Supporting Information

Zero-Dimensional Carbon Dots Enhance Bone Regeneration, Osteosarcoma Ablation and Clinical Bacterial Eradication

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Experimental Section

Synthesis and characterization of the carbon dots (CD). Citric acid (3 g) and glycine (3 g) were placed in 10 mL deionized water with ultrasonication to form a transparent suspension. The mixture was heated in a domestic microwave oven (800 W) for 3 min and the suspension solution changed into a brown color. Then the solution was centrifuged to remove the unreacted citric acid. The obtained solution was concentrated and then dialyzed against deionized water for one day.

The morphology of the CD was analyzed by using TEM. A drop of the CD solution was placed onto a carbon-coated copper grid and viewed with high-resolution transmission electron microscopy (HRTEM, JEOL 2100, Japan). The Zeta potential measurements were determined with the Zetasizer Nano Z (Malvern, UK). Photothermal effect of the CD was measure by being irradiated with NIR laser (808 nm, 1 W/cm^2, BWT, Beijing, China) for 10 min and the temperature change was carefully recorded every 10 s.

Preparation of CS/nHA/CD and CS/nHA scaffolds. Scaffolds were prepared by dissolving CD (10, 20, and 30 mg) in 10 mL of 2% acetic acid and stirring to create CD-containing solutions at various concentrations (1, 2, and 3 mg/mL). 150 mg of nHA powder (20 nm, DKnano, Beijing, China) and 100 mg of CS powder (Sigma, USA) were successively added into the CD solution under constant stirring to form a homogenous solution. The mixture was poured into 48-well plates, frozen overnight at −80 °C, and freeze dried in a lyophilizer (Alpha1-4 LSC Plus, Christ) until dry. Then, the scaffolds were neutralized by soaking them in 0.5 M NaOH and washing

them with distilled water repeatedly; then, they were lyophilized again. The CS/nHA scaffolds were fabricated in the same manner but in the absence of CD in the initial CS/nHA mixed solution.

Characterization of the scaffolds. The morphology and pore size of the scaffolds were examined using SEM. Scaffold samples were dried under a vacuum, gold coated, and examined with SEM (S-3000N, Hitachi). The morphology of the scaffolds (CS/nHA and CS/nHA/CD composites) was further studied by using TEM. FTIR spectra of the scaffolds were measured on a PerkinElmer 580B IR spectrophotometer using the KBr pellet technique.

Photothermal performance of the scaffolds was evaluated by a series of *in vitro* and *in vivo* studies. First, 10 mg of scaffolds in wet state were irradiated for 10 min by an NIR (808 nm, 1 W/cm^2) laser, and the temperature changes were recorded by an IR camera (FLIR ONE, FLIR) every 1 min. Then, 10 mg of scaffold was soaked in 500 μL of PBS in a 24-well plate with 10 min of continuous irradiation (808 nm, 1 W/cm^2); the temperature change was carefully recorded every 10 s during this process. Finally, 10 mg of scaffold was implanted under the tumor center in tumor-bearing nude mice (generated by injection of UMR-106 osteosarcoma cells) and irradiated under NIR laser (808 nm, 1 W/cm^2) for 10 min; the hyperthermia effect in the tumor site was carefully recorded every 1 min.

To assay the scaffold integrity, the CS/nHA/CD scaffolds were immersed in 1 mL NaCl solution with ionic strength (0, 0.1, 0.5, 1, 1.5 and 2 M) and incubated at 37 °C. After 2, 4, 6, 12, and 24 h incubation, the supernatants were collected and the optical

densities (OD) were analyzed at 333 nm (characteristic absorbance of CD in this study) by a microplate reader (Multiskan GO, Thermo Scientific).

Biocompatibility. Scaffold cytotoxicity was determined by Cell Counting Kit-8 (CCK-8) assay. 5×10^4 rat bone marrow mesenchymal stem cells (rBMSCs, Cyagen Biosciences) were incubated with the scaffolds (10 mg) at 37 °C. L-Dulbecco's Modified Eagle Medium (L-DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) was removed on the respective days. After culturing for 1, 3, and 5 days, the medium was discarded. 500 μL of CCK-8 solution (10%) was added to each well; the plates were incubated for 2 h, and the OD was determined at 450 nm using a microplate reader.

Healthy human blood containing EDTA was diluted with normal saline in a ratio of 4:5 by volume. CS/nHA/CD scaffolds and CS/nHA scaffolds were dipped in 1 mL of normal saline and incubated at 37 °C for 30 min. 1 mL of deionized water and PBS was used as a positive control and negative control, respectively. Then, 0.2 mL of diluted blood was added to each sample, and the mixtures were incubated at 37 °C for 1 h. Next, all samples were centrifuged, and the supernatant was collected. ODs of the supernatants were determined at 545 nm using a microplate reader (Multiskan GO, Thermo Scientific).

Cell morphology in the scaffolds was observed by SEM. After culturing for 1 day, scaffolds loaded with rBMSCs were washed 3 times with PBS, and then fixed with 3% glutaraldehyde for 4 h at 4 °C. Then, the scaffolds were dehydrated in a graded ethanol series (30, 50, 70, 90, and 100%). The cell-loaded scaffolds were dried under

a vacuum, gold coated, and examined with SEM (S-3000N, Hitachi).

Real-time polymerase chain reaction (RT-qPCR). The expression of focal adhesion pathway genes in rBMSC cells was evaluated by RT-qPCR. rBMSCs (5×10^4 cells) were seeded in 10 mg of CS/nHA/CD or CS/nHA scaffolds and cultured for 24 h. Total RNA of the cells was isolated using a TRIZOL reagent (Thermo Scientific). Chloroform was added to isolate the RNA in the aqueous phase. The upper colorless aqueous phase was transferred to a new 1.5-mL tube, and isopropanol was added to precipitate the RNA. Finally, the RNA pellets were washed with 75% ethanol and dissolved in water treated with the RNase inhibitor diethyl pyrocarbonate. RNA concentrations were determined on a NanoDrop 2000 spectrophotometer (Thermo Scientific). Approximately 1 mg of RNA from each sample was reversed transcribed into complementary DNA (cDNA) using the RNA-to-cDNA master mix kit (Applied Biosystems) following the manufacturer's instructions. The expression level of genes, including focal adhesion kinase (FAK), paxillin (PXN), and vinculin (VCL), was quantified using Rotor-gene Q (Qiagen) with SYBR® Premix ExTM Taq II (TaKaRa). The cycling protocols were set as follows: 95 °C for 15 min, followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s. The results were analyzed using Rotor-Gene Real-Time analysis software 6.0. The relative mRNA expression level of each gene was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and determined using a cycle threshold value.

The *in vitro* osteogensis of the scaffolds was also studied by evaluating the expression of osteogenesis-related genes using RT-qPCR with the same protocol

described above. rBMSCs (5×10^4 cells) were seeded in 10 mg of CS/nHA/CD or CS/nHA scaffolds and cultured for 7 and 14 days within osteogenic medium (Cyagen Biosciences). Then, gene expression levels of runt-related transcription factor 2 (RUNX2), type I collagen (COL-I), osteocalcin (OCN), osteopontin (OPN), and alkaline phosphatase (ALP) were quantified. The forward and reverse primers of the selected genes are listed in Table S1.

Table S1. Primer sequences used for RT-qPCR.

Gene	Primer sequence	
	Forward	Reverse
FAK	5′-GCAATGGAACGAGTATTAAAGGTCTT-3′	5′-GGCCACGTGCTTTACTTTGTG-3′
PXN	5′-CTCTGAACTTGACCGGCTGTTA-3′	5′-CCCCCCAAGGGAGTGTTATT-3′
VCL	5′-GTGGCGACGGCACTACAGA-3′	5′-ACCGACTCCACGGTCATCTACT-3′
RUNX2	5′-TCCAACCCACGAATGCACTA-3′	5′-GAAGGGTCCACTCTGGCTTTG-3′
COL-I	5′-GCAGGGTTCCAACGATGTTG-3′	5′-AGGAACGGCAGGCGAGAT-3′
OCN	5′-CAATAAGGTAGTGAACAGAC-3′	5′-CTTCAAGCCATACTGGTCT-3′
OPN	5’-TCCAAAGCCAGCCTGGAAC-3’	5’-TGACCTCAGAAGATGAACTC-3’
ALP	5’- GTCATCATGTTCCTGGGAGA-3’	5’-GGCCCAGCGCAGGAT-3’
GAPDH	5′-CATGGCCTTCCGTGTTCCTA-3′	5′-CCTGCTTCACCACCTTCTTGAT-3′

In vitro tumor ablation. CS/nHA/CD and CS/nHA scaffolds seeded with UMR-106 cells were irradiated with or without an 808 nm laser (1 W/cm^2) for 10 min after co-culturing for 24 h. Then, a standard CCK-8 assay was carried out to determine cell viability, and SEM was used to observe cell morphology.

Live/dead staining was carried out for viability staining studies, and the UMR-106 cell-seeded scaffolds (24 h seeding) were irradiated with or without 808 nm laser (1 W/cm^2) for 10 min. The medium was removed, and the adherent cells were subjected to live/dead staining following the manufacturer's protocol (Sigma, USA). The cells were photographed using CLSM (LSM 700, ZEISS).

In vitro antibacterial properties. The antibacterial properties of scaffolds were evaluated using clinically collected gram-positive *S. aureus* and gram-negative *E. coli*. After being cultivated in lysogeny broth (LB) medium (1% w/v tryptone, 0.3% w/v yeast extract, and 0.5% w/v $NaCl_2$) under shaking at 200 rpm and 37 °C for 12 h, the bacteria were adjusted to a concentration of 2×10^5 CFU/mL in the antibacterial assay. Different scaffolds (10 mg) were introduced to 500 μL of bacterial suspension, then were treated with or without 10 min of NIR irradiation (1 W/cm^2). Bacterial suspension without scaffolds served as the control. The resultant suspensions were incubated in a biochemical incubator at 37 °C for 24 h. The viable number of bacteria in PBS was quantified by standard serial dilution. A culture suspension of 100 μL from each tube was uniformly spread on LB agar plates, and the number of viable bacterial colonies was counted after incubation at 37 °C for 24 h. The antibacterial rate was determined using the following formula:

Antibacterial rate (%) = $(N_{control} - N_{scaffold}) / N_{control} \times 100$

$N_{control}$ is the average number of bacteria in the control sample (CFU/sample), and $N_{scaffold}$ is the average number of bacteria in the test samples (CFU/sample).

In vivo osteoinductivity. All animal studies were approved by the Institutional Animal Care and Use Committee of Guangzhou General Hospital of Guangzhou Military Command. Scaffolds weighing 10 mg were seeded with 5×10^4 rBMSCs and incubated in osteogenic medium at 37 °C. The medium was changed every 2 days until 7 days, then the medium was replaced with FBS-free L-DMEM.

In vivo osteoinductivity was studied in adult male Sprague-Dawley rats (250-300 g,

n=5). The rats were anesthetized, and their lower limbs were shaved and sterilized with iodine and ethanol, then draped in a sterile manner. A 1 cm incision was made on the lateral thigh of the rat. Intermuscular spaces were bluntly dissected to expose the muscle pouch of gluteus maximus muscle. The rBMSC-loaded CS/nHA/CD scaffold was implanted into the muscle pouch. The incision was closed in two layers with nylon sutures in subcutaneous tissues and the skin. This procedure was repeated in the opposite limb of the same rat with an implanted CS/nHA scaffold.

The rats were euthanized with a pentobarbital overdose to harvest the implanted scaffolds at 4 weeks after implantation. New bone formation in the scaffolds was detected with Micro-CT (Aloka). The scan parameters were scanning resolution of 48 μm; rotation angle of 360°; and voltage of 80 kV. The obtained CT images were transferred into three-dimensional reconstructed images and the BMD (mg/cm^3) in the scaffolds was measured.

The harvested samples were fixed with 4% formaldehyde, then dyed with H&E and Masson's trichrome staining. The histological morphology of the samples was observed under inverted microscope (BX51, Olympus).

In vivo tumor therapy. Adult male BALB/c nude mice were purchased from the Medical Experimental Animal Center of Guangdong Province. A suspension of 5×10^6 UMR-106 cells was injected subcutaneously into the back (1 cm above the base of the tail) of nude mice to establish the tumor-bearing mouse model. When tumors grew to 7~10 mm in diameter, the nude mice were randomly divided into 4 groups with 5 mice each: 1) CS/nHA/CD+NIR; 2) CS/nHA/CD; 3) CS/nHA+NIR; and 4) CS/nHA.

Briefly, the mice were anesthetized by intraperitoneal injection of 3% pentobarbital solution. The back was sterilized with iodine and ethanol, then draped in a sterile manner. A 1 cm incision was made at the right edge of tumor, then 10 mg of scaffold was implanted exactly under the tumor tissue. After implantation, an 808 nm NIR laser (1 W/cm^2) was applied to the tumor site for 10 min. The changes in tumor volumes of all groups were carefully recorded every other day for 14 days using the following formula: 1/2 × larger diameter × (smaller diameter)^2. After 14 days, the mice were over anesthetized for euthanasia, and the scaffolds and tumor tissues were peeled and fixed in a 4% formaldehyde solution (pH=7.0).

The tissues were processed with routine paraffin embedding. 5 μm thick sections were cut and placed on glass slides, followed by H&E staining and Ki-67 IHC staining. Finally, the pathological sections were observed using an inverted microscope (BX51, Olympus).

In vivo antibacterial properties. The CS/nHA/CD or CS/nHA scaffolds were implanted into the muscle pouches of SD rats (n=5 in each group), and 10 μL of clinical *S. aureus* or *E. coli* (1 × 10^7 CFU/mL in PBS) was delivered into the muscle pouches; then, 10 min of NIR irradiation (1 W/cm^2) was applied. The rats were euthanized 1 week after implantation, and the harvested samples were immediately put into 1 mL of normal saline and ultrasonicated for 5 min to shake the bacteria off of the samples. Then, 100 μL of the bacterial solution was uniformly spread on LB agar plates. The number of bacterial colonies was counted after incubation at 37 °C for 24 h. The samples were fixed with 4% formaldehyde, dyed with H&E staining and

Giemsa staining, and observed using an inverted microscope (BX51, Olympus).

Statistical analysis. Analysis was performed using SPSS 19.0 software (IBM). One-way analysis of variance was performed. The *in vivo* osteoinductivity and antibacterial studies were analyzed by paired t-tests. The significance level was $p<0.05$.

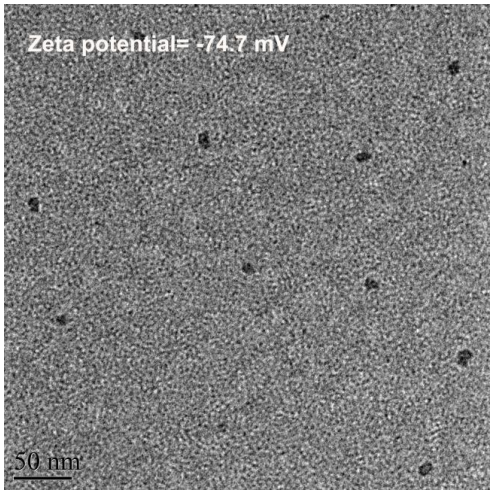

Figure S1. Morphology and zeta potential of CD.

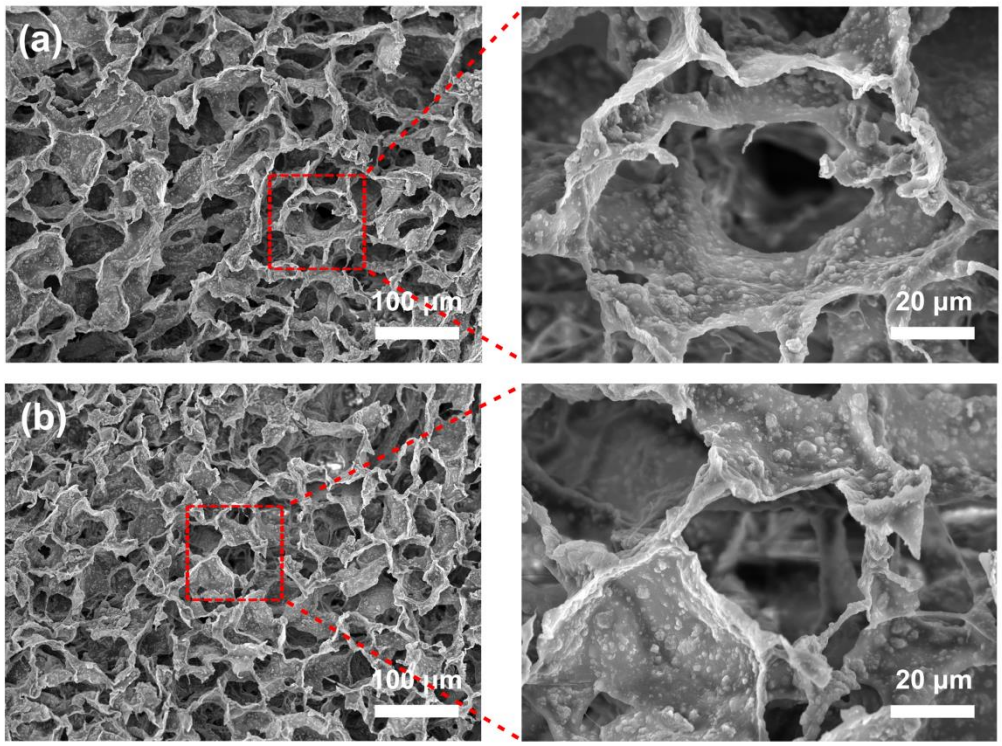

Figure S2. SEM analysis of the scaffolds. (a) CS/nHA/CD scaffold made with 2 mg/mL CD; (b) CS/nHA/CD scaffold made with 1 mg/mL CD.

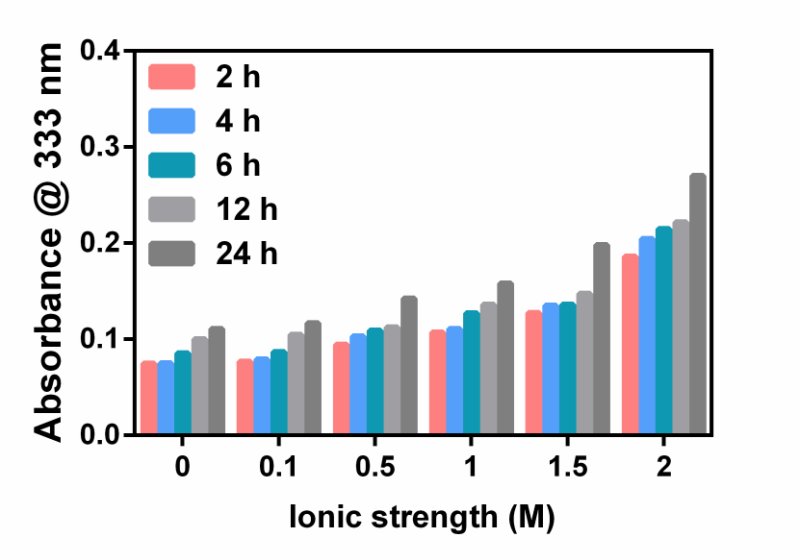

Figure S3. Effect of ionic strength on scaffold integrity.

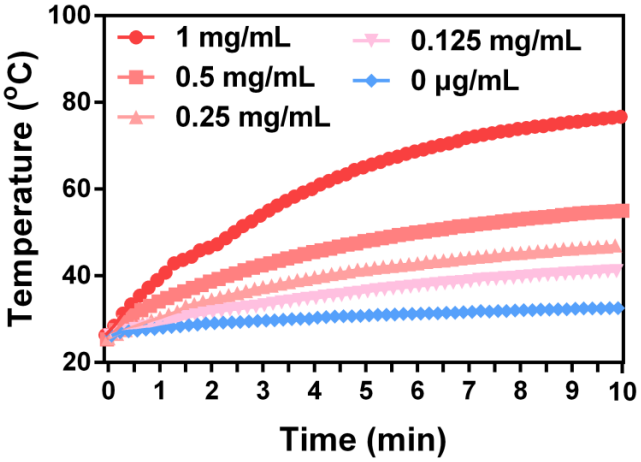

Figure S4. Photothermal effects of CD *in vitro*.

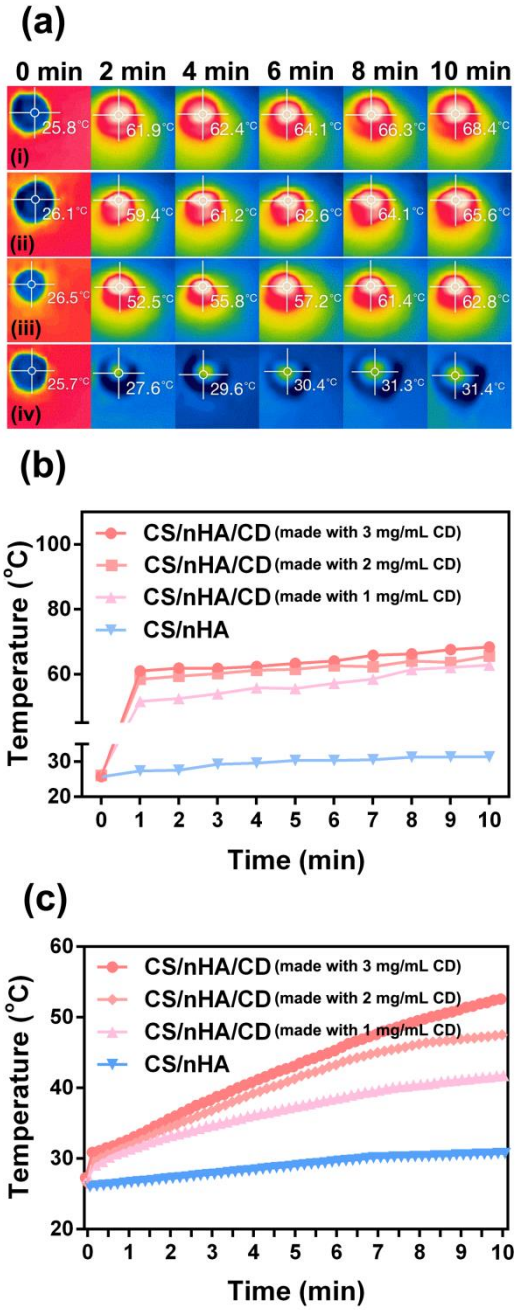

Figure S5. *In vitro* photothermal effect of the scaffolds. (a) IR image of the scaffolds in a wet state captured every 2 min (from left to right) under 1 W/cm^2, 808 nm laser irradiation for 10 min. (i-iv) CS/nHA/CD scaffold made with 3 mg/mL CD, CS/nHA/CD scaffold made with 2 mg/mL CD, CS/nHA/CD scaffold made with 1 mg/mL CD, and CS/nHA scaffold, respectively. (b) Quantitative temperature changes of the scaffolds in a wet state under 1 W/cm^2, 808 nm laser irradiation for 10 min

derived from the IR camera measurements shown in (a). (c) Quantitative temperature changes of the PBS solution around the soaked scaffolds under 1 W/cm^2, 808 nm laser irradiation for 10 min. CS/nHA/CD scaffold made with 3 mg/mL CD was chosen in the further study due to its optimum photothermal effect for tumor and infection photothermal therapy.

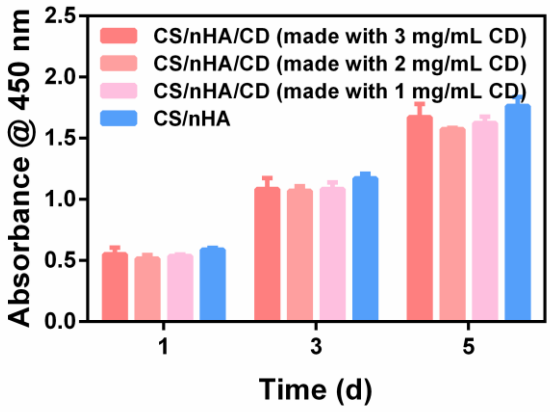

Figure S6. Cytotoxity of the scaffolds measured by CCK-8 assay.

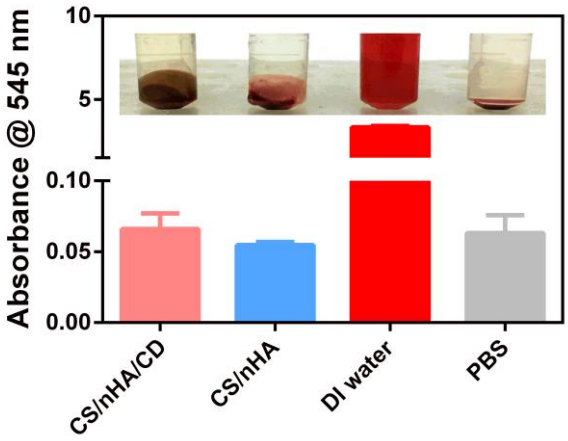

Figure S7. Hemolysis tests of the scaffolds. Inset images (from left to right): CS/nHA/CD scaffold, CS/nHA scaffold, DI water, and PBS.

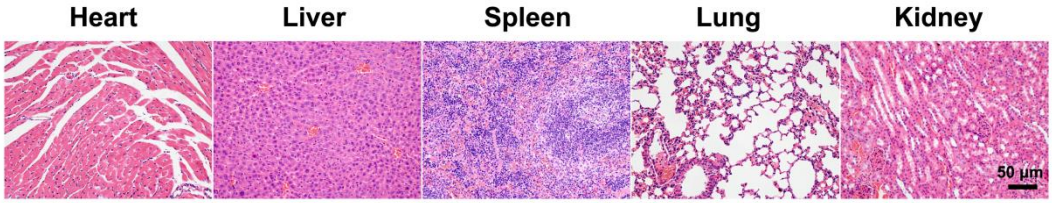

Figure S8. H&E staining of the main organs in tumor-bearing mice after 14 days of implantation of CS/nHA/CD scaffold.

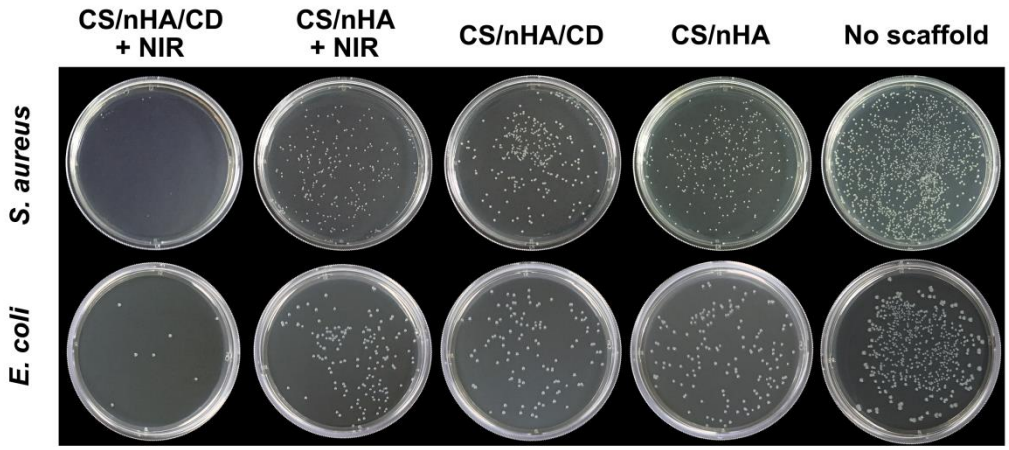

Figure S9. Numbers of colonies in the presence of the different treatments against clinically relevant *S. aureus* and *E. coli in vitro.*