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Supplementary Information

Bottom-up approach to build osteon-like structure by cell-laden photocrosslinkable hydrogel

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Materials

Poly (ethylene glycol) dimethacrylate polymer (PEGDMA, Mw=1000 Da), Gelatin (Porcine Type A, Cat No.G2500), Methacrylic anhydride (MA), PKH26/67, 3-(trimethoxysilyl)propyl methacrylate (TMSPMA), Propidium iodide (PI), Fluorescein diacetate (FDA) and 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, I 2959) were obtained from Sigma-Aldrich. Rabbit anti-osteocalcin (OCN)/PE, rabbit anti-von Willebrand factor (VWF)/FITC and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Beijing Biosynthesis Biotechnology Co. LTD. The UV light source (Omnicure S1500) was purchased from EXFO Photonic Solutions Inc. Photomasks were designed by using AutoCAD 2007 and printed on transparencies with 20,000-dpi resolution from Qingyi Precision Maskmaking (Shenzhen) Co., LTD. All other chemicals were acquired from Chengdu Kelong Chem Co unless otherwise specified.

Preparation of prepolymer solutions

PEGDMA solution was prepared by mixing 20% (wt/wt) of PEGDMA, 80% (wt/wt) of Dulbecco's Phosphate Buffered Saline (DPBS) and 1% (wt/wt) of I2959, then stirred until the PEGDMA and photoinitiator completely dissolved.

GelMA (methacrylated gelatin) was synthesized as the following steps. 10 g of gelatin were dissolved in 100 mL of DPBS (pH 7.4) and stirred at 50℃. A volume of 10 mL of methacrylic anhydride was added slowly. The mixture was stirred for 3 hours at approximately

50℃, and then diluted with 500 mL DPBS. Lastly, the solution was dialyzed against distilled water using a 12-14KDa cutoff dialysis tubing at 37℃ for 1 week, and freeze-dried until a constant weight was reached. Gel-MA solution was prepared by mixing 5% (wt/wt) GelMA, 95% (wt/wt) of Dulbecco's Phosphate Buffered Saline (DPBS) and 0.1% (wt/wt) I 2959, then stirred until the GelMA and photoinitiator completely dissolved.

Fabrication of osteon-like structures

In method 1 (**Fig. S1A**), namely "circle-and-cross", after pipetting 180μL of PEGDMA prepolymer solution on the middle of a glass slide on which three spacer slides (with a thickness of 150μm) were placed to control the height of the gels, a cover slide was put on the solution. Subsequently, a photomask was placed on the top slide and microgels formation was induced by exposing the prepolymer to UV light for 27s (360–480 nm; 7.9mW/cm^2). After first crosslinking, the glass slide with microgels was thansferred into a petri dish containing 25mL of mineral oil and the microgel arrays were manually assembled by swiping a syringe needle along the surface of the slide straightly. A second crosslinking was performed to stabilize the final constructs composed of circle-shaped microgel assemblies and cross-shaped microgel assemblies for 7s.

In method 2 (**Fig. S1B**), namely "layer-by-layer", we used GelMA as the material. The circle-shaped microgels were formed as mentioned in method 1 by 20s of UV lighting instead. The glass slide with the microgels was then placed on another prepolymer solution, and a relevant photomask covering the circle part was placed on the slide. At last, the solution was exposed to UV light for 20s. The single hydrogel layer was formed and separated from the glass slide to be manually assembled layer by layer into a larger structures stabilized by 5s of UV lighting.

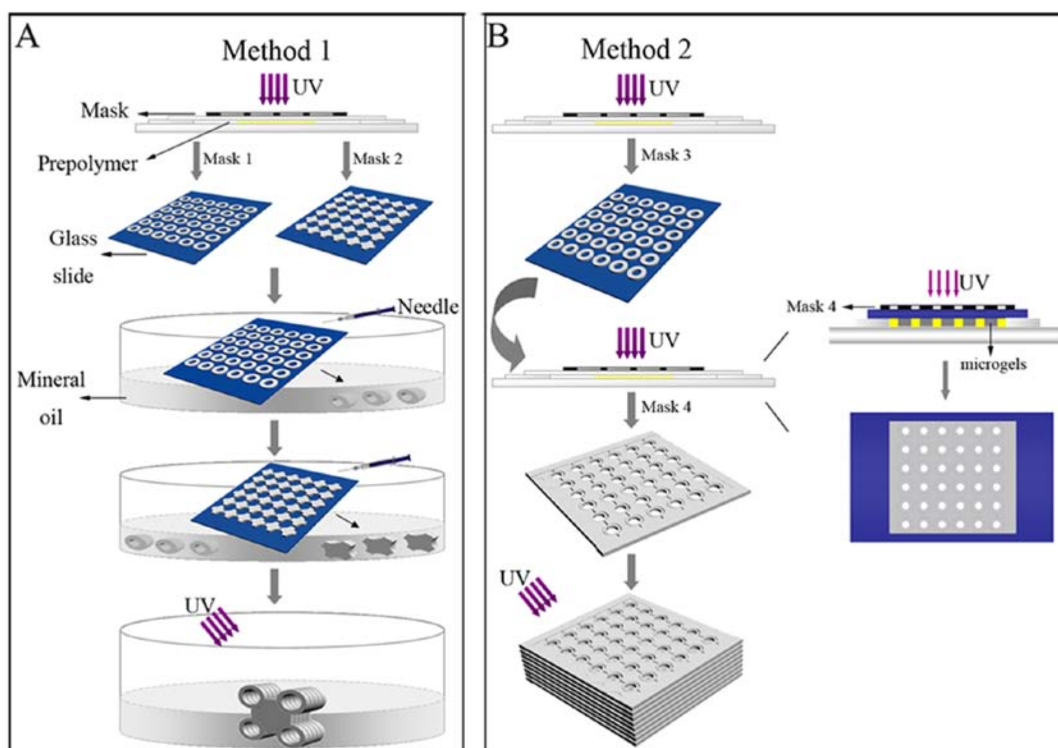

Fig. S1 Schematic illustration of fabricating the osteon-like structures (A-“circle-and-cross” method, B-“layer-by-layer” method).

Perfusion test

To test the connectivity of the channels within osteon-like microgel assemblies, Rhodamine B dye solution was perfused into these channels and the whole process was observed under fluorescent microscopy (Leicactr 4000), in the meanwhile, to verify mass transfer among the constituting microgels. As shown in **Fig. S2**, a TMSPMA treated glass slide was used to stabilize the constructs onside, and the two needles with 0.5mm diameter inserted into the two sides of the construct to act as an inlet and outlet were just fit for the channel with the same diameter. Rhodamine B dye solution was perfused at an average flow rate of 15 μl/min and the distribution images of the dye in the microchannels were taken. Because of the limited view of our microscopy device, we merely perfused one of the four channels in “circle-and-cross” assembled structures and a channel next to the edge in “layer-by-layer” assembled structures for a clear view of the perfusion process.

The methodology of using TMSPMA was described as the followings:

1. Put a glass beaker in a bucket of ice (Caution: this is a very exothermic reaction releasing a lot of heat), weigh 50 g of NaOH pellets, slowly add 450 mL of distilled water and

have all pellets dissolved.

2. In the same beaker, place the slides in a staggered manner, make sure all of them contact with the NaOH solution.

3. Cover the beaker with a pyrex dish (Caution: do not use aluminum foil to cover as NaOH reacts with aluminum producing gaseous side products)

4. Let it sit overnight in the fume hood.

5. Discard the 10% NaOH solution into the proper waste bottle. Then with gloves, thoroughly rinse and rub each slide one by one under distilled water.

6. Dip each slide in 3 distinct 100% reagent alcohol baths and let it air dry (faster under the hood).

7. Wrap the glass slides with aluminum foil and bake for 1h at 80˚C.

8. TMSPMA treatment (under the fume hood):

a. Stack slides vertically and poor 3 mL of TMSPMA on top of the stack using a syringe.

b. After 30 min, flip the stack upside down to get even coating.

9. Cover the beaker with aluminum foil and bake the whole assembly in the beaker overnight at 80˚C.

10. Clean the slides in reagent alcohol again with 3 baths and air dry.

11. Wrap the glass slides with aluminum foil and bake again for 1-2 hours at 80˚C.

12. Store the TMSPMA coated glass slides wrapped in aluminum foil at room temperature.

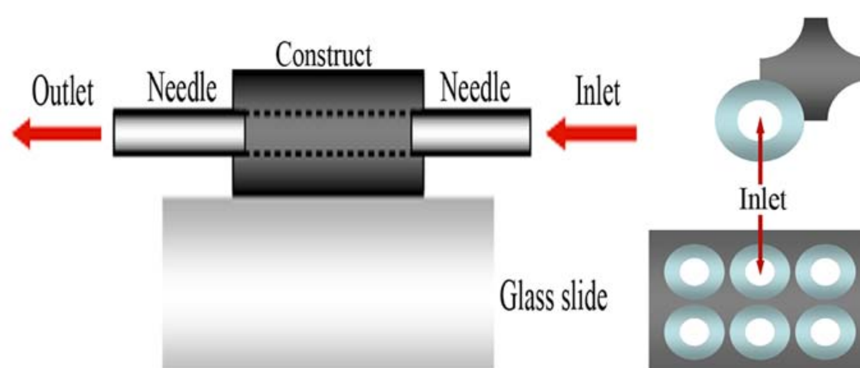

Fig. S2 Schematic illustration of the perfusion process. Platform of perfusion is mainly composed of a glass slide to stabilize microgels and two needles functioning as inlet and outlet inserted into the two ends of the assembly.

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Cell culture and encapsulation

HUVECs and MG 63 came from CCTCC (China Center for Type Culture Collection).

All cells were cultured in a standard cell culture incubator in a 5% CO2 atmosphere at 37°C. MG63 and HUVECs were separately maintained in Dulbecco's modified eagle medium (DMEM; Gibco) supplemented with 10% FBS, 1% Penicillin/Streptomycin changed every 2 days.

To encapsulate HUVECs and MG63 cells within the PEGDMA/GelMA solution, the cells were trypsinized, counted and resuspended in prepolymer solution before polymerization at a specific relevant concentration.

Confocal image analysis

To check the availability of assemble processes for biological applications in both methods, cell-laden microgels and assemblies were collected after each step in assembly procedure and analyzed for cell viability. Cells within hydrogel were stained by Live/Dead dyes (1 μL of FDA and 1 μL of PI) in 1mL of DPBS for 2 min and visualized under a confocal laser scanning microscope (CLSM, Leica-TCS-SP5, German) to get fluorescence images for quantitatively analysis using Image Pro software (Media Cybernetics, Bethesda, MD). Cell viability was determined for cells within microgles which were formed by prepolymer solution being exposed to a first UV light, immersed in mineral oil, and then exposed to UV light for a second time (**Fig. S3**).

To study further cell behaviors, cell-laden assemblies in “layer-by-layer” method were collected after being cultured for 1, 3, 5 and 7 days in cell culture medium, and cell morphology was characterized by being stained with Live/Dead dyes and analyzed via confocal images (**Fig. S4**).

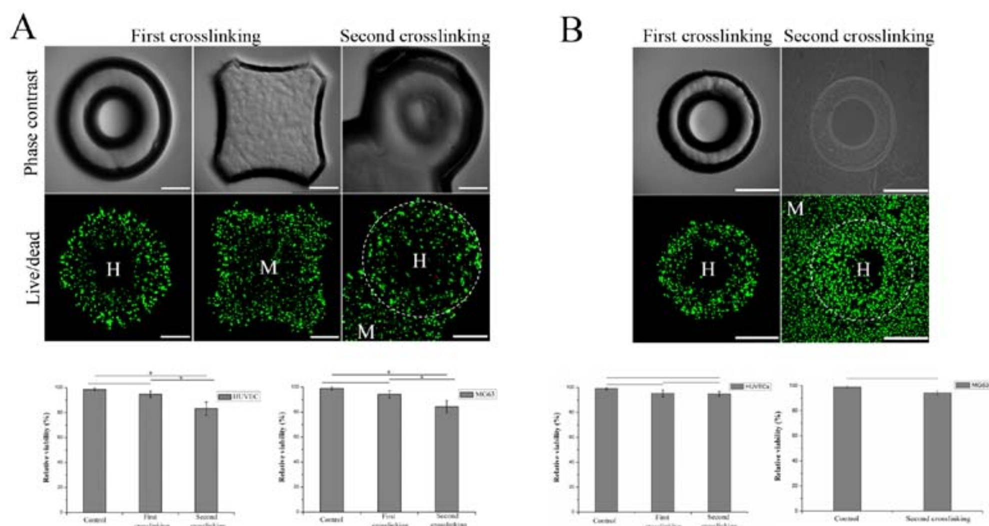

Fig. S3 Cell viability during assembly process. (A) Phase-contrast andfluorescent images of the cell-laden microgels and assemblies after first and second crosslinking, and quantitative statistics of cell viability during each step in the "circle-and-cross" method. (B) Phase-contrast and fluorescent images of the cell-laden microgels and single hydrogel layer aftefirst and second crosslinking, and quan titative statistics of cell viability during each step in the "layer-by-layer" method. Scale bars: 500 μm. Cell seeding density: $1{\times}10^{7}$ cells/ml (A), $4{\times}10^{6}$ cells/ml (B). Values represent mean and standard deviation (N = 5). $P < 0.05$. * denotes significant difference. M stands for MG63 and H stands for HUVECs.

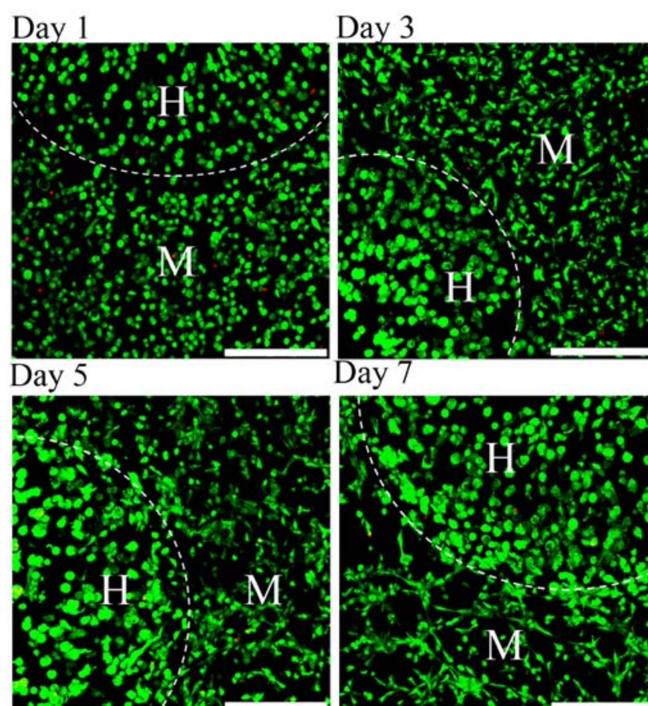

Fig. S4 Higher magnification confocal images of single cell-laden hydrogel layer in "layer-by-layer"

method after being cultured for 1, 3, 5 and 7 days to show cell morphology and viability. Scale bars: 250 µm. Cell seeding density: 4×10^{6} cells/ml. M stands for MG63 and H stands for HUVECs.

Gene expression by quantitative RT-PCR

Osteogenesis and vasculogenesis-related gene expressions of patterned cells in layer-by-layer method were investigated by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). After 4 and 7 days, total RNA was extracted from samples using Trizol reagent (Invitrogen, USA), and then converted into complementary DNA (cDNA) using ReverTra Ace® qPCR RT Kit (Toyobo, Japan). The sequences of primers for alkaline phosphatase (ALP), vascular endothelial growth factor (VEGF), collagen Ⅰ (COL-Ⅰ), osteocalcin (OCN) and GAPDH genes were given in **Table 1**. Quantitative real-time PCR reaction was performed using CFX96™ real-time PCR detection system (Bio-Rad, CFX960) with SsoFast™ EvaGreen® Supermix (Bio-Rad). GAPDH was used as the housekeeping gene to normalize results. The ΔΔCt-value method was used to calculate the relative expression values. All samples were analyzed in triplicate.

Table 1. Primers sequences for target genes

Symbol	Primers
GAPDH	**5'-GGCATGGACTGTGGTCATGAG-3'**
	5'-TGCACCACCAACTGCTTAGC-3'
Alkaline phosphatase	**5'-GCTGGCAGTGGTCAGATGTT-3'**
	5'-CTATCCTGGCTCCGTGCTC-3'
Vascular endothelial growth factor	**5'-TTGCCTTGCTGCTCTACCTCCA-3'**
	5'-GATGGCAGTAGCTGCGCTGATA-3'
Osteocalcin	**5'-TTGGACACAAAGGCTGCAC-3'**
	5'-CTCACACTCCTCGCCCTATT-3'
Collagen type Ⅰ	**5'-CACACGTCTCGGTCATGGTA-3'**
	5'-AAGAGGAAGGCCAAGTCGAG-3'

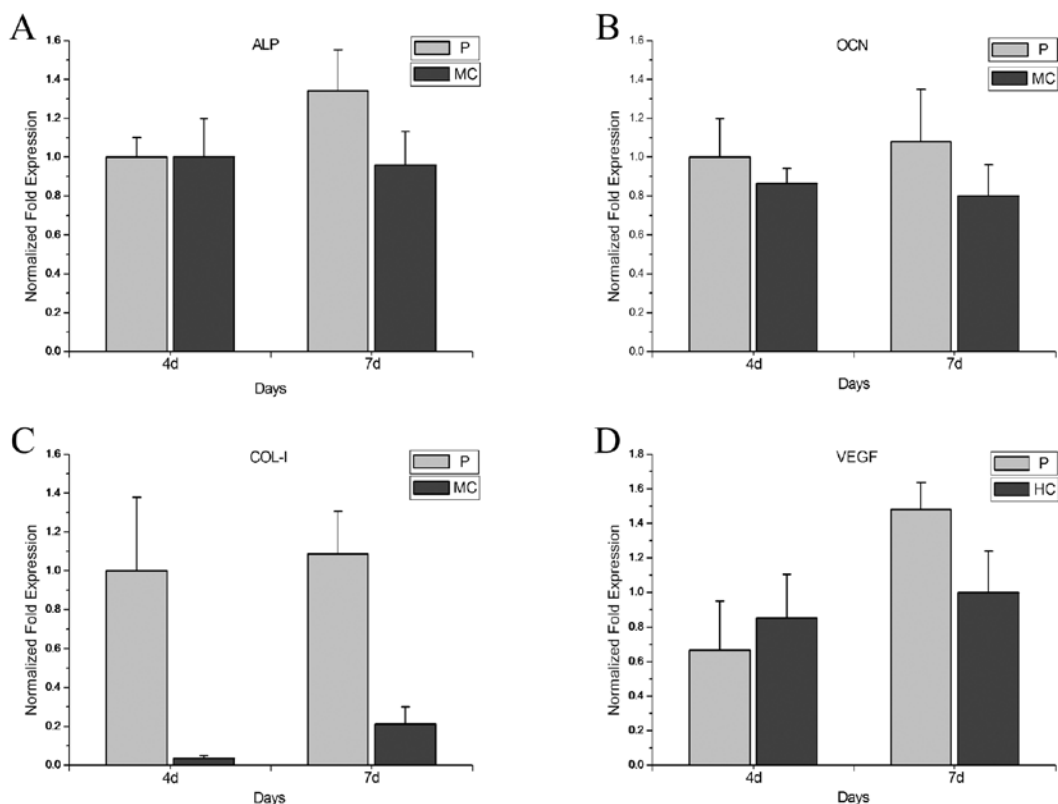

Fig. S5 Quantitative gene expressions of MG63 and HUVEC cells cultured in hydrogel after 4 and 7 days by real-time RT-PCR. The expression levels were normalized to the expression of GAPDH as an internal control. (A) ALP, (B) OCN, (C) COL-I and (D) VEGF. P stands for MG63 and HUVEC in patterned hydrogel. MC stands for MG63 in controlled hydrogel (unpatterned, MG63 only) and HC stands for HUVEC in controlled hydrogel (unpatterned, HUVEC only).

Alkaline phosphatase (ALP), collagen Ⅰ (COL-Ⅰ) and osteocalcin (OCN) are markers of osteogenic differentiation, while vascular endothelial growth factor (VEGF) is a marker of vasculogenic differentiation. In the patterned hydrogel, all the gene expressions gradually increased with culture time. As for ALP, OCN and VEGF, the expression was similar or a little higher compared with the control. The expression of COL-Ⅰ was significantly higher than the control indicating the enhanced osteogenic differentiation of MG63 in this patterned system. This implied that the MG63 cells patterned in the hydrogel kept the normal or even increased differentiation along osteogenic linage, and the patterned HUVEC cells kept normal differentiation along vasculogenic linage. This result proved this patterned system supported the osteogenesis of MG63 as well as the vasculogenesis of HUVEC.

Immunostaining of cells in layer-by-layer patterned system

After culture for 7 days, the assembled cell-laden structures were frozen and sectioned (6 μm in thickness), then stained with rabbit anti-OCN/PE and rabbit anti-VWF/FITC to examine the osteogenesis of MG63 and vasculogenesis of HUVEC relatively. Cells were costained to visualize the nuclei by DAPI blue, and examined using a fluorescent microscopy.

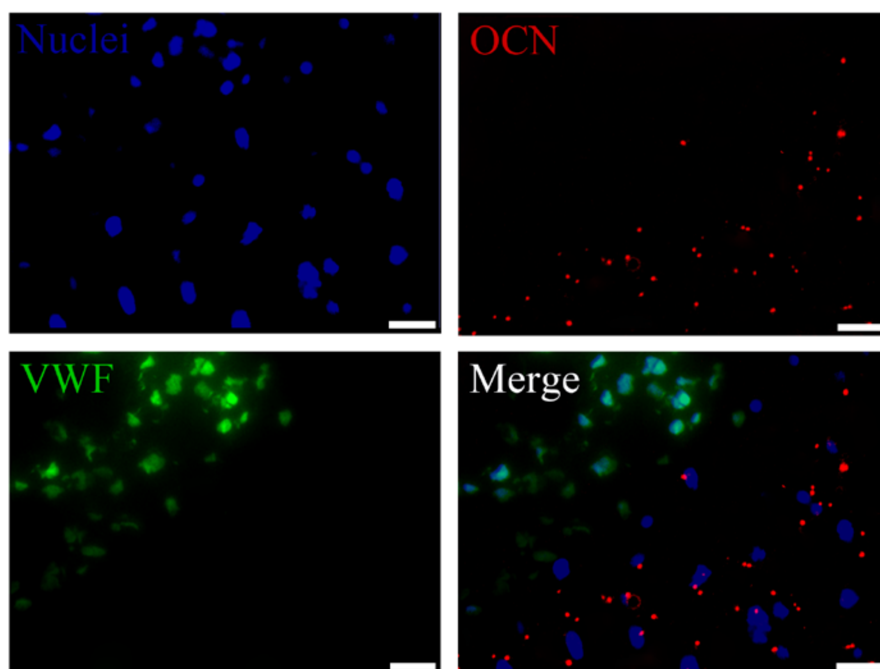

Fig. S6 Representative immunostaining fluorescence images of MG63/HUVEC patterned system. MG63 was stained with rabbit anti-OCN/PE (red), HUVEC was stained with rabbit anti-VWF/FITC (green) and nuclei were stained with DAPI (blue). Scale bars: 50 μm.

VWF is a marker of potent endothelial mitogens inducing angiogenesis, and its positive staining would stand for the distribution of HUVEC and the vasculogenic parts; osteocalcin (OCN) is a marker of osteogenesis, and its positive staining would stand for the bony parts in the patterned system. Except the blue staining of nuclei, positive green staining of VWF was in the inside circle indicating the vasculogenic differentiation of HUVEC, while positive red staining of OCN was in the outside area indicating the osteogenenic differentiation of MG63. This result further proved the initial appearance of the osteon-like structure with designed both bony and vascular structure.

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Data analysis

Data from at least three independent experiments were analyzed and values were represented as mean ± standard error of means. The t-test was used to analyze the statistical significance of the data. Values with a p value less than 0.05 were considered statistically significant.