# Supporting Information

## Regulation of bone regeneration by chiral modified hydroxyapatite/chitosan scaffolds

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## Experimental and Methods

**Reagents and Materials.** All chemicals were of analytical grade. Trisodium hydrogen pyrophosphate ($Na_3HP_2O_7$), lanthanum nitrate ($La(NO_3)_3 \cdot 6H_2O$), chitosan ($(C_{6n}H_{11}N_nO_{4n})$), and sodium hydroxide (NaOH) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Hydrogen peroxide ($H_2O_2$) was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. Glacial acetic acid ($CH_3COOH$) was purchased from Tianjin Xinbote Chemical Reagent Co., Ltd. Glutaraldehyde ($C_5H_8O_2$) was purchased from Guangdong Wengjiang Chemical Reagent Co., Ltd. Dichloromethane ($CH_2Cl_2$) was purchased from Tianjin Damao Chemical Reagent Factory. Poly (L-lactide) ($(C_6H_8O_4)$) was purchased from Shanghai MacLean Biochemical Technology Co., Ltd.

**Apparatus.** Fourier transform infrared (FT-IR) analyses were performed on an EQUINOX-55 spectrometer. Scanning electron microscope (SEM) images were collected on a Hitachi S-4800, allowing observation of the microscopic morphology of different prepared materials. Transmission electron microscopy (TEM) was recorded on a Tecnai G2 F20 microscope. The x-ray diffraction (XRD) patterns were carried out on a Ultima IV diffractometer using Cu Kα radiation.

**Synthesis of HAP.** The boiled yak bone strips were soaked in a 10wt% NaOH solution for 12 h, then soaked in a $H_2O_2$ solution for 24 h, and dried at 60 °C for 3 h. Then they were placed in a muffle furnace, heated to 800 °C for 6 h, and the bone strips were taken out and crushed into bone powder with a crusher. They were soaked in a 0.09 M $Na_3HP_2O_7$ solution (1 g/mL) and reacted at 70 °C for 72 h. After the reaction was completed, they were centrifuged and dried at 60 °C. The bone powder was placed in a muffle furnace and calcined at 1200 °C for 1 h, and sieved with a 100-mesh sieve to obtain hydroxyapatite (HAP).

**Synthesis of La/HAP.** 5.0234 g of HAP was added to 1 M La(NO3)3·6H2O solution and then reacted at 70 °C for 72 h. After the reaction, the material was centrifuged and dried. The material was then calcined at 900 °C for 3 h, ground, and sieved with a 200-mesh sieve to obtain La/HAP.

**Synthesis of PLLA/La/HAP.** Different amounts of poly (L-lactide) (PLLA) (0, 0.0505, 0.1546, 0.2632, 0.3763, and 0.4945 g) were dissolved in 50 mL of dichloromethane and heated to 35 °C. 5 g of HAP powder was dispersed in 50 mL of dichloromethane solution. Then, the PLLA solution was added to the HAP suspension and stirred for 0.5 h to obtain PLLA/La/HAP-0, PLLA/La/HAP-1, PLLA/La/HAP-3, PLLA/La/HAP-5, PLLA/La/HAP-7, and PLLA/La/HAP-9.

**Synthesis of PLLHC-X materials.** 4.5530, 4.5990, 4.6938, 4.7927, 4.8957 and 5.0033 g samples were weighed and dispersed in 0.1 M HAc solution. Then 0.4 g chitosan (CS) powder was added to the above 6 groups of dispersions and stirred for 4 h to obtain a uniform light yellow slurry. 2 mL of 0.1 wt% glutaraldehyde solution was added to 6 beakers respectively. Each uniform colloidal liquid was thinly spread on a watch glass and freeze-dried for 48 h. The freeze-dried samples were completely immersed in 1 M NaOH solution. After soaking for 24 h, they were washed several times until the solution was neutral. The obtained samples were defined as PLLHC-0, PLLHC-1, PLLHC-3, PLLHC-5, PLLHC-7, and PLLHC-9.

**Bone marrow mesenchymal stem cells (rBMSCs) were inoculated into different experimental groups.** The freeze-dried scaffold material was cut into 2 mm thick discs with a diameter of about 8 mm using a sharp blade, and the material was sterilized using low-temperature ethylene oxide, soaked in serum-free culture medium for 12 h, and the culture medium was absorbed with sterilized filter paper and placed in a 24-well plate. Phosphate buffered saline (PBS) was added to the material 4 h before inoculation of cells. After culturing the 2nd to 3rd generation of bone marrow mesenchymal stem cells, the medium was discarded and the cells were washed twice with PBS solution. The cells were digested with trypsin and beaten into single cells. The cells were counted using a cell counting plate and the concentration of the cell suspension was adjusted to 1.0×106 cells/mL or 2.5×106 cells/mL. For the experimental group used to measure cell proliferation and adhesion, 20 μL of a cell suspension with a concentration of 1.0×106/ml was added to each material. For the experimental group used to detect the osteogenic differentiation of bone marrow mesenchymal stem cells induced by PCR and immunofluorescence staining, 20 μL of

a cell suspension with a concentration of 2.5×106/mL was added to each material. After the cells adhered to the material for 3 h, 1 mL of 10% fetal bovine serum (FBS) complete culture medium was added to a 24-well plate and cultured in a 5% CO2, 37°C incubator, with the medium changed every 2 to 3 days.

**The morphology of rBMSCs adhering to different groups of materials.** After culturing for 2 days, the cell-material complex was removed, washed twice with PBS, and fixed with 2.5% glutaraldehyde for 2 h. Finally, it was dehydrated with 30%, 50%, 75%, 85%, 95% and 100% ethanol gradient solutions in sequence, critical point dried, sprayed with gold, and observed with SEM.

**Live and dead cell staining.** 1 mL of the corresponding sample working solution was added to different groups. Sample treatment cells were taken from rBMSC cells in the logarithmic growth phase and the cells were counted. The cell concentration was adjusted and cultured overnight in a 37°C constant temperature incubator to allow the cells to adhere to the wall. rBMSCs were treated according to the above grouping and culture for 2 days. Reagent A (Calcein-AM) and reagent B (PI) 10 times were diluted with dye diluent (solution C). 985.5 μL PBS was mixed with 10 μL diluted reagent A and 4.5 μL reagent B and used immediately. They were washed once with PBS to remove excess serum. Staining solution were added and incubated at room temperature in the dark for 15 min. Finally, they were washed three times with PBS to terminate staining, observed the results and took photos.

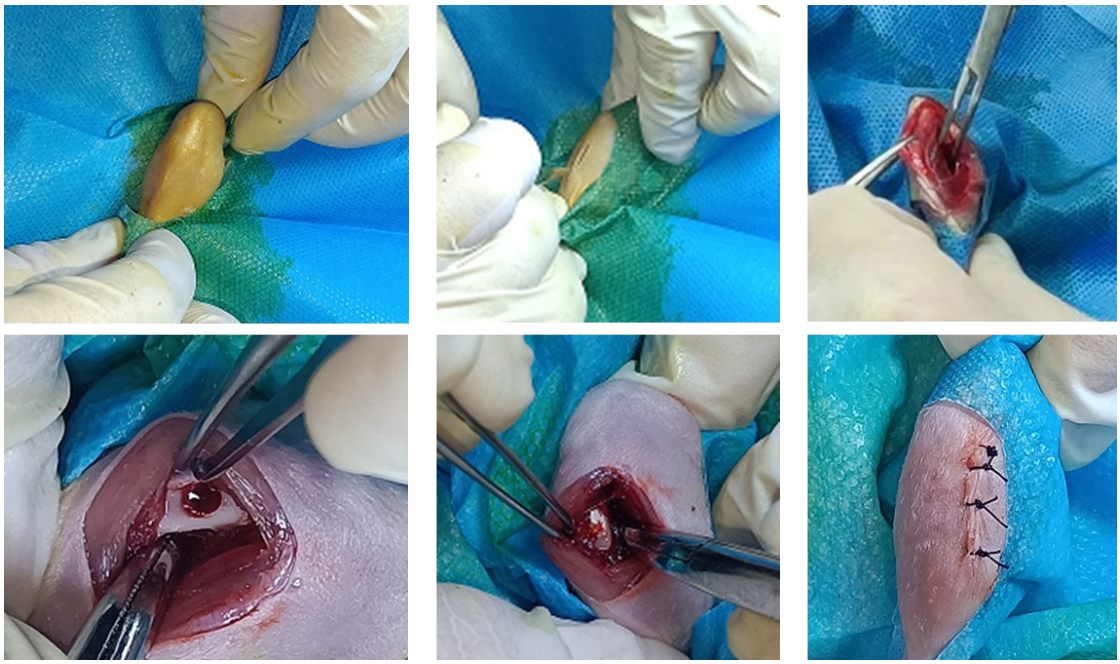
**Alizarin red staining.** rBMSCs cells in the logarithmic growth phase were seeded in a 24-well plate at an appropriate density, and were treated accordingly according to the purpose of the experiment to set up a control group and an experimental group. After culturing for 21 days, the working solution was discarded and the cells were fixed with 4% paraformaldehyde. After fixation, they were washed once with PBS and stained with Alizarin red for about 10 min. Then, they were washed 1-2 times with distilled water and photographed under a microscope. After the filming, 1 mL/well of 10% cetylpyridinium chloride was added, shaken for 1 min, 100 μL of the solution was transferred to a 96-well plate, and the absorbance at 562 nm was detected on a microplate reader.

**Alkaline phosphatase (ALP) activity assay.** Cells were inoculated on materials of different groups at 1, 7, and 14 days old, the culture medium was removed, and the samples were washed twice with PBS for 5 min. Trypsin was added to digest the cells on the materials, and the samples were centrifuged at 1000 rpm for 8 min. Each group of cells was placed in an EP tube with a certain amount of double distilled water, and directly placed in liquid nitrogen for 3-5 s. The cells were immediately taken out and transferred to a -20°C refrigerator for 20-30 s, and then taken out and thawed at room temperature. After thawing, the cells were placed in liquid nitrogen again, and this process was repeated 3 times to disrupt the cells. Buffer and matrix solution were added to the sample and mixed thoroughly, and place in a water bath for 15 min. The colorimetric reagent was added, and the absorbance of each well was measured using an ELISA instrument (wavelength 520 nm). Double distilled water was added as a blank control group. The alkaline phosphatase (ALP) activity of the sample to be tested was calculated based on the known concentration of phenol standard application solution. Three parallel samples were set for each sample. The total protein concentration of the samples was detected using a BCA kit. The calculation formula of ALP activity in the sample is:

(U/gprot) = (measured OD value - blank OD value) / (standard OD value - blank OD value) × phenol standard concentration / protein concentration of the sample to be tested.

**Creation of tibial defect and experimental grouping in rats.** All in vivo experiments were reviewed and approved by the Ethics Review Committee of Lanzhou University of Technology. All animal experiments were performed using inhalation anesthesia. Sprague-Dawley (SD) rats were randomly divided into groups, each weighing 200 g, with 6 rats in each group, half of which were male and half were female. The animals were weighed before surgery to monitor body weight, and anesthesia was performed with an intraperitoneal injection of 10% chloral hydrate (0.3 mL). After successful anesthesia, the right limbs were uniformly prepared, and the rats were fixed on the operating table in a supine position. The surgical area was disinfected with 2.5% povidone-iodine and 75% ethanol, and a drape was laid to observe the knee

joint of the rat. The knife was inserted into the medial vertical bone surface 2 cm below the knee joint to form a longitudinal incision of about 1 cm, and the skin and subcutaneous tissue were separated to fully expose the tibial bone surface. Then, a 3 mm diameter ball drill was used to drill holes using an implanter at 900 RPM, and 4 °C saline was used to cool the holes to prevent bone necrosis. Different groups of scaffold materials were implanted in the holes, and the surgical area was sutured in layers with 4-0 sutures and covered with wound dressings. Ceftriaxone sodium (2 mg/kg body weight) was given postoperatively to prevent infection. After implantation 4 and 8 weeks, the SD rats were anesthetized by overdose of 10% chloral hydrate and the tibia was dissected for further analysis (Scheme S1).



Scheme S1. Establishment of rat tibial defect model and intraoperative external images after implantation of materials

**Histological analysis.** The dissected sections were decalcified, fixed, and embedded in paraffin. Paraffin sections were obtained using a hard tissue microtome (Leica SP1600; Leica Biosystems, Nussloch, Germany) with a section size of 10 μm. After deparaffinization, the sections were rinsed five times and then stained with hematoxylin and eosin (H&E), Masson, CD31, and RUNX2. Images The sections were observed under a microscope and captured with a digital camera (Olympus BX51; Olympus Corporation, Tokyo, Japan).

**Micro-CT examination.** After Micro-CT scanning, the fixed samples in each group were three-dimensionally reconstructed using Dragonfly 2024.1 software (Object Research Systems, Canada). The internal microscopic structure of the sample is clearly presented through three-dimensional reconstruction technology, significantly improving the image visualization effect. Among them, a hollow cylindrical area with a radius of 0.5 mm and an axial extension of 5 mm was set as the region of interest (ROI) with the implanted stent as the central axis. Three-dimensional bone morphometric analysis was used to qualitatively analyze bone volume/total tissue volume (BV/TV), trabecular separation/spacing (Tb.Sp), and trabecular thickness (Tb.Th).

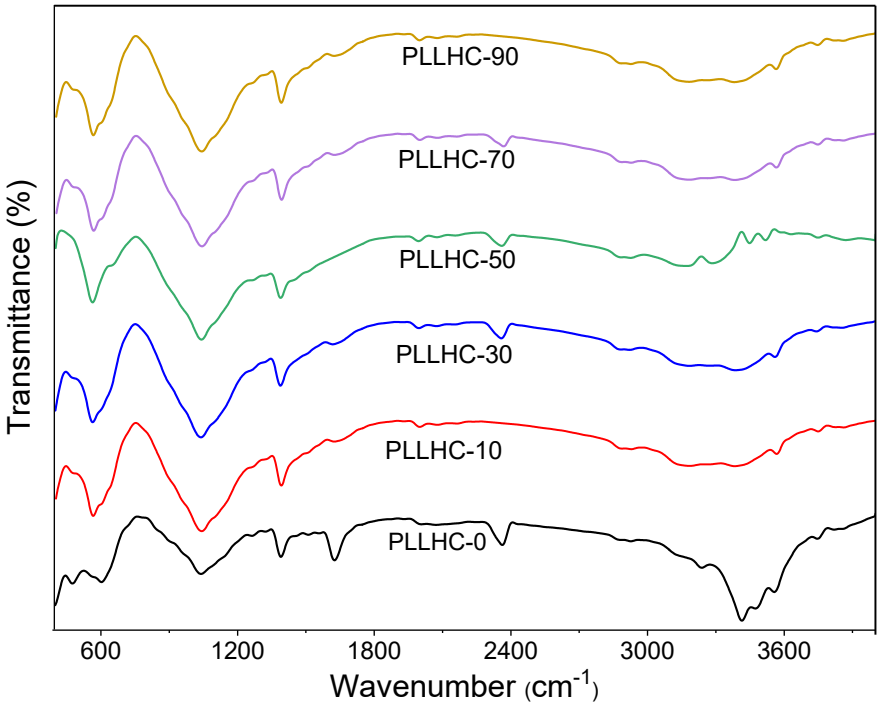



Figure S1. FT-IR spectra of PLLHC-0, PLLHC-1, PLLHC-3, PLLHC-5, PLLHC-7 and PLLHC-9

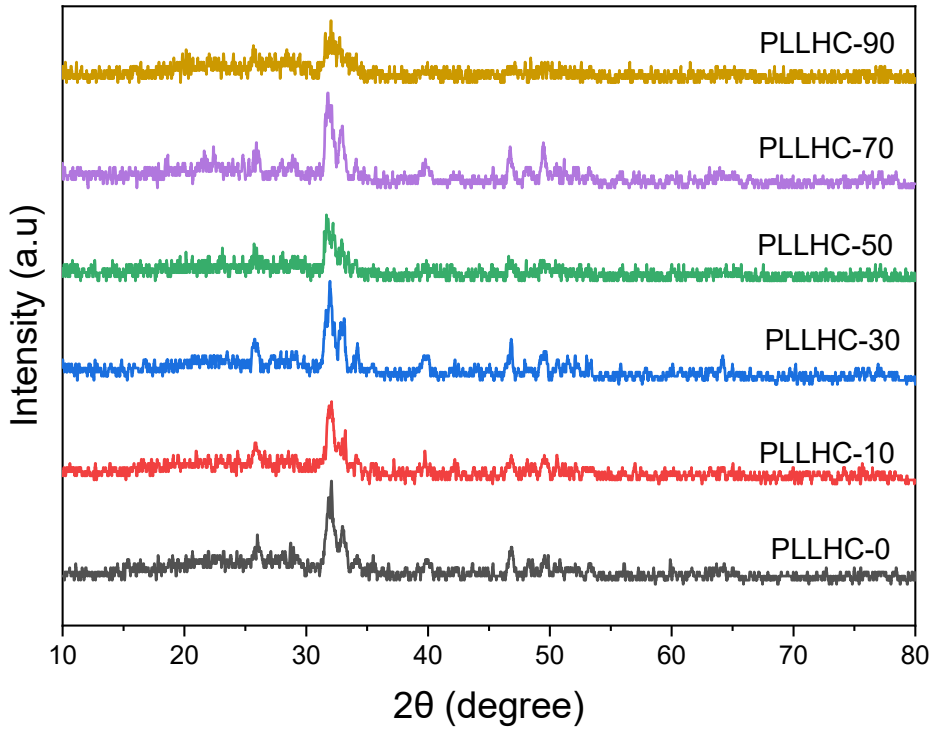



Figure S2. XRD pattern of PLLHC-0, PLLHC-1, PLLHC-3, PLLHC-5, PLLHC-7 and PLLHC-9

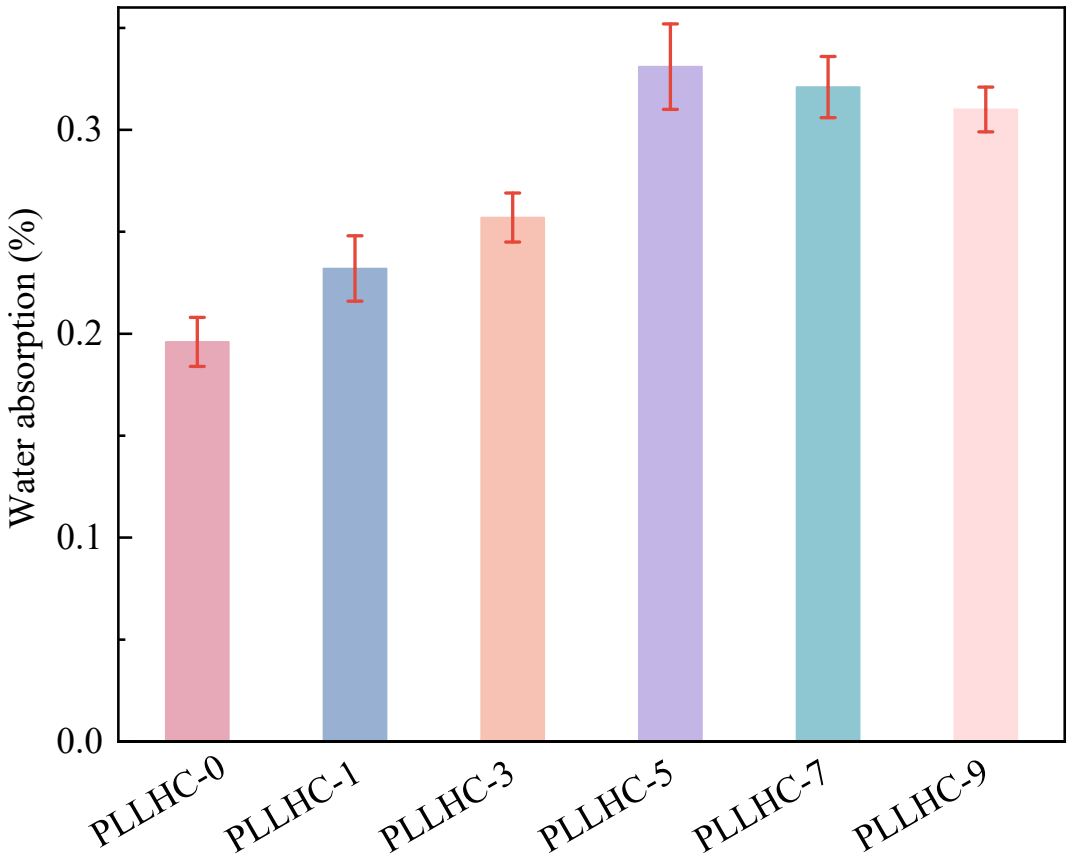



Figure S3. Water absorption of PLLHC-0, PLLHC-1, PLLHC-3, PLLHC-5, PLLHC-7 and PLLHC-9

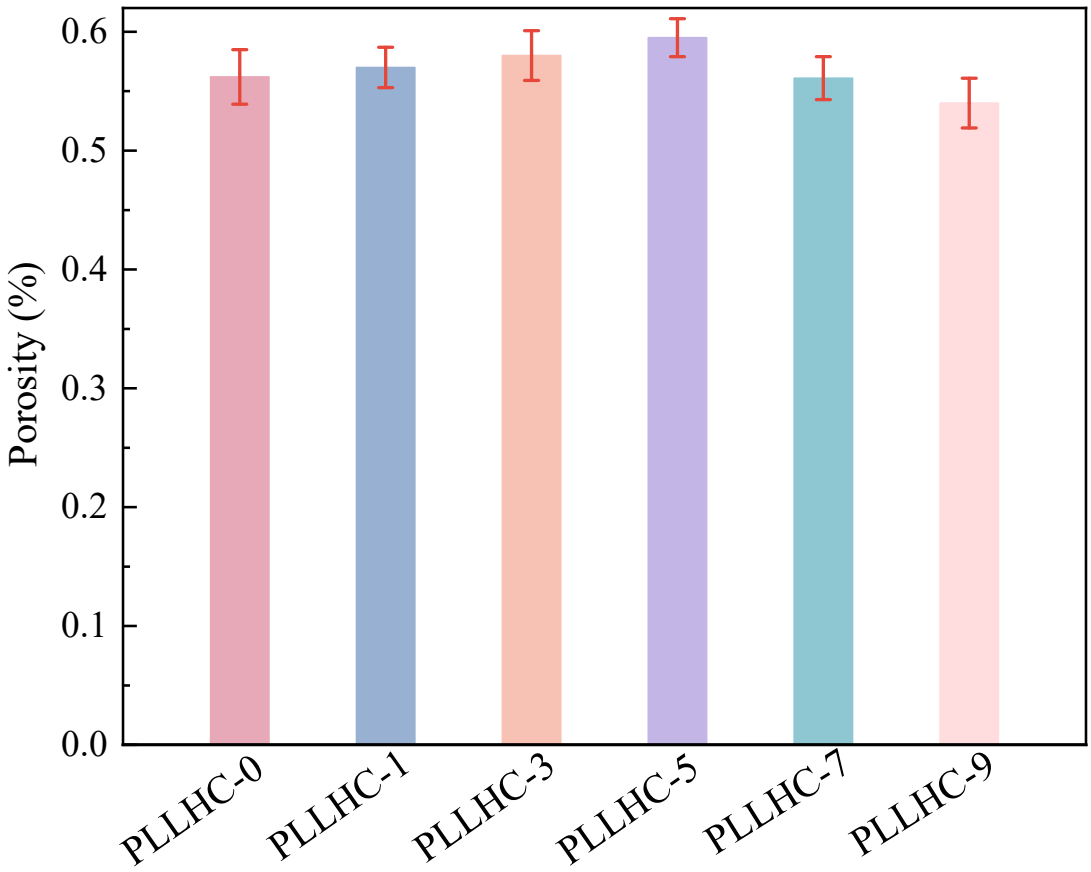



Figure S4. Porosity of PLLHC-0, PLLHC-1, PLLHC-3, PLLHC-5, PLLHC-7 and PLLHC-9

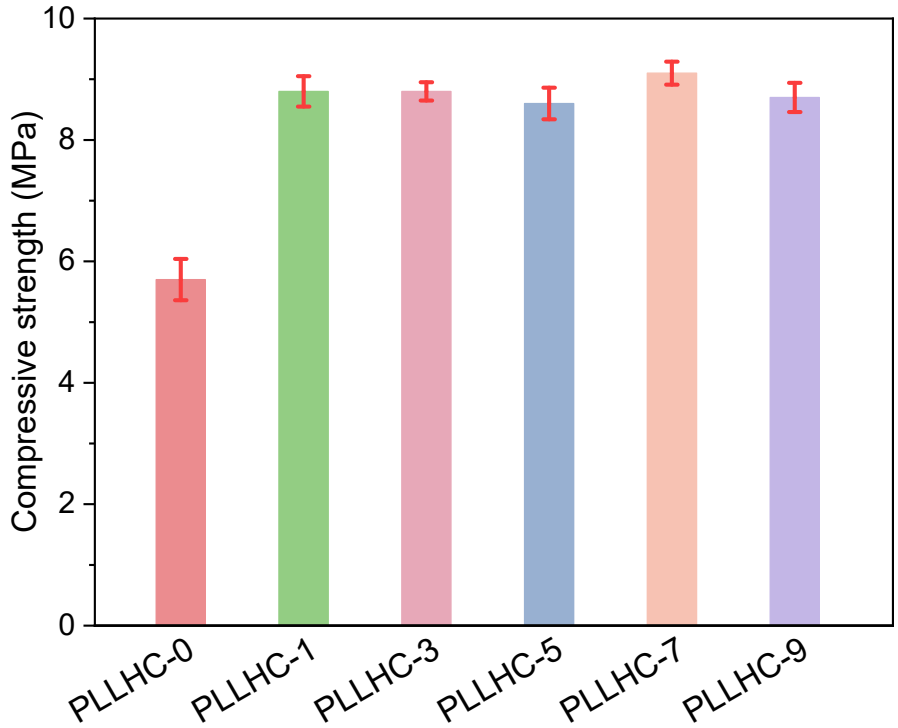



Figure S5. Mechanical properties PLLHC-0, PLLHC-1, PLLHC-3, PLLHC-5, PLLHC-7 and PLLHC-9