

Electronic Supplementary Information (ESI)
Experimental details for the communication

Synergistic Acceleration in the Osteogenesis of Human Mesenchymal Stem Cells by Graphene Oxide-Calcium Phosphate Nanocomposites

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Materials: Igepal CO-520, cyclohexane (99%), *N,N*-dimethylformamide (99.8%), calcium chloride dihydrate (99.99%), Pluronic[®] F-127 (BioReagent), sodium phosphate dibasic ($\geq 99.0\%$), and molecular biology grade water were purchased from Sigma (Sigma, St. Louis, MO) and used without any additional purification. Single layer graphene oxide (GO) was purchased from Nanocs Inc (New York, NY). The growth medium (Thermo Scientific[™] HyClone[™] AdvanceSTEM[™] mesenchymal stem cell expansion kit) and osteogenic differentiation medium (Thermo Scientific[™] HyClone[™] AdvanceSTEM[™] mesenchymal stem cell osteogenic differentiation kit) for mesenchymal stem cells, 0.25% HyClone[™] Trypsin, Phosphate buffered saline solution (PBS, BioReagent), HyClone[™] antibiotic antimycotic solution (containing 10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of Amphotericin B per mL), total protein extraction reagent (Thermo Scientific[™] Pierce[™] M-PER[™] Mammalian Protein Extraction Reagent), Pierce[™] BCA Protein Assay Kit, and phosphate colorimetric assay kit (Abcam[®]) were purchased from Fisher Scientific (Pittsburgh, PA).

Colorimetric MTT assay was performed using the Cell Proliferation Kit I (MTT) from Roche Diagnostics GmbH (Mannheim, Germany). The osteogenesis quantitation kit from Millipore (Millipore, Temecula, CA) was used for quantitative Alizarin Red Staining (ARS) assay. The antibody for osteocalcin (OCN) and FITC labelled secondary antibody were purchased from Santa Cruz Biotechnology (Dallas, TX). Fluoroshield[™] with DAPI histology mounting medium was from Sigma-Aldrich (St. Louis, MO). Alexa 488 labeled antibody to alkaline phosphatase (ALP) was ordered from BD Biosciences (Franklin Lakes, NJ).

Cells: Human bone marrow mesenchymal stem cells (hMSCs) were purchased from Fisher Scientific (Thermo Scientific[™] CET). The growth medium and the osteogenic differentiation medium for hMSCs from Fisher Scientific were supplemented with 1% HyClone[™] antibiotic antimycotic solution before usage and are still referred to growth medium (GM) and osteogenic medium (OM), respectively in this paper. The hMSCs were incubated in the GM until 80%

confluence, harvested with 0.25% Trypsin, subcultured and expanded. Cells at passages 2-3 were used for all the experiments.

Experimental Details:

Neutralization of GO: The GO purchased from Nanocs Inc. was highly acidic (pH 3~4). It was neutralized with equal volume of 0.25 M NaOH and centrifuged at 12,000 rpm for 2 minutes. The pellet was washed three times with distilled water and re-suspended in 0.015 M phosphate buffer saline (PBS).

Synthesis of GO Nanoparticles and GO-CaP Nanocomposites: GO-CaP and CaP nanoparticles were synthesized via double reverse microemulsion procedure as reported.⁷ Briefly, Two 10 mL glass vials were prepared with (A) 200 μ L of 100 mM CaCl₂, and (B) 200 μ L of 60 mM Na₂HPO₄ containing 1 mg neutralized GO, respectively. Next, 2.65 mL of cyclohexane containing 30 vol % of Igepal CO-520 was added to each vial. Fluids in both vials were stirred at high speed (800 rpm) for 20 minutes on stirring hot plates to obtain clear microemulsion A and B. The microemulsion B was slowly dropped into microemulsion A and stirred for 5 minutes to completely mix both emulsions and to allow for calcium phosphate precipitate formation in nano sized droplets (microemulsion C). Pluronic[®] F-127 (50 μ L, 1% W/V) was slowly added to the microemulsion C by drops, followed by 20 minutes stirring to stabilize the GO-CaP nanoparticles. Brown fluffy precipitate of Pluronic[®] coated GO-CaP particles were observed after the emulsion was disrupted by addition of three times volume of ethanol. The solution was centrifuged at 6,000 rpm, washed three times with ethanol and water (molecular biology grade), respectively.

The fabrication of Pluronic coated CaP nanoparticles followed the same procedure as above described except that microemulsion B contained no GO. Once synthesized, the particles are stored in water (molecular biology grade) for maturation at room temperature in a Biosafety cabinet.

Transmission Electron Microscopy (TEM) Characterization: TEM images were collected from freshly made GO-CaP and CaP particles, and the particles after one and two weeks maturation using a JEOL 1200 TEM (JEOL USA Inc., Peabody, MA). The substrates were dispersed in water and dropped onto Formvar/carbon 300 mesh EM specimen grid (Fisher Scientific, Pittsburgh, PA) for imaging. Eighty keV was used to avoid beam damage to the particles.

Raman Spectroscopy: Twenty μ L of GO-CaP, CaP and neutralized GO in water was dried on calcium fluoride slide. Raman spectra were then collected from the dried film using a confocal Raman microscope (Renishaw Invia, Gloucestershire, England) with 785 nm near infrared laser. All the spectra were baseline corrected using custom written Matlab scripts.

Cytotoxicity Evaluation: The cytotoxicity of Pluronic[®] F-127 coated GO-CaP, CaP and uncoated GO toward hMSCs was evaluated by testing the cell viability using colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay kit (Roche Diagnostics GmbH, Mannheim, Germany). Ten thousand hMSCs were seeded in each well of 96-well plates with GM. After 24 h of incubation, the medium was changed into GM containing various

concentration of GO, CaP, and GO-CaP. After 72 h, the nanoparticle-containing medium was removed and cells were washed with PBS three times. Fresh medium was added, followed by 10 μ L MTT reagent per well at 37 °C for 4 h according to the manufacturer's protocol. The absorbance was read at a wavelength of 550 nm with Microplate Reader (Biotek Instruments, Winooski, VT). The assay was run in 96-well plates with six repeats per treatment.

Alizarin Red Staining (ARS) and Quantification: The extent of osteogenic differentiation of hMSCs was evaluated by ARS assay which quantifies the amount of mineralization arising from bone nodule formation. Forty thousand hMSCs were seeded in each well of 24-well plates and allowed to attach overnight. The medium was changed to control or particle-containing OM after 24 h, and then every 3 days for the remaining culture period. The final concentration of GO-CaP in the medium was 10.5 μ g/ml containing around 0.5 μ g/ml GO and 10 μ g/ml CaP. The same concentration of individual components, i.e. GO and CaP, was employed for comparison. Blank wells (without cells) with particles-containing OM as well as cells cultured in particles-containing GM were used as negative control. At two, three and four weeks after particle treatment, the amount of induced calcification was quantified following the protocol of the Osteogenesis Quantitation Kit from Millipore. After washing off excessive dyes, white light images were acquired from each well using a tissue dissection microscope (Carl Zeiss Microscopy, Thornwood, NY) before dissolving the stained calcium in acid for quantification. Normalized calcium concentration was calculated by ratio-ing the quantified calcium concentration from ARS to the total protein content.

In a separate experiment, the hMSCs in 24-well plates were treated with OM containing 0.5 μ g/ml GO, 10 μ g/ml CaP, 10.5 μ g/ml GO-CaP, and concurrent 0.5 μ g/ml GO plus 10 μ g/ml CaP (GO+CaP), respectively. The particle-containing OM was changed every 3 days. After two weeks, the calcium content in each well was quantified and normalized to the total protein content in parallel plates.

Phosphate Quantification: After 2 and 3 weeks of particle treatment in OM and GM (negative controls) in parallel plates, the cells were lysed using cell lysis reagent (Pierce™ M-PER™ mammalian protein extraction reagent) then centrifuge at 13,000 rpm for 15 min. The pellet was washed 3 times with water, followed by thorough mixing with 200 μ l 10% acetic acid. After centrifuging another time, the supernatant was tested for phosphate content using a quantitative phosphate assay kit (Abcam®, Fisher Scientific, Pittsburgh, PA).

Total Protein Assay: Total protein was extracted from parallel plates (with ARS assay) using Pierce™ total protein extraction reagent and quantified using Pierce™ BCA protein assay kit with bovine serum albumin as a standard.

Immunofluorescence imaging: The osteogenesis of hMSCs was examined by immunostaining of osteoblast markers osteocalcin (OCN, Santacruz biotechnology, TX) and alkaline phosphatase (ALP, BD Bioscience, San Jose, CA) after two weeks of incubation. 20,000 hMSCs were seeded on 8-well chamber slides (Lab-Tek™, Fisher Scientific, Pittsburgh, PA) with GM. After 24 h, the medium was changed to control OM or particle-containing OM, and then changed every 3 days. After two weeks, the cells were washed with PBS twice, fixed with 4% paraformaldehyde for 10

minutes at room temperature, permeabilized with 0.1% Triton-X100 for 10 min, and blocked with 1% BSA/10% normal goat serum for 1 h at room temperature. For OCN staining, cells were incubated with primary antibody (1:200) to OCN for 1 h at room temperature, washed three times with PBS, and then incubated with goat anti-mouse FITC labelled IgG (1:200) for 2 h followed by two washes of PBS. For ALP staining, cells were incubated with Alexa 488 labeled primary antibody to ALP (1:500) for 1 h at room temperature and washed three times with PBS. Cover slips were mounted with Prolong[®] gold antifade mounting medium containing DAPI nuclear stain (Life Technologies, Grand Island, NY) onto the slides. Immunofluorescence images were acquired with a Nikon Eclipse fluorescence microscopy (Nikon, Tokyo, Japan).

Results:

Quantification assays of calcium and phosphate content in mineral deposition have been conducted on hMSCs cultured with GO, CaP, and GO-CaP treatments in OM as well as cells with treatments in GM. Significant calcification is observed with all treatments in OM as shown by ARS images in Figure S3 after 2, 3 and 4 weeks. The hMSCs incubated with particles in GM showed no positive calcium staining with ARS. Figure S4 demonstrated phosphate abundance in the mineral deposition through quantitative phosphate assay after 2 and 3 weeks of treatment. The results are in agreement with the calcium quantification, following the order of GO-CaP > CaP >> GO > control, while no phosphate deposition observed in the GM groups.

Supplemental Figures:

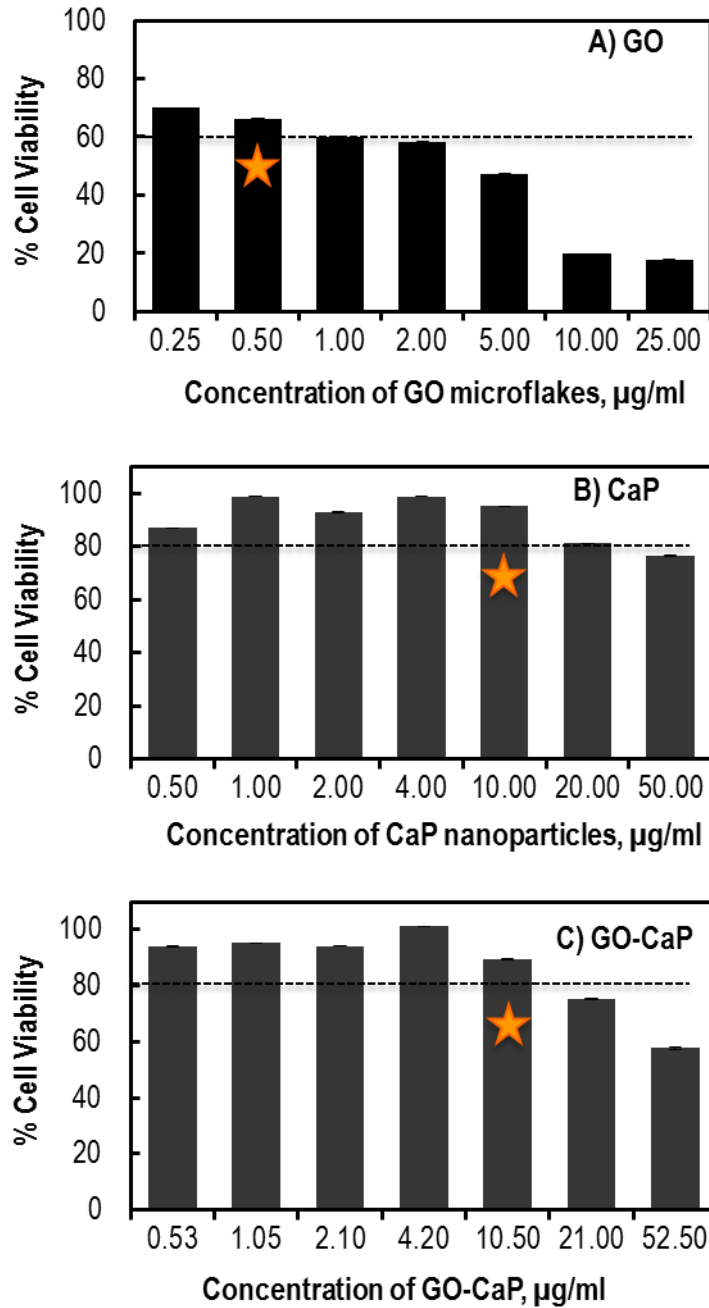


Figure S1: Cell viability of hMSCs by MTT assay (ratioed to control) with 72 hours treatments by GO microflakes (A), CaP nanoparticles (B), and GO-CaP nanocomposites (C). The star signs mark the concentrations of particles selected for usage in the current study.

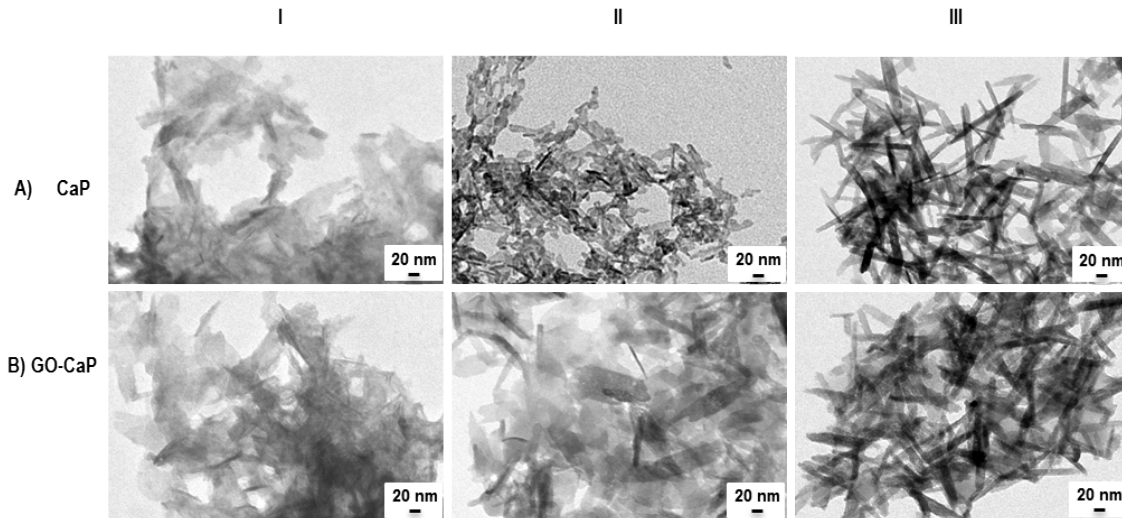


Figure S2: TEM images of CaP nanoparticles (A), and GO-CaP nanocomposites (B) at freshly made (I), 1 week (II) and 2 weeks (III) after fabrication. Scale bars indicate 20 nm.

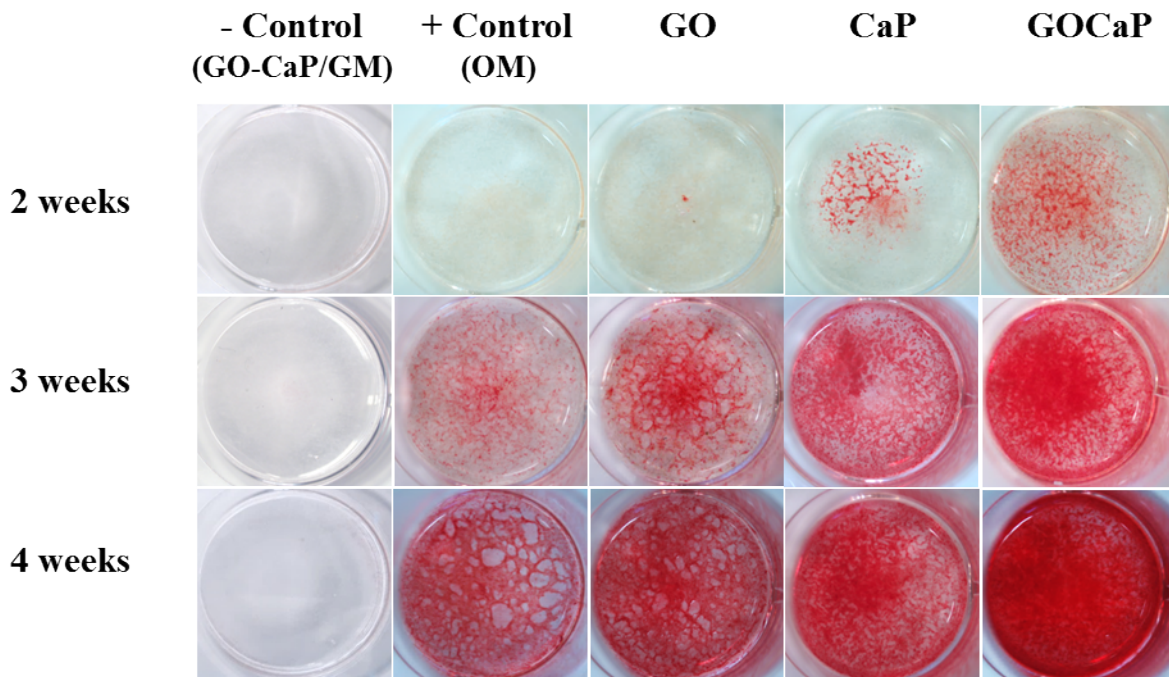


Figure S3: White light images of Alizarin red stained cell cultures after 2, 3, and 4 weeks treatment with GO, CaP, and GO-CaP, the nontreated positive controls (in OM) and the negative controls with GO-CaP in GM. Calcium deposits are stained in red in the images.

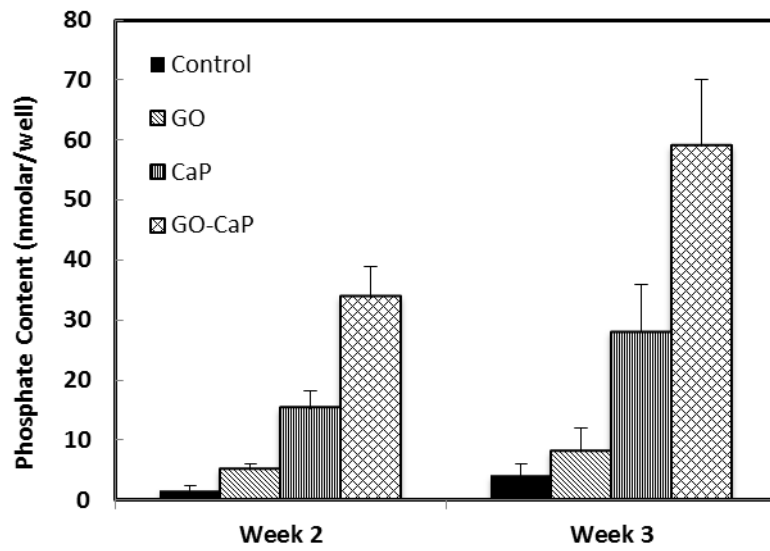


Figure S4: Phosphate deposition in hMSC cultures with GO, CaP and GO-CaP treatments in OM, and with OM only (control) after 2 and 3 weeks of treatments.