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Supporting Information

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Monodisperse Branched Molybdenum-Based Bioactive Nanoparticles Significantly Promote Osteogenic Differentiation of Adipose-Derived Stem Cells

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Experimental section

1. Materials

Tetraethyl orthosilicate (TEOS), ammonium molybdate ($H_8MoN_2O_4$) were supplied by Energy Chemical (Shanghai, China). Cetylpyridinium bromide (CPB), triethylphosphate (TEP), calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), Urea, cyclohexane, isopropanol, ammonium hydroxide ($NH_3{\cdot}H_2O$) and ethyl alcohol (99% ETOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of reagent grade purity and were used without further purification.

2. Synthesis of Mo-rBGNs

Here cetylpyridinium bromide and isopropanol were respectively used as a main template and a co-template agent to control the monodispersed nanoscale morphology and radial porous structure of BGNs. And TEOS should be dripped into the reactive solution to achieve the goal of complete dispersion. The presence of urea was also for the catalytic hydrolysis of TEOS. In addition, $Ca(NO_3)_2{\cdot}4H_2O$ and $H_8MoN_2O_4$ were dissolved in water and hot ammonia solution (pH=9) respectively and then added into the reaction system.

2. Biomineralization activity assessment of Mo-rBGNs

Briefly, 10 mg of 0Mo-rBGNs, 2Mo-rBGNs, 5Mo-rBGNs, and 10Mo-rBGNs powders were soaked into 10 mL of simulated body fluids (SBFs) and kept in an orbital shaker (model IKA KS 4000) at 120 rpm and fixed temperature of 37 ^{o}C for 1, 3 and 7 d. At every selected time point, the samples were removed from SBFs by centrifugation at 10000 rpm for 10 min and washed twice with DW before drying in oven at 60 ^{o}C for 10 h. The composition and chemical structure of formed powders were determined by XRD and FTIR to detect the HA phase formation. The morphology of HA nanocrystals on xMo-rBGNs nanoparticles was subsequently observed by TEM.

3. *In vitro* cellular-biocompatibility assessment of Mo-rBGNs

ADSCs were obtained from ATCC (American Type Culture Collection). In particular, the cells were cultured in growth medium (DMEM, Invitrogen) containing 20% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Thermo Fisher Scientific) in a humidified atmosphere at 5% CO_2 and 37 ^{o}C. Then, ADSCs were seeded into a 96-well plate at 3500 cells/well and incubated in the same culture conditions for 24 h. Afterwards the cells were respectively treated with xMo-rBGNs at concentrations of 50, 100, 150 or 200 μg mL^{-1} for 24 h or 72 h. 0Mo-rBGNs were used as a control. At the predetermined time points, the medium was removed from each well, 100 μL of alamar blue reagent in DMEM (10%v/v) was added to each well, which was then incubated at 37 ^{o}C for 4 h. Subsequently, the optical density (OD) was measured at 530 nm excitation and 600 nm emission using a microreader (Spectramax; Molecular Devices, Sunnyvale, CA, USA). Untreated cells were used as controls, and the results were converted to cell viability according to the equation:

Relative cell viability (%) = OD_{Sample} / $OD_{Control}$ × 100%

Briefly, after incubation for 24 h, the ADSCs were washed by PBS (Dulbecco, Thermo Fisher Scientific) and incubated with 100 μL of LIVE/DEAD solution (calcein-AM / ethidium homodimer) for further 40 min at 37 ^{o}C, and then washed thrice with PBS. The cell fluorescence imaging of live and dead cells was investigated under confocal laser scanning microscopy (CLSM, model Olympus FV300). In the cell viability tests, the solution without nanoparticle was used as the negative control (Blank).

3. Cellular biominerialization and osteogenic differentiation analysis

3.1 Alkaline phosphatase (ALP) activity

In brief, ADSCs were seeded on 24-well plates at a density of 2 × 10^4 cells/mL and cultured with DMEM, supplemented with 20% FBS and 1% penicillin/streptomycin in an atmosphere at 37 °C and 5%

CO_2. After being incubated for 24 h, the culture DMEM was extracted from the 24-well plates and the same volume of differentiation medium containing 10 nM dexamethasone, 10 mM β-glycerophosphate, 0.28 M ascorbic acid and different concentrations of xMo-rBGNs (0, 50, 100, 150 and 200 μg mL^{-1}) was replenished. The differentiation medium was changed every 3 d during culture period. At each regular time point, the cells after digestion by Trypsin-EDTA (GIBCO) solution were collected into a 1.5 mL tube and washed twice with PBS by centrifugation at 3000 rpm for 2 min. After mixing cells with the lysis solution, the mixture were standing for 10 min and then centrifuged at 12000 rpm for 15 min at 4 ℃ to collect supernatant. The ALP activity was also standardized to the total protein content measured by the Pierce BCA protein assay kit (Thermo Fisher Scientific). The absorbance was assayed through a microplate reader at a wavelength of 405 nm to exhibit the level of ALP activity.

3.2 Immunofluorescence staining of osteogenic proteins

At sampling time point, cells incubated by osteoinductive medium and xMo-rBGNs were were washed with PBS, fixed with 4% paraformalclehyde and then treated with 0.1% Triton at room temperature for 45 min, blocked with 1% bovine serum albumin for 30 min, and incubated with two kinds of primary antibodies including COL I (1:1,000; Abcam, Cambridge, UK) or BSP (1:200; Abcam) at 4 ℃ for 12 h. After washing thrice by PBS, the cells were further incubated with the appropriate secondary antibodies (Alexa 594; Thermo Fisher Scientific) in a dark atmosphere for 1 h at 37 ℃. Images were observed by the fluorescence microscope ((IX53, Olympus).

3.3 qRT-PCR assay of osteogenic genes

Briefly, Tripure (Roche) was performed to extract total RNA from ADSCs after incubation with osteoinductive medium and xMo-rBGN for 7 or 14 d, and a reverse transcription reagent kit (Takara) was used to prepare single-stranded cDNAs from 0.5 μg RNA. The qRT-PCR samples consisted of 7.5 μL

SYBR Green supermix (Applied Biosystems 7500; Bio-rad) with 0.5 μL cDNA, 0.3 μL forward primer, 0.3 μL reverse primer and 6.4 μL double distilled water (DDW). And qRT-PCR was performed on a fast-real-time PCR system (Applied Biosystems 7500). The cycle parameters were: 95 °C for 2 min, followed by cycling 40 times at 95 °C for 15 s, and 60 °C for 1 min. Following the instructions of the manufacturer, GAPDH was used as housekeeping gene and the primers for qRT-PCR are shown in Table S1.

Table caption

Table S1 The primers for qRT-PCR.

Figure captions

Figure S1 Morphology evaluation of Mo-rBGNs. (a-d) Low magnification TEM images of 0Mo-rBGNs (a), 2Mo-rBGNs (b), 5Mo-rBGNs (c) and 10Mo-rBGNs (d) (scale bar: 500 nm).

Figure S2 EDS mapping of 10Mo-rBGNs. (a) Overlay EDS mapping of elements in 10Mo-rBGNs; (b) EDS mapping of O in 10Mo-rBGNs; (c) EDS mapping of Si in 10Mo-rBGNs; (d) EDS mapping of Ca in 10Mo-rBGNs; (e) EDS mapping of P in 10Mo-rBGNs; (f) EDS mapping of Mo in 10Mo-rBGNs. Scale bar: 1 μm.

Figure S3 TEM images of Mo-rBGNs after biomineralization for 1 d. (a-d) High magnification TEM images of 0Mo-rBGNs (a), 2Mo-rBGNs (b), 5Mo-rBGNs (c) and 10Mo-rBGNs (d) (scale bar: 100 nm); (e-h) TEM images of 0Mo-rBGNs (e), 2Mo-rBGNs (f), 5Mo-rBGNs (g) and 10Mo-rBGNs (h) (scale bar: 200 nm); (i-l) Low magnification TEM pictures of 0Mo-rBGNs (i), 2Mo-rBGNs (j), 5Mo-rBGNs (k) and 10Mo-rBGNs (l) (scale bar: 500 nm).

Figure S4 TEM images of Mo-rBGNs after biomineralization for 3 d. (a-d) High magnification TEM

images of 0Mo-rBGNs (a), 2Mo-rBGNs (b), 5Mo-rBGNs (c) and 10Mo-rBGNs (d) (scale bar: 100 nm); (e-h) TEM images of 0Mo-rBGNs (e), 2Mo-rBGNs (f), 5Mo-rBGNs (g) and 10Mo-rBGNs (h) (scale bar: 200 nm); (i-l) Low magnification TEM pictures of 0Mo-rBGNs (i), 2Mo-rBGNs (j), 5Mo-rBGNs (k) and 10Mo-rBGNs (l) (scale bar: 500 nm).

Figure S5 TEM images of Mo-rBGNs after biomineralization for 7 d. (a-d) High magnification TEM images of 0Mo-rBGNs (a), 2Mo-rBGNs (b), 5Mo-rBGNs (c) and 10Mo-rBGNs (d) (scale bar: 50 nm); (e-h) TEM images of 0Mo-rBGNs (e), 2Mo-rBGNs (f), 5Mo-rBGNs (g) and 10Mo-rBGNs (h) (scale bar: 100 nm); (i-l) TEM pictures of 0Mo-rBGNs (i), 2Mo-rBGNs (j), 5Mo-rBGNs (k) and 10Mo-rBGNs (l) (scale bar: 200 nm); (m-p) Low magnification TEM pictures of 0Mo-rBGNs (m), 2Mo-rBGNs (n), 5Mo-rBGNs (o) and 10Mo-rBGNs (p) (scale bar: 500 nm).

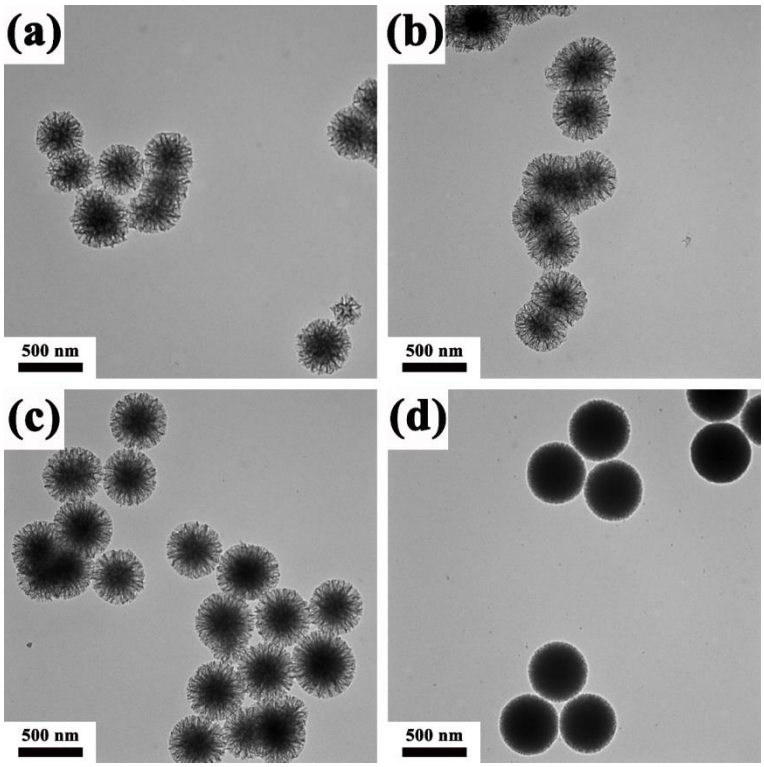
Figure S6 Fluorescence images of cells after culture with Mo-rBGNs for 3 d at 40 μg mL^{-1} (A), 120 μg mL^{-1} (B) and 200 μg mL^{-1} (C) (scale bar: 100 μm).

Figure S7 Relative expression of osteoblastic marker gene during osteogenic differentiation following induction by Mo-rBGNs with 30 μg mL^{-1} and 90 μg mL^{-1} on day 7 and 14. (a, b) Relative expression of Col I (a) and Bsp (b) on day 7; (c, d) Relative expression of Col I (c) and Bsp (d) on day 14. *$p < 0.05$; **$p < 0.01$.

Table S1 The primers for qRT-PCR.

Genes	Forward primer(5’-3’)	Reverse primer(5’-3’)
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Collagen-I	CACCCAGAGTGGAGCAGTG	GCAGGTTTCGCCAGTAGAG
Bsp	CAGGTGAAGGAGAGAGCGTC	CACTAGGAGCGGTGGTTGTC

3. Figures



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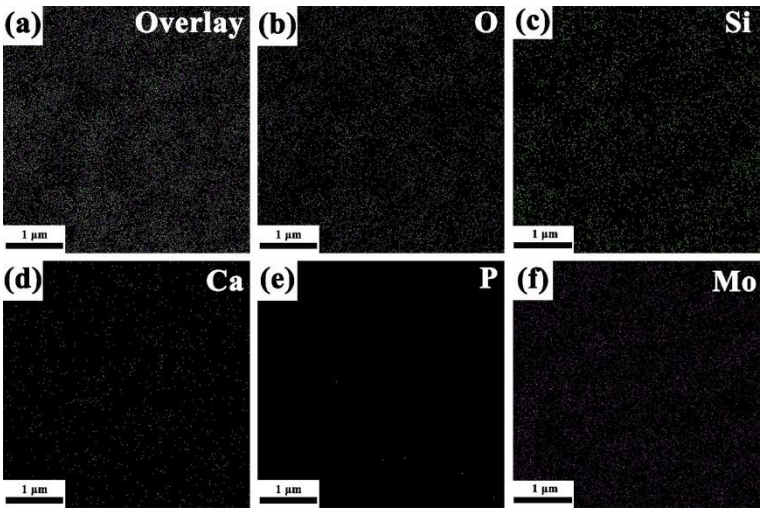

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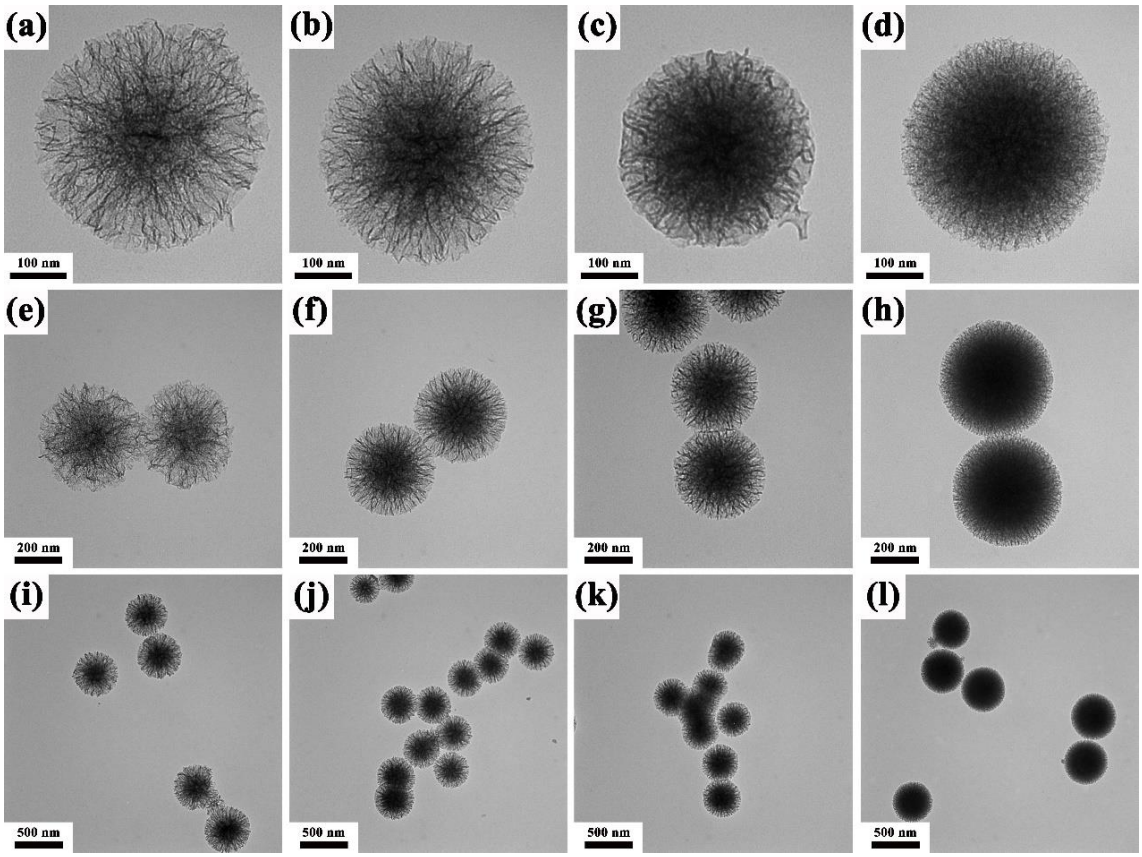

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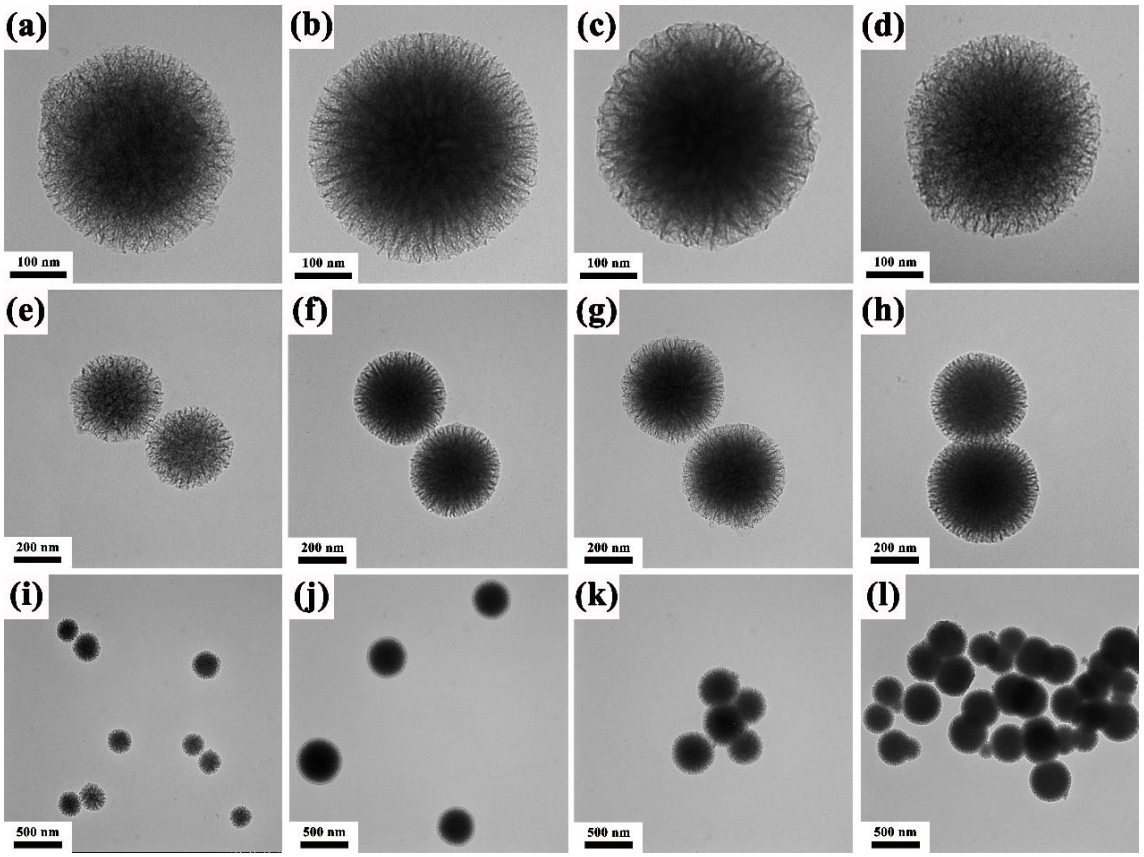

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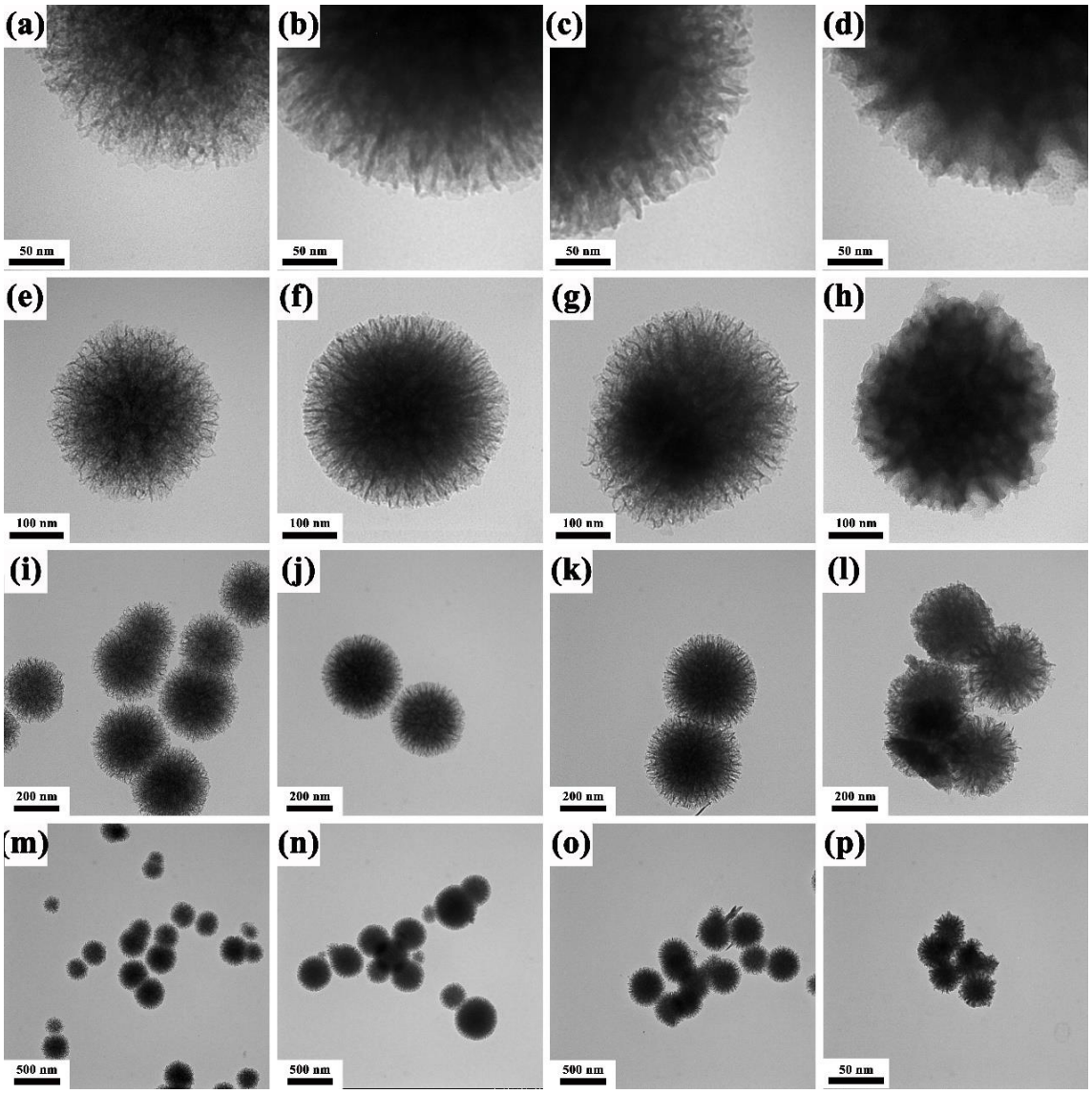
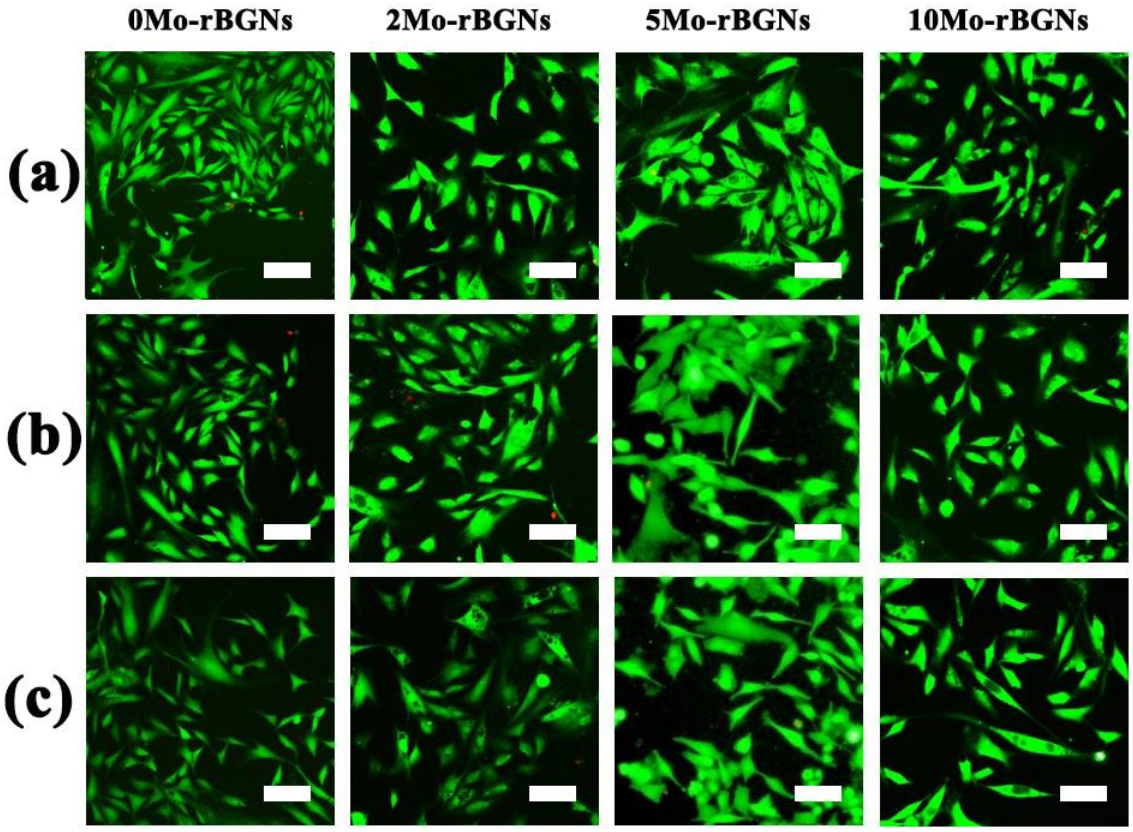


Figure S5 TEM images of Mo-rBGNs after biomineralization for 7 d. (a-d) High magnification TEM images of 0Mo-rBGNs (a), 2Mo-rBGNs (b), 5Mo-rBGNs (c) and 10Mo-rBGNs (d) (scale bar: 50 nm); (e-h) TEM images of 0Mo-rBGNs (e), 2Mo-rBGNs (f), 5Mo-rBGNs (g) and 10Mo-rBGNs (h) (scale bar: 100 nm); (i-l) TEM pictures of 0Mo-rBGNs (i), 2Mo-rBGNs (j), 5Mo-rBGNs (k) and 10Mo-rBGNs (l) (scale bar: 200 nm); (m-p) Low magnification TEM pictures of 0Mo-rBGNs (m), 2Mo-rBGNs (n), 5Mo-rBGNs (o) and 10Mo-rBGNs (p) (scale bar: 500 nm).



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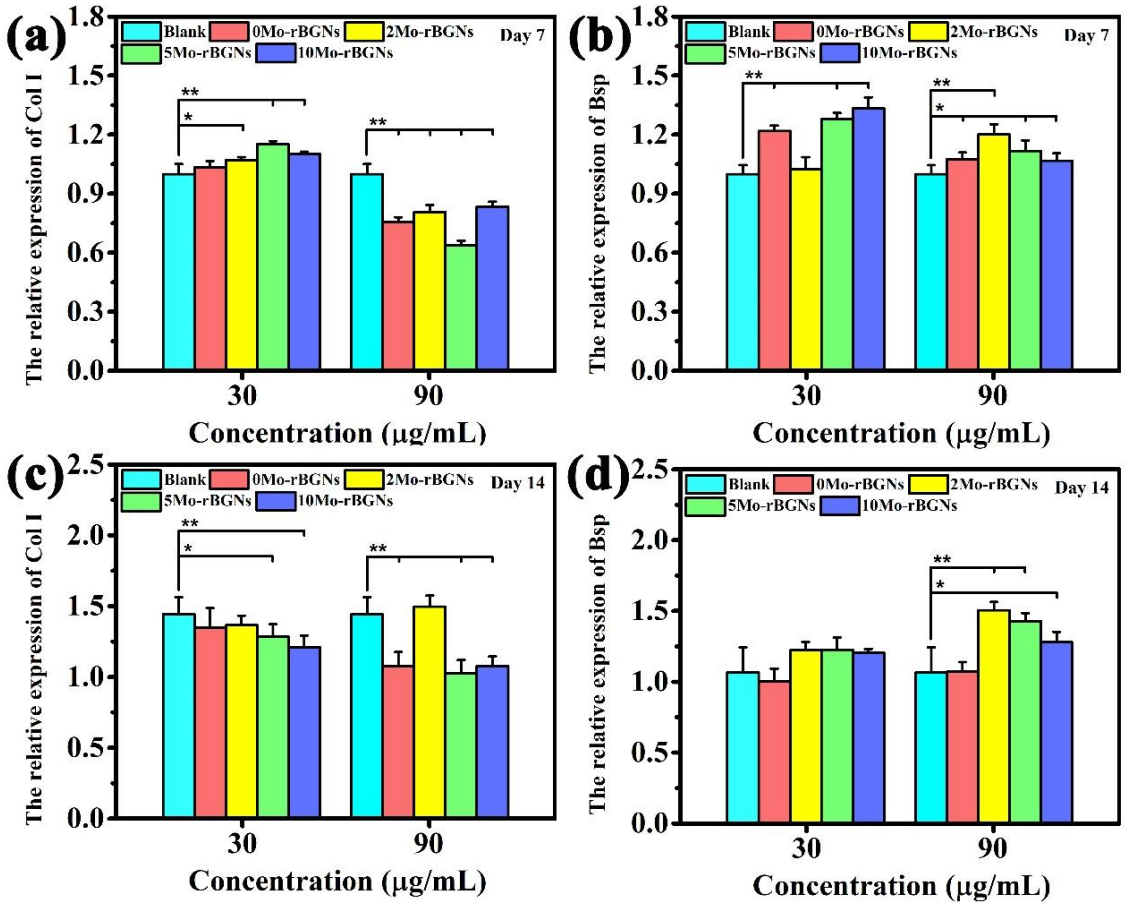

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