

## **†Electronic Supplementary Information (ESI)**

### ***In situ* Gold Nanoparticle Growth on Polydopamine-coated 3D-printed Scaffolds Improves Osteogenic Differentiation for Bone Tissue Engineering Applications: *In Vitro* and *In Vivo* Studies**

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## **Experimental procedures**

### **Materials**

Polycaprolactone (MW 45 kDa), Trizma<sup>®</sup> hydrochloride, 3-hydroxytyramine hydrochloride (dopamine hydrochloride), and Gold (III) chloride hydrate (99.995% trace metals basis) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS), trypsin-EDTA, and penicillin–streptomycin were purchased from Gibco BRL (Invitrogen Co. Ltd, Carlsbad, CA, USA). Osteogenic medium used in the current study comprised DMEM containing 10% FBS, 1% penicillin–streptomycin, 10 mM  $\beta$ -glycerol phosphate disodium salt hydrate (Sigma-Aldrich, St. Louis, MO), 300  $\mu$ M ascorbic acid (Sigma-Aldrich), and 0.1  $\mu$ M dexamethasone (Sigma-Aldrich). Deionized and distilled water (DDW) was obtained from an ultrapure water system (Puris-Ro800; Bio Lab Tech., Korea). All other reagents and solvents were of analytical grade and used without further purification, unless specified.

### **Fabrication of the 3D-printed PCL scaffolds**

The 3D-printed PCL scaffold manufacturing process has been previously described.<sup>1, 2</sup> PCL scaffolds were prepared using a 3D bio-printing system (laboratory-made system at the Korea Institute of Machinery and Materials). The printing instrumentation consisted of a 3D bio-printing system equipped with a three-axis X-Y-Z translation stage, dispenser, nozzle, compression/heat controller, and software system (Fig. 1). The dispenser was covered with a heating jacket to melt the PCL polymer. PCL pellets were melted at 85°C in a heating dispenser, and 3D plotting strands were produced by the 3DP system. Once the PCL had melted, a continuous air pressure of 300 kPa was applied to the dispenser, which extruded the 3D strand, and this was plotted layer-by-layer. The nozzle size was 400  $\mu$ m, and the strand

distance was 400  $\mu\text{m}$  in a square pattern.

### **Preparation of PDA-coated PCL scaffolds (PCLD) and growth of GNPs growth**

3D PCL scaffolds were immersed in dopamine hydrochloride solution (2 mg/mL in 10 mM of Tris buffer, pH 8.5) at room temperature. After 2 h, the PDA-coated 3D PCL scaffolds were washed with fresh DDW 3 times followed by vortexing in Tris-HCl solution overnight. Scaffolds were then washed with fresh DDW and dried under nitrogen gas to form the final PCL–PDA (PCLD) scaffolds. The GNPs were directly reduced onto the PCLDs from  $\text{HAuCl}_4$  solution (0.1, 0.5, 1, and 2 mM/mL in DDW) with stirring for 12 h at room temperature. After the GNP growth, samples were washed 3 times with DDW. PCLD scaffolds containing GNPs grown in 0.1, 0.5, 1, and 2 mM gold solutions were abbreviated as PCLDG0.1, PCLDG0.5, PCLDG1, and PCLDG2 in this study.

### **Characterization of cell proliferation on 3D-printed scaffolds**

Human adipose tissue-derived mesenchymal stem cells (hADSCs) and self-production company growth medium were purchased from CEFO (Cefobio, Seoul, Korea). The hADSCs were cultured in CEFO growth medium with supplement in a 5%  $\text{CO}_2$  incubator at 37°C. Prior to use in biological assessments, the manufactured scaffolds were sterilized on a clean bench under UV light overnight. hADSCs were then drop seeded onto the scaffolds at a density of  $2 \times 10^4$  cells per well in 50  $\mu\text{L}$  of media. After 2 h, each well was filled with CEFO growth medium. Cell proliferation was determined at 1, 4, and 7 days and evaluated ( $n = 4$ ) using an EZ-Cytox cell viability assay kit (Daeil Lab Service Co., Ltd, Seoul, Korea). The absorbance of the medium was measured at 450 nm using a microplate reader (ELISA, Bio-Rad, Hercules, CA, USA). These experiments were repeated in triplicate.

### **ALP activity of scaffolds**

Scaffolds were seeded and incubated, as described above, using  $5 \times 10^4$  cells per well (section 2.4). The medium was changed every 2 days, and ALP activity was evaluated after 7 days of culture. For the ALP activity, cells attached to the scaffold were washed twice with DPBS and detached using trypsin. The harvested cells were lysed for 30 min on an ice bath using 1× RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, and 1 mM EDTA] containing a protease inhibitor cocktail (Boehringer Mannheim GmbH, Germany). Each of the lysates was centrifuged at 13000 rpm at 4°C for 15 min to remove cell debris. After centrifugation, the supernatant was collected and reacted with p-nitrophenol phosphate solution (pNPP, Sigma) in a 5% CO<sub>2</sub> humidified incubator at 37°C for 30 min. The reaction with pNPP was then quenched by the addition of 50 µL of 1 M NaOH, and the quantity of p-nitrophenol was measured by absorption at 405 nm using a microplate reader. A calibration curve was generated using standard p-nitrophenol solutions. The quantity of p-nitrophenol produced by the cultured cells was obtained by comparing the absorption results against the calibration curve. The enzymatic activity was represented as µM of reaction product (p-nitrophenol) per min per µg of total cellular protein. These experiments were performed in triplicate.

### **Immunofluorescence of scaffolds for RUNX2 gene expression**

Immunostaining was performed to examine the gene expression of the cells under different scaffold environments. Scaffolds were seeded and incubated, as described above, using  $5 \times 10^4$  cells per well (section 2.4). The medium was changed every 2 days. The immunofluorescence was examined after 7 days of culture. After DPBS washing, the

cellurized scaffolds were fixed with 3.7% formaldehyde at 4°C for 20 min. After washing twice with DPBS, samples were washed with 0.5% Triton X-100 (in PBS) at room temperature for 10 min and then blocked using 1% bovine serum albumin (in PBS) for 30 min at room temperature. Samples were then incubated with an Alexa Fluor® 488 labeled-antibody against RUNX2 (1:200 in 1% BSA; Abcam, Cambridge, UK, Cat. #ab215954) for 1 h at room temperature followed by DPBS washing following the manufacturer's procedure. After DAPI staining, the fluorescently labeled scaffolds were examined using a fluorescence microscope (Olympus IX71, Japan).

### ***In vivo* implantation of scaffolds in a rabbit calvarial defect model**

Six male New Zealand white rabbits (18–25 weeks old, 3 kg) were used and housed in specific, pathogen-free facilities. The animals were cared for and treated in accordance with guidelines established by the Seoul National University Institutional Animal Care and Use Committee. This study conformed to the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines for pre-clinical studies. The guidelines regarding the care of animal research subjects were strictly followed, and the research was approved by the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital, Korea (IACUC No. BA1708-230/074-01).

The surgical procedure was performed while the animals were under general anesthesia using Zoletil (15 mg/kg; Zoletil50, Virbac S.A., Carros, France) and Xylazine (5 mg/kg; Rompun, Bayer Korea, Ansan, Korea). The animals were intramuscularly injected with 30 mg/kg cefazolin (Chongkundang Pharm, Cheonan, Korea) before the surgical procedures. The surgical field was scrubbed with a povidone–iodine solution, a midline incision was made over the calvarium, and a full-thickness flap elevated. A 15mm-sized calvarial defect is

considered to be a critical-size defect in a rabbit. If we planned to evaluate bone regeneration by PCLDG itself, we would compare the results with a sham surgery group as a negative control group in 15 mm critical-sized defects. However, we aimed to assess the effect of GNP accumulation on the PCL scaffolds in bone regeneration around the scaffolds. We created 8-mm defects and applied all three (PCLDG, PCLD, and PCL) scaffolds in an animal. Calvarial defects with an 8-mm diameter were created using a trephine bur (3i Implant Innovation, West Farm Beach, Florida, USA) and a low speed engine under sterile saline irrigation. Scaffolds were applied into the calvarial defects according to following experimental groups: (1) PCL group, (2) PCLD group, and (3) PCLDG1 group. The incisions were sutured in layers with 5-0 chromic gut and 4-0 silk. All the animals received intramuscular injection of Gentamycin 10 mg (Jeil Pharmaceutical, Daegu, Korea) and subcutaneous injection of Ketoprofen (Tradol, Jeil Pharmaceutical, Daegu, Korea) 3 mg for 3 days after surgery. All animals were sacrificed at 4 weeks after surgery, and block sections, including the grafted sites, were harvested and fixed in neutral buffered 10% formalin solution at low temperature.

### **Microcomputed tomography ( $\mu$ CT) analysis**

$\mu$ CT scans of tissue samples were obtained using a SkyScan 1173 (SkyScan, Kontich, Belgium). After reconstruction of images using a computer program (Nrecon Ver. 1.7.0.4, Kontich, Belgium), the regenerated bone volume and defect tissue volume were morphometrically measured using the CT analyzer software (SkyScan, Kontich, Belgium). The percentage of mineralized bone volume relative to the defect tissue volume (BV/TV) was measured using software (CT-analyzer; SkyScan, Kontich, Belgium) with a lower gray threshold level of 65.<sup>3</sup>

### **Histological analysis**

The specimens were decalcified with a 10% EDTA solution for 2 weeks, dehydrated through a series of ethanol solutions of increasing concentration, and embedded in paraffin. We obtained 5- $\mu$ m-thick coronal sections through the center of the circular defects were obtained and stained with hematoxylin and eosin. The prepared specimens were examined using light microscopy. After microscopic examination, an image of each slide was captured using a digital camera, and the resulting images were saved to a computer for histomorphometric analysis. The new bone fraction within the defect (%) was outlined in the magnified histological images and calculated as the ratio of the area of newly formed bone divided by the area of the whole defect using an automated image analysis system (Tomoro Scope Eye 3.5 Image Analyzer; Techsan Digital Imaging, Seoul, Korea).

### **Analysis equipment**

The morphology of the scaffolds was observed using SEM (Hitachi S-4700, Japan) at an acceleration voltage of 15 kV. All samples were sputter-coated with platinum for 10 minutes. XPS was performed using a K-Alpha 89 (Thermo Electron, UK) to confirm the surface chemistry. TGA was carried out using a TGA Q5000IR (TA Instruments, USA). The scaffolds were analyzed in a nitrogen atmosphere at a heating rate of 10°C/min at a temperature range of 25°C–800°C.

### **Statistical analysis**

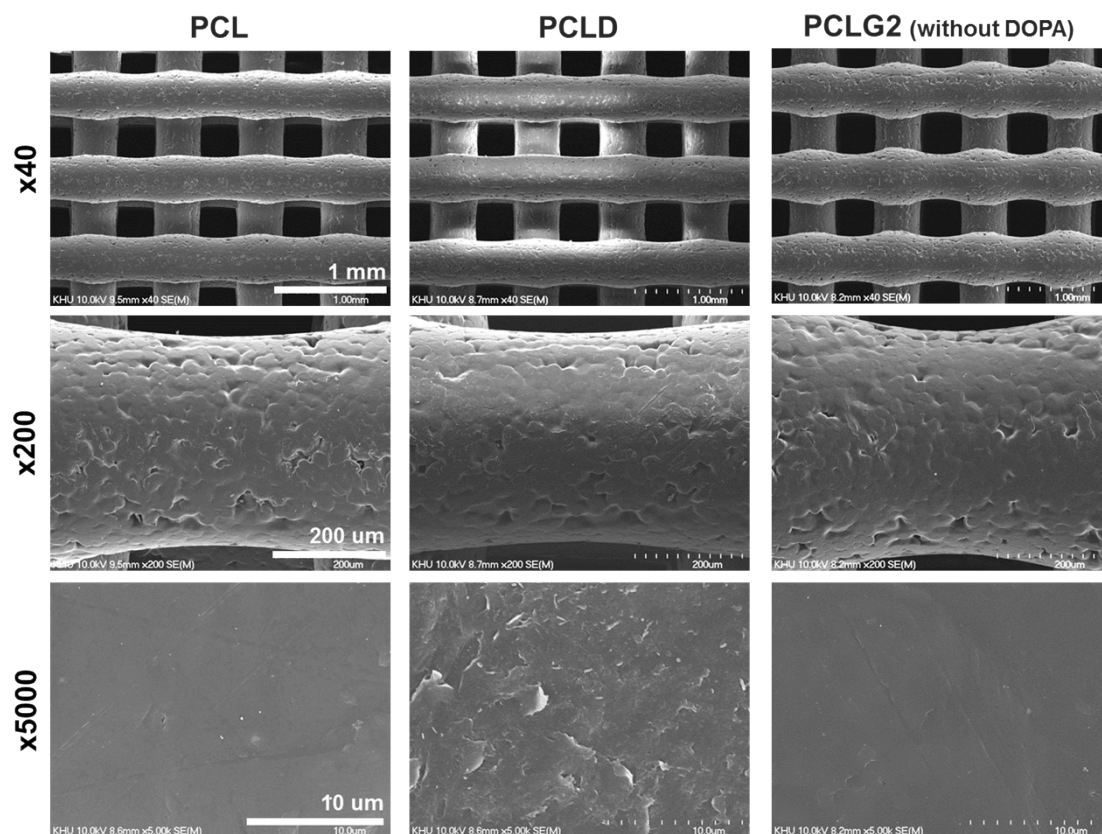
All values are given as mean  $\pm$  standard deviation. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). All statistical analyses were performed using one-way ANOVA with Tukey's post-hoc tests (ALP activity and *in vivo*

testing) and two-way ANOVA with Bonferroni's multiple comparison post-hoc tests (cell proliferation testing). *P*-values <0.01 were considered to be statistically significant.

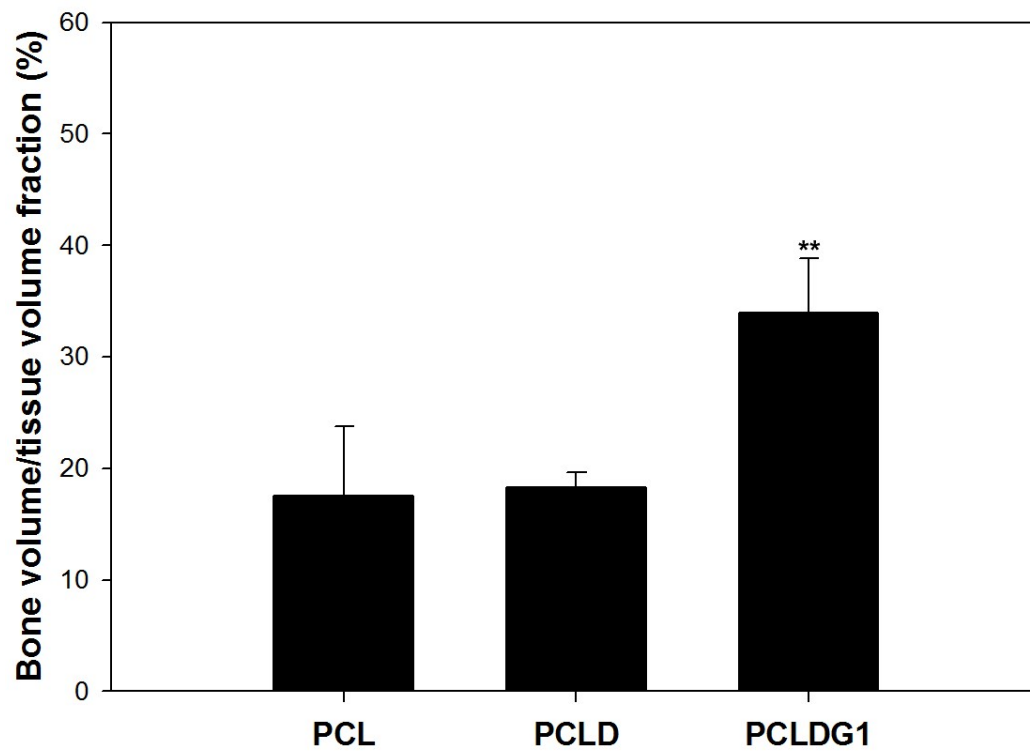
## References

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**Fig. S1.** Scanning electron microscopy images of bare PCL and PDA-coated scaffolds and a 2 mM  $\text{HAuCl}_4$ -treated bare PCL scaffold.



**Fig. S2.** Area quantification of newly formed bone over 4 weeks. The percentage of mineralized bone volume relative to the defect tissue volume (BV/TV) was obtained from uCT analysis. “\*\*” indicates a significant difference compared with other groups.