Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information

Microspheres Embedded Hydrogel Construct - Binary Delivery of Alendronate and BMP-2 for Superior Bone Regeneration

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S1. Decellularization and histological analysis of native bone and decellularized bone extracellular matrix

Porcine tibia was collected; cancellous bone was separated from compact bone followed by washing with phosphate-buffered saline (PBS containing 0.1 % w/v gentamicin). The bone segments were crushed into small pieces (~ 3-4 mm) in presence of liquid nitrogen. Bone granules were demineralized by immersing them in 0.5 N HCL (25 ml/g bone) in a rotating condition (300 rpm) for 24 h. The bone granules were rinsed thoroughly with distilled water to remove acid from the surface and subjected for lipid extraction in chloroform and methanol mixture (1:1) for 1 h. After lipid extraction, the granules were washed with methanol and distilled water followed by lyophilisation for 24 h. Decellularized was performed with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (Invitrogen, Gibco) at 37 °C for 24 h. Trypsin removed all cellular components from native bone matrix (NBM) and the resultant material was termed as decellularized bone ECM (DBM). DBM was thoroughly rinsed twice with PBS and finally with 1% antibiotic-antimycotic solution followed by freeze drying for 24 h for future use.

Histological evaluation of NBM and DBM were performed after fixing them with 4% paraformaldehyde (PFA) for 24 h at room temperature. NBM and DBM were dehydrated with graded alcohol series, xylene treatment, and embedded in paraffin to obtain tissue block. Tissue sections of NBM and DBM were stained with hematoxylin and eosin (H & E), 4, 6-diamidino-2-phenylindole (DAPI, Invitrogen, USA), and Masson's trichome (MT, Sigma) to confirm successful decellularization process. Stained sections were observed under microscope (ZEISS, Germany) and pictures were captured.

S2. DNA quantification from native bone and decellularized bone extracellular matrix

DNA isolation kit (QIAamp, Qiagen, USA) was used to extract total DNA from NBM and DBM to confirm complete removal of cellular components in DBM after decellularization process. The extracted DNA from both NBM and DBM were quantified using DNA quantification kit (Sigma-Aldrich, USA) as per manufacturer's instructions. Further, isolated DNA from both samples was resolved with 1% agarose gel along with a standard DNA ladder (1 kb). Ethidium bromide (EtBr) was used to indicate DNA fragment in NBM/DBM.

S3. Swelling study

Gelatin microspheres were immersed into the PBS for 4 h at room temperature. Dried and wet microspheres were observed under microscope and average diameter of the prepared microspheres was measured. The swelling ratio was calculated by the formula:

$$\text{Swelling ratio} = \frac{Diameter\ of\ wet\ microsphere}{Diameter\ of\ dry\ microsphere}$$

Also, the swelling study was performed with different hydrogel constructs and the result was provided in Fig. S5.

$$\%\ \text{Swelling} = \frac{Weight\ of\ swollen\ hydrogel - Weight\ of\ dry\ hydrogel}{Weight\ of\ dry\ hydrogel} \times 100$$

S4. Hemolytic assay

BOC, BOC/ALN, BOC/BMP, and BOC/ALN/BMP hydrogel constructs were evaluated for hemolysis assay. In brief, human blood was collected and erythrocytes were separated by centrifuging at 1800 rpm for 15 min. Then, 0.2 ml of erythrocytes was diluted with 9.8 ml of

saline to obtain an erythrocyte suspension. The hydrogel constructs (5 mm diameter) were submerged into 1 ml of diluted cell suspension and incubated for 1 h at 37 °C. Similarly, gelatin microspheres were transferred to 96 well plates at 2 mg per well and immersed into 200 µl of diluted cell suspension and incubated for 1 h at 37 °C for hemolysis assessment. Subsequently, hydrogels and microspheres were removed from diluted blood samples and the supernatant was collected after centrifugation at 1800 rpm for 15 min. The absorbance of the supernatant was measured at 540 nm using a microplate reader (Multiskan, Thermo Scientific, USA). 0.1% Triton-X 100TM was taken as positive control and 0.9% saline was taken as negative control.

Percentage hemolysis was calculated as:

$$\% H = [(A_S - A_N)/ (A_P - A_N)] \times 100$$

where, AS, AN, and AP are the absorbance of hydrogel samples, the negative control and the positive control respectively.

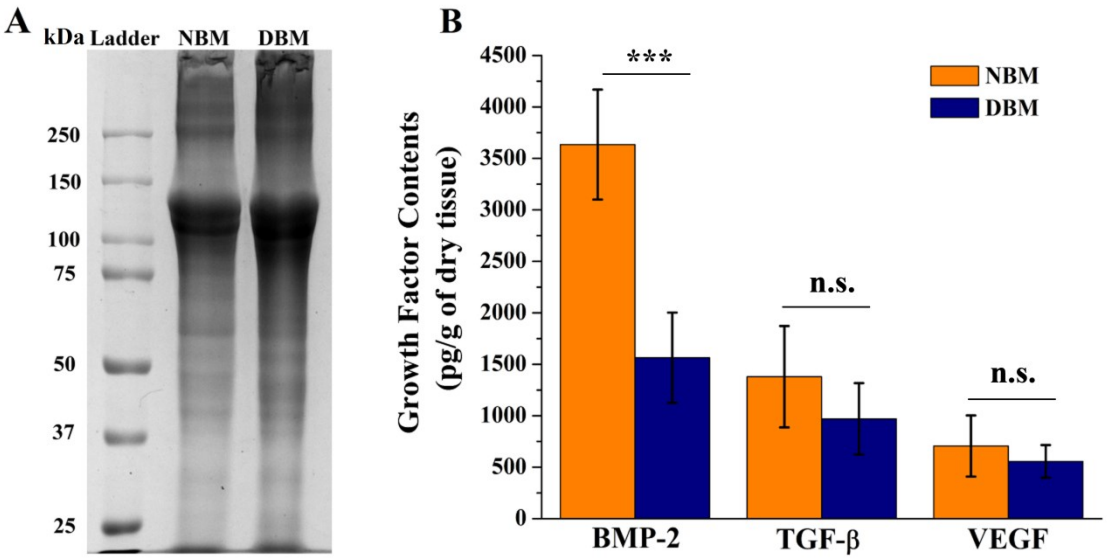
S5. Isolation of human amniotic membrane derived stem cells (HAMSCs)

For human amniotic stem cell isolation, placentas were collected in a sterile container and brought to the laboratory. Then, the placentas were repeatedly washed and disinfected with Hank's balanced salt solution (HBSS; Gibco, USA) containing antibiotic antimycotic solution. The amniotic membrane was dissected from the chorion and subjected to 0.05 % trypsin–EDTA solution (Gibco, USA) for 1 h. Further, the supernatent was discarded, followed by digesting the tissue with Earle's balanced salt solution (EBSS; Gibco, USA) containing 2 mg/ml type IV collagenase (Gibco, USA) for 60 min. HAMSCs were collected after centrifuging the digested solution at 1500 rpm for 10 min and cultivated in Dulbecco's modified eagle's media containing low glucose medium (supplemented with 10% FBS, 1% antibiotic-antimycotic solution; Gibco,

USA) at humidified environment (37 °C, 5% CO_2). Cells were passaged with TrypLE™ (Gibco, USA) and cells at passage two were used for experiments.

Table S1. Primer Sequences for RT-PCR

Primer Sequences			
Genes	**Forward 5' - 3'**	**Reverse 5' - 3'**	**Amplicon Size (bp)**
GAPDH	CCATGGAGAAGGCTGGGG	CAAAGTTGTCATGGATGACC	195
COL I	CAACCTCAAGAAGGCCCT	TTACAGGAAGCAGACAGGGC	250
OPN	CCAGAGTGCTGAAACCCA	TTAATTGACCTCAGAAGATGCACT	250
OCN	ATGAGAGCCCTCACACTCCTC	GCCGTAGAAGCGCCGATAGGC	294



Results:

Fig. S1: SDS-PAGE of total extracted protein from NBM and DBM (A), Growth factor detection of BMP-2, TGF-β, and VEGF by ELISA (B). Y-error bars represent standard deviation. Triple asterisks signify $P < 0.001$.

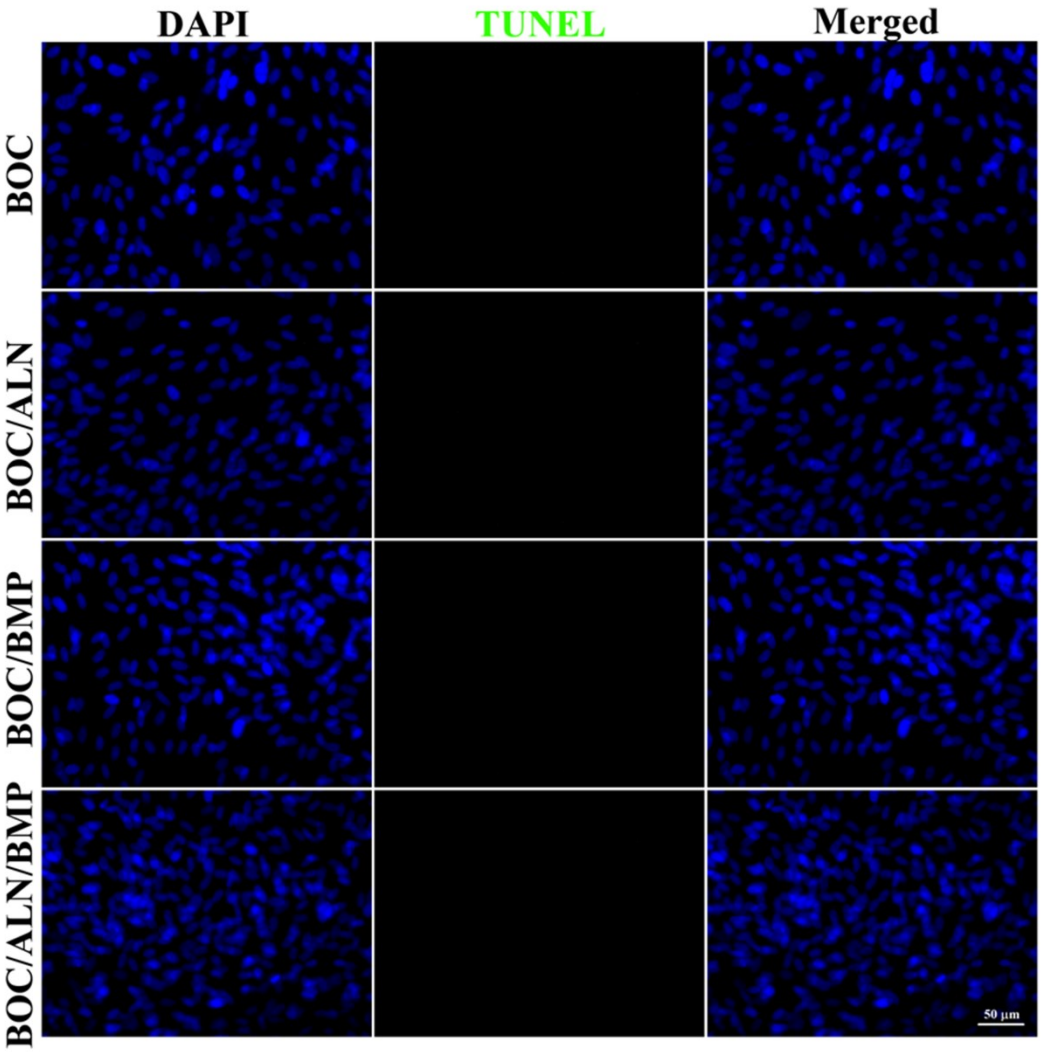


Fig. S2: Apoptosis analysis on the surface of hydrogel constructs at 7 days by TUNEL assay. Blue represents DAPI staining. (Image captured at 20X, Scale bar indicates 50 µm).

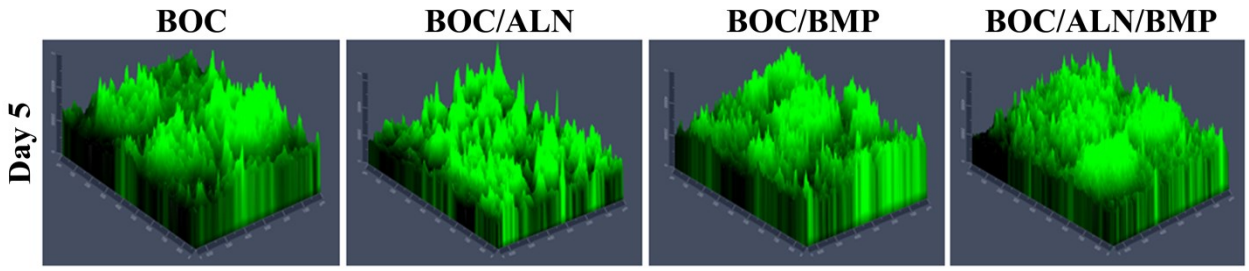


Fig. S3: HAMSCs penetration within the hydrogel constructs at 5 days captured in 2.5D (Image taken at 10 X).

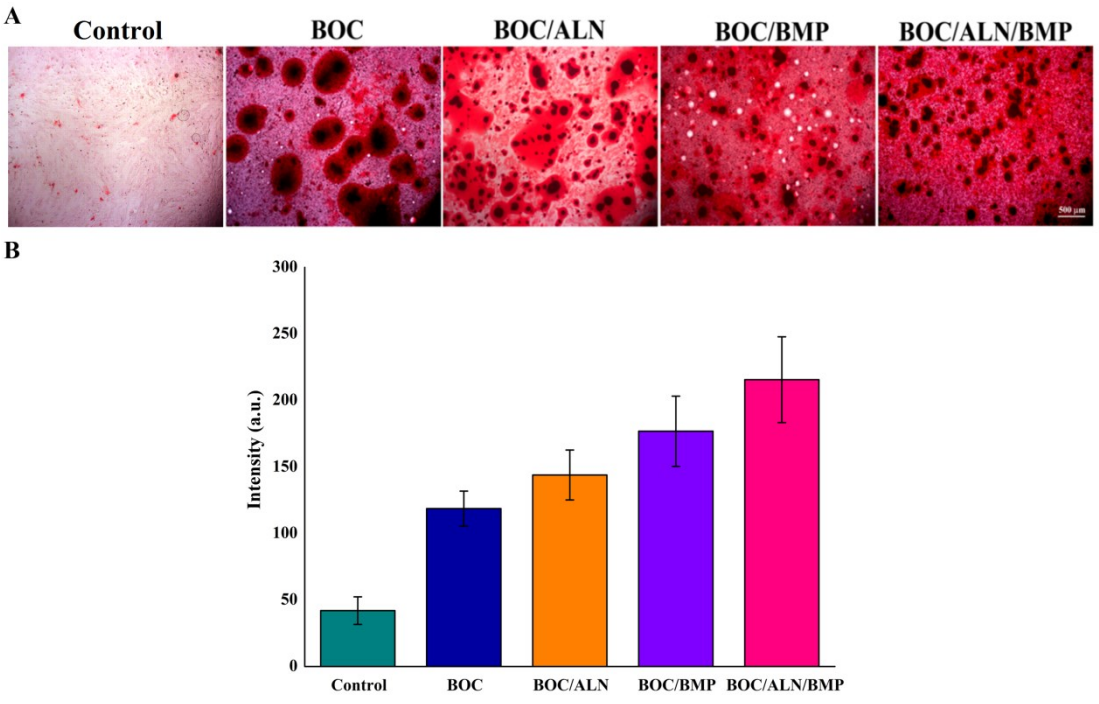
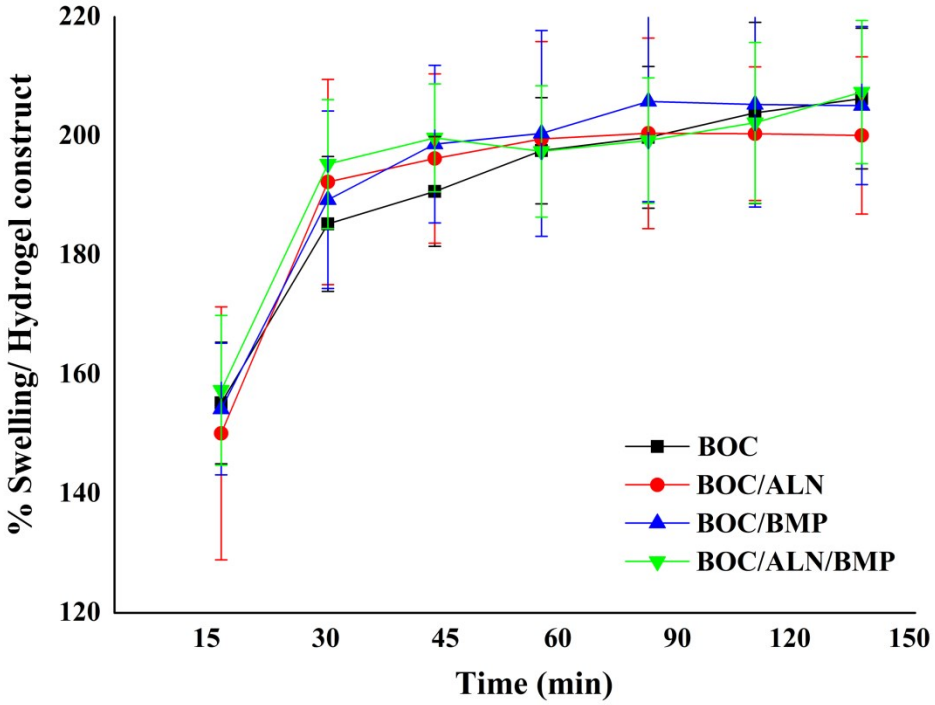


Fig. 

S4:

Representative image of Alizarin red-stained hydrogel constructs after 14 days of osteogenic induction (A) and their respective intensities (B).

Fig. S5: Representative swelling study of different hydrogel constructs in PBS at 37 °C.