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Supporting Information

Magnesium-based layered double hydroxides nanosheets: a new bone repair material with unprecedented osteogenic differentiation performance

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Experimental Section

1.1 Materials

Chemicals used in this study were analytical-grade and obtained from commercial sources without any further purification. Magnesium nitrate ($Mg(NO_3)_2 \cdot 6H_2O$), ytterbium(III) nitrate pentahydrate ($Yb(NO_3)_3 \cdot 5H_2O$), aluminium nitrate ($Al(NO_3)_3 \cdot 9H_2O$), sodium hydroxide (NaOH), sodium nitrate ($NaNO_3$), formamide (CH_3NO) and ammonia solution ($NH_3 \cdot H_2O$), were purchased from Aladdin Chemical Co., Ltd. (Shanghai, China). Total RNA extraction kit, Centrifuge tubes, FBS and Penicillin/Streptomycin were purchased from Gibco (Thermo Fisher Scientific, Waltham, USA). Magnesium, magnesium oxide, tricalcium phosphate (TCP) and hydroxyapatite (HA) were purchase from Aladdin Chemical Co., Ltd. (Shanghai, China). MC3T3-E1 cells and α-minimum essential medium-nucleotide (α-MEM-N) were obtained from the National Infrastructure of Cell Line Resource (NICLR, China). Periostin antibody from rabbit (bs-4994R) and anti-rabbit IgG/HRP antibody from goat (074-15-06) were purchased from Bioss (Beijing, China) and Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, USA), respectively. The CCK-8 reagent was obtained from Dojindo (Kumamoto, Japan). Ultrapure water was obtained from a Milli-Q Millipore system (Thermo Fisher Scientific, Waltham, USA).

1.2 Synthesis of LDHs monolayer nanosheets

A solution was prepared by mixing $Mg(NO_3)_2 \cdot 6H_2O$ (3 mmol), $Al(NO_3)_3 \cdot 9H_2O$ (1.2 mmol) and $Yb(NO_3)_3 \cdot 5H_2O$ (0.3 mmol) in 47.5 mL of deionized water. Thereafter, 20 mL of formamide was added to this solution and stirred for 30 min, during which 2.5 mL of ammonia solution was added slowly with stirring. The mixture was then transferred to a Teflon-lined stainless-steel autoclave and heated at 120 °C for 30 min. The resulting product was centrifuged and washed thoroughly 4x with deionized water and ethanol. To collect LDH monolayer nanosheets in the supernatant, the obtained pellet was re-dispersed in deionized

water and centrifuged again at 4,000 *g*. The residual formamide was removed by dialysis (8 kDa) in deionized water for 48 h.

1.3 Drug loading

A stock solution of AL (1 mg/mL) was prepared. To determine the drug loading capacity, AL was added to the LDH solution (1 mg/mL) with various mass ratios from 0.25:1 to 4:1. After stirring for 6 h, free AL was removed by centrifugation at 12,000 *g* for 20 min and the obtained AL/LDHs were re-dispersed in PBS for further study.

1.4 Determination of LC and EE

The concentration of AL was determined spectrophotometrically, where cerium (IV) was used to oxidize AL for further determination. First, a standard curve of ceric solution against AL concentration was made. 1.5 mL of ceric sulphate (dissolved by 0.5 M sulphuric acid) was added to 5 mL of AL solution (AL concentrations ranging from 1 mg/mL to 10 mg/mL) for 2 h at 25 °C, the amount of the consumed ceric equivalent to the concentration of AL was determined by measuring the absorbance of ceric solution (as blank) against the test solution at 320 nm. Using this standard curve, the concentration of non-encapsulated AL in the supernatant after the drug loading experiment was calculated. The LC and EE values of AL were calculated by the equations below:

$$LC = (W_{Fed} - W_{Non-encapsulated}) / W_{Yb-LDH} \times 100\% \quad (1)$$

$$EE = (W_{Fed} - W_{Non-encapsulated}) / W_{Fed} \times 100\% \quad (2)$$

Where W_{Fed} is the total mass of initial added drug; $W_{Non-encapsulated}$ represents the mass of unencapsulated drug in supernatant after centrifugation; W_{Yb-LDH} is the total mass of added LDHs for the drug loading.

1.5 ITC measurements

A Nano ITC (TA Instruments, New Castle, USA) was used in the ITC measurements. The procedure was performed as follows: AL suspension (0.25 mL, 1.0×10^{-3} mol/L) was added

dropwise through a calorimeter injection syringe into LDH solution (1.2 mL, 1.0×10^{-4} mol/L) in an ITC cell at 25 °C. The titration gap was set at 300 s to achieve full heat equilibration. The titration curves were obtained by an independent model. The three thermodynamic parameters (enthalpy change ΔH, entropy change ΔS and affinity constant K_A) were analyzed using NanoAnalyze™ software (TA Instruments).

1.6 Sample characterization

The powder X-ray diffraction patterns were recorded on an XRD-6000 diffractometer (Shimadzu Corp., Kyoto, Japan) with a Cu Kα source, and a 10°/min scan step between 3° and 70°. HRTEM images were obtained using JEM-2100 (JEOL Ltd., Tokyo, Japan) with an accelerating voltage of 200 kV. The thickness was recorded with an atomic force microscope (AFM, Veeco, NanoScope IIIa) in a tapping mode. The ICPS-7500 (Shimadzu Corp.) recorded the inductively coupled plasma (ICP) emission spectroscopy to determine the chemical composition of LDH samples. XPS spectra were studied by PHIQ2000 X-ray photoelectron spectroscopy. A Mastersizer 2000 laser particle size analyzer (Malvern Panalytical Ltd., Malvern, UK) was used to analyze the particle size distribution. FT-IR spectra were recorded on a Vector 22 spectrophotometer (Bruker Corporation, Billerica, USA) from 3200-400 cm^{-1} at a resolution of 2 cm^{-1}. The UV-vis-NIR absorption spectra were recorded on U-3000 spectrophotometer (Shimadzu Corp.) from 300-900 nm with a slit width of 1.0 nm. A Siemens Inveon device (Munich, Germany) was employed in micro-CT measurements.

1.7 Preparation of liquid extracts

Liquid extracts were prepared according to the International Organization for Standardization (ISO 10993-12). Step 1: the complete medium (CM) was prepared with 89% (v/v) α-MEM-N, 10% (v/v) FBS, and 1% (v/v) penicillin/streptomycin solution; Step 2: LDHs, AL/LDHs, and AL were diluted to 100 μg/mL with CM and incubated in a constant temperature carbon dioxide incubator (CTCDI, MCO-175, Panasonic Healthcare Holdings

Co., Ltd. Tokyo, Japan) for 24 h at 37 °C, relative humidity of 95% and 5% carbon dioxide; Step 3: the solutions were centrifuged at 12,000 *g* for 10 min, and the supernatants (liquid extracts) were transferred to a sterilized 50 mL centrifuge tube, and stored at 4 °C for subsequent experiments.

1.8 Biocompatibility *in vitro*

A suspension (15,000 cells/mL) of MC3T3-E1 cells was prepared after the MC3T3-E1 cells grew into a logarithmic growth phase. Afterwards, the cell suspension was inoculated into a 24-well plate with 15,000 cells/well for 12 h to allow the cells to fully adhere to the wells. Subsequently, 1 mL of LDHs, AL/LDHs, and AL liquid extracts were added to designated cells for further incubation. The CCK-8 assay was carried out on Day 0, 1, 3, and 5, and OD values at 450 ± 5 nm were measured by a multifunctional full-wavelength microplate reader (Varioskan Flash; Thermo Fisher Scientific)[1]. Moreover, the flow cytometry assay was carried out on Day 5 measured by FACSCanto plus (BD Biosciences).

1.9 Osteogenic differentiation of MC3T3-E1 cells *in vitro*

2 mL of MC3T3-E1 cell suspension was added to two 6-well plates with 40,000 cells/well and then plates were placed into the CTCDI overnight to allow the cells to fully adhere to the wells. The time point of full adhesion was marked as Day 0. On Day 0, 2 mL of fresh liquid extract and CM were added to each well correspondingly; the solutions in the wells were completely replaced every other day. On Day 7, the total RNA extraction kit was used to extract total RNA from the cells and the detailed steps are as follows: cells were washed twice with PBS and then trizol reagent (Life Technologies Corporation, Carlsbad, USA) was added to the wells (1 mL/well). After 15 min, the trizol reagent containing lytic cells was transferred to a microtube and vigorously mixed with 200 μL of chloroform (Merck KGaA, Darmstadt, Germany). After centrifugation at 12,000 *g* for 15 min at 4 °C (Legend Micro 17R; Thermo Fisher Scientific), the supernatant was collected to perform the total RNA isolation (Ambion Inc., Austin, USA) based on the manufacturer's protocol. A NanoDrop spectrophotometer

(Thermo Fisher Scientific, USA) was used to measure the concentration of extracted RNA, which was subsequently transcribed to cDNA using a high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, USA). The transcriptional levels of *Alp*, *Runx2,* and Collagen I were compared between LDHs, AL/LDHs, AL, and the blank control groups [6]. Specific sequences of primers were designed with Primer (version 5.0, Premier Biosoft International, USA) (Table S1). The relative gene expression was calculated with the ΔΔcT method.

1.10 Alizarin Red S staining and ALP staining assays

On Day 12, the liquid extract or CM was totally removed, and the cells in each well were washed twice with 2 mL of PBS. Afterwards, the cells were fixed with 2 mL of 4% paraformaldehyde (Gibco, USA) for 15 min. After washing 3x with distilled water, each well was incubated with 2 mL of Alizarin Red S solution (Solarbio Life Science, Beijing, China) for 15 min or 2 mL of ALP staining solution (Solarbio Life Science, Beijing, China) for 20 min. Subsequently, all cells were washed 3x with distilled water and observed with an Eclipse80i microscope (Nikon, Minato, Japan).

1.11 Western blot assay

The cell proteins were collected using RIPA Lysis and Extraction Buffer (Invitrogen, Carlsbad, CA, cat. no. 89901). The protein concentration was measured by PierceTM Rapid Gold BCA Protein Assay Kit (Invitrogen, Carlsbad, CA, cat. no. 53225). Approximately 30 μg of protein was loaded with SDS-PAGE gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane on ice. Then, the PVDF membrane was blocked with 5% dilute skim milk in TBST for 45 min at room temperature. After washing with TBST, the membrane was incubated with primary antibodies overnight. Subsequently, TBST was used to wash the membranes three times, and secondary antibodies were then incubated with the membranes at room temperature for 45 min. The target bands were visualized using enhanced chemiluminescence (ECL, Thermo Fisher Scientific, USA) technology and the optical

densities of the bands were analyzed with Bio-Rad image analysis software (Bio-Rad, Hercules, USA). *Gapdh* was used as the reference gene. The primary antibodies used in the present study were anti-RUNX2 (1:1000, Abcam, #ab23981) and anti-Osteocalcin (1:1000, Abcam, #ab13421).

1.12 FITC-labeled phalloidin staining

Fluorescein isothiocyanate (FITC)-labeled phalloidin staining was carried out on Day 7. Initially, trypsin-EDTA (0.05%) (Gibco, USA) was used to digest the cells, and then the digested cells were seeded into new confocal dishes (Thermo Fisher Scientific, USA). The cells were stained by the phalloidin working solution and 4',6-diamidino-2-phenylindole (DAPI) containing anti-fluorescence quenching agent. Finally, fluorescence observation was performed with a confocal microscope (Leica Camera AG, Wetzlar, Germany). FITC excitation/emission filters (Ex/Em=496/516 nm) and DAPI excitation/emission filters (Ex/Em=364/454 nm) were selected.

1.13 Establishment of the animal model of osteonecrosis

The experiments were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. The protocol was approved by the Animal Care and Use Committee of Peking Union Medical College Hospital (PUMCH). 65 healthy and mature female New Zealand white rabbits with an average weight of 2.5 kg were purchased from the animal room of PUMCH. All rabbits were lived in a temperature-controlled room (25 °C), with a 12 h light/dark cycle and a relative humidity of 40–60%. After 1 week for acclimatization, 60 rabbits were anaesthetized by injecting 3% pentobarbital (1 mL/kg) into the ear vein; 5 rabbits in the blank control group underwent no treatment. After the greater trochanter of the femur was exposed, a bone knife was used to locate a point approximately 5.5 mm below the greater trochanter. A 3 mm diameter Kirschner wire (K-wire) was placed at the located point at an angle of 45 degrees with the femur. A tunnel was drilled from the located point to the center of the femoral head with a depth of 25-30 mm. The

freezing steps were as follows and were repeated five times: the K-wire was soaked in liquid nitrogen for more than 10 s, and then inserted into the tunnel for at least 10 s. Afterwards, 1.6 mg of LDHs (8 mg/mL), or 1.6 mg of AL/LDHs (8 mg/mL), or 0.2 mL of PBS (1X) was injected into the corresponding tunnel. In addition, the iliac crest on the right side of the rabbits in the positive control group was harvested and cut into appropriate size (approximately 2 mm) to fill the tunnels. All rabbits were injected twice intra-muscularly with 200,000 units of penicillin (Shengwang Corp, Shandong, China) immediately after surgery and 24 h postoperatively (Figure S10).

1.14 Biological safety *in vivo*

2 mL of peripheral blood was taken through the ear vein at Weeks 0, 2, 4 and 8 postoperatively and placed into a vacuum blood collection tube (Solarbio Life Science, Beijing, China). The blood sample was vibrated upside down for 5 times and then allowed to settle for 5 min. After centrifugation at 3000 *g* for 10 min, the supernatant was transferred to a 1.5 mL sterile centrifuge tube (Axygen Scientific Inc., Union City, USA). The concentrations of Mg and Al ions in the serum were quantified using ICP-MS (iCAP Q; Thermo Fisher Scientific, USA). Rabbit liver, kidney, and spinal cord were harvested and then soaked in neutral formalin (Solarbio Life Science, Beijing, China). Subsequently, sections were sliced to 50 μm thickness by rotary microtome (RM2255; Leica). Histological structures were stained by H&E staining and verified with the Eclipse 80i microscope.

1.15 Micro-CT and Immunohistochemical staining analysis

The rabbits were sacrificed by air embolism at specific time points and whole femurs were harvested and soaked in neutral formalin. The femurs of the rabbits were scanned *via* micro-computed tomography (micro-CT; Siemens, Germany) to evaluate the volume of regenerated bone and BMD. After two analytical areas were confirmed based on the drilled tunnel and femoral head (Figure S11), quantitative analysis of newly regenerated bone in the analytical areas was carried out using Inveon research workplace software (Version 2; Siemens,

Germany) with threshold bone density values from 500-1300 HU. After micro-CT scanning, the femur samples were decalcified, embedded in paraffin, and the targeted areas were sliced to 50 μm thickness by the rotary microtome. Sections were stained with H&E and anti-periostin antibody to detect new bone regeneration *via* a microscope. The regenerated collagen fibers had been quantitatively analyzed in H&E sections using Image J 1.52v software (https://imagej.nih.gov/ij/notes.html).

1.16 Statistical analysis.

Data were expressed as mean ± standard deviation (s.d.). Statistical comparisons were made by unpaired Student's t-test (between two groups) and one-way ANOVA (for multiple comparisons) followed by Tukey's post-test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

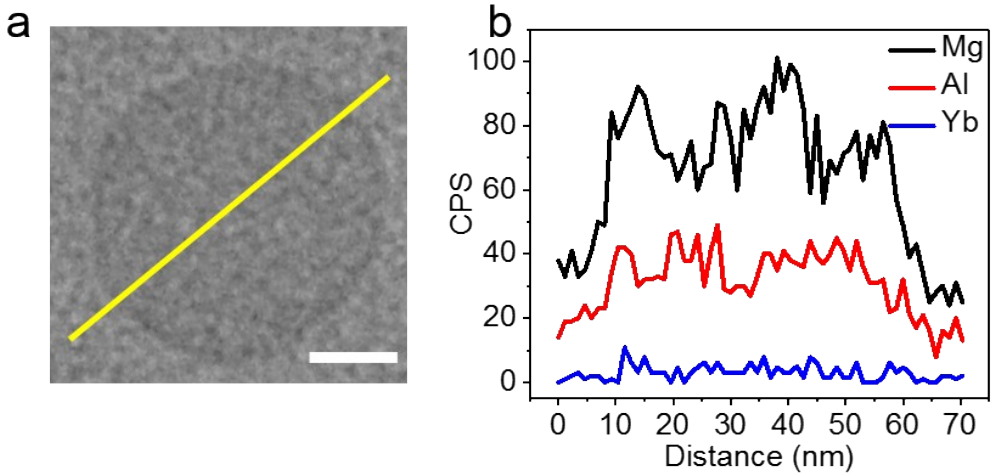

Figure S1. Elemental EDX line profiles of LDH nanosheets for Mg, Al, and Yb, respectively. Scale bar: 20 nm.

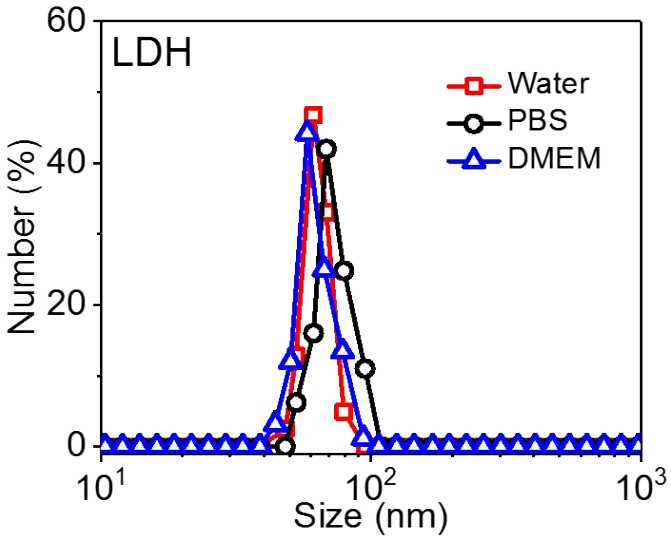

Figure S2. Size distribution of LDH nanosheets in water, PBS, and DMEM culture medium, respectively.

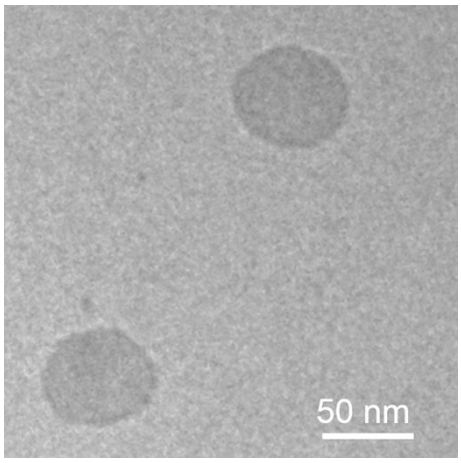

Figure S3. HRTEM image of AL/LDHs sample.

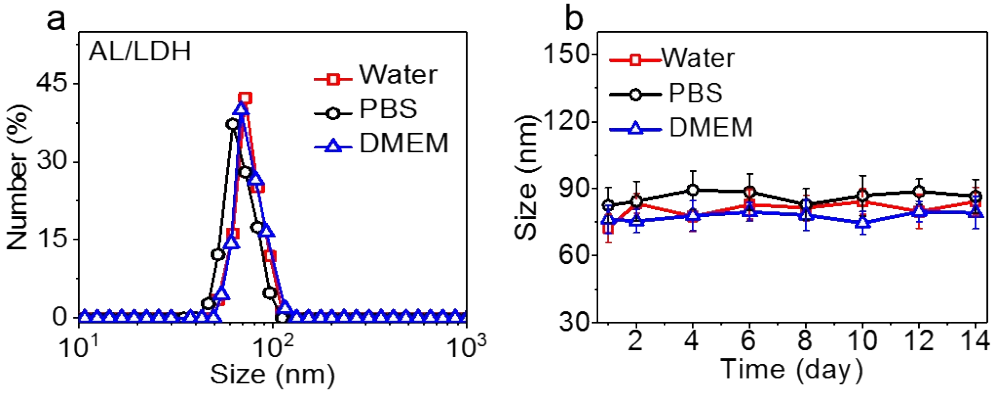

Figure S4. (a) Size distribution of AL/LDHs in water, PBS, and DMEM culture medium, respectively. (b) Hydrodynamic diameter of AL/LDHs in water, PBS, and DMEM respectively for 14 d. Error bars stand for ± s.d. (n = 3).

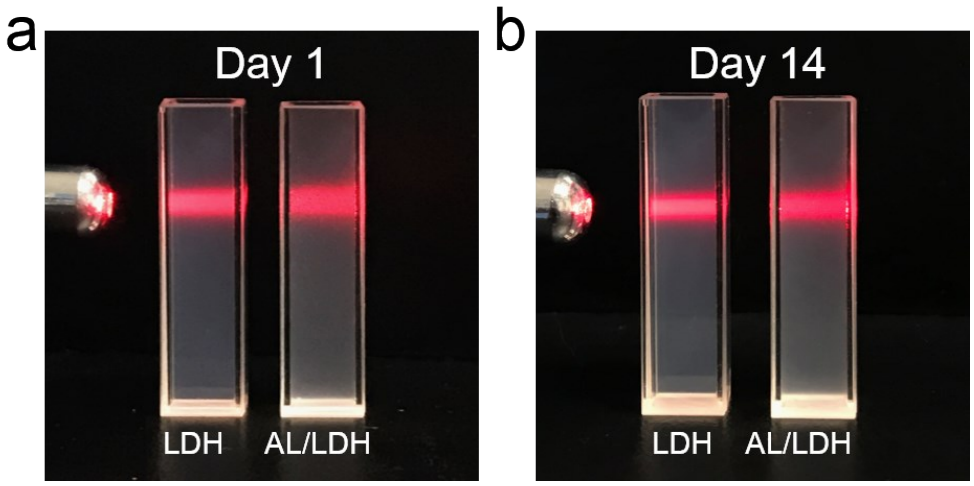

Figure S5. Tyndall effect of LDHs and AL/LDHs in aqueous solution on Day 1 and Day 14.

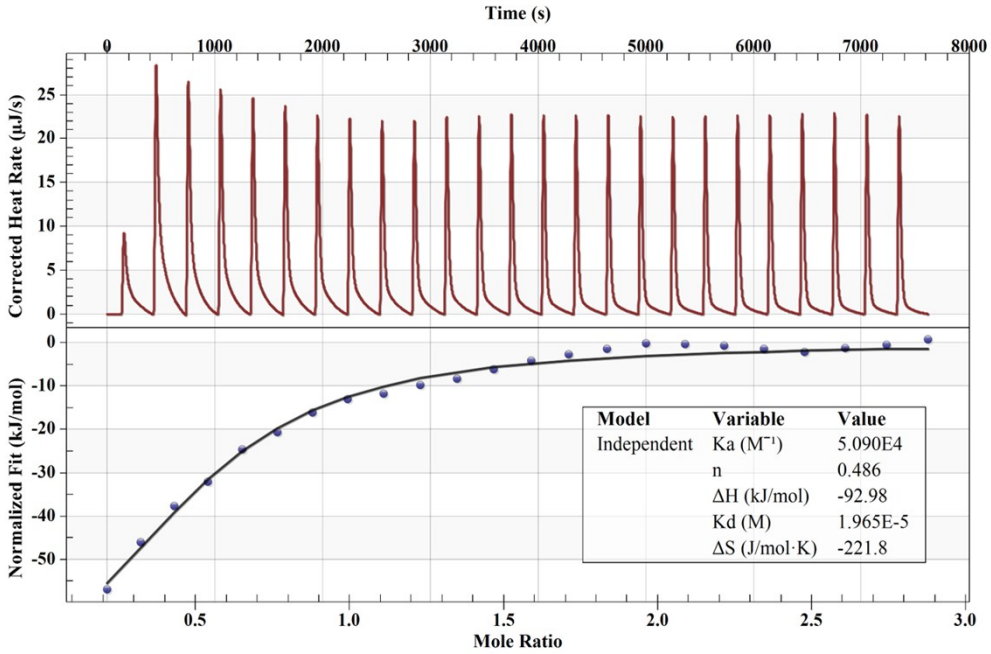

Figure S6. ITC titration measurements for AL (1.0×10^{-4} mol/L) and LDHs (1.0×10^{-3} mol/L).

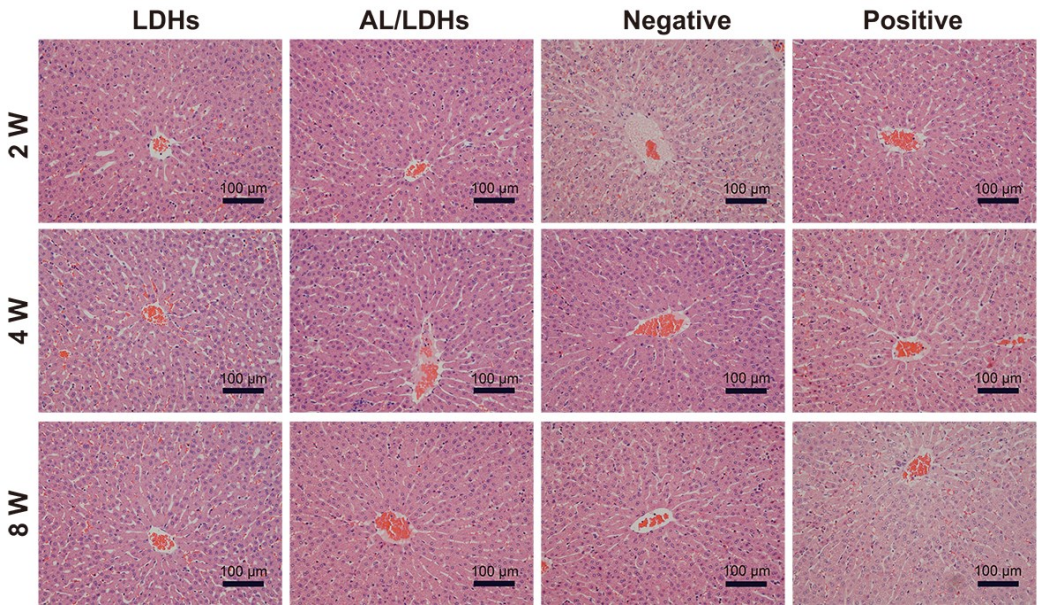

Figure S7. Tissue structure of liver without obvious difference at any time point or groups. Scale bar: 100 μm.

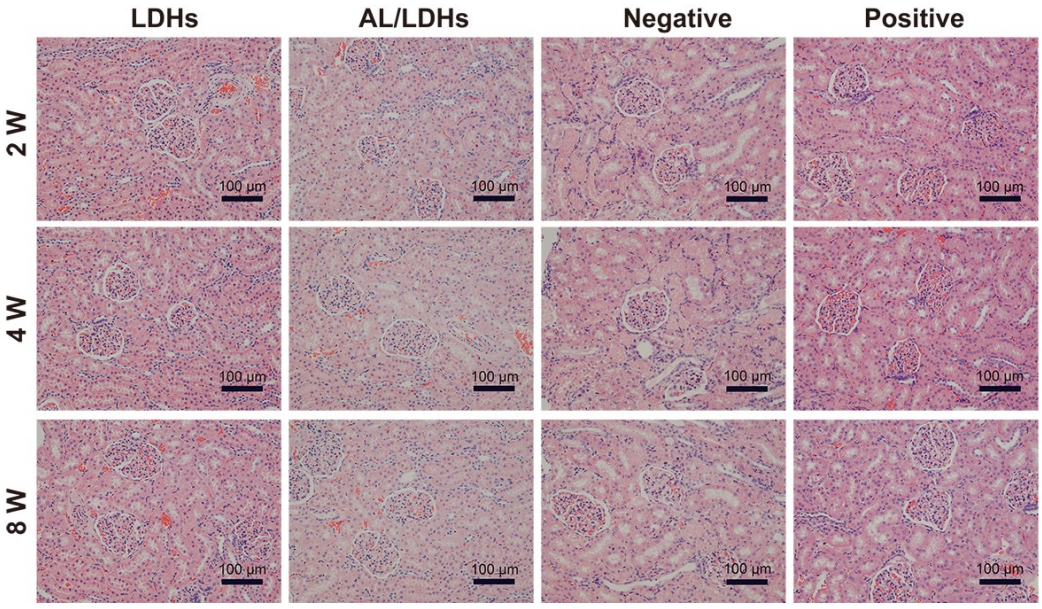

Figure S8. Tissue structure of kidney without obvious difference at any time point or groups. Scale bar: 100 μm.

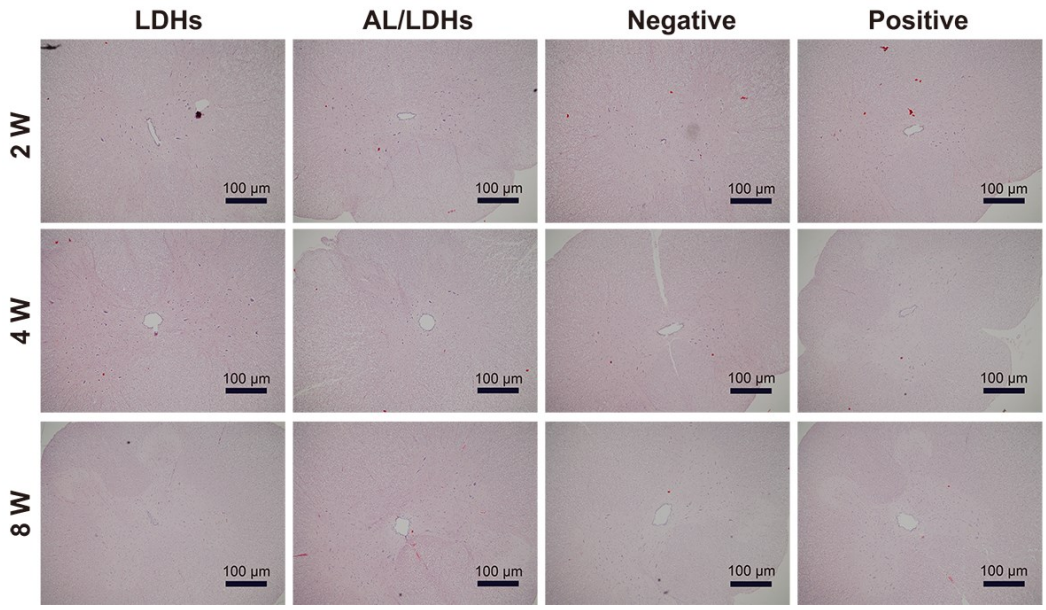

Figure S9. Tissue structure of spinal cord without obvious difference at any time point or groups. Scale bar: 100 μm.

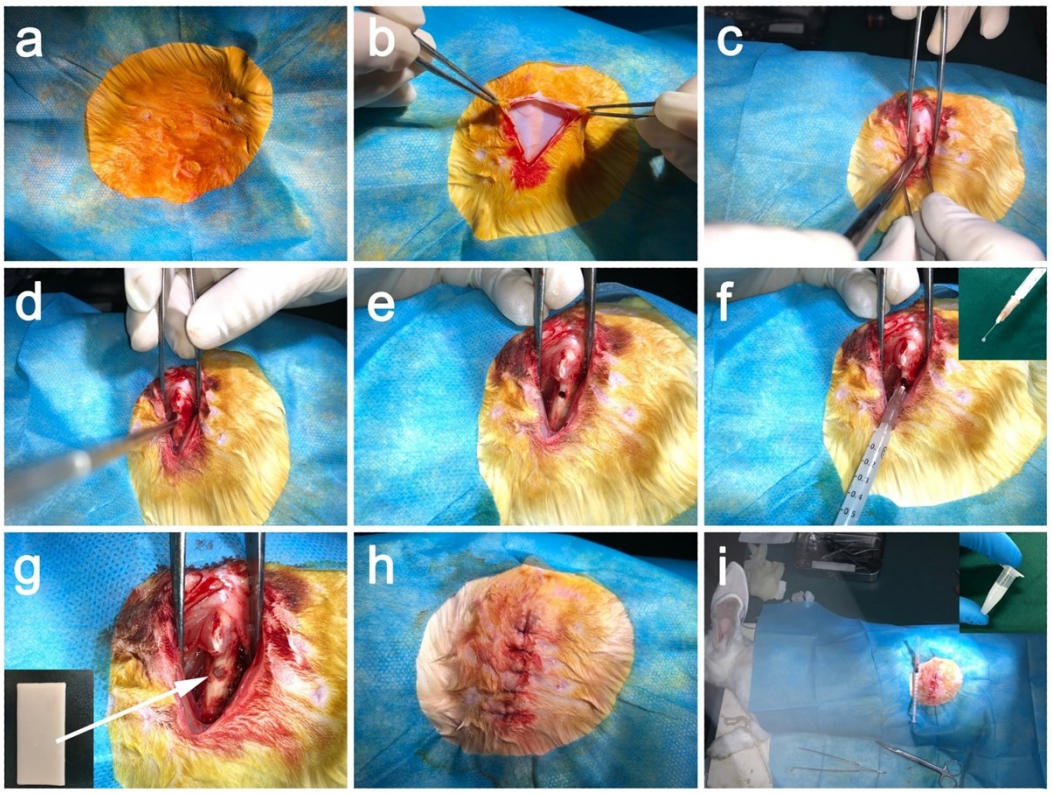

Figure S10. The whole process of surgical operation. (a) disinfect; (b) cut open; (c) position; (d) drill and freeze; (e) the entrance of the tunnel; (f) inject material; (g) seal inlet with bone wax; (h) stitch; (i) general photo after surgery.

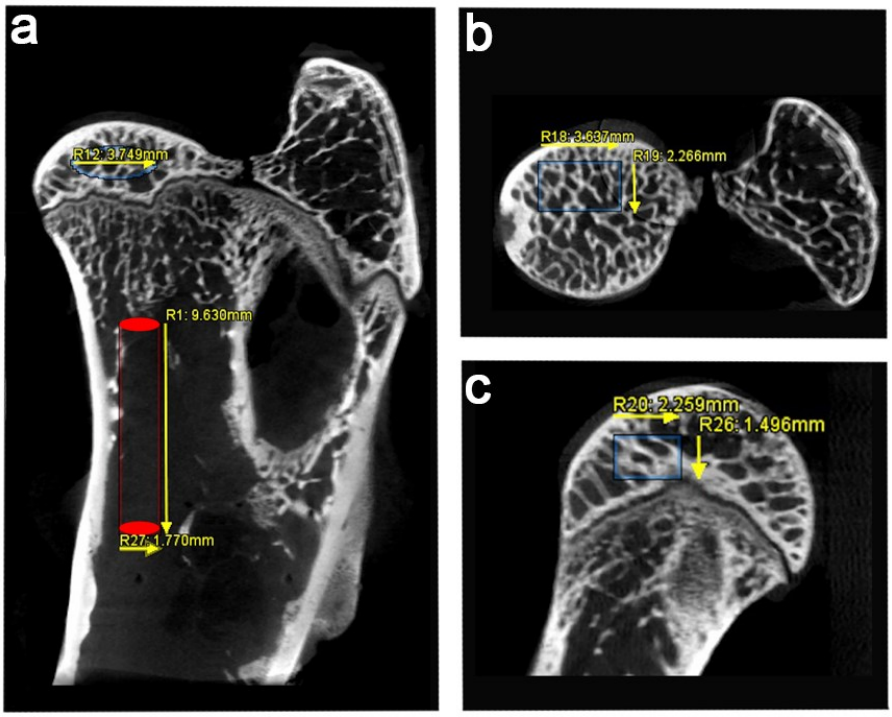

Figure S11. The analysis parameters of relevant areas were shown above: (a) sagittal cross-section view; (b) coronal cross-section view; (c) cross-section screenshot. The analytical volume of femoral shaft and femoral head is 24 mm^3 and 10 mm^3 respectively.

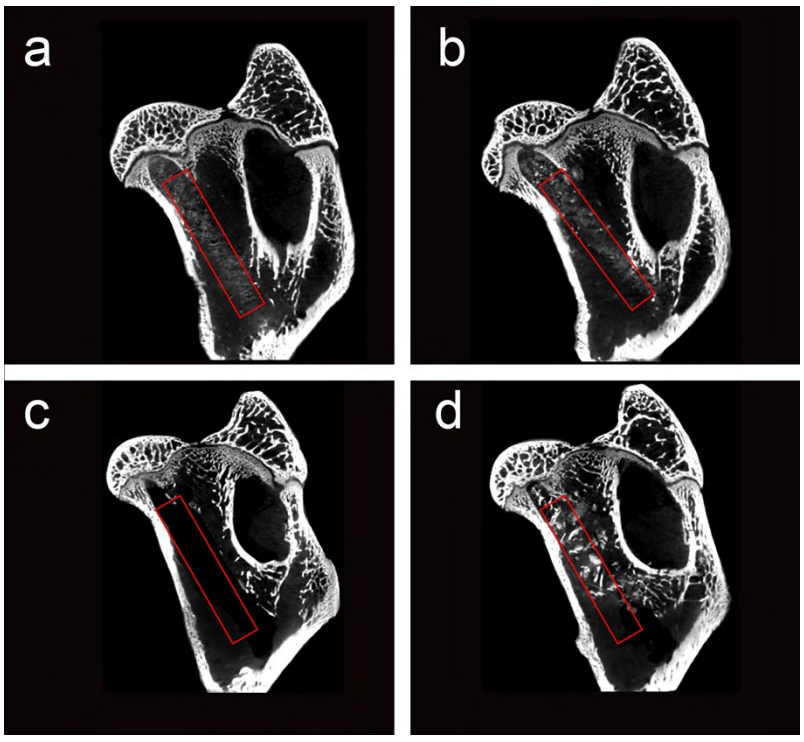

Figure S12. Immediate postoperative imaging results after the materials were injected into the corresponding tunnels: (a) LDHs group, (b) AL/LDHs group, (c) negative control group, (d) positive control group.

Table S1. Primer sequences of *Alp*, *Runx2*, Collagen I, and *Gapdh*.

Primer Name	Primer Sequence（5'-3'）	Gene ID
m*Alp*-F	TTACGCTCACAACAACTACCAG	11647
m*Alp*-R	GGAGGCATACGCCATCAC	
m*Runx2*-F	CCTACCAGCCTCACCATAC	12393
m*Runx2*-R	GTCTACTGACATCAGCTACCG	
m*ColA1*-F	CGCCATCAAGGTCTACTGC	12842
m*ColA1*-R	CGGGAATCCATCGGTCAT	
m*Gapdh*-F	AGGTCGGTGTGAACGGATTTG	14433
m*Gapdh*-R	TGTAGACCATGTAGTTGAGGTCA	

Reference

1 A. T. Neffe, B. F. Pierce, G. Tronci, N. Ma, E. Pittermann, T. Gebauer, O. Frank, M. Schossig, X. Xu, B. M. Willie, M. Forner, A. Ellinghaus, J. Lienau, G. N. Duda and A. Lendlein, *Adv. Mater.*, 2015, **27**, 1738–1744.