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Facet-dependent of Fe_3O_4 for Enhancing Osteogenic Differentiation of BMSCs

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The synthesis of Fe_3O_4 nanoparticles

The flower-like Fe_3O_4 nanoparticles were fabricated *via* a simple solvothermal process. Briefly, the 0.875 g sodium dodecyl sulfate (SDS, Keshi, China) and 7 mmol $FeCl_3 \cdot 6H_2O$ (Keshi, China) were dissolved together into 70 mL ethylene glycol (Keshi, China) with ultrasonication for 15 min. And then 5.25 mL 2-Aminoethanol (Keshi, China) was added into the solution with magnetic stirring vigorously for 30 min. Whereafter, the solution was transferred into a 100 mL Teflon-lined autoclave for 12 h at 200 ℃ and then cooled naturally at room temperature. The black product was washed with ethanol and ultrapure water for several times and freeze-dried for further use. With the same procedure, the spherical Fe_3O_4 nanoparticles could be obtained with 10 mmol $FeCl_3 \cdot 6H_2O$, 6 mL 2-Aminoethanol and 70 mL ethylene glycol.

Characterization of Fe_3O_4 nanoparticles

The phase structure of particles was obtained by X-ray diffraction spectra (XRD, Shimazu XRD-6100, Cu-Kα radiation, $\lambda = 1.5406$ Å) operating from $2\theta = 10°$ to 80° at a scan rate of 5° min^{-1}. The morphology was obtained using transmission electron microscope (TEM, JEOL JEM-2100 F) with an accelerating voltage of 200 kV. Raman spectra were obtained using a Raman spectrometer (Horiba LabRAM HR Evolution) and laser wavelengths of 532 nm were used with output laser power of 400 μW or 100 mW. X-ray photoelectron spectroscopy (XPS) analysis was performed on a Thermo Scientific K-Alpha electron spectroscopy using an Al anode as the excitation source. The magnetic property was assessed with a vibrating sample magnetometer (VSM, LakeShore7404) at room temperature and the magnetic field ranged from -2T to 2T. After the samples were degassed at 120 ℃ for 8 h, the specific surface area of samples was analyzed by the ASAP2460 automatic physical adsorption instrument.

Animal Experiment

All animals were purchased from Charles River Laboratories. Mice were acclimatized to the environment of the animal facility for at least 7 days prior to the experiments. The animal experiments were carried out in compliance with the guidelines of the Institutional Animal Care and Use Committee of Army Medical University, which oversees conformity with national law (Regulations on the Administration of

Laboratory Animals). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Army Medical University (AMUWEC20226282).

Haemolytic Analysis

The whole blood from Balb/c mice were isolated into centrifuge tube wetted by heparin sodium and then were centrifuged at 1500 rpm for 10 min. The erythrocytes were collected and washed with saline for 3 times. Then 40 μL of erythrocytes were added into 960 μL of PBS containing 100 μg Fe_3O_4 nanoparticles. PBS and ultrapure water were used as negative and positive controls. After incubated at 37 ℃ for 120 min, the absorbance of supernatant at 540 nm was measure and the hemolysis rate was calculated by the equation (1):[1]

$$hemolysis\ (\%) = (A_S - A_n) / (A_p - A_n) \times 100\% \quad (1)$$

where A_s, A_n and A_p represent the absorbance of the supernatant after incubating the erythrocyte with Fe_3O_4 PBS and ultrapure water, respectively.

Isolation and culture of bone marrow mesenchymal cells (BMSCs)

The BMSCs were isolated from Sprague Dawley rat (100 g).[2] Firstly, the tibias and femurs of rats were isolated after sacrificed with overdose of sodium pentobarbital. And then the muscles, ligaments and excess tissue on the bone were discarded. Subsequently, the medullary cavities were flushed with DMEM/F-12 (Gibco) medium supplemented with fetal bovine serum (FBS, 10% v/v, Gibco) to obtain BMSCs. The isolated cells were cultured on culture dishes at 37 °C in a humidified, 5% CO_2 atmosphere and the non-adherent cells were washed out with PBS after 24 h. The adherent cells were BMSCs and regarded as passage 0 (P0). The cells were harvested at appropriate confluence by 0.25% (w/v) trypsin ethylene diamine tetra-acetic acid (EDTA) solution.

Quantitative real-time PCR (qRT-PCR)

The BMSCs in P2 - P4 were seed in 6-well plate (10^5 cells per well) and cultured for 24 h. Then Fe_3O_4 nanoparticles were added with final concentration of 100 μg/mL and the group without Fe_3O_4 nanoparticles was identified as control. The culture medium was changed every 2 days. After incubation for 7 days, total RNA was extracted from

cells using Trizol (Takara, Japan). cDNA was obtained using All-In-One 5X RT MasterMixTM (ABM, China) and qRT-PCR test was conducted using the BlasTaqTM 2X qPCR MasterMix (ABM, China). The quantification of gene expression was performed with $2^{-\Delta\Delta Ct}$ method. The primer sequences used were listed in Table S1.

Alkaline phosphatase (ALP) staining

Stem cells were seeded in 24-well plate (3×10^4 cells per well) and treated as described for qRT-PCR test. After incubation for 10 days, cells were fixed by paraformaldehyde (Beyotime, China), washed with PBS and stained by ALP kit (Solarbio, China).

Calculation details

Spin-polarized density functional theory (DFT) calculation was carried out using the Perdew-Burke-Ernzerhof (PBE) generalized gradient approximation (GGA) functional with CASTEP code in materials studio. The energy cutoff was set to 500 eV. And the convergence criteria were maximal force of 0.05 eV/Å, energy change of 2×10^{-5} eV/atom, maximal stress of 0.1 GPa and maximal displacement of 0.002 Å. Monkhorst-Pack grid k-points of $1 \times 2 \times 1$ were used to calculate the electronic structure. The absorption energy (E_{ad}) was calculated by equation (2):[3]

$$E_{ad} = E_{A/F} - E_A - E_F \quad (1)$$

where the $E_{A/F}$, E_A, and E_F are the total energy of amino acid on Fe_3O_4, amino acid and Fe_3O_4 respectively.

To obtain the energy barrier, the linear synchronous transition/quadratic synchronous transit (LST/QST) tools were employed.

Statistical analysis

All experiments were carried out at least in triplicate and the statistical analysis were performed using one-way ANOVA or two-way ANOVA to determine the significance.

Table S1. Primer sequences used for qRT-PCR test.

Gene name	Primer forward	Primer reverse
Runx2	AGTTTGGCAGCTCAGAGGAG	GCTTCTGAGATGGGTCAGGC
Col-1	GCGAAGGCAACAGTCGATTC	ACTGTCTTGCCCCAAGTTCC
OPN	AGTTTGGCAGCTCAGAGGAG	GCTTCTGAGATGGGTCAGGC
18S	CATTCGAACGTCTGCCCTAT	GTTTCTCAGGCTCCCTCTCC

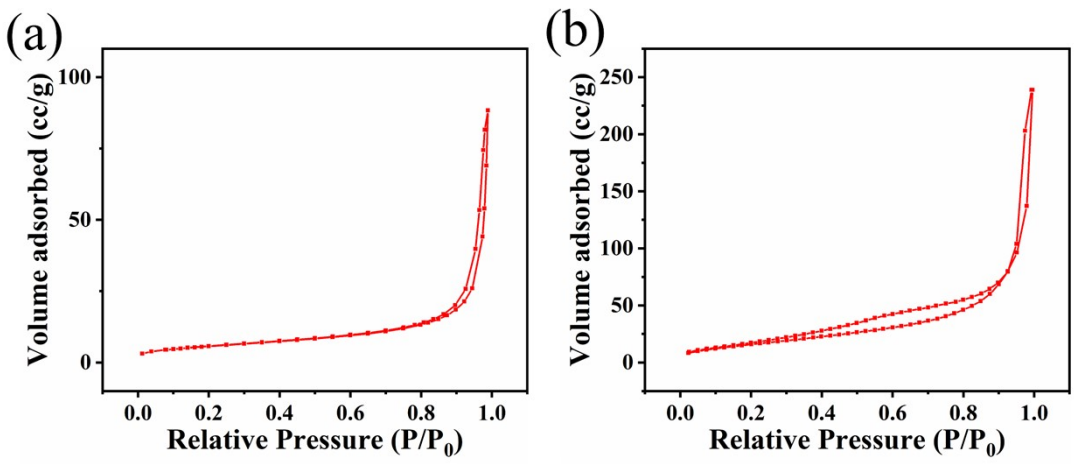

Figure S1. The nitrogen adsorption/desorption isotherms of (a) S-Fe_3O_4 and (b) F-Fe_3O_4.

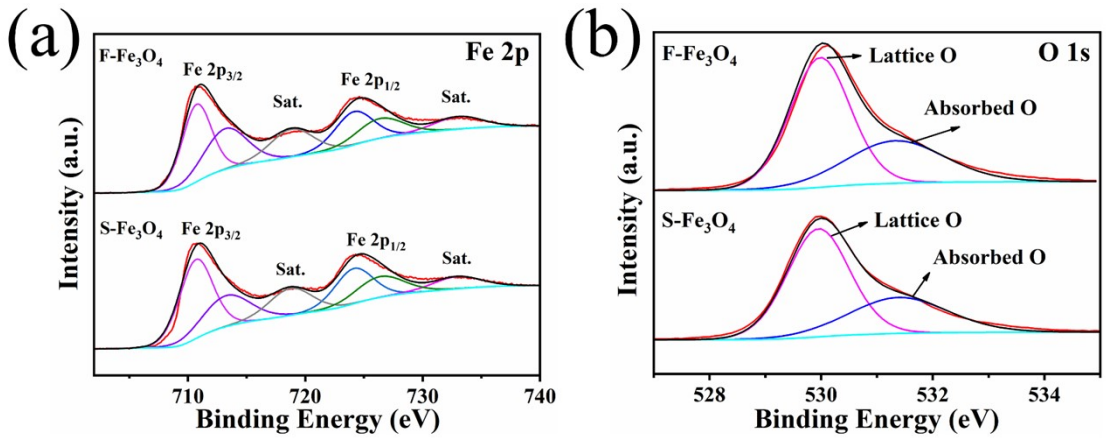

Figure S2. XPS spectra of Fe_3O_4 of (c) Fe 2p and (d) O 1s.

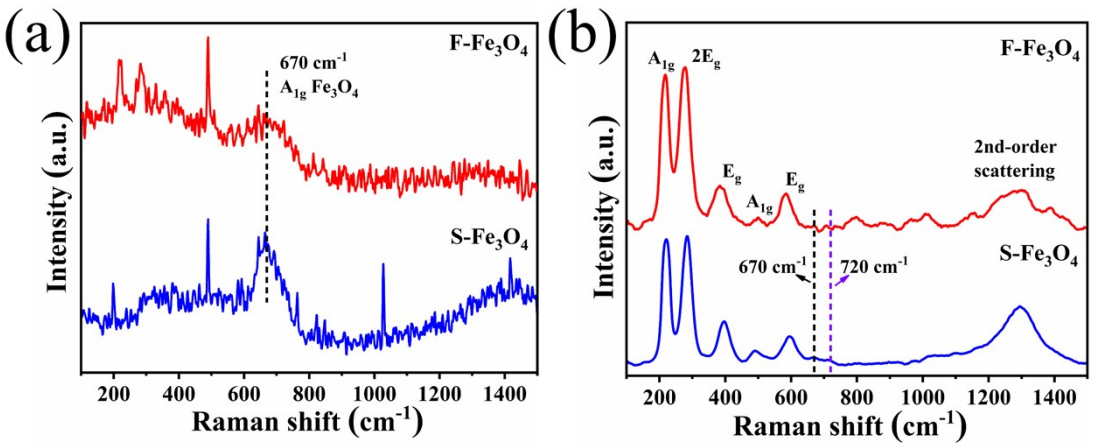

Figure S3. The Raman spectra collected from Fe_3O_4 with output laser power of about (a) 400 μW and (b) 100 mW.

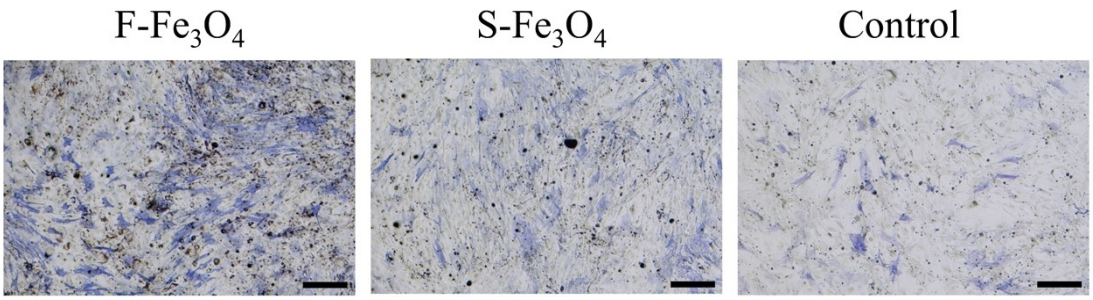

Figure S4. ALP staining (scale bar: 500 μm) images of stem cells cultured in different environments for 10 days.

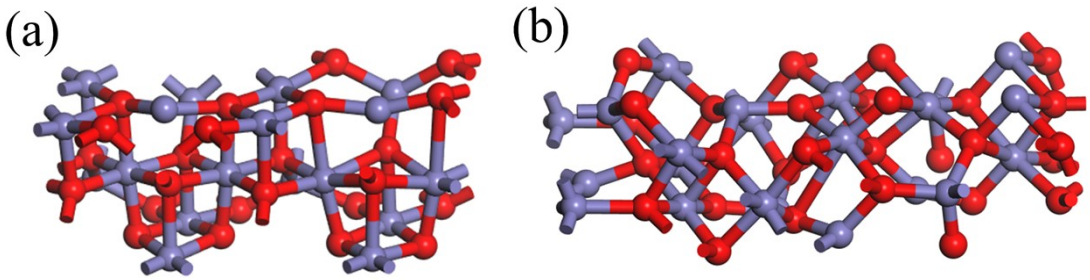

Figure S5. The exposed (a) (400) and (b) $(4\bar{2}2)$ facets of Fe_3O_4. The red and purple atoms are O and Fe atoms.

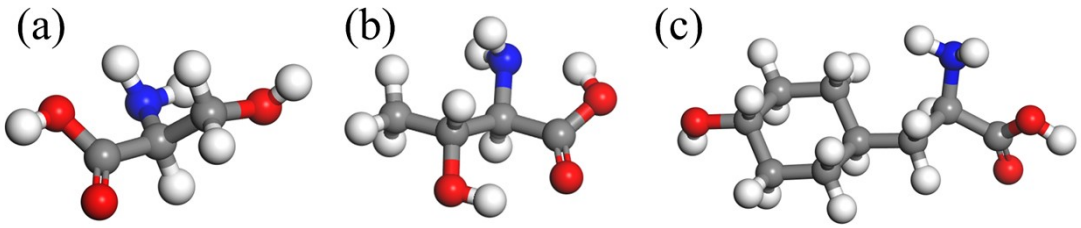

Figure S6. The optimized structure of (a) serine, (b) threonine and (c) tyrosine. The white, grey, red and blue atoms are H, C, O and N atoms.

Reference:

1 D. Sun, X. Pang, Y. Cheng, J. Ming, S. Xiang, C. Zhang, P. Lv, C. Chu, X. Chen, G. Liu and N. Zheng, *ACS Nano*, 2020, **14**, 2063-2076.

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3 F. Wang, L. Mao, X. Wei and J. Mao, *Chem.Eng. J.*, 2022, **450**.