Supplementary Information

Synergistic osteogenesis promoted by magnetically actuated nano-mechanical stimuli

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Characterization of magnetic nanoparticles

FT-IR characterization and thermal stability

Fourier-transform infrared spectroscopy (FT-IR) was measured on a Bio-Rad Win-IR spectrophotometer (Watford, UK) using the potassium bromide (KBr) slice method. The amounts of oleic acid (OA) capped on magnetic nanoparticles' surfaces were measured by thermogravimetric analysis (TGA) (TA Instruments TGA500, USA). The samples were heated from 25°C to 800°C at a rate of 10°C /min under N_2

flow.

Crystal structure and morphology Analysis

X-ray diffraction (XRD) patterns of the native samples were obtained using a D8 Advance diffractometer (Bruker Co., Germany) by Cu Kα radiation (λ = 0.154Å). The morphology and size of the magnetic nanoparticles were detected using a FEI Tecnai G2 S-Twin transmission electron microscope (TEM). Carbon-coated duplex copper meshes (Beijing XXBR Technology Co., Ltd) were used to prepare samples. The samples were dispersed in chloroform at a concentration of 0.02 mg/mL by ultrasonication, dripped onto the copper mesh and dried at room temperature. Particle size distributions were evaluated with NIH Image J software. Dynamic light scattering (DLS) measurements were implemented by a Zetasizer Nano ZS90 (Malvern Instruments, Southborough, MA) equipped with a He/Ne ion laser (λ = 633 nm) and 173 degree scattering angle. The nanoparticles were dispersed in chloroform at a concentration of 0.1 mg/mL, and then the sizes of nanoparticles were tested.

Magnetism and dispersibility

The magnetic properties of magnetic nanoparticles and subsequent IO-OA/PLGA nanocomposites were measured using a Quantum Design-MPMS-XL7 vibrating sample magnetometer (VSM) at the temperature of 300 K. The dispersion property of magnetic nanoparticles in chloroform was determined by observing their precipitated phenomenon at different times with a concentration of 100 mg/mL. And the dispersibility of magnetic nanoparticles in the PLGA matrix was determined by AFM and TEM under the same Fe content (2.75%, w/w).

Cytotoxicity measurement

The cytotoxicity of the magnetic nanoparticles was assessed by using extraction liquor of materials in MC3T3-E1 cells and measuring the relative cell growth rate using the Cell Counting Kit-8 (CCK-8) assay. The extraction liquor of 200 mg/mL IO-OA NPs (IO NPs) was obtained by immersing the particles in culture medium for 24 h on a 37°C shaking table according to national standards. The cells were firstly cultured in 96-well tissue culture plates (Costar, Corning, USA) at density of 1×10^4

cells per well for 24 h. Then, the original medium was substituted with successive dilutions of extraction liquor, and the cells were cultured for another 24 h. Osteoblasts cultured in extraction liquor were observed with an optical microscope. Finally, the extraction liquor was replaced with 100 μL fresh medium containing 10% CCK-8 (7sea biotech). After 2 h incubation, the optical density (OD) at 450 nm was measured with a Multifunction microplate scanner (Infinite M200, Tecan, Switzerland). The culture medium without extraction liquor was used for a control experiment, and results were averaged over three parallel samples. The images of osteoblasts cultured with different particles' original leaching liquor were obtained by optical microscope. In addition, the viability of cells exposed to different concentrations of nanoparticles for 24 h was also measured to evaluate the material cytotoxicity. Nanoparticles were dispersed in culture medium by ultrasonication for several hours and then diluted to a final concentration of 1000, 500, 250, 125 or 62.5 μg/ml. These dispersions were used in cell experiments, as described above. The morphology of osteoblasts treated with nanoparticles at a concentration of 62.5 μg/ml was observed under a light microscope.

Mechanical properties

The mechanical properties of the composite scaffolds were obtained using a universal mechanical testing machine (Instron 1121, UK). The compressive elastic modulus and the compression strength of the dry scaffolds were tested at a crosshead rate of 2 mm/min at 25°C room temperature. The compressive elastic modulus was calculated as the slope of the first linear region (5-10%) of the stress strain curve. Five parallel samples were measured for each condition.

Choice of magnetic field intensity

The primary establishment of magnetic field intensity was based on previous studies. Moderate-intensity SMF (1 mT ~ 1 T) have been identified as capable to affect biological behavior of multiple cell lines, including MC3T3-E1 cells [J. Dent. Res., 2003, 82, 962 - 966; Biomaterials, 2011, 32, 7831 - 7838; Biomaterials, 2018, 183, 151 - 170]. For instance, moderate SMF (72 ~ 144 mT) has been recently applied to osteogenesis of MG-63 cells, and the results showed an improved cell proliferation

and enhanced osteogenic differentiation. [Sci. Rep., 2018, 8, 16270] Qiu et al. have demonstrated that the expression levels of bone-associated genes are significantly upregulated *in vitro*, and a study of rat model of bone defect also indicated the greater ability of SMF (50 ~ 150 mT) to accelerate bone regeneration and osseointegration *in vivo*. [FASEB J., 2019, 33, 6069-6081] On the basis of above reports, 70 ~ 80 mT SMF was employed to conduct the cell experiments.

Biological assessment of nanocomposites

Cytotoxicity analysis

Cytotoxicity of nanocomposites was evaluated using extraction liquor of materials in osteoblasts and measured the cell viability by the CCK-8 assay. The extraction liquor of 6 cm^2/mL IO-OA/PLGA nanocomposites with various IO-OA NPs mass contents (0%, 0.5%, 1%, and 5% w/w) as prepared were obtained and used to process cell experiments according to aforementioned method.

Cell adhesion and proliferation assays

The thin films of materials on glass coverslips (d = 14 mm, 30 mm) were prepared by traditional experiment method in our laboratory. Briefly, the round siliconized cover slides were prepared by dealing with 2% trimethylchlorosilane/$CHCl_3$ (v/v) solution, and then calcined for 4 h at 180°C. IO-OA /PLGA nanocomposites with different IO-OA NPs contents (0%, 0.5%, 1%, 5% w/w) were dissolved in chloroform with a concentration of 3% (w/v). Thereafter, 8 μL or 15 μL solution was added dropwise to a siliconized glass coverslip to form a thin film by evaporation for 3 h in the air and drying under vacuum for 48 h at 25°C. The cover slides coated with the composites were placed on 24-well (6-well) culture plates (Costar), and then sterilized by 75% alcohol along with UV for 2 h. After PBS (pH = 7.4) washing for three times, 2×10^4 (10×10^4) MC3T3-E1 cells were seeded into each well of the 24-well (6-well) plate containing the coated cover slides and cultured with DMEM at different times with and without SMF. The medium was replaced by fresh culture medium every 2 days. After the cells were cultured for fixed time, 30 μL CCK-8 was added into each culture well. Then, the cells were incubated

for another 2 h. Subsequently, 200 μL solution was transferred to 96-well culture plate, and further measured by a Multifunction microplate scanner at 450 nm. PLGA was used as control group, and results were averaged over three parallel samples.

Cell morphology assays

Cell attachment and morphology were investigated using fluorescence isothiocyanate (FITC, Sigma) staining after the cells were incubated for 0.5, 1, and 4 days with and without SMF. The staining method was performed as follows: The cells on each well were fixed with 4% paraformaldehyde (PFA) solution for 15 min at room temperature, and rinsed three times with PBS. Then, the cells were stained with FITC (0.5 mg/mL in DMSO) for 10 min at 37°C and then washed repeatedly. Subsequently, nuclear staining was executed using 4, 6-diamidino-2-phenylindole (DAPI, Sigma, USA). The cells were cultured in 2 μL/mL DAPI/PBS staining solution for 1 min at room temperature, and then washed with PBS for three times. Finally, the morphology of the cells was observed under a Fluorescence reverse microscope (TE-2000U, Nikon, Japan).

ALP activity assay

Alkaline phosphatase (ALP) staining was applied to assess the activity of ALP in MC3T3-E1 cells grown on different substrates based on the manufacturer's instructions. When cultured for 10 days with and without SMF, the cells were washed three times with PBS, fixed with 4% PFA for 15 min, and washed with PBS again. Thereafter, the cells were immersed in 500 μL ALP dye (Beyotime Biotechnology, Inc) for at least 12 h at room temperature under dark conditions. The extra ALP dye was washed away with PBS and then the purple color intensity was observed by a light microscope (TE2000U, Nikon).

Alizarin red staining for calcium deposition

Alizarin red staining was applied to evaluate mineral deposition in MC3T3-E1 cells cultured on different materials based on a previously published protocol. When incubated for 14 days and 21 days with and without SMF, the cells were washed three times with PBS, fixed with 4% PFA for 15 min, and washed with PBS again.

Afterwards, the cells were immersed in 1% (w/v) Alizarin Red S (ARS, Sigma) solution for 30 min at 37°C. The excess ARS dye was flushed out with PBS and the content of calcium-rich deposits was qualitatively evaluated according to the red color intensity observed by a light microscope (TE2000U, Nikon). Calcium quantification was executed using 10% cetylpyridinium chloride (CPC) solution. ARS stained membrane films were rinsed with PBS and then treated with 1 mL CPC solution for 1 h to desorb calcium ions. Absorbance was measured at 540 nm by a multifunction microplate scanner.

Quantitative real-time polymerase chain reaction

MC3T3-E1 cells cultured on various substrates were incubated for 14 days with and without SMF. The expression of osteogenesis-related genes was quantitatively evaluated via real-time PCR. The total RNA of the cells cultured on different substrates was extracted using TRIzol Reagent (Invitrogen, Thermo Fisher, USA) on the basis of the manufacturer's manual. The purity and concentration of RNA were assessed by Nanodrop Plates (Infinite M200, Tecan, Switzerland). The mRNAs of all the samples were reversely transcribed according to the description in Prime Script RT Reagent Kit with gDNA Eraser RR047A (TaKaRa, Japan). The expression of osteogenesis-related genes was quantified using SYBR Premix Ex Taq RR420A (TaKaRa, Japan). Gene-specific primers containing glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alkaline phosphatase (ALP), osteocalcin (OCN), bone morphogenetic protein 2 (BMP2) and Piezo type mechanosensitive ion channel component 1 (Piezo1) were designed by the primer design software of beacon 5.0 as shown in Table S1. Real-time PCR analysis was implemented using Stratagene Mx3005P Real-time PCR System (Agilent Technologies Inc., USA) and the gene expression levels were acquired by the threshold cycles (Ct). Relative transcript quantities were calculated through using the ΔΔCt method. GAPDH was used as a reference gene and was amplified as well as the target genes from the same cDNA samples. The difference of the Ct value between the sample and GAPDH was defined as the ΔCt. The difference in the ΔCt of the cells grown on the experimental groups

relative to the control group cells was defined as the ΔΔCt. The fold change in gene expression was expressed as 2-ΔΔCt.

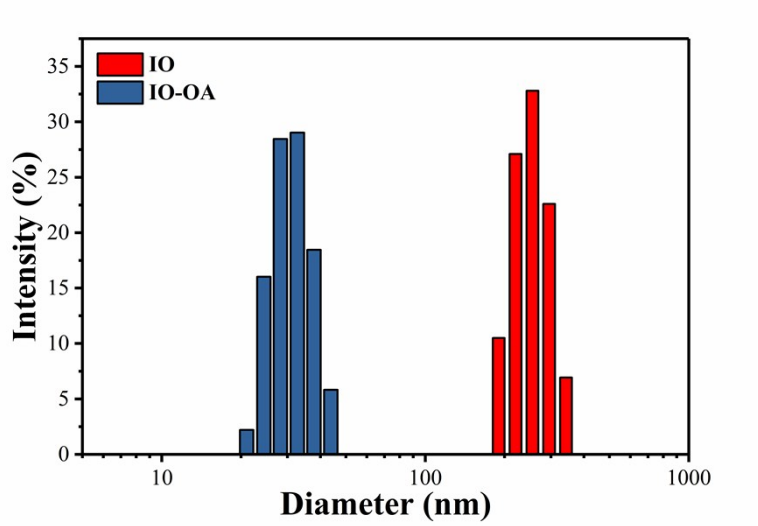

Fig. S1 Size distribution histograms of IO and IO-OA NPs measured in chloroform.

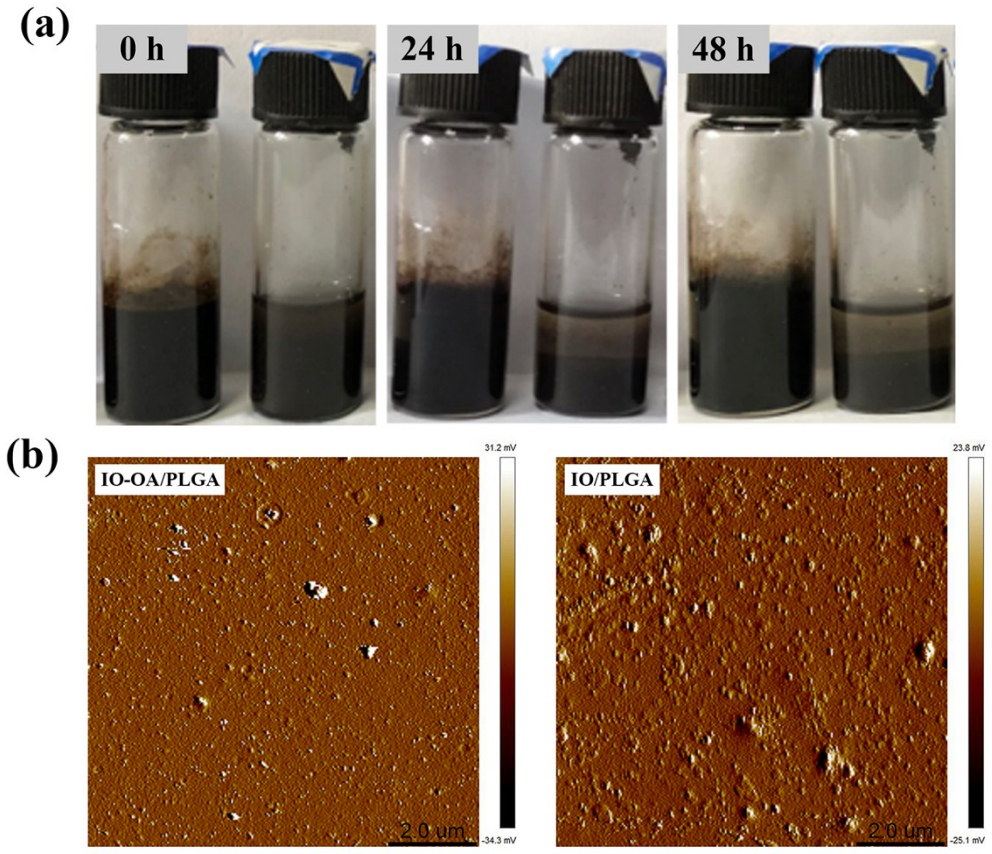

Fig. S2 (a) Images of IO-OA (left) and IO (right) in chloroform at different times after ultrasonication. (b) AFM images of IO-OA/PLGA and IO/PLGA films under the same Fe content (2.75%, w/w). Scale bar: 2 µm.

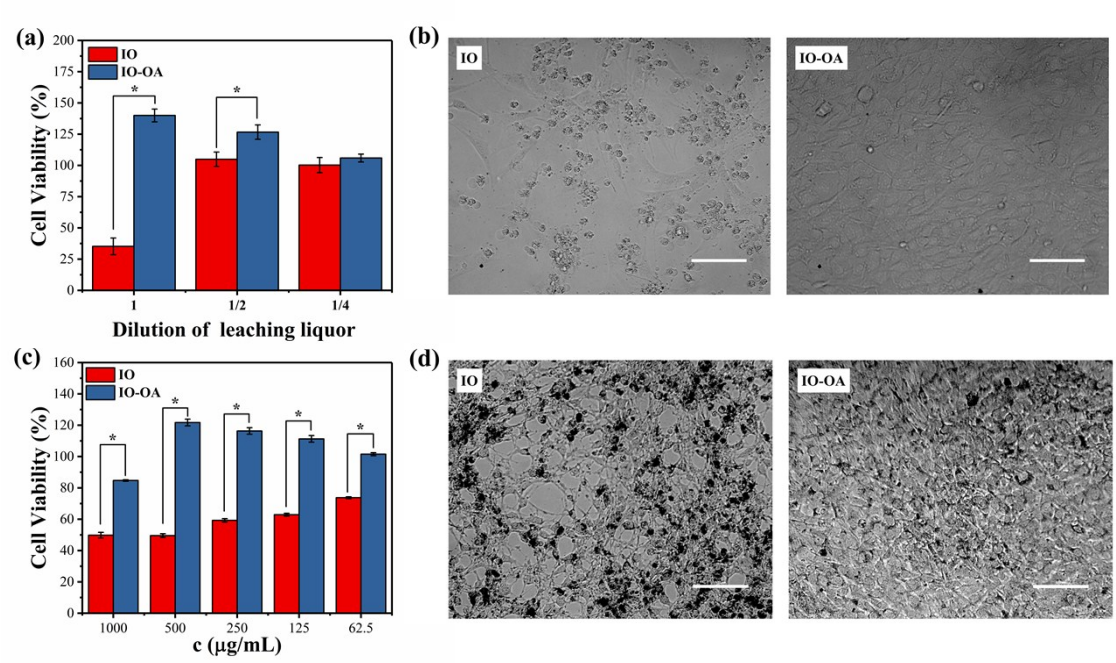

Fig. S3 (a) Cytotoxicity of magnetic nanoparticles' leaching liquor on MC3T3-E1 cells detected by the CCK-8 assay. (b) Light microscopy images of osteoblasts cultured in the presence of different particles' original leaching liquor for 24 h. Scale bar: 100 μm. (c) Viability of MC3T3-E1 cells after being exposed to nanoparticles for 24 h with different concentrations. (d) Light microscopy images of osteoblasts incubated with nanoparticles at a concentration of 62.5 μg/ml. Scale bar: 100 μm. * indicates statistically significant difference ($p < 0.05$).

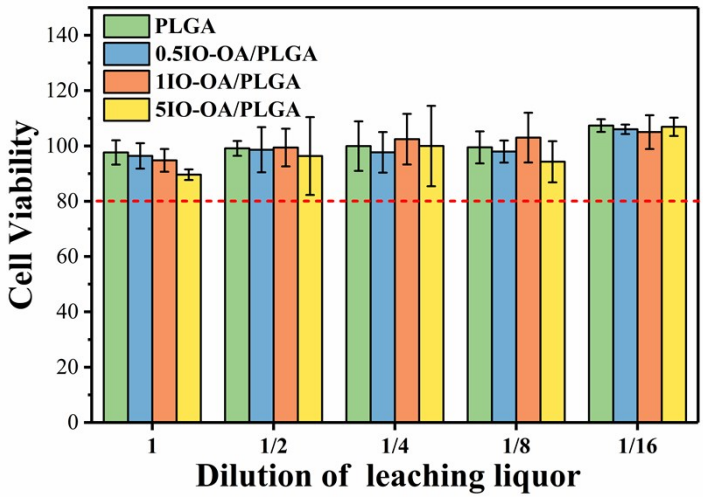

Fig. S4 Cytotoxicity of IO-OA/PLGA nanocomposites' leaching liquor on osteoblasts detected by the CCK-8 assay.

Table S1. Primary sequences of GAPDH, ALP, OCN, BMP-2 and Piezo1

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
GAPDH	TGAACTAACACAGAGGAGGATCAG	GCTTAGGGCATGAGCTTGAC
ALP	CCAGAAAGACACCTTGACTGTGG	TCTTGTCCGTGTCGCTCACCAT
OCN	AAGCAGGAGGGCAATAAGGT	TTTGTAGGCGGTCTTCAAGC
BMP2	GAAGCAAGGTGTCTCCAAG	TCCGCTGTTTGTGTTTCG
Piezo1	ATCGCCATCATCTGGTTCCC	TGGGGTGTGAATGGCACAAT