization during in-vitro degradation of homogeneous and three-layer gradient scaffolds

3.4 Crystalline Structure

XRD was performed to characterize the crystal structure of the homogeneous scaffolds and three-layer gradient scaffolds. XRD curves from both groups (**Figure 10**) exhibited characteristic peaks dominated by PLGA at approximately 20° and HA-related diffraction peaks near 32° [14]. Compared with the homogeneous scaffold, the three-layer gradient scaffold displayed a broader PLGA diffraction peak, indicating a more distributed PLGA phase. This aligned with the varied PLGA ratios across the three distinct layers of the gradient scaffold. On the other hand, the three-layer gradient scaffold developed a much sharper HA diffraction peak. This sharp peak confirms higher HA content within the gradient scaffold design. Collectively, these XRD results verified that the structural and compositional features of the two types of scaffolds are consistent with our material design, and further demonstrated that the three-layer gradient scaffold possessed a more complex crystallographic nature. At week 4, the homogeneous scaffold retained its main XRD features, with only slight changes in the PLGA halo at $\sim 20^\circ$ and the HA peaks at $\sim 32^\circ$, consistent with its slower degradation. In contrast, the gradient scaffold showed a broader PLGA diffraction halo around 20° , indicating a more disordered PLGA phase after degradation. This can be attributed to the faster hydrolysis of PLGA in the gradient scaffold, which caused chain scission and reduced the amount of intact PLGA.

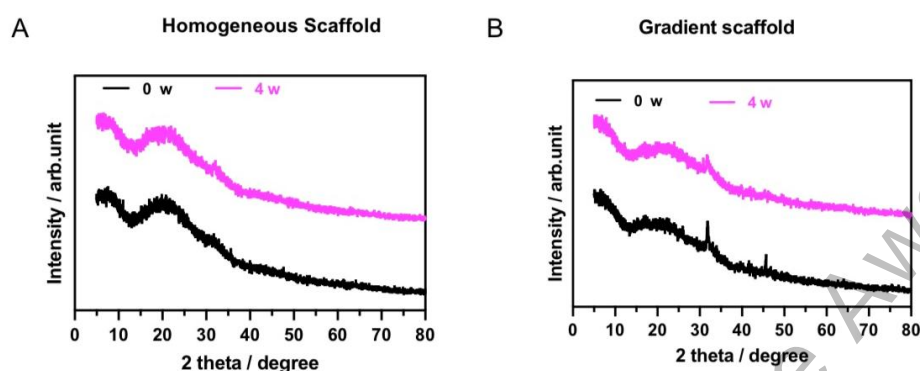


Figure 10. XRD profiles of the homogeneous and three-layer gradient scaffolds

3.5 Degradation Behavior

Macroscopic optical photographs were obtained for homogeneous and three-layer gradient scaffolds following 0, 2, and 4 weeks of in-vitro degradation. In addition, mass loss curve of both scaffolds, in which the remaining weight percentage was calculated, was recorded over the 4-week in-vitro degradation period.

The optical images in **Figure 11A** showed progressive structural breakdown for both scaffold groups over the degradation period. From the mass loss curve in **Figure 11B**, both scaffold groups exhibited continuous mass reduction throughout the 4-week test, indicating that degradation occurred in both scaffolds. At week 4, the three-layer gradient scaffold retained approximately 40% of its initial mass, whereas the homogeneous scaffold maintained around 60% of its original mass. This observation demonstrates that the three-layer gradient scaffold underwent faster degradation compared with the homogeneous scaffold. This outcome aligns with our design rationale: the outer layer of the gradient scaffold with a 35:65 PLGA ratio will degrade faster than the homogeneous scaffold with a 50:50 PLGA ratio. The results verified that the degradation rate of PLGA-based scaffolds can be tuned by adjusting the PLGA monomer ratio.

Over the 4-week degradation period, the three-layer gradient scaffold exhibited rapid early-stage mass loss, which could open interconnected pore channels to facilitate cell migration, nutrient diffusion and new-tissue growth. This favorable early-stage rapid degradation is enabled by the layered architecture of the gradient scaffold: the outer layer

degrades fast, while the inner layers are designed to degrade more slowly to preserve structural support for maturing newly-formed bone tissue. In contrast, the homogenous scaffold adopts a uniform PLGA ratio throughout. To maintain long-term structural integrity, it must degrade at a slower overall rate, which consequently restricts its capacity to support cell migration and nutrient infiltration.

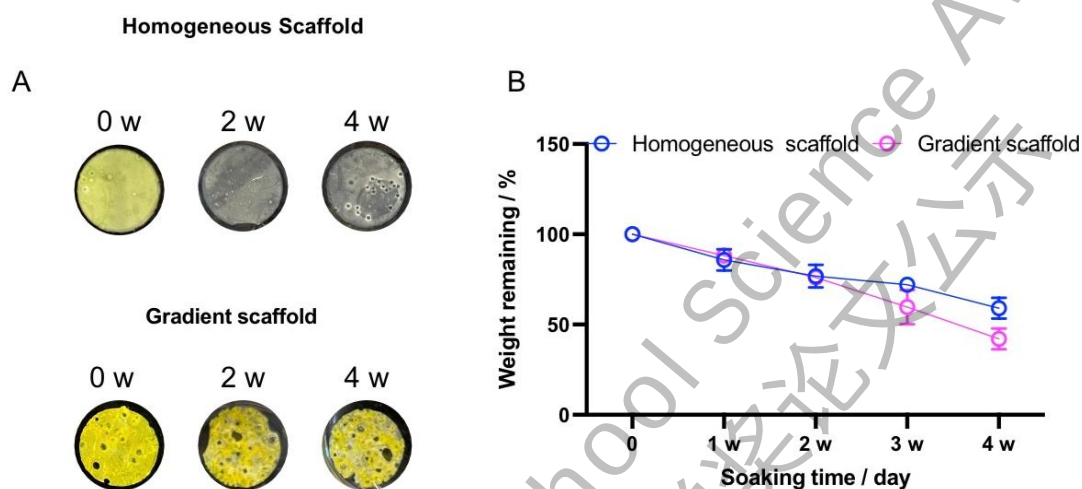


Figure 11. In- vitro degradation performance of homogeneous and three-layer gradient scaffolds

3.6 pH-responsive Drug Curcumin Profile

Curcumin release efficiency of homogeneous and three-layer gradient scaffolds was measured under pH 5.5 PBS simulating inflammatory bone microenvironment and pH 7.4 PBS simulating normal body microenvironment over 168 h using UV- Vis spectroscopy (**Figure 12**).

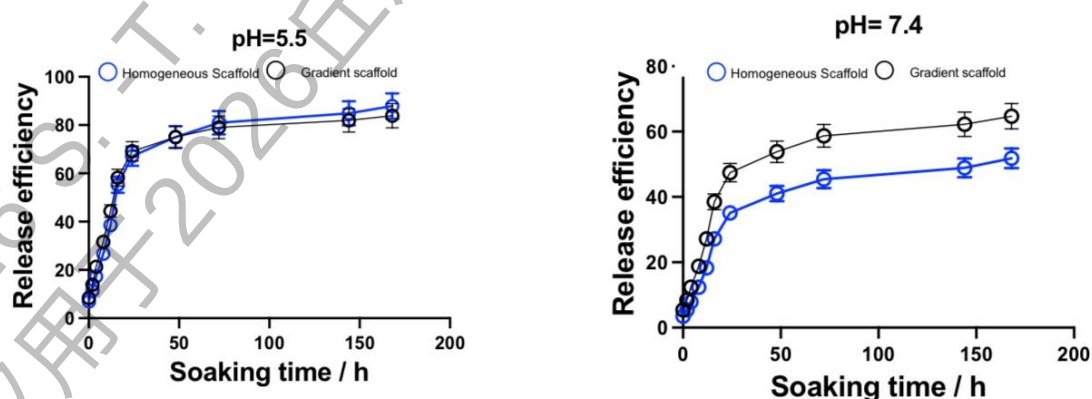


Figure 12. Curcumin release behavior under different pH conditions for homogeneous and three-layer gradient scaffolds

The three layer gradient scaffold exhibited prominent pH-responsive curcumin release behavior. At acidic pH 5.5 PBS, the three gradient scaffold achieved approximately 85-88% of cumulative curcumin release by 168 h, whereas under physiological neutral pH 7.4 PBS, drug release was substantially suppressed to around 50% at the same time point. In contrast, the homogeneous scaffold showed much less pH-dependent responsiveness. Despite that it achieved a cumulative curcumin release rate comparable to that of the three-layer gradient scaffold under acidic conditions, its curcumin release rate reached approximately 63% at neutral pH by 168 h, substantially higher than that of the gradient scaffold. This pH-responsive release profile endows the three-layer gradient scaffold with a key advantage: drug release can be tailored to match the physiological microenvironment.

Curcumin release from scaffolds is jointly governed by molecular diffusion and PLGA matrix erosion. Acidic conditions accelerate PLGA degradation and theoretically promote drug release [15]. In this study, both scaffolds exhibited high total curcumin release at pH 5.5 PBS. The main group-specific distinction appeared under physiological pH 7.4 PBS, where the three-layer gradient scaffold effectively restrained drug release. This pH-dependent release behavior aligned with the material design intention: limiting premature drug leakage under healthy neutral tissue condition while retaining sufficient curcumin payload for subsequent inflammatory-phase intervention. The stratified internal structure of the three-layer gradient scaffold limits the occurrence of water penetration into the inner curcumin-loaded layer and thus hinders outward diffusion of embedded curcumin under normal physiological environment [16]. Such structure enables the three-layer gradient scaffold to adapt to the dynamic microenvironment during bone repair and supports sustainable targeted therapeutic function. Its ability to preserve therapeutic drug release under healthy tissue microenvironments also prevents potential cytotoxic effects as high concentrations of bioactive agents can impair normal cell viability in non-inflamed tissue [17]. As such, compared with the homogeneous scaffold, the three-layer gradient scaffold possesses remarkable advantages of pH-responsive drug release behavior and sustained drug release for bone regeneration.

3.7 Osteogenic Differentiation

The immunofluorescence assay targets two key osteogenic proteins: RUNX2, an early

osteogenic marker [18] and ALP, a mid-stage osteogenic marker [19]. Immunofluorescence staining was performed on homogeneous and three-layer gradient scaffolds at week 0, 2 and 4. RUNX2 was labelled with green fluorescence, ALP was labelled in magenta, and cell nuclei were stained blue. Higher fluorescence intensity means stronger osteogenic protein expression (Figure 13).

At week 0, both scaffold groups displayed similar cell density and nearly undetectable RUNX2 and ALP fluorescence signals, confirming identical starting conditions for cell differentiation. As culture proceeded, fluorescence brightness for both RUNX2 and ALP increased in both groups. Notably, the three-layer gradient scaffold exhibited stronger and denser green RUNX2 and magenta ALP signals compared with the homogeneous group at week 2 and week 4. On the other hand, cells cultured on three-layer gradient scaffolds also showed more spread out and clustered cellular morphology.

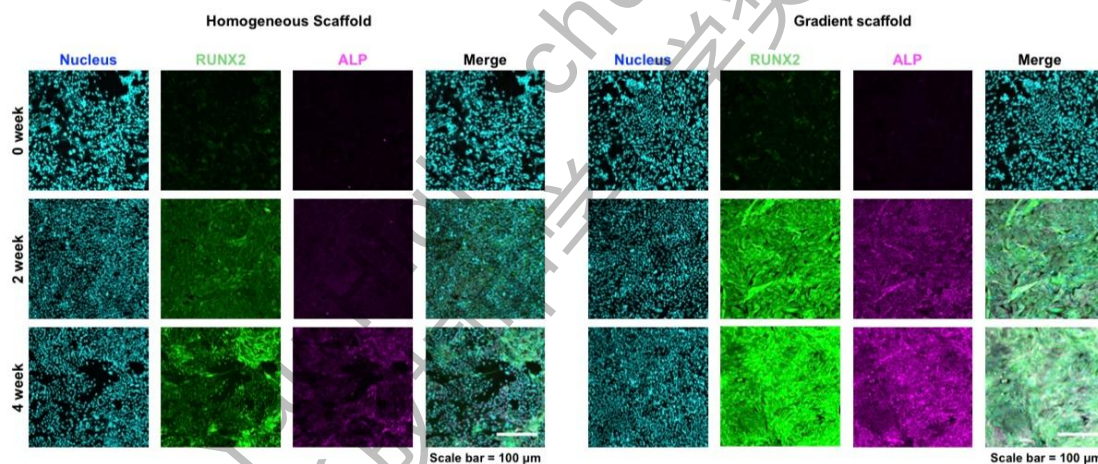


Figure 13. Immunofluorescence analysis of osteogenic markers for homogeneous and three-layer gradient scaffolds

Quantitative analysis of immunofluorescence was conducted to compare fluorescence intensity of RUNX2 and ALP on homogeneous and three-layer gradient scaffolds. At week 0, no significant difference was detected for either marker between the two groups, indicating comparable starting conditions. Mean fluorescence intensity of RUNX2 and ALP increased over time in both scaffold groups. At week 2 and week 4, the three-layer gradient scaffold showed significantly higher RUNX2 intensity (**** $p < 0.0001$) compared with the homogeneous scaffold. Similarly, significant differences of ALP intensity appeared at week 2

(** $p < 0.01$) and became more pronounced at week 4 (**** $p < 0.0001$). These quantitative results matched the visual observation from immunofluorescence images (**Figure 14**).

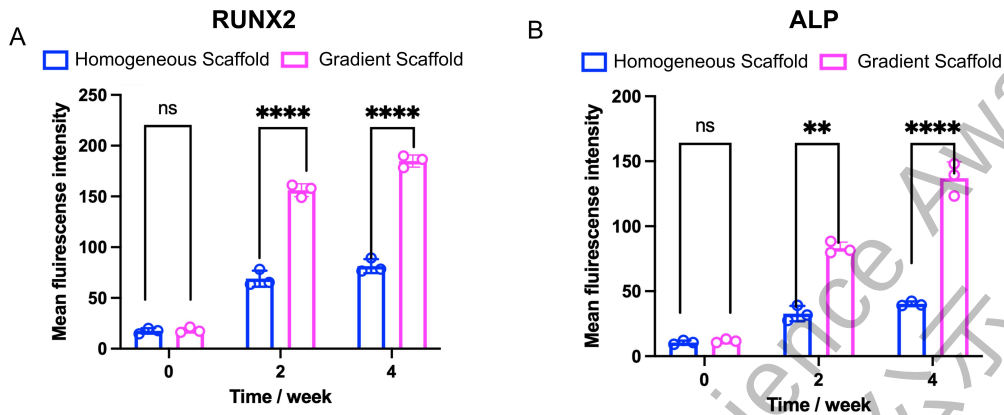


Figure 14. Quantitative fluorescence intensity of osteogenic markers for homogeneous and three-layer gradient scaffolds

In addition, qPCR was performed to quantify the fold change of RUNX2 mRNA and ALP mRNA on homogeneous and three-layer gradient scaffolds (**Figure 15**). At week 0, no significant difference of RUNX2 mRNA and ALP mRNA levels was observed between the two groups confirming comparable initial gene expression status. RUNX2 mRNA and ALP mRNA level increased over time in both groups. At week 2 and week 4, the three-layer gradient scaffold displayed significantly higher RUNX2 mRNA and ALP mRNA fold change compared with the homogeneous group (**** $p < 0.0001$).

Since both scaffolds share the same starting cell conditions, the enhanced osteogenic protein expression of the three-layer gradient scaffold, observed in both immunofluorescence staining images and quantitative analysis of fluorescence intensity, indicates that the layered structural design provides physical cues that effectively promote osteogenic differentiation of seeded cells. The qPCR transcriptional results are consistent with immunofluorescence results, further confirming that the layered structure up-regulated RUNX2 and ALP at transcription level which further translated into abundant RUNX2 and ALP protein. Collectively, these molecular observations provide support for superior osteogenic differentiation performance of the three-layer gradient scaffold.

The strong osteogenic performance can be attributed to the distinctive porous architecture of the gradient scaffold, as evidenced by morphological observations in 3.1 and porosity

quantification in 3.2. Compared with homogeneous scaffold, the layered configuration, higher porosity, and irregular larger pores in the gradient scaffold creates a better porous microenvironment that facilitates cell infiltration into the scaffold interior, efficient nutrient transport and subsequent new-tissue ingrowth [20].

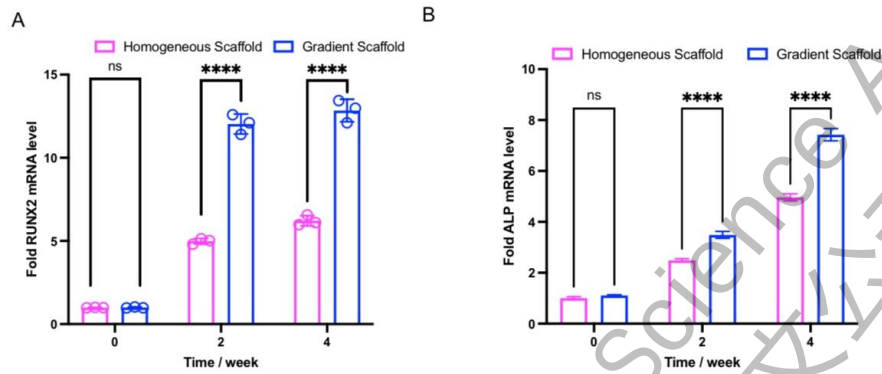


Figure 15. qPCR gene expression analysis of osteogenic markers for homogeneous and three-layer gradient scaffolds

3.8 Anti-inflammatory Effect

Immunofluorescence staining was used to evaluate M1 macrophage polarization of three different experiment groups. Confocal laser scanning microscope was used to observe and analyze the fluorescence intensity of M1 (**Figure 16**).

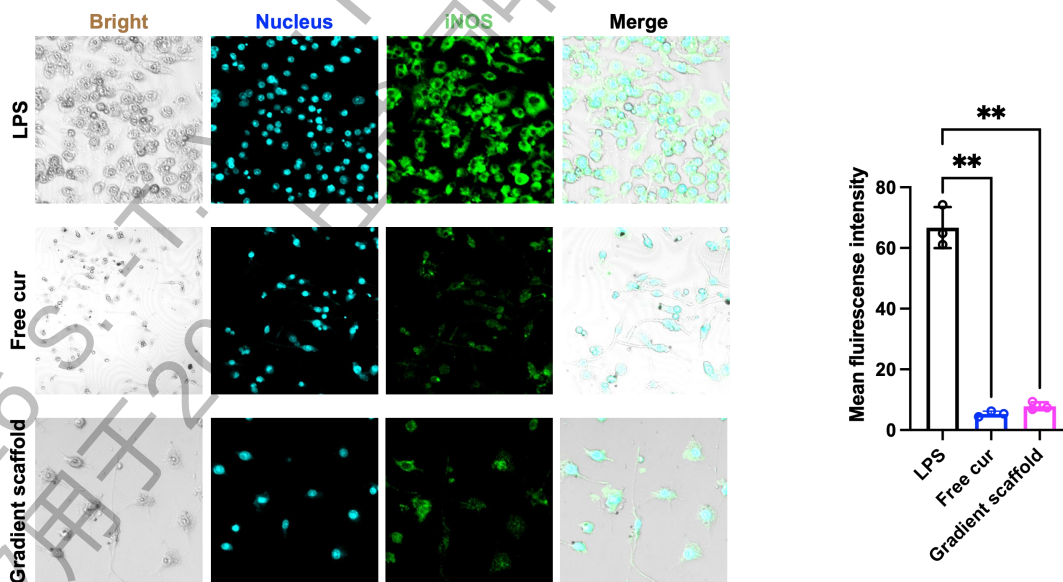


Figure 16. Immunofluorescence analysis of macrophage polarization (M1)

The LPS group, RAW264.7 cells cultured in medium with only LPS, showed strong

fluorescence, indicating obvious pro-inflammatory M1 activation. The free curcumin group (RAW264.7 cells cultured in medium with LPS and free curcumin) and the gradient scaffold group (RAW264.7 cells cultured in medium with LPS and curcumin released from the three-layer gradient scaffold) both showed significantly reduced intensity of fluorescence. This proved that curcumin successfully inhibited LPS-induced M1 inflammatory responses. On the other hand, the gradient scaffold group showed similar anti-inflammatory effects compared with free curcumin group, which indicates that the curcumin released from the scaffold remains biologically functional and can effectively suppress pro-inflammatory macrophage activation [21].

Immunofluorescence staining was used to evaluate anti-inflammatory M2 macrophage polarization with CD206 as a specific M2 marker. The LPS group showed very low CD206 expression, both as observed from the immunofluorescence images and in the quantitative fluorescence intensity bar graph. The free curcumin group exhibited only a slight increase of CD206 intensity, with no significant difference from the LPS group. In contrast, the gradient scaffold group presented a remarkably higher CD206 signal, showing strong M2 polarization. The prominent M2 polarization is favorable for alleviating inflammation and creating a favorable microenvironment for bone healing (**Figure 17A**).

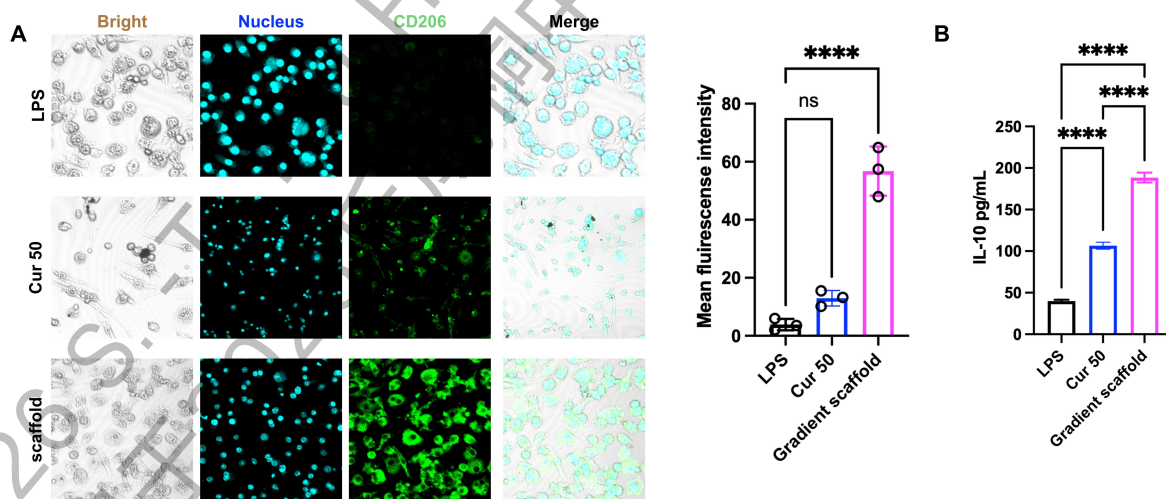


Figure 17. Immunofluorescence analysis of macrophage polarization (A) and ELISA analysis of macrophage (B) of M2

To further verify the M2-polarizing effect, ELISA was applied to detect the secretion of IL-10, a key anti-inflammatory cytokine mainly secreted by M2-typed macrophages. As shown

in **Figure 17B**, the gradient scaffold group produced markedly higher IL-10 levels compared with the LPS group and the free curcumin group. These ELISA findings corroborated the immunofluorescence results, demonstrating that the three-layer curcumin-loaded gradient scaffold effectively drives macrophages towards the anti-inflammatory M2 phenotype and thus possesses a strong advantage of reducing inflammation, supporting tissue repair and promoting bone regeneration. [22]

Interestingly, unlike the gradient scaffold, free curcumin failed to induce obvious M2 polarization under the current experimental conditions, which was an unexpected result. One possible explanation for this phenomenon is that the curcumin extract from the gradient scaffold was collected after 24-hour soaking of the composite scaffold. During the incubation period, HA within the scaffold may have partially dissolved, potentially releasing calcium and phosphate ions into the medium. These ions have been reported to activate the calcium-sensing receptor (CaSR) and downstream Wnt/ β -catenin signaling in macrophages, which could in turn contribute to M2-type anti-inflammatory polarization. Therefore, the M2-polarizing effect of the gradient scaffold extract may not be solely attributable to curcumin and could also involve HA-derived ionic cues [23]. However, this remains speculative because Ca/P release, CaSR activation, and Wnt/ β -catenin signaling were not directly measured in this study. Further experiments using HA-free scaffolds, curcumin-free gradient scaffolds, and CaSR/Wnt inhibitors are needed to verify this possibility.

4 Future Plan

An unexpected observation was found in this study: the gradient scaffold group exhibited superior performance in promoting anti-inflammatory M2 polarization compared with the free curcumin group. This phenomenon suggests that the immunomodulatory capacity of the gradient scaffold may not originate solely from the released curcumin. Chemical cues from HA and physical cues provided by the architecture of the three-layer gradient scaffold might also be able to contribute to anti-inflammatory effects [24]. To further verify the relative contributions of these different factors, future experiments can adopt direct cell seeding onto the gradient scaffold instead of using curcumin extracts from the scaffold. This will allow us to comprehensively assess the anti-inflammatory effects of the whole scaffold system. Multiple

control groups will be designed to decouple these effects: a curcumin-free gradient scaffold to separate drug-mediated effects from material-derived effects, and a gradient scaffold without HA incorporation to distinguish the effect from HA and that from curcumin.

In addition, this study monitored the degradation behavior of the gradient scaffold over 4 weeks, which only reflected the early stage of degradation of the three-layer gradient scaffold. In follow up work, we will extend degradation monitoring to 12 weeks, to match the full timeframe for typical natural bone healing. With longer observation window, we can record detailed long-term changes, including scaffold mass retention, the progressive disintegration of the layered structure and the remaining structural support. According to these data, we can further tune the PLGA ratio to optimize scaffold design, ultimately developing a biomaterial that better recapitulates natural bone healing.

Moreover, all biological tests in this project were conducted using isolated cell samples under in-vitro conditions. Such cell-only experiments cannot fully recapitulate the complexity of in-vivo bone repair. To further validate the practical performance of the gradient scaffold, in-vivo animal experiments are necessary in future work. By implanting the gradient scaffold into real injury sites, we will evaluate its behavior and functions under authentic physiological environments and the host's native immune system. These in-vivo results will provide more robust evidence and move our findings closer to clinical application for bone-defect treatment.

5 Conclusion

In conclusion, we developed a curcumin-loaded three-layer gradient PLGA/HA scaffold that outperformed homogeneous scaffolds for bone repair, as its layered porous structure and tunable PLGA/HA ratios enabled favorable cell infiltration and staged degradation while significantly enhancing RUNX2 and ALP expression at both protein and mRNA levels; the scaffold also exhibited pH-responsive curcumin release, reducing premature leakage at pH 7.4 but allowing sufficient release under inflammatory pH 5.5, and effectively suppressed M1 macrophage polarization, promoted M2 polarization, and increased IL-10 secretion, indicating that this scaffold integrates staged degradation, osteogenic induction, and immunomodulation and represents a promising candidate for complex bone defect repair that warrants further in vivo validation.

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Upon advancing to high school, I shared my research interest with Mr. Liao Hui from the school's STEM Office. He was the person who helped me the most in this study. He encouraged me to read research papers about biodegradable composite scaffolds for bone regeneration. He gave me valuable ideas about how to conduct literature review and how to narrow down to my core research question. He guided me in theoretical learning, introduced me to data analysis methods, and provided insightful comments on my research plan. His expertise in biology and chemistry enabled him to provide ample support whenever I faced technical challenges in this study. He engaged me in in-depth academic discussions and actively cultivated my critical thinking. In addition, he gave me valuable suggestions for my paper writing. I sincerely thank Mr. Liao for his selfless and continuous encouragement, guidance, and support throughout the entire journey of this study, which greatly improved my academic literacy and research capabilities.

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