Biomimetic Gelatin Methacrylate/Nano Fish Bone Hybrid Hydrogel for Bone Regeneration via Osteoimmunomodulation

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Experimental Section

Preparation and characterization of NFB

First, grass carp with meat was weighted, and then mixed with tap water at a mass ratio of 1:3 (fishbone: water). Then, the mixture was heated at 121°C for 1 h using a pressure cooker. After pressure cooking, the mixture was repeatedly washed with hot water (around 80°C) for the removal of flesh, floating fat and other impurities, and then the white solid was collected. Next, the NFB was fabricated according to the previous report.[1] In brief, the white solid was crushed into powders using a mincing machine, and then mixed with ice water at a mass ratio of 1:10. Subsequently, the mixture was micronized with a bone grinding machine at a speed of 2000 rpm, followed by the addition of equivalent 0.3% trilatone solution. The mixture solution was stirred at room temperature for 24 h with trilatone solution changed every 8 h. After that, the mixture was thoroughly rinsed with a large amount of distilled water till the absence of bubbles and then decellularized bone mud was obtained. Next, the water content of decellularized bone mud was detected. Finally, bone mud with a water content of 5% was poured into the wet high -energy ball mil and stirred at a speed of 3000 rpm and under a ball mill filling rate of 85% and grinding ball diameter of 0.5 mm. After 6 h, nano fishbone solution was obtained and lyophilized for further reservation and utilization. Size distribution of the NFB was analyzed by dynamic light scattering (DLS, Malvern Zetasize NanoZS90, UK). The scanning electron microscope (SEM) was used to determine the morphology of the NFB. Zeta potential was also measured by DLS. The element composition of the NFB was analyzed by a field emission scanning electron microscope equipped with a detector for energy dispersive X-ray spectroscopy and X-ray diffractometer (XRD).

Fabrication of Gel-MA/NFB hydrogels

The Gel-MA/NFB hydrogels were prepared by the photopolymerization of NFB and Gel-MA in an aqueous solution with an initiator. In brief, 10% (w/v) freeze dried Gel-MA and 3% (w/v) NFB were fully dissolved in distilled water with 1% (w/v) Irgacure 2959 photoinitiator (Sigma-Aldrich). The mixture was then transferred to a custom-made 24-well mold (diameter 10 mm and thickness 5 mm for each well). Then, the mixture solution was irradiated with a long-wavelength UV lamp (Intell-ray 400, Uvitron, 365 nm, 400 W) for 1~2 min at room temperature at a 5~10 cm distance. The resultant hydrogels were removed from the mold and freeze dried in vacuum at -50°C for 2 days prior to further characterization. All other preparation conditions were same.

Characterization of hydrogels

Interior morphology of hydrogels

The porosity and interior morphology of Gel-MA/NFB hydrogels were researched by SEM. First, the swollen hydrogel samples were reached their maximum swelling ratio in distilled water at room temperature, quickly frozen in liquid nitrogen and

then freeze-dried under vacuum at -50°C for at least 2 days until all the solvent was removed. The freeze-dried hydrogel samples were then cut carefully and fixed on aluminum stubs and sputtered with gold for 30 s for interior morphology observation with a SEM instrument.

Equilibrium swelling ratio and swelling kinetics of hydrogels

Since biocompatibility apparently depends on water content, characterization of the amount of imbibed water in the swollen gel is important.[2] The swelling kinetic of Gel-MA/NFB hydrogel was tested at 37°C over a period of 72 h. The dry hydrogels were weighed before the test, and then they were immersed in 10 mL of PBS solution. Before weighing, the excess surface water of hydrogel samples was removed by filter papers at each measurement time point. The swelling ratio (Q_t) of the hydrogels, at time t, is calculated as a function of time: $Q_t=[(W_t-W_0)/W_0]\times100\%$ (W_t: the weight of the hydrogel at time t, W_0: the weight of the corresponding dry hydrogel at t = 0). All swelling ratio results were obtained from triplicate samples and data were expressed as the mean ± standard deviation.

In vitro biodegradation of Gel-MA/NFB hydrogels

In vitro biodegradation of the Gel-MA/NFB hybrid hydrogels was evaluated with phosphate buffer (PBS, PH=7.4). The dry hydrogels were weighed before the test, and then soaked in 10 mL of PBS buffer and incubated 24 h at 37°C. At each test point, the hydrogels were removed and washed with distilled water for three times, follow by dried for 24 h at 37°C to remove the residual water and recorded the weight of hydrogels. The PBS buffer was refreshed every other day. The degradation percentage (DP) was determined by the following weight equation: $DP=(W_0-Wt)/W_0\times100\%$, where W_0 represents the initial dry hydrogels weight and Wt is the weight at time t. The DP was expressed as the mean ± standard deviation (n=3).

Rheological measurements

The rheological properties of Gel-MA/NFB hydrogels were carried out by a rheometer (HAAKE, Thermo, Germany). A plate-plate geometry with a diameter of 20 mm and plate-to-plate distance of 1mm was used in all tests. At first, all hydrogel samples were fixed on the plate, and then the measurements of mechanical spectra were recorded in a constant strain mode, with a deformation of 0.05 maintained over the frequency range of 0.1-10 Hz (rad/s) at 37°C. The temperature dependence of Storage modulus (G′) and loss modulus (G″) was measured with temperature scan ranging from 15 to 40°C (heating rate 1.75°C min^{-1}) at a constant frequency of 1 Hz and a constant strain (γ= 0.05, 1.88 mrad).

Cell culture

In this study, the NIH 3T3 cells, hDPSCs cells, and RAW264.7 cells were used. The murine sourced macrophage cell line RAW264.7 cells were cultured in α-MEM supplemented with 10% heat-inactivated fetal bovine serum and 1% (v/v) penicillin/streptomycin (P/S) in a humidified CO_2 (5%) incubator with the temperature maintained at 37°C. NIH 3T3 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (P/S) at 37°C with a 5% CO_2 humidified atmosphere. RAW 264.7 cells were passaged by a cell scraper after reaching 80% confluence, while NIH 3T3 cells were passaged by trypsinization. The hDPSCs utilized in the studies were collected and cultured as previously reported.[3] Healthy human third molars were extracted from 12 adults (18–20 years of age; 6 males and 6 females) at the Department of Oral and Maxillofacial Surgery, the Affiliated Stomatological Hospital of Sun Yat-sen University. Informed consent was obtained from all the participants, and approval was granted by the Sun Yat-sen University Research Ethics Committee. Briefly, pulp tissues were isolated from the teeth and digested with type I collagenase (3 mg/mL) and 4 mg/mL dispase (Gibco-BRL, Grand Island, NY, USA) at 37°C for 30 min. After that, the cells were cultured in α-MEM (Gibco-BRL) supplemented with 10% of fetal calf serum (Gibco-BRL), 100 μg/mL streptomycin, 100 U/mL penicillin (HyClone, Logan, UT, USA), and 5 mmol/L glutamines (Gibco-BRL) at 37°C and 5% CO_2. All the hDPSCs isolated from 12 adults were mixed together for all the experiments.

Proliferation and attachment of NIH 3T3 on Gel-MA /NFB hydrogels

The live/dead cell staining assay and methyl thiazolyl tetrazolium (MTT) viability assay were used to research the *in vitro* cytotoxicity of the hydrogels. For the live/dead staining, the Gel-MA/NFB hydrogels were cut into round shapes with a diameter that just filled the well of a 24-well cell culture plate and immersed into the 75% alcohol for 4h, then sterilized under UV light (in the DMEM) for at least 2 h before being put into the 24-well cell culture plates. After that the hydrogels were washed twice by PBS buffer and DMEM. Then, the hydrogels were placed in the 24-well plate and the cells were seeded at a density of 1×10^4 cells per well and incubated at 37°C for 30min (to prevent cells from slipping away from hydrogel to the culture dish) and then added the medium. At day 1, 3 and 5, cells were stained with 1 μg/mL Calcein-AM and PI (Propidium iodide, Sigma-Aldrich, 1 μg/mL) at room temperature for 30 min to label the living and the dead cells, respectively. After that the cell vitality on the hydrogel surface was imaged by a fluorescence microscope. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay was used to evaluate effect of Gel-MA/NFB hydrogels extracts on the proliferation of NIH 3T3 cells. NIH 3T3 cells were seeded in a density of 1×10^3 cells per well (96 well plate) and cultured overnight at 37°C under 5%CO_2. The culture medium was subsequently removed and replaced by material extract medium. At days 1, 3 and 5, 20 μL of MTT solution (5 mg/mL in PBS) was added into each well. The MTT medium solution was removed after 4 h incubation and 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. After being vortexed for 8 min, quantitative detection of the samples was done on a microplate reader to measure the absorbance at the wavelength of 490 nm.

Proliferation of RAW264.7 cells with Gel-MA /NFB hydrogels

MTT assay was performed to evaluate the proliferation of RAW 264.7 cells with Gel-MA/NFB hydrogels extracts. RAW 264.7 cells were seeded in a density of 10^4 cells per well (96 well plate) and cultured overnight. The culture medium was subsequently removed and replaced by material extract medium. At day 1 and 2, 20 μL of MTT solution (5 mg/mL in PBS) was added to the medium in each well. The MTT/medium solution was removed after 4 h and 150 μL of DMSO was added to dissolve the formazan crystals. Quantitative detection was done on a microplate reader to measure the absorbance at the wavelength of 490 nm.

Osteoimmunomodulatory effects of Gel-MA/NFB hydrogels on macrophages

RAW264.7 cell morphology on NFB or Gel-MA/NFB hydrogels

RAW264.7 cells were seeded in 6 well plates at a density of 1.5×10^5 cells per well. After 24 h, the culture medium was removed and replaced by 3 mL of α-MEM (with 10% heat-inactivated fetal bovine serum) containing NFB particles in different concentrations (0, 200, 300, 500 and 1000 μg/mL) or Gel-MA/NFB hydrogels. The cells were incubated for 24 h, the medium was removed and the cells were washed with PBS three times and then fixed with 4% paraformaldehyde at room temperature for 20 min, after that, the cells were permeabilized using 1% Triton X-100 for 5~10 min, and then were washed with PBS twice. Then, the cells were incubated with Rhodamine phalloidin (5 μg/mL) for 30 min to stain the cytoskeletons and were washed with PBS twice. In the end, 4′,6-diamidino-2-phenylindole (DAPI, 5 μg/mL) was added to stain the nuclei for 5~10 min. Images were captured by a Fluorescence microscope.

TRAP staining of RAW264.7 cells

Tartrate-resistant acid phosphatase (TRAP) is a metalloenzyme highly expressed in activated macrophages and osteoclast cells. This acid phosphatase can be detected by naphthol AS-Bi phosphoric acid in conjunction with diazonium salts. Therefore, the effect of Gel-MA/NFB hydrogels on the differentiation and maturation of RAW264.7 macrophages into multinucleated osteoclasts was investigated. The RAW264.7 cells were plated at a density of 1×10^4 cells/well in 48-well plates for 24 h and then incubated with α-MEM (containing 10% FBS, 1% P/S, Gel-MA/NFB hydrogels) for 3 days to stimulate RAW264.7 cells into macrophage-like cells. TRAP staining was performed to on days 3 after the induction of macrophage phenotype, following the protocol (BestBio, China). TRAP buffer without tartrate solution was applied as control. After staining, the 48-well plates were photographed by a Nikon digital camera. The TRAP-positive cells with three or more nuclei (multinucleate cells) were counted from multiple image frames and statistically analyzed.

Flow cytometry

Expression of macrophage surface markers CCR7 (M1 marker) and CD163 (M2 marker) were detected by flow cytometry. After 3 days of stimulation by material, the cells were physically detached and centrifuged. The obtained cells were washed with PBS. Samples were then incubated with CCR7 antibodies (Biolegend, PE) or CD163 antibodies (Biolegend, PE) at 37°C for 30~45 min to label CCR7 or CD163 respectively. The blank group incubated with no antibodies. The treated cells were analyzed by flow cytometer and data analysis were performed using Flowing Software.

Inflammatory response of macrophages on Gel-MA/NFB hydrogels

RAW 264.7 cells were seeded on different Gel-MA/NFB hydrogels in 6 well plate at a density of 1×10^5 cells per well and incubated for 4 d. Then, the culture medium of macrophages was collected to examine the expression of Tumor necrosis factor α (TNF-α), Interleukin 1β (IL-1β), Interleukin 6 (IL-6), Oncostatin M (OSM) and Interleukin 10 (IL-10) by ELISA kits (CUSABIO, China). Gene expression of inflammatory cytokine (TNF-α, IL-18, IL-1β, OSM and IL-6) was assessed by reverse-transcription quantitative PCR (RT-qPCR). After culture medium collection, the culture plates were washed with PBS and then the total RNA was extracted by TRIZOL reagent. Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using GoScript™ Reverse Transcription System. RT-PCR was performed to quantify gene expression levels by means of the SYBR Premix Ex Taq (Takara). The primers used in this section is shown in Table S1. GADPH was used as a housekeeping gene. RAW264.7 cells cultured with complete medium served as the control groups.

Osteogenic differentiation evaluation of macrophage-conditioned medium

The mineralization of hDPSCs

To research the effect of the Gel-MA/NFB hydrogel-stimulated macrophage on the osteogenic differentiation of hDPSCs. Conditioned medium was prepared by culturing RAW264.7 cells (1×10^5 cells/well, 6-well plate) on Gel-MA or Gel-MA/NFB hydrogels for 4 d, and then the supernatants were collected and mixed with complete DMEM medium (10% FBS, 1% P/S) at a ratio of 1:2 (labeled as condition medium) for the following cell culture. Alizarin Red staining was used to identify mineralization nodule. The hDPSCs cells were seeded on the hydrogels in 24-well plates at a density of 1×10^4 cells per well containing conditioned medium or complete medium. After stimulation for 6 d, the condition medium was replaced by osteogenesis induction medium (OIM) (DMEM with 10% FBS, 1%P/S, 10 mM b-glycerophosphate, 50 μg/ml ascorbic acid, and 10nM dexamethasone) and was refreshed every two days. After 4, 7, 14 days, the OIM was removed and the cells were washed with PBS, follow by fixation by in 4% paraformaldehyde for 20 min. After being rinsed with PBS three times, the cells were stained with a solution containing 2% Alizarin Red S at pH (4.1~4.3, Sigma-Aldrich) for 30 min. Then, the cells were washed with PBS four times before images acquired with a light microscope. The groups without conditioned media and samples were used as the blank group. Quantitative results of the alizarin red staining were analyzed by image J. The groups without materials were used as the control group. The Gel-MA group treated with condition medium and Gel-MA hydrogel. The Gel-MA/1%NFB group treated with Gel-MA/1%NFB hydrogel and condition medium. The Gel-MA/3%NFB group treated with Gel-MA/3%NFB hydrogel and condition medium. The Gel-MA/5%NFB group treated with Gel-MA/5%NFB hydrogel and condition medium.

Alkaline phosphatase activity of hDPSCs

Alkaline phosphatase activity of hDPSCs was used to access the effect of the Gel-MA/NFB hydrogels-stimulated macrophage on the osteogenic differentiation of hDPSCs. hDPSCs cells were seeded on the hydrogels in 24-well plate at a density 1×10^4 cells per well with conditioned medium or complete medium. After culture for 6 days, the conditioned medium was removed and replaced by OIM. The ALP activity assay was performed after differentiation 4, 7, 14 days of induction. Steps are as follows, the medium was removed and the cells were washed with PBS. Then, the cells were detached from the hydrogels by trypsinization, followed by centrifugation for 5 min at 1200 rpm. Next, 1% Triton X-100 with PMSF (150 μL) was added to lyse the cells for 30 min and the mixture was centrifuged at 12000 rpm for 5~10 min at 4°C. 50 μL of working solution was mixed with 50 μL supernatant samples, which was subsequently evaluated by the Alkaline Phosphatase Assay Kit (Beyotime Biotechnology)

following the manufacturer's instructions. The ALP activity was calculated as the changed optical density (OD) values. The groups without conditioned media and samples were used as the blank group. The groups without materials were used as the control group. The Gel-MA group was treated with condition medium and Gel-MA hydrogel. The Gel-MA/1%NFB group was treated with Gel-MA/1%NFB hydrogel and condition medium. The Gel-MA/3%NFB group was treated with Gel-MA/3%NFB hydrogel and condition medium. The Gel-MA/5%NFB group was treated with Gel-MA/5%NFB hydrogel and condition medium.

Osteogenesis-related genes and protein Expression

The expression of osteogenesis-related genes and proteins was also detected by RT-qPCR and ELISA kits to evaluate the effect of the Gel-MA/NFB hydrogels-stimulated macrophage on the osteogenic differentiation of hDPSCs. The hDPSCs cells were seeded on the hydrogels in 6-well plates at a density 2×10^4 cells per well with conditioned medium or complete medium. After culture for 6 days, the conditioned medium was removed and replaced by OIM. After 4, 7, 14 days, the culture medium was centrifuged (1200 rpm for 2 min) to collect supernatants and examine the concentrations of osteogenesis-related proteins and angiogenesis-related proteins containing type I collagen (COL-1), human vascular endothelial growth factor (VEGF) and runt-related transcription factor 2 (RUNX-2) by ELISA kits (CUSABIO, China) according to the manufacturer's instructions. The RT-qPCR was employed to evaluate the mRNA expression of osteogenic genes. The total RNA of the cultured hDPSCs was extracted by a TRIZOL reagent (Invitrogen). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using GoScript™ Reverse Transcription System. RT-PCR was performed to quantify gene expression levels by means of the SYBR Premix Ex Taq (Takara). Expression levels of the osteogenesis-related genes including COL-1, VEGF, RUNX-2, and Osteopontin (OPN) was quantified. GADPH was used as the housekeeping gene. The forward and reverse primers of the selected genes were listed in Table S1. The groups without conditioned media and samples were used as the blank group. The groups without materials were used as the control group. The Gel-MA group was treated with condition medium and Gel-MA hydrogel. The Gel-MA/1%NFB group was treated with Gel-MA/1%NFB hydrogel and condition medium. The Gel-MA/3%NFB group was treated with Gel-MA/3%NFB hydrogel and condition medium. The Gel-MA/5%NFB group was treated with Gel-MA/5%NFB hydrogel and condition medium.

Rat model of bone defects

All animal procedures were approved by the Animal Care and Use Committee of Sun Yat-sen University (GuangZhou, China). Sprague-Dawley rats (250~280 g, ~4 weeks old) were anesthetized by abdominal cavity injection using the chloral hydrate (30 mg/kg). A midline incision was made to raise a full-thickness flap and expose the parietal bone. Two full-thickness craniotomies defects (5 mm diameter) were created by using a bone trephine drill (Traus 90) with the irrigation of sterile saline to prevent heat injuries. A cylinder with a diameter of 5 mm and a thickness of 1 mm Gel-MA/NFB hydrogel or Gel-MA hydrogel was placed into the clean defect site of the animals. Finally, the incision was closed with sutures. The animals were sacrificed 4 weeks' post-surgery and the bone tissues were collected and immersed in 4% buffered paraformaldehyde. A microcomputed tomography (micro-CT) system (Skyscan 1172) was applied to examine the samples and the volume of the newly formed bone (BV/TV), bone mineral density (BMD) were determined by the auxiliary histomorphometric software (CTAn).

Histological and immunohistochemical analysis

For histological analysis, the bone tissues were decalcified in 10% ethylenediaminetetraacetic (EDTA) acid for one month, dehydrated in a graded series of alcohol, and finally embedded in paraffin. The specimens were cut at a 4~5 μm thickness in the sagittal direction along the artificial defect and the cranial bone. Histological observation was performed with hematoxylin and eosin (H&E) and Masson staining (Servicebio). Semi-qualification of the new bone formation was performed by analyzing H&E images by using Image J. Additionally, immunohistochemical assessment was used to evaluate the expression of COL-1, VEGF, and OPN and inflammatory cytokines (TNF-α, IL-6, IL-1β, IL-10), this further confirmed the immune reaction caused by Gel-MA/NFB hydrogels.

Statistical Analysis

All statistical computations were performed using SPSS software, and the statistical significance was analyzed using one-way ANOVA. All the data are shown as means ± standard deviation, and the level of significance was set at $P < 0.05$.

Tables and Figures Captions

Table S1 Primer pairs used in the RT-qPCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
TNF-α	AGCCGATGGGTTGTACCTG	ATAGCAAATCGGCTGACGGT
IL-6	TGTTCTCTGGGAAATCGTG	CAAGTGCATCATCGTTGTTCATAC
IL-1β	ATGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
OSM	ATGCAGACACGGCTTCTAAGA	TTGGAGCAGCCACGATTGG
GADPH	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT
IL-18	GACTCTTGCGTCAACTTCAAGG	CAGGCTGTCTTTTGTCAACGA
RUNX-2	GGTACTTCGTCAGCATCCTATCA	GTCAGCGTCAACACCATCATT
OPN	TGACCAGAGTGCTGAAACCCA	TTCCTGACTATCAATCACATCGG
COL-I	ACGAAGACATCCCACCAATCA	CAGATCACGTCATCGCACAAC
VEGF	GGCGGCCTTCGCTTACTC	GGCTGCTTCTTCCAACAATG

Table S2 Atomic proportion (%) in NFB particles.

Element	Wt%	Wt% Sigma
C	0.15	0.00
O	71.04	0.26
Na	0.44	0.12
Mg	0.80	0.09
Al	0.19	0.07
P	8.19	0.10
K	0.00	0.05
Ca	19.21	0.19
Total:	100.00	

Figure S1. Images of Gel-MA hydrogels and Gel-MA/NFB hydrogels.

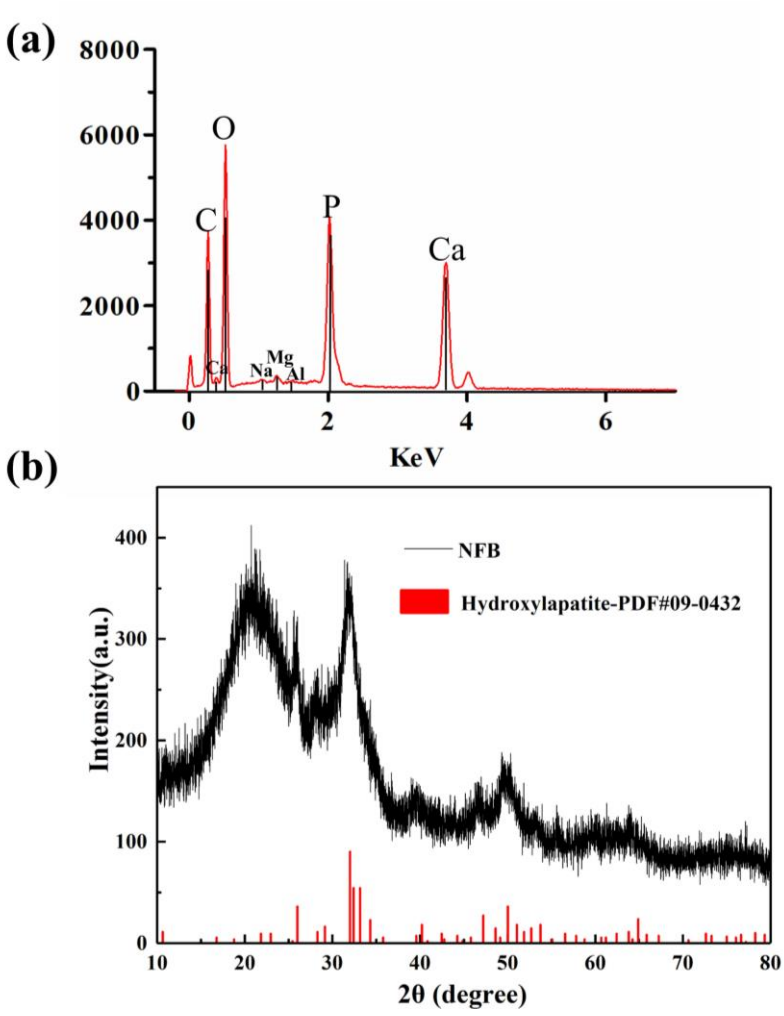

Figure S2. (a) EDS spectra of nano fish bone (NFB); (b) XRD spectra of NFB.

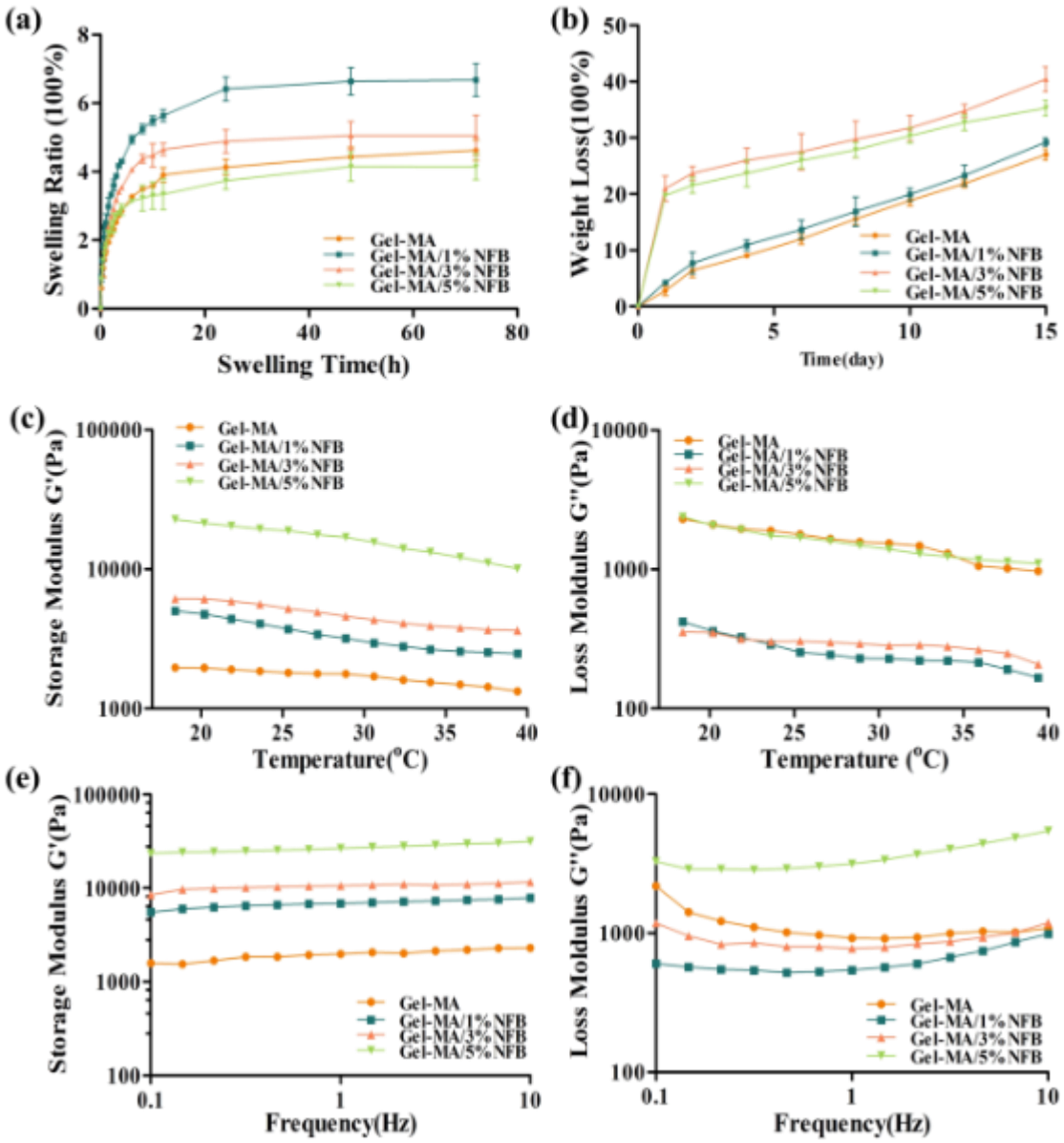

Figure S3. Characterization of Gel-MA/NFB hydrogels. (a) Swelling kinetics of the Gel-MA hydrogels and Gel-MA/NFB hydrogels in PBS at 37°C; (b) *In vitro* biodegradation of Gel-MA/NFB hydrogels in PBS at 37°C; (c, d) Effect of frequency on storage modulus (G′) and loss modulus (G″) with Gel-MA hydrogels and Gel-MA/NFB hydrogels; (e, f) Effect of temperatures on storage modulus (G′) and loss modulus (G″) with Gel-MA hydrogels and Gel-MA/NFB hydrogels.

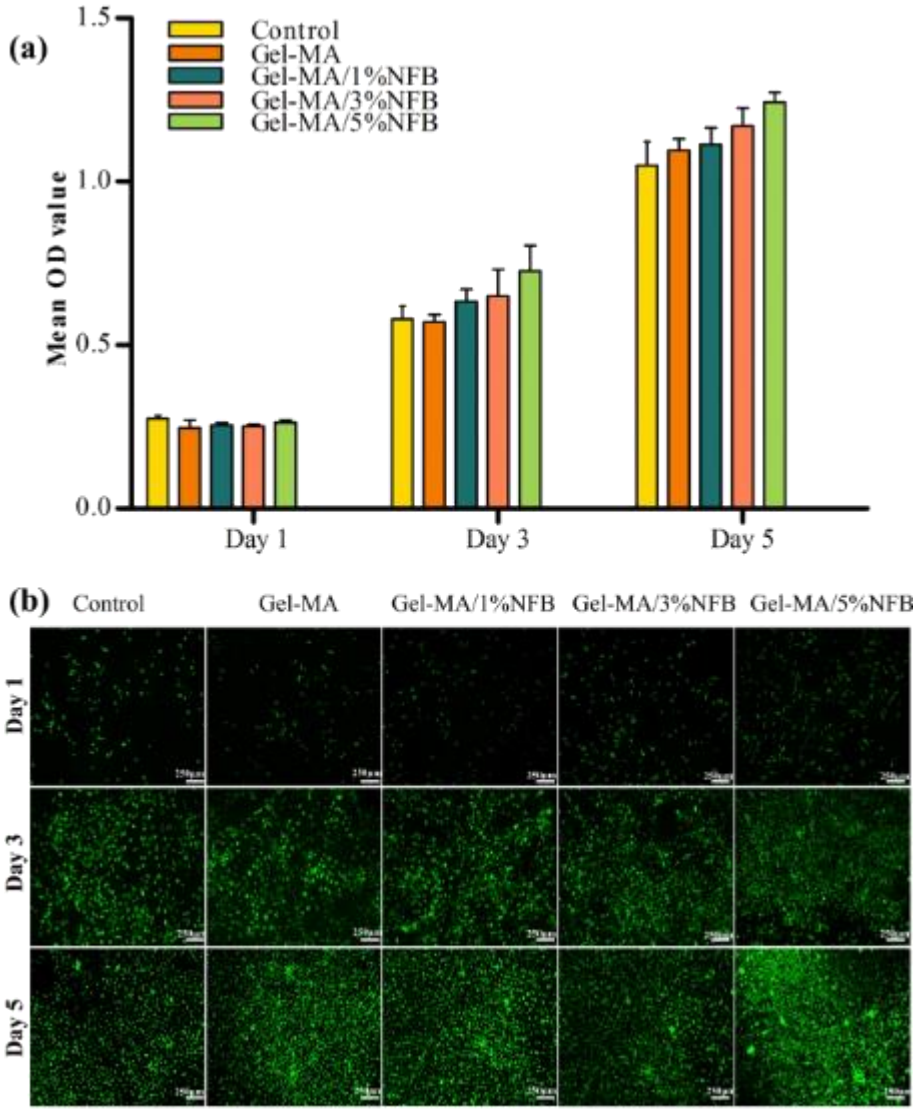

Figure S4. A cytocompatibility assay performed on hydrogels. (a) MTT assay; (b) live/dead staining assay to evaluate the viability of NIH 3T3 cultured with a Gel-MA hydrogel or a Gel-MA/NFB hydrogel.

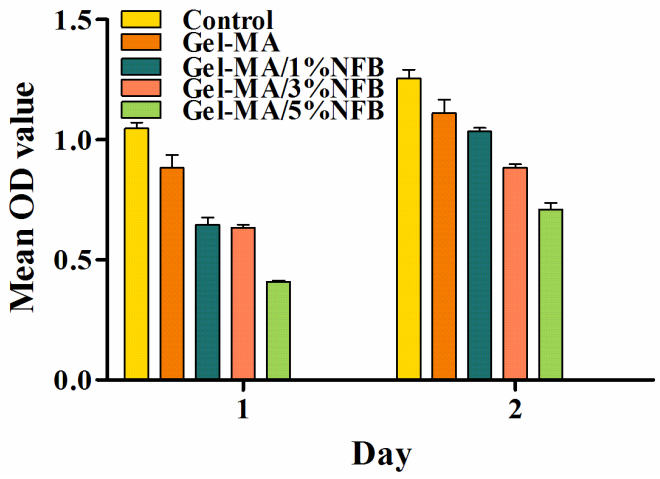

Figure S5. The viability of macrophages (RAW264.7 cells) by MTT analysis cultured with Gel-MA/NFB hydrogels extracts.

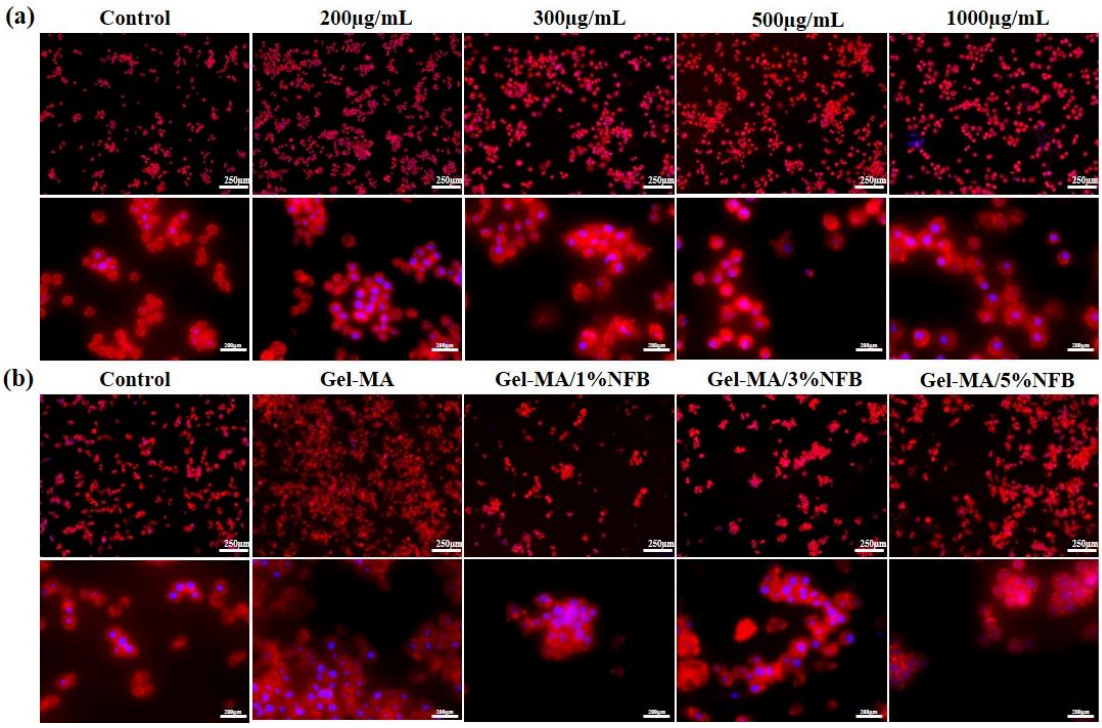

Figure S6. Inflammatory response of macrophages cultured on Gel-MA hydrogels or Gel-MA/NFB hydrogels. (a) The cell morphology of macrophages on NFB was stained with DAPI (blue, nuclei) and Rhodamine phalloidin (red, cytoskeleton); (b) The cell morphology of macrophages on Gel-MA/NFB hydrogels was stained with DAPI and Rhodamine phalloidin.

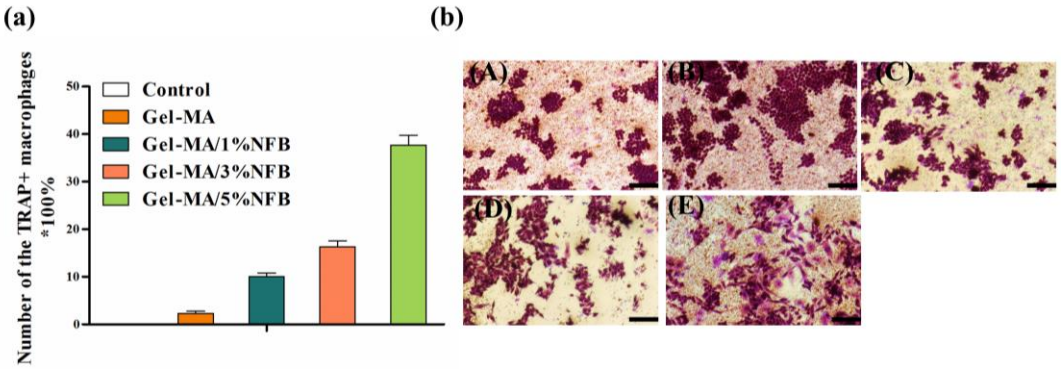

Figure S7. (a) Quantitatively analysis the percentage of TRAP-positive cells; (b) Typical TRAP staining images of RAW264.7 monocytes cultured on Gel-MA hydrogels or Gel-MA/NFB hydrogels. Scale bar = 100 μm; (A) control; (B) Gel-MA; (C) Gel-MA/1%NFB; (D) Gel-MA/3%NFB; (E) Gel-MA/5%NFB.

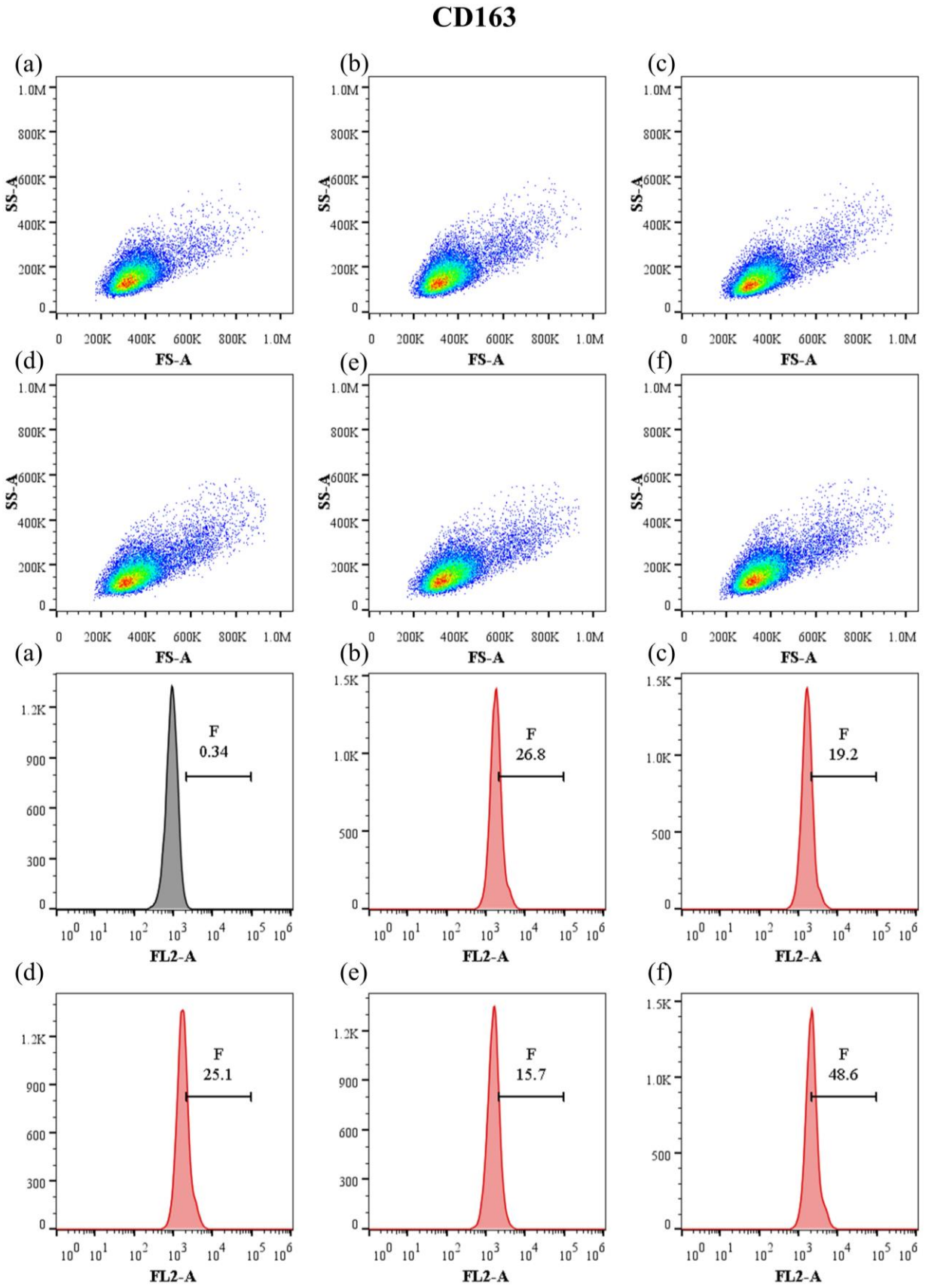

Figure S8. Representative images of surface markers CD163 of RAW264.7 analyzed by flow cytometry: (a) Blank; (b) Control; (c) Gel-MA; (d) Gel-MA/1%NFB; (e) Gel-MA/3%NFB; (f) Gel-MA/5%NFB.

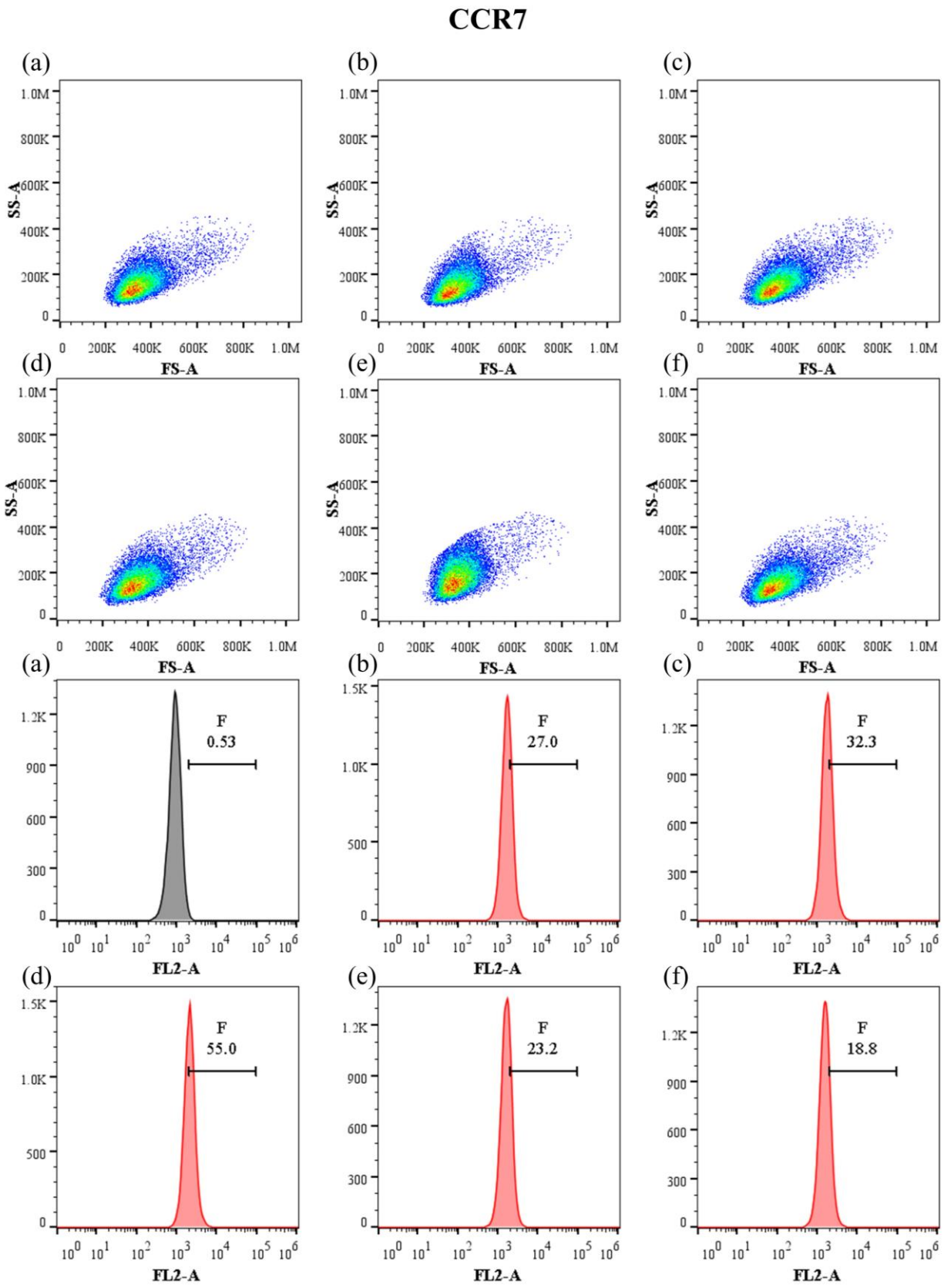

Figure S9. Representative images of surface markers CCR7 of RAW264.7 analyzed by flow cytometry: (a) Blank; (b) Control; (c) Gel-MA; (d) Gel-MA/1%NFB; (e) Gel-MA/3%NFB; (f) Gel-MA/5%NFB.

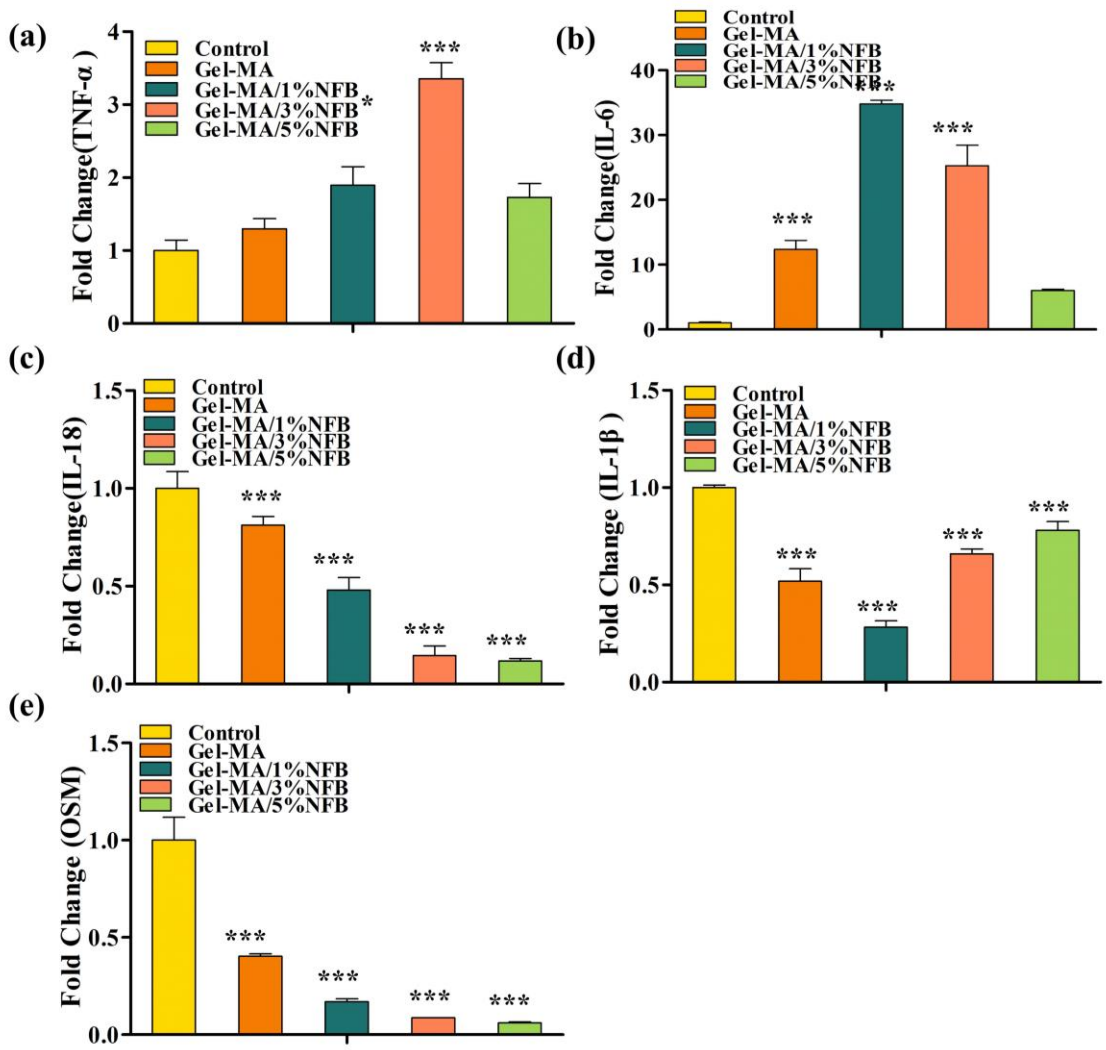

Figure S10. Effects of Gel-MA hydrogels or Gel-MA/NFB hydrogels on the expression of inflammatory genes (TNF-α, IL-6, IL-18, IL-1β, OSM) detected by RT-qPCR. The data were normalized to GADPH expression (*p < 0.05, **p<0.01, *** p<0.001) as compared with the control group.

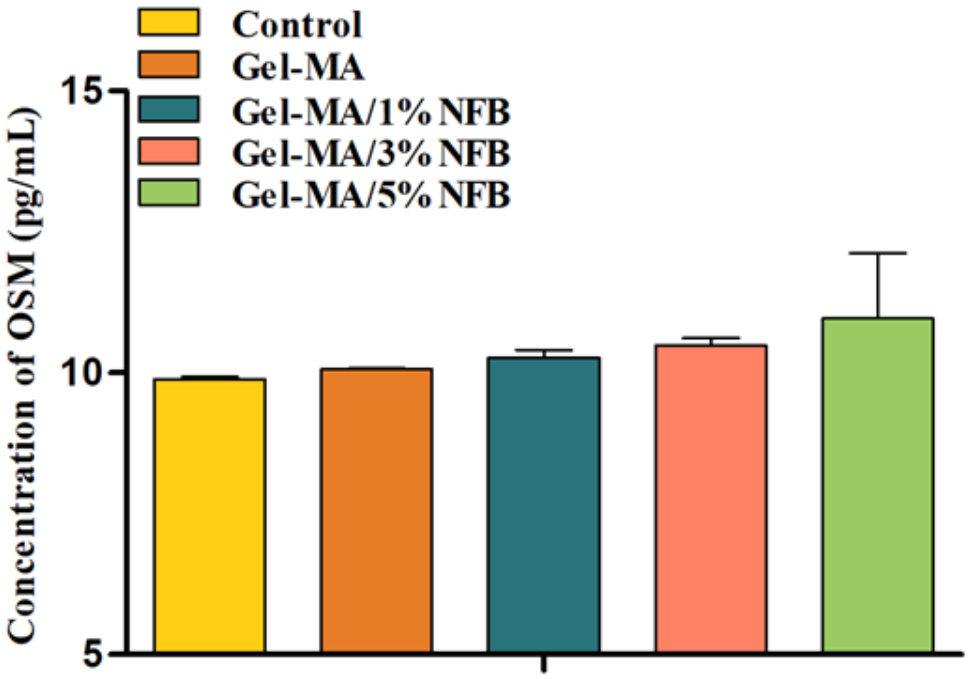

Figure S11. Effects of Gel-MA hydrogels or Gel-MA/NFB hydrogels on the expression of inflammatory proteins (OSM) detected by ELISA.

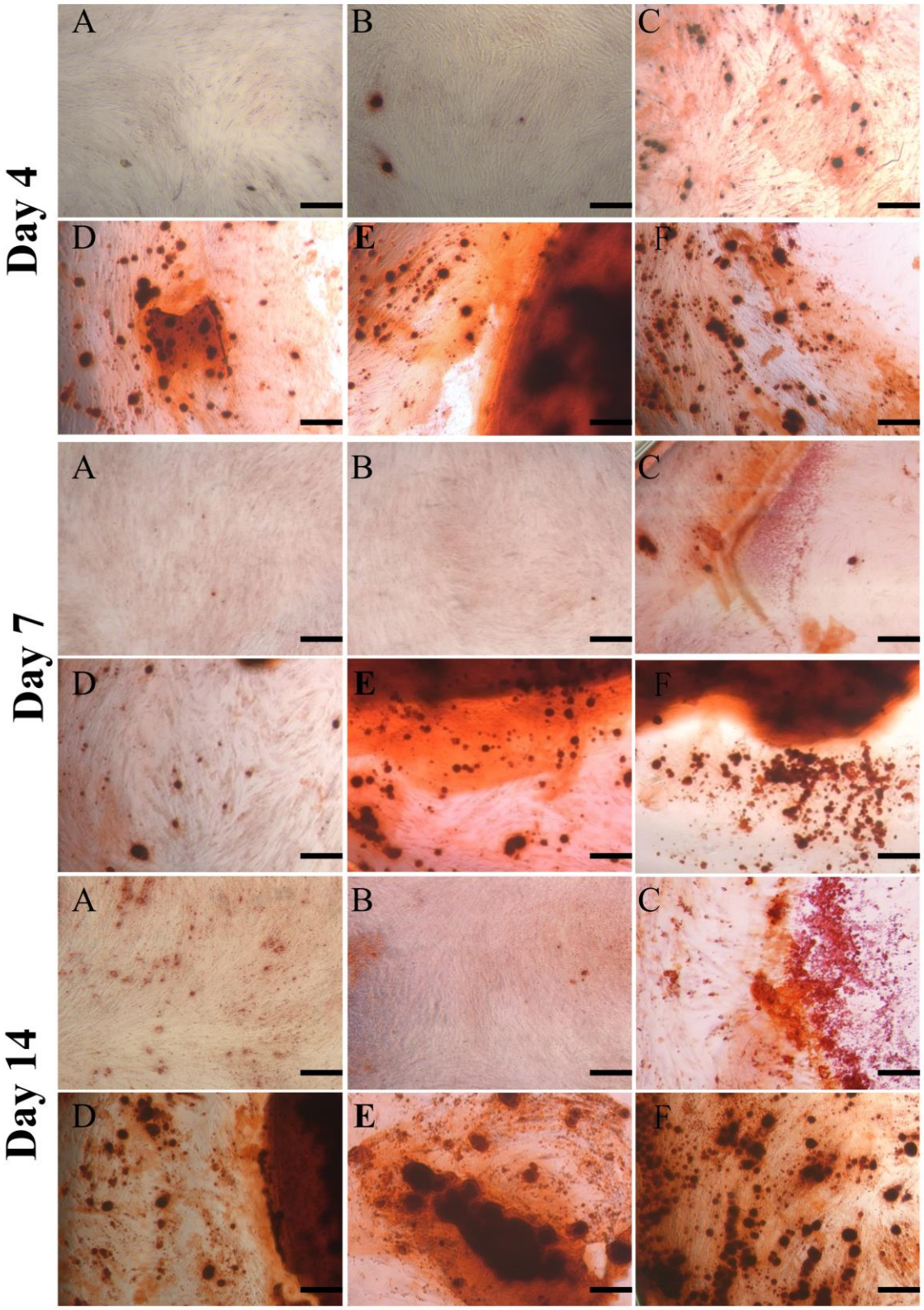

Figure S12. Alizarin red staining was used to demonstrate the mineralized nodules formed by hDPSCs' exposure to media conditioned by macrophages cultured on Gel-MA hydrogels or Gel-MA/NFB hydrogels. Scale bar = 100 μm. a) Blank; b) Control; c) Gel-MA; d) Gel-MA/1%NFB; e) Gel-MA/3%NFB; f) Gel-MA/5%NFB.

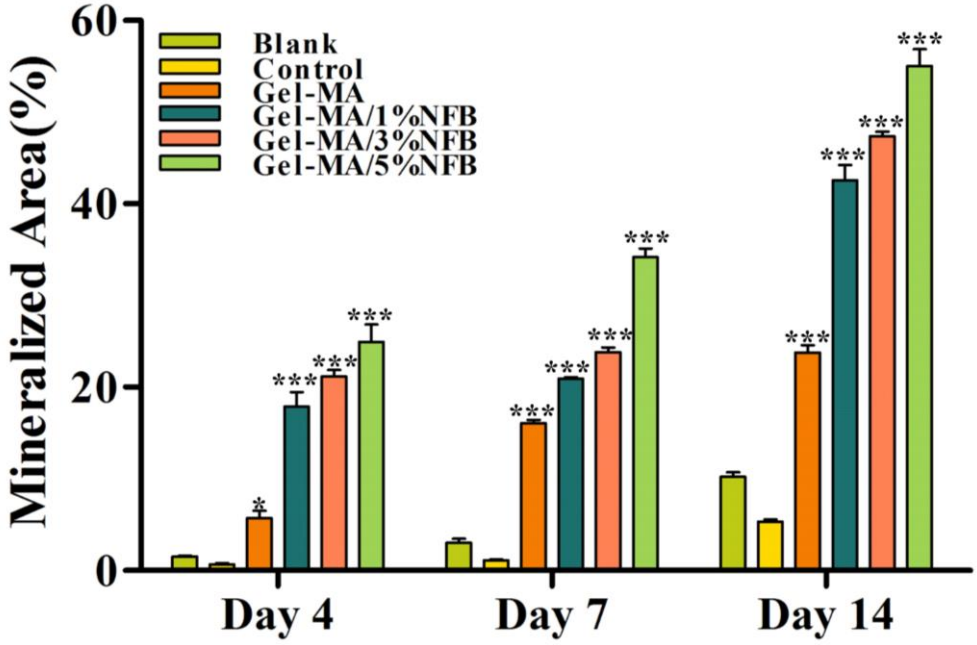

Figure S13. Image J analysis of the mineralized area of a culture dish.

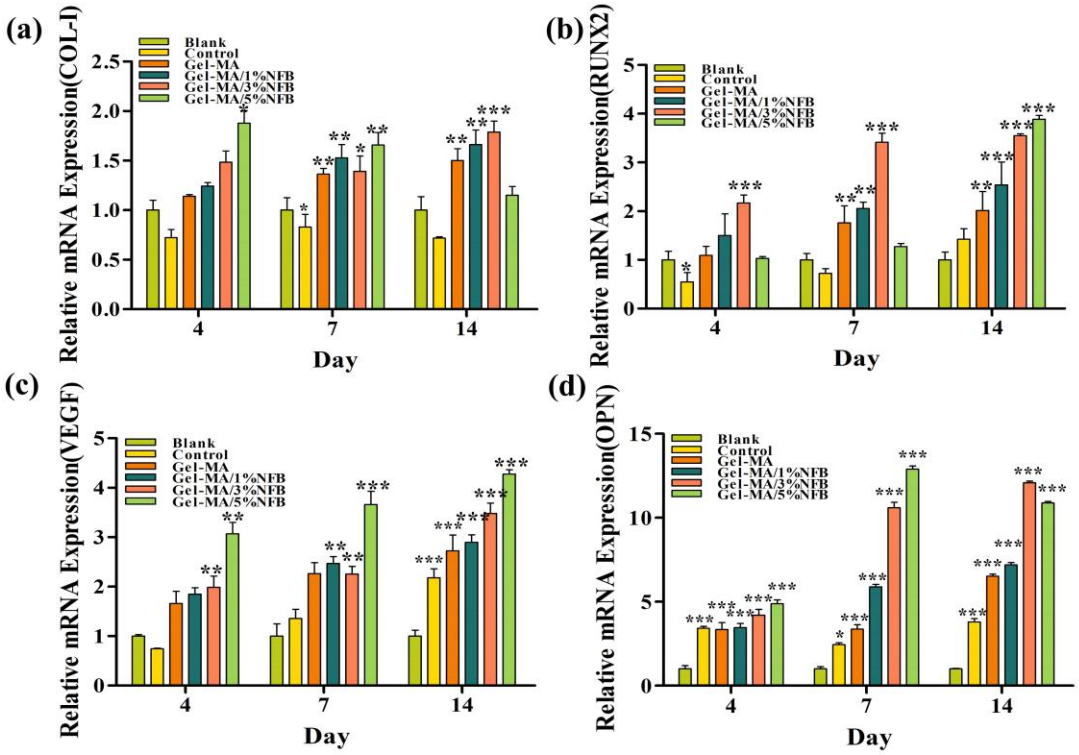

Figure S14. Effects of macrophage-conditioned medium of RAW264.7s osteogenic-related gene and angiogenic-related gene expression of hDPSCs after culturing for 4, 7, 14 days (COL-I, RUNX-2, VEGF, OPN) detected by RT-qPCR. The data were normalized to GADPH expression (*p < 0.05, **p<0.01, *** p<0.001) as compared with the blank group.

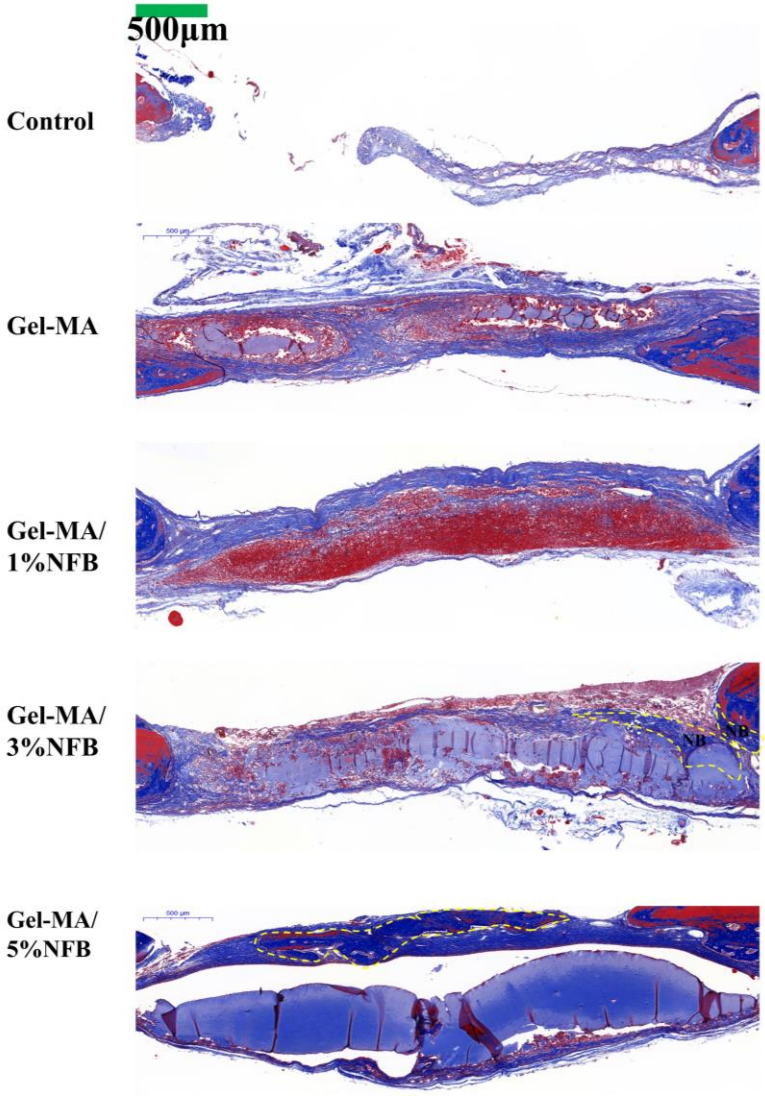

Figure S15. Masson staining of bone defect section at postoperative 4 weeks.

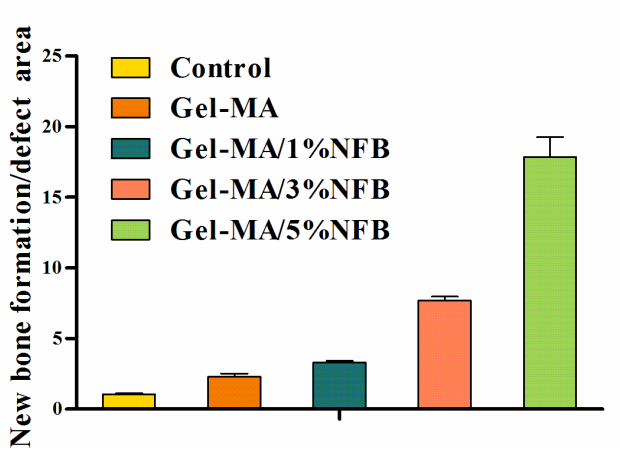

Figure S16. Quantitative analysis of H&E staining with Image J.

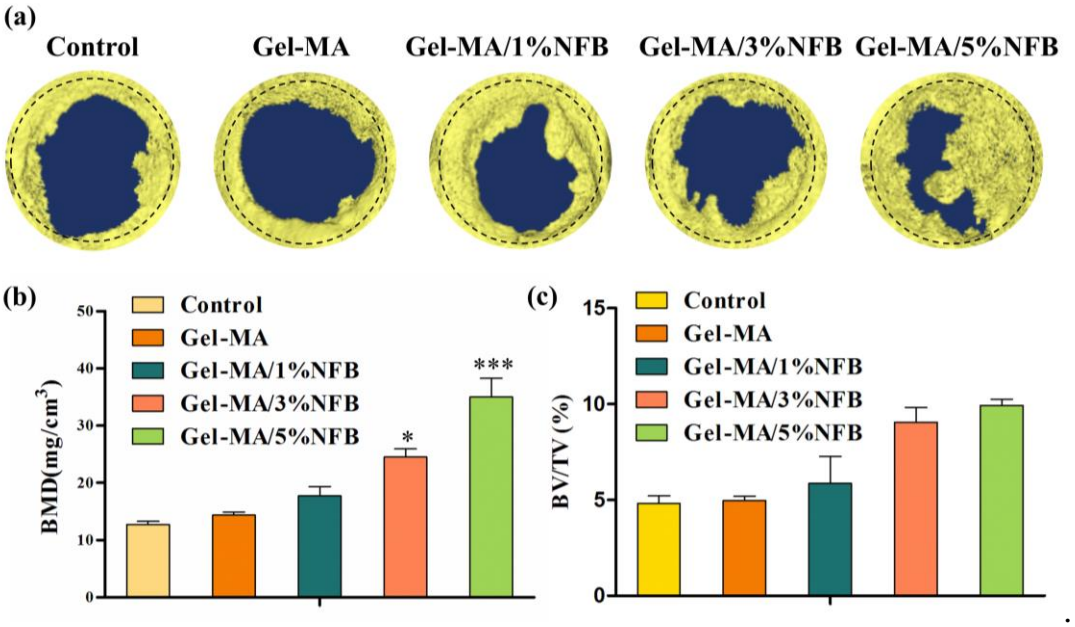

Figure S17. Micro-CT imaging and analysis of harvested craniums obtained from Sprague–Dawley rats after treatments for 4 weeks. (a) 3D reconstruction of micro-CT images of the cranium based on the density variations of osseous tissue. (b-c) Micro-CT quantification of the mineralized areas, as assessed by BMD, BV/TV. (*$p < 0.05$, **$p < 0.01$, *** $p<0.001$).

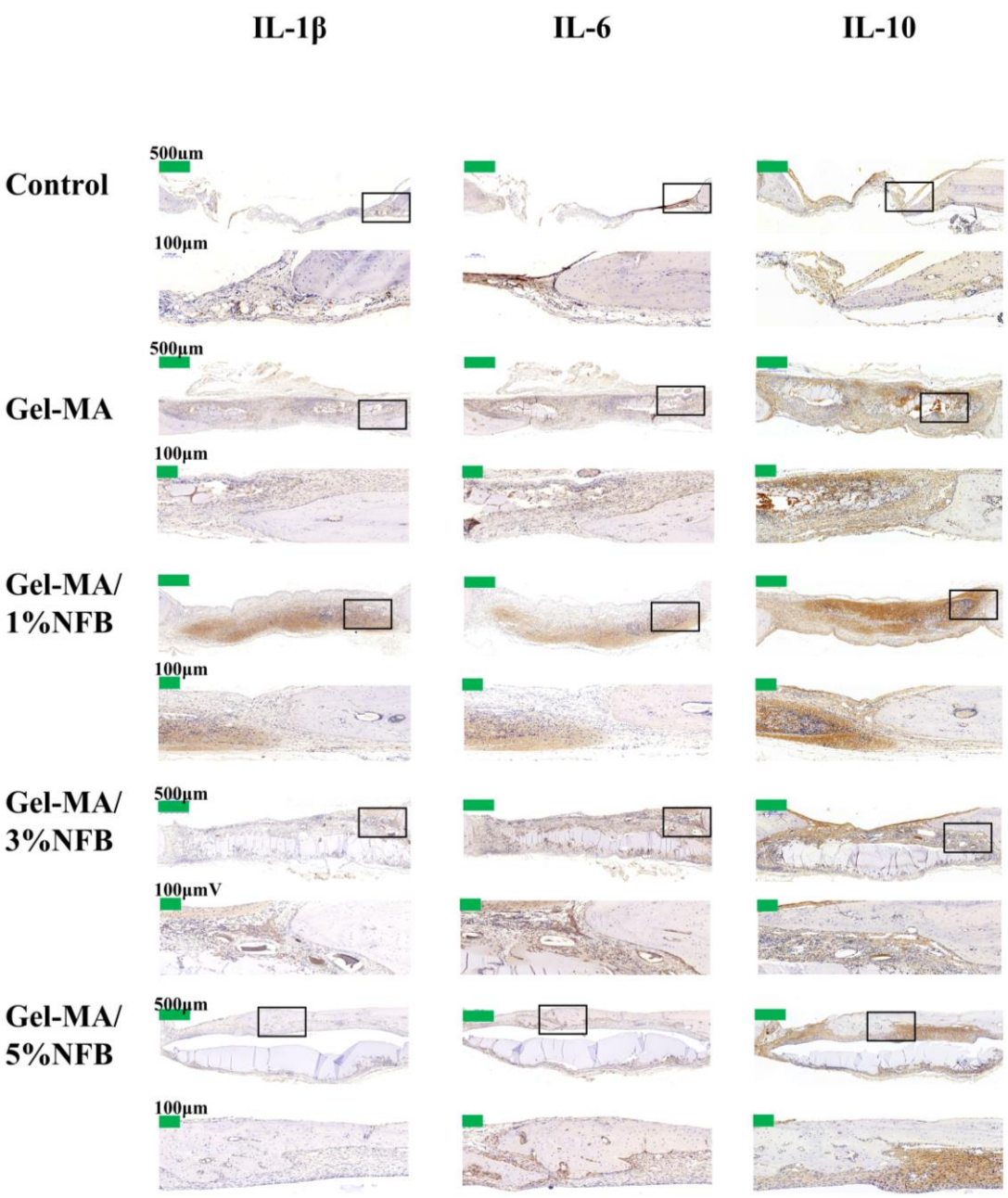

Figure S18. Immunohistochemical staining of bone defect section at postoperative 4 weeks.

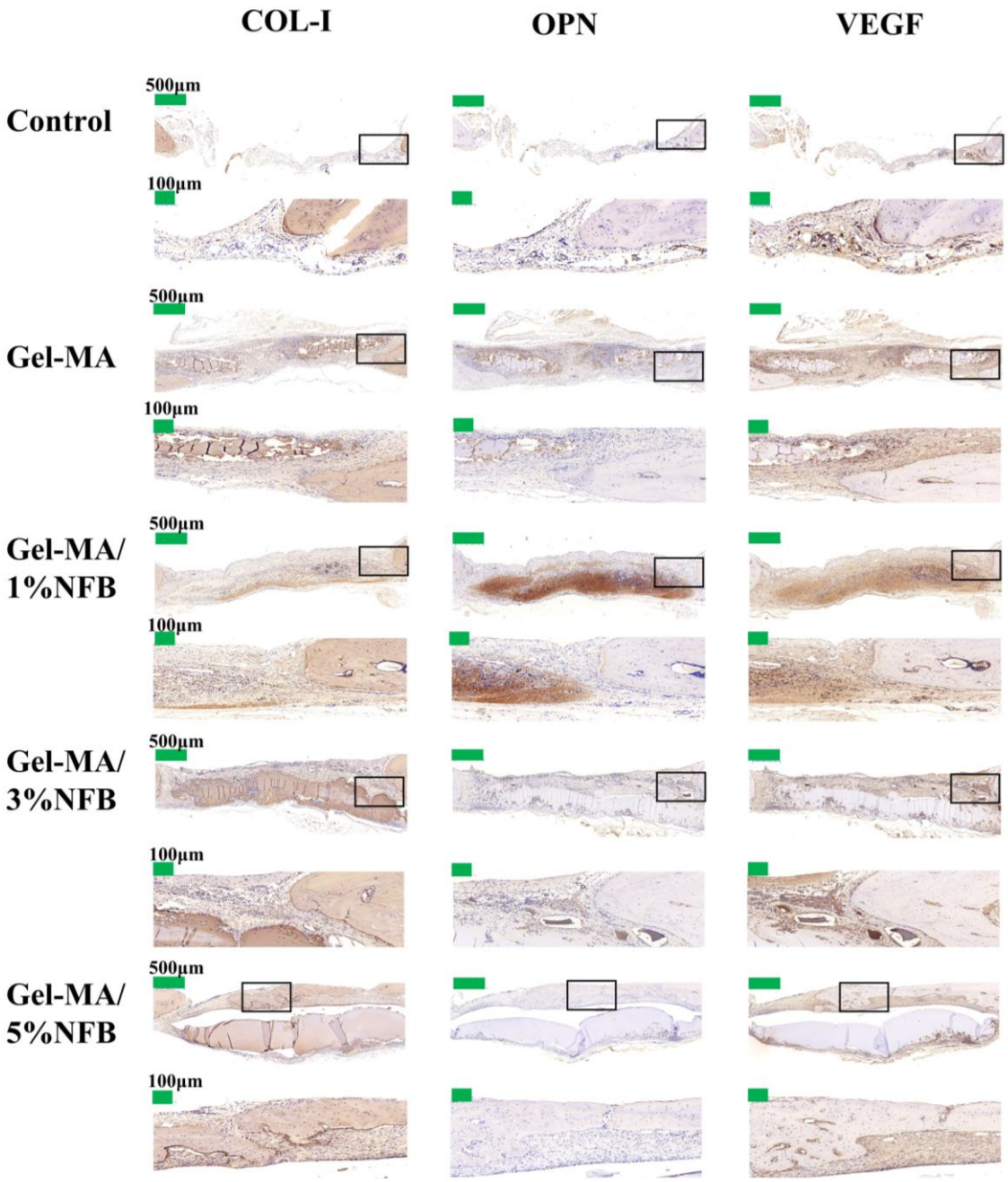

Figure S19. The bone regeneration 4 weeks after surgery: Immunohistological staining of COL-1, OPN and VEGF for bone formation after 4 weeks.

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