Supporting information for

Near-Infrared Light Triggered Porous AuPd Alloy Nanoparticles to Produce Mild Localized Heat for Accelerating Bone Regeneration

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Experimental Details

Preparation of porous AuPd alloy nanoparticles.

For porous AuPd alloy nanoparticles synthesis, an aqueous solution of chloroauric acid ($HAuCl_4$, 10 mM, 0.2 mL) aqueous solution and sodium tetrachloropalladate (Na_2PdCl_4, 10 mM, 0.2 mL) were added into a vial, and followed by adding hexadecylpyridinium chloride aqueous solution (HDPC, 9 mg, 1 mL). After mixing evenly, the newly prepared ascorbic acid (AA, 0.6 M, 0.3 mL) aqueous solution was quickly added into the above mixed uniform solution under gentle stirring. Finally, the mixed solution was kept stirring at 35 ºC for 3 hours. Collection of products through centrifugation and washed in turn with water, 75% alcohol and PBS solution.

NIR laser-induced heat conversion.

0.5 ml of PBS containing different Au concentrations of porous AuPd alloy nanoparticles was added into quartz cuvettes, then a fiber-coupled continuous semiconductor diode laser (MDL-H-808nm-5W-17080741) with a power density of 2 W cm^{-2} was used to irradiate the solution for 5 min. The laser spot was 1 cm in diameter which could cover the entire surface of the solution. A digital thermometer (TES 1319A-K type) was used to real-time monitor the temperature increase.

In vitro cytotoxicity assays.

Water-soluble etrazolium salts, 2-(2-methoxy-4-nitorophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H assay

(WST-8, Dojindo) were used to analyze the cytotoxicity of porous AuPd alloy nanoparticles. First, the preosteoblasts-MC3T3-E1 cells were inoculated into a 96-well plate (1×10^4 cells per well) in normal culture medium consisting of α-modified eagle medium (α-MEM) and 10% (v/v) fetal bovine serum (FBS) at 37 °C in a humidified atmosphere consisting of 5% CO_2. After 12 h of inoculation, porous AuPd alloy nanoparticles with different Au concentrations (0, 25, 50, 100, and 200 μg ml^{-1}) were added, and five multiple holes were set for every sample. After 12, 24 and 48 h of culture, the extracts were replaced with 180 ul culture medium and 20 ul of the WST-8 reagent at 37 ℃, 5% CO_2 for 1h. Finally, the optical density was determined at 490 nm by an enzyme linked immunosorbent assay Reader (Biotek-mQuant, Bio-Tek Instruments).

In vitro **photothermal experiments.**

The preosteoblast-MC3T3-E1 cells were inoculated into the 96-well plates at a density of 1×10^4 cells per well in α-modified eagle medium (α-MEM) consisting of 10% (v/v) fetal bovine serum (FBS), 100 U mL^{-1} streptomycin, and 100 U mL^{-1} penicillin in a humidified 5% CO_2 atmosphere at 37 °C and incubated overnight. 20 wells containing preosteoblast-MC3T3-E1 cells were randomly divided into 4 groups and subjected to variable conditions. In these four groups, groups 1 and 3 were only added PBS, and groups 2 and 4 were added porous AuPd alloy nanoparticles at 50 μg ml^{-1}. After incubation with the porous AuPd alloy nanoparticles for 24 h, the medium was removed and washed with PBS for 3 times, and the fresh culture medium was added, then

groups 1 and 2 did not accept additional treatment and still maintained normal growth; and groups 3 and 4 received 808 nm laser irradiation (2 W cm^{-2}), simultaneously, the temperature of the preosteoblast-MC3T3-E1 cells was monitored through an infrared thermal imaging camera (FLIR C2, USA). After irradiation for 3 min, the laser would be turned off to bring the temperature of the cells down to normal to avoid damaging cells (Supplementary Video 1), this process was repeated 3 times. After irradiation, at different time points after incubation (i.e., 12, 24, and 48 h), the cells were washed by PBS for three times followed by removing of culture medium. Then, 180 ul culture medium and 20 ul of the WST-8 reagent was added into each well and incubated for 1 h at 37 ℃, 5% CO_2. Finally, the optical density value at 490 nm was determined.

After 8 days of culture, MC3T3-E1 cells were washed with PBS and lysed in RIPA lysis buffer (Beyotime Biotechnology, China) containing 1% PMSF (Roche, Basel, Switzerland). After determine the protein concentration by BCA Protein Assay Kit (Thermo Fisher Scientific), the membranes were blocked with 5% degreased milk in PBS (w/v) with 0.1% Tween20 and then incubated with rabbit anti-BMP2 (1:2000, Abcam, Cambridge, UK) and rabbit anti-Hsp47 (1:5000, Abcam, Cambridge, UK) antibodies overnight at 4°C. Finally, the membranes were incubated with 1:10000 HRP conjugated goat anti-rabbit IgG (Abbkine, Redlands, CA, USA) after washed by PBS with 0.1% Tween20.

RNA Sequencing Analysis for DEGs, GO and KEGG.

RNA-sequencing analysis was used to evaluate the change of mRNA profiles between cells in the appropriate temperature-treated (experimental group, cells treated with porous AuPd alloy nanoparticles and laser irradiation) and non-appropriate temperature-treated (control group, neither porous AuPd alloy nanoparticles nor laser irradiation) groups. According to the protocols provided by manufacturer, total RNA was extracted by Trizol reagent (Invitrogen Carlsbad, CA, USA). After enrichment of eukaryotic and prokaryotic mRNA, the enrichment mRNA was fragmented and then reverse transcripted into cDNA with random primers. After cDNA fragments were ligated to Illumina sequencing adapters, the ligation products were amplified and sequenced using Illumina HiSeq2500 by Gene Denovo Biotechnology Co (Guangzhou, China).

DESeq2 software was used to determine the DEGs between experimental group and control group.[1] The criteria for differentially expressed genes/transcripts was set as fold change ≥ 2 and false discovery rate below 0.05 (FDR < 0.05).

Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were carried out to evaluate the molecular or biological functions of DEGs and identify enriched metabolic pathways in DEGs comparing with the whole genome background. Also, FDR ≤ 0.05 was set as a threshold to calculate p-value.

In vivo photothermal experiments.

All animal procedures were approved by the Animal Care and Use Committee of Wuhan University. Eighteen healthy male SD rats (4 weeks old and mean body weight, 250 g) were anesthetized by isoflurane inhalation, and a skin incision was made along the mid-shaft of the skull to expose the cranial bone, then the full-thickness defects (8 mm in diameter) were created on both sides of the cranial bone by a trephine bur. All the defects were divided into the following three groups (n=12): (1) PBS (100 μl) as the negative control group, (2) PBS (100 μl) with 808 nm laser irradiations as the positive control group, and (3) porous AuPd alloy nanoparticles (0.08 mg Au kg^{-1}, 100 μl) with 808 nm laser irradiation as the experimental group. To have a high enough particle concentration in the defect, PBS and porous AuPd alloy nanoparticles solution was all directly injected into the space between the surface of the cranial defects and the skin. At 0.5 h post-injection, 808 nm laser (2 W cm^{-2}) was used to irradiate the defect site for 3 min in the positive control group and the experimental group, and the temperature of the defect under irradiation was monitored by an infrared thermal imaging camera (FLIR C2, USA). After irradiation for 3 min, the laser would be turned off to bring the temperature of the defect down to the body temperature, and then carried out the second 3 minutes of irradiation, this process was repeated 10 times. This above treatment process was carried out every five days over a period of 6 weeks. At 3 and 6 weeks after surgery, the cranial samples were harvested and

scanned by micro-CT (Skyscan1176, Belgium) to evaluate the regeneration of newly-formed bone in the defect area after animals were sacrificed by carbon dioxide (CO_2). At 2 h before euthanasia, rats were injected with bromodeoxyuridine (BrdU, 100 mg kg^{-1}) (Sigma) intraperitoneally to characterize tissue or cells with actively proliferation ability. After generating the 3D images of the cranial defect samples, we assessed the quality of the regenerated bone in the regions of interest (ROI). After the micro-CT scanning examination, all the cranial samples were decalcified with 10% ethylenediaminetetraacetic acid (EDTA), dehydrated with graded ethanol solutions, embedded in paraffin, cut into 4 μm sections, and stained with Masson's trichrome staining for histological observation.

Immunostaining of osteocalcin and BrdU were also performed on paraffin-embedded sections of the cranial samples. After quenched the endogenous peroxidase activity with 3% H_2O_2 and blocked with 4% bovine serum albumin (BSA) for 60 min at room temperature, sections were incubated with primary antibody against osteocalcin (1:400, Abcam) and BrdU (1:200, Cell Signaling Technology) over night at 4 °C. To detect the primary antibody, a secondary horseradish peroxidase conjugated antibody (Maxim biotechnology, Fuzhou, China) was added for 30 min at room temperature. The nucleus was counterstained with hematoxylin, and all sections were observed under a light microscope.

Histology examination.

Six weeks after the first injection of porous AuPd alloy nanoparticles, major organs (i.e., heart, liver, spleen, lung, and kidney) from the experimental and control group were harvested and fixed in 10% neutral buffered formalin, processed routinely into paraffin, sectioned at 4 μm, stained with haematoxylin and eosin (H&E), and examined by digital microscopy.

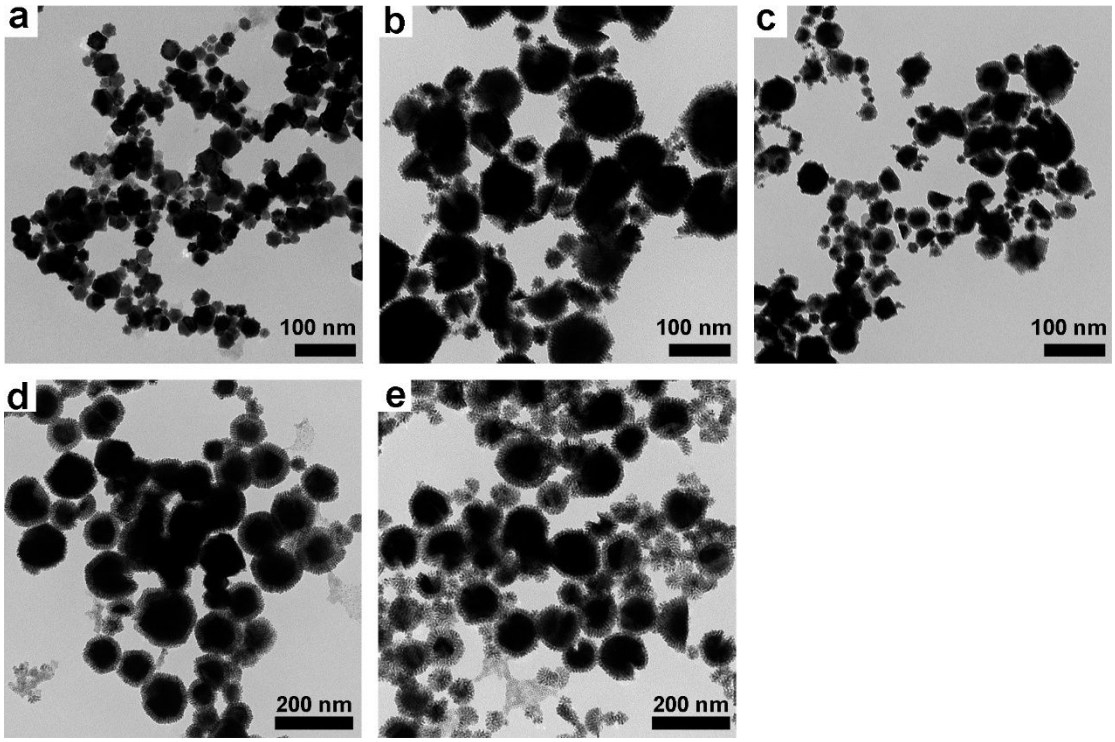

Figure S1. TEM images of porous AuPd alloy nanoparticles under the molar ratios between Pd and Au is (a) 0.5:2, (b) 1:2, (c) 1.5:2, (d) 2:2, and (e) 3:2.

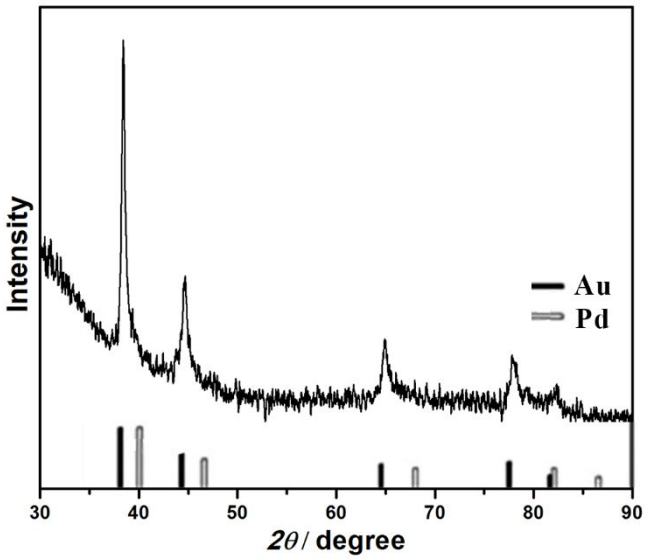

Figure S2. XRD pattern, bars show the calculated values for Au (black, JCPDS no. 04-0784) and Pd (gray, JCPDS no. 05-0681).

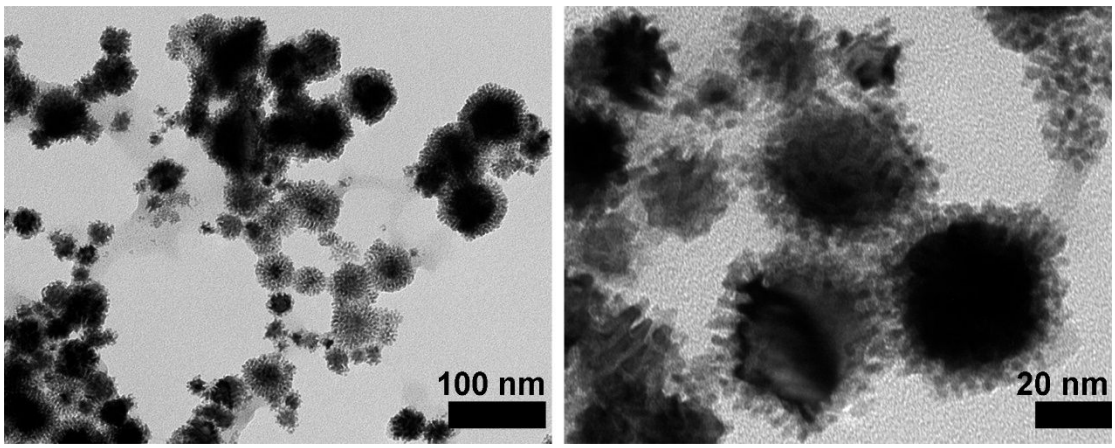

Figure S3. Representative TEM images of the products collected from the reaction with the similar condition used in the synthesis of porous AuPd alloy nanoparticles changing ascorbic acid into ascorbic acid sodium salt.

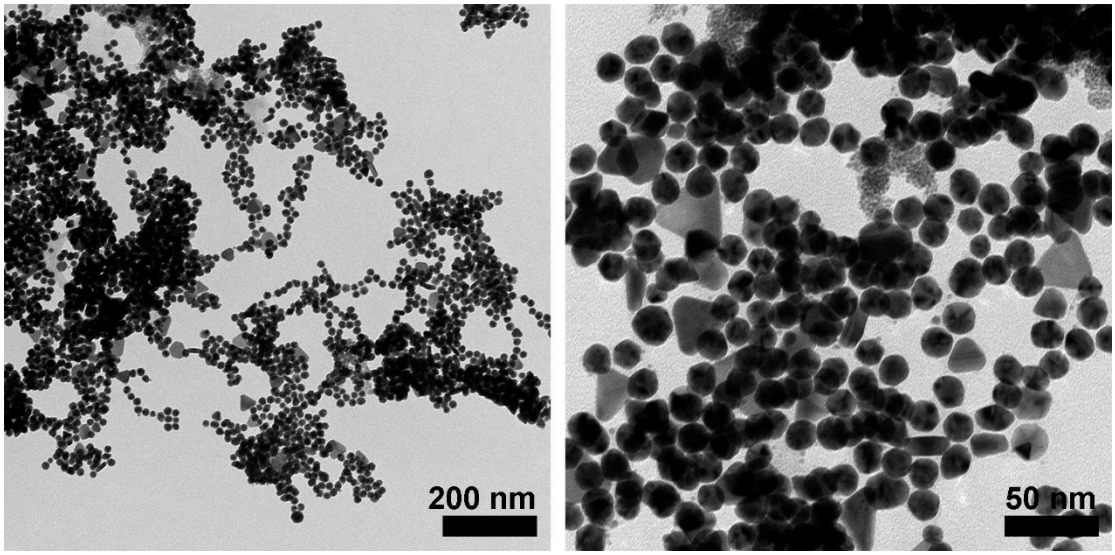

Figure S4. Representative TEM images of the products collected from the reaction with the similar condition used in the synthesis of porous AuPd alloy nanoparticles changing ascorbic acid into phloroglucinol.

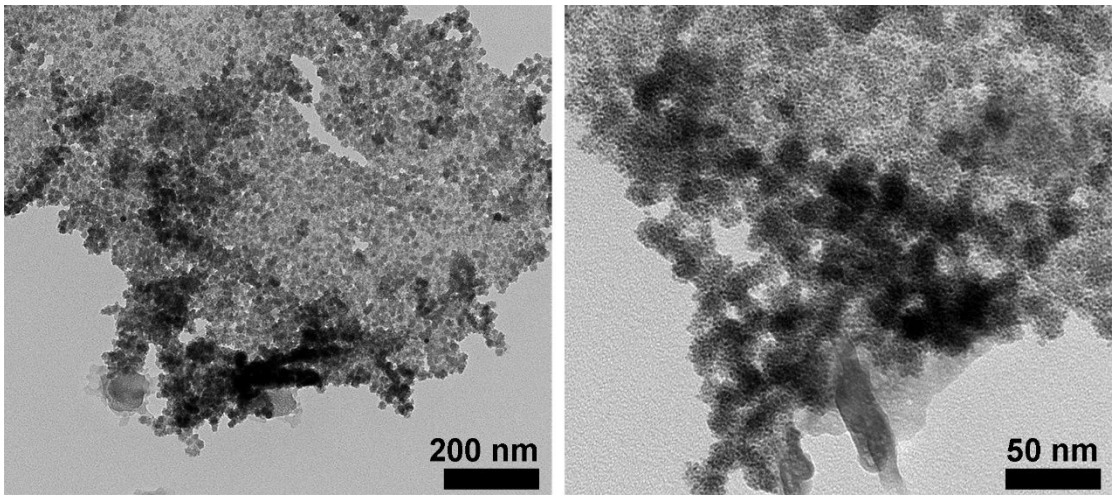

Figure S5. Representative TEM images of the products collected from the reaction with the similar condition used in the synthesis of porous AuPd alloy nanoparticles changing ascorbic acid into citric acid.

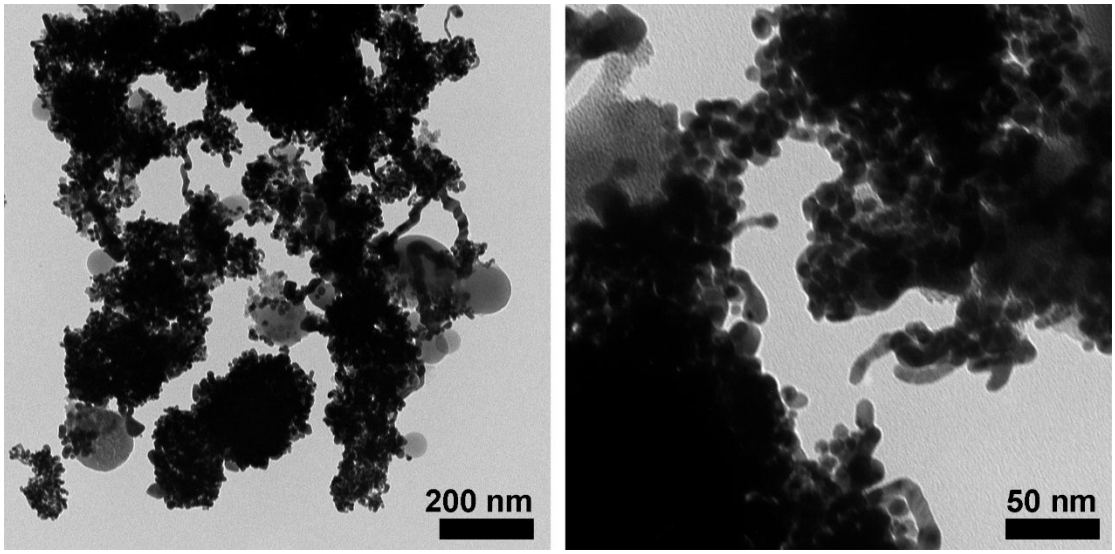

Figure S6. Representative TEM images of the products collected from the reaction with the similar condition used in the synthesis of porous AuPd alloy nanoparticles changing ascorbic acid into sodium citrate.

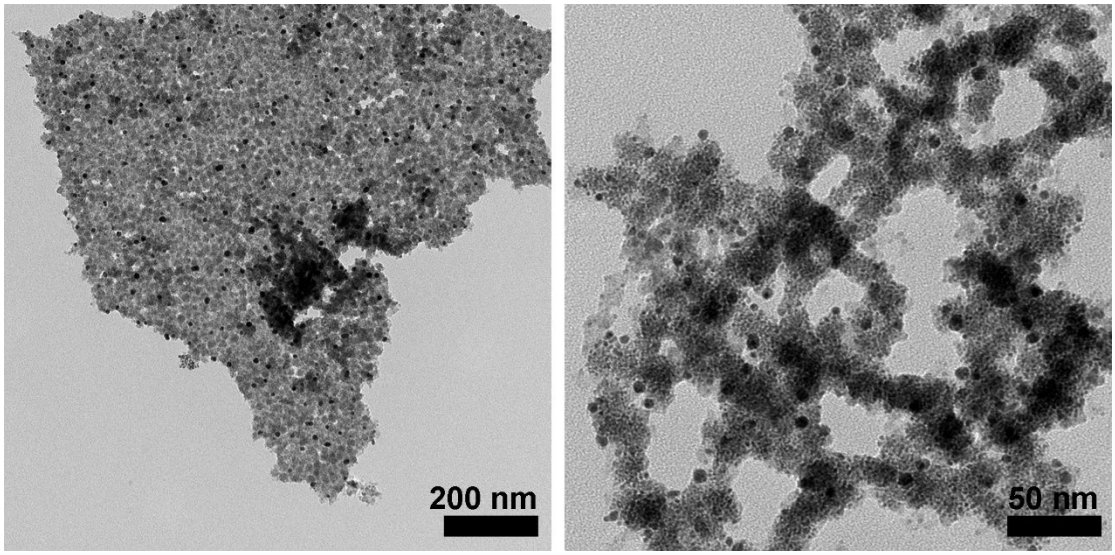

Figure S7. Representative TEM images of the products collected from the reaction with the similar condition used in the synthesis of porous AuPd alloy nanoparticles changing ascorbic acid into glucose.

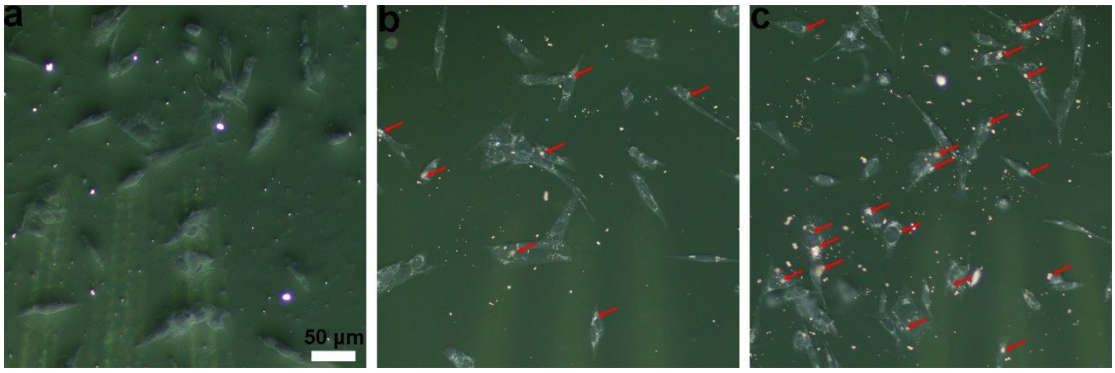

Figure S8. Optical dark field images of preosteoblast-MC3T3-E1 cells incubated with porous AuPd alloy nanoparticles at different Au concentration. (a) 0 μg ml^{-1}; (b) 50 μg ml^{-1}; (c) 100 μg ml^{-1}; The red arrows indicate the bright clusters.

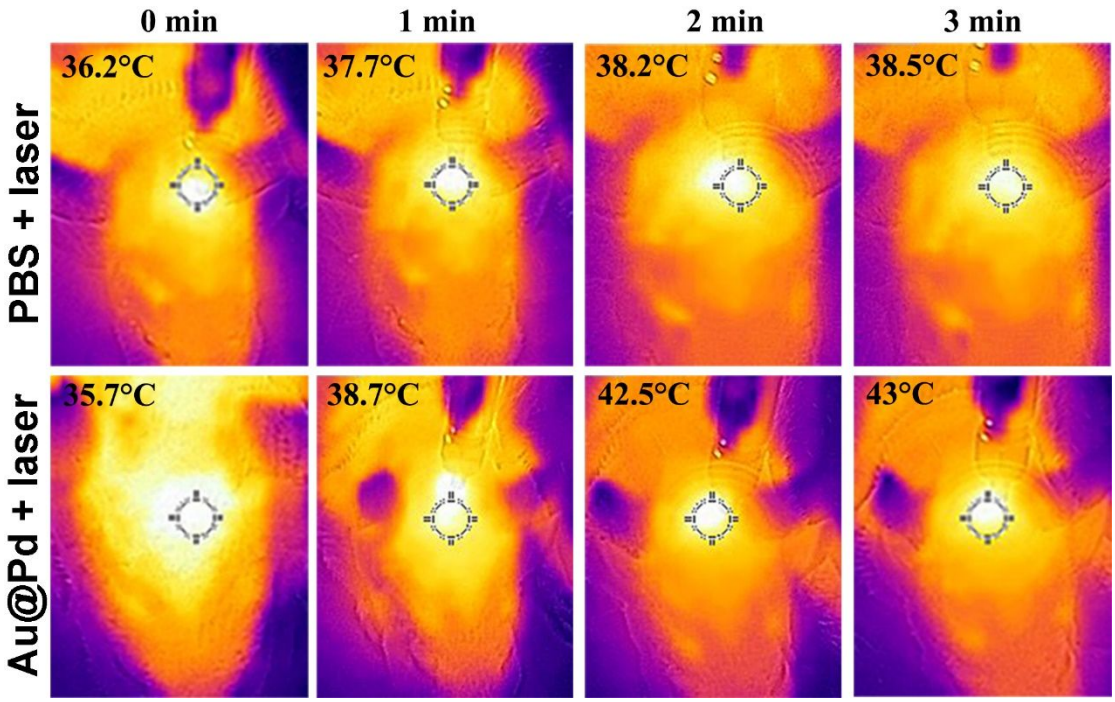

Figure S9. Infrared thermographic maps in the cranial defect-bearing rat irradiated by the 808 nm laser (2 W cm^{-2}) for 3 min at 0.5 h after separate injection with 100 μl of PBS and porous AuPd alloy nanoparticles (0.08 mg Au

kg^{-1}) solution.

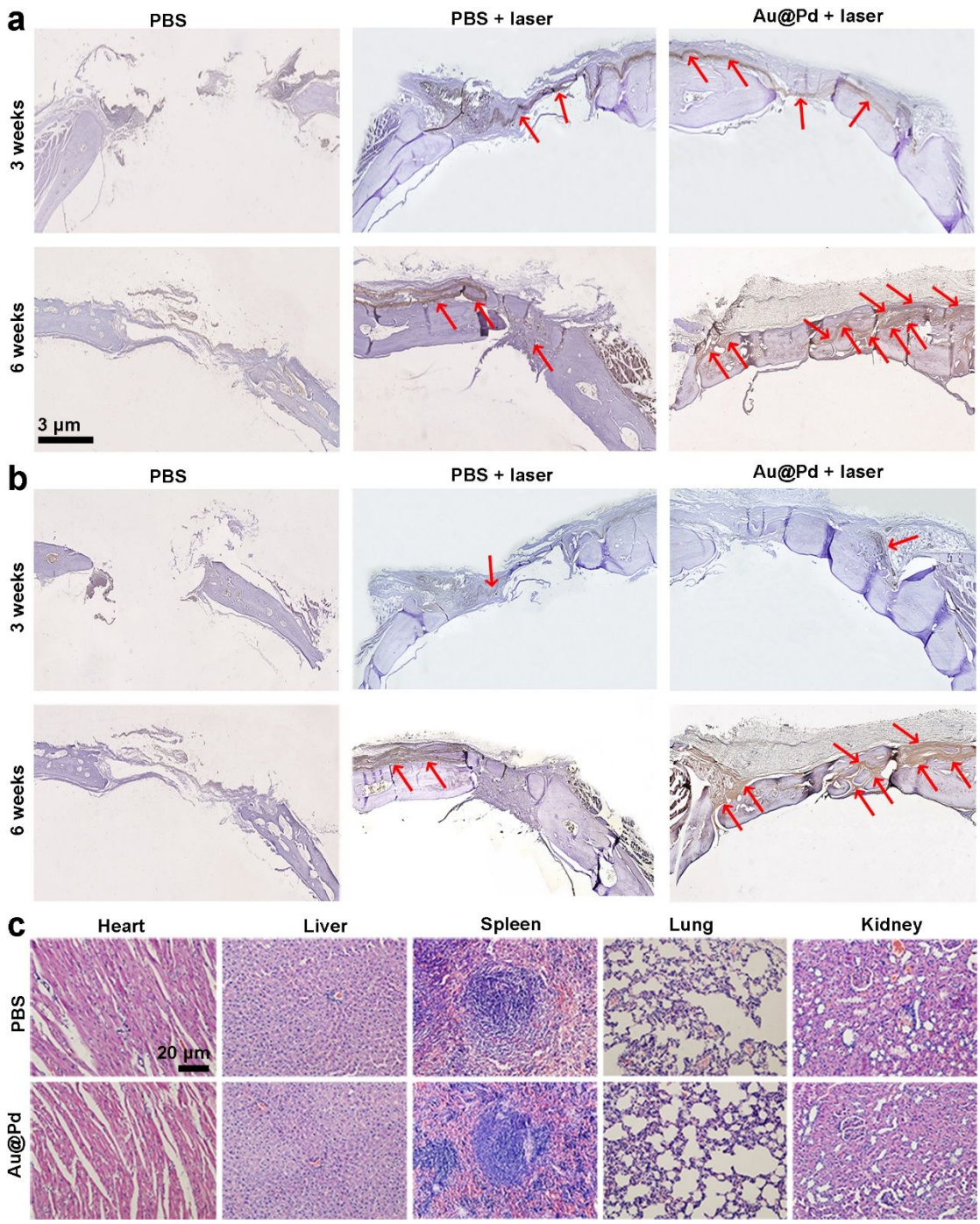

Figure S10. Immunostaining of (a) cell proliferation marker BrDu and (b) osteoblast differentiation marker osteocalcin at 3 and 6 weeks. The red arrows refer to BrDu and osteocalcin, respectively. (c) Hematoxylin and eosin (H&E) staining of the main organs after porous AuPd alloy nanoparticles injection.

SI References

(1) Love, M. I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biol.* **2014,** *15,* 550.