Electronic Supplementary Material (ESI) for Nanoscale.

This supplementary information was **updated** on **15th May 2020.**

Modulated podosome patterning in osteoclasts by fullerenol nanoparticles disturbs the bone resorption for osteoporosis treatment

The previous supplementary information contained errors in Figures S6 and S8. The hematoxylin and eosin (H&E) staining results in these figures were incorrect, and have been replaced. The original images that they replace are copied below for future reference. The captions associated with these images remain unaltered.

Please contact Nanoscale@rsc.org with any inquiries, citing the DOI: doi.org/10.1039/D0NR01625J

Figure S6:

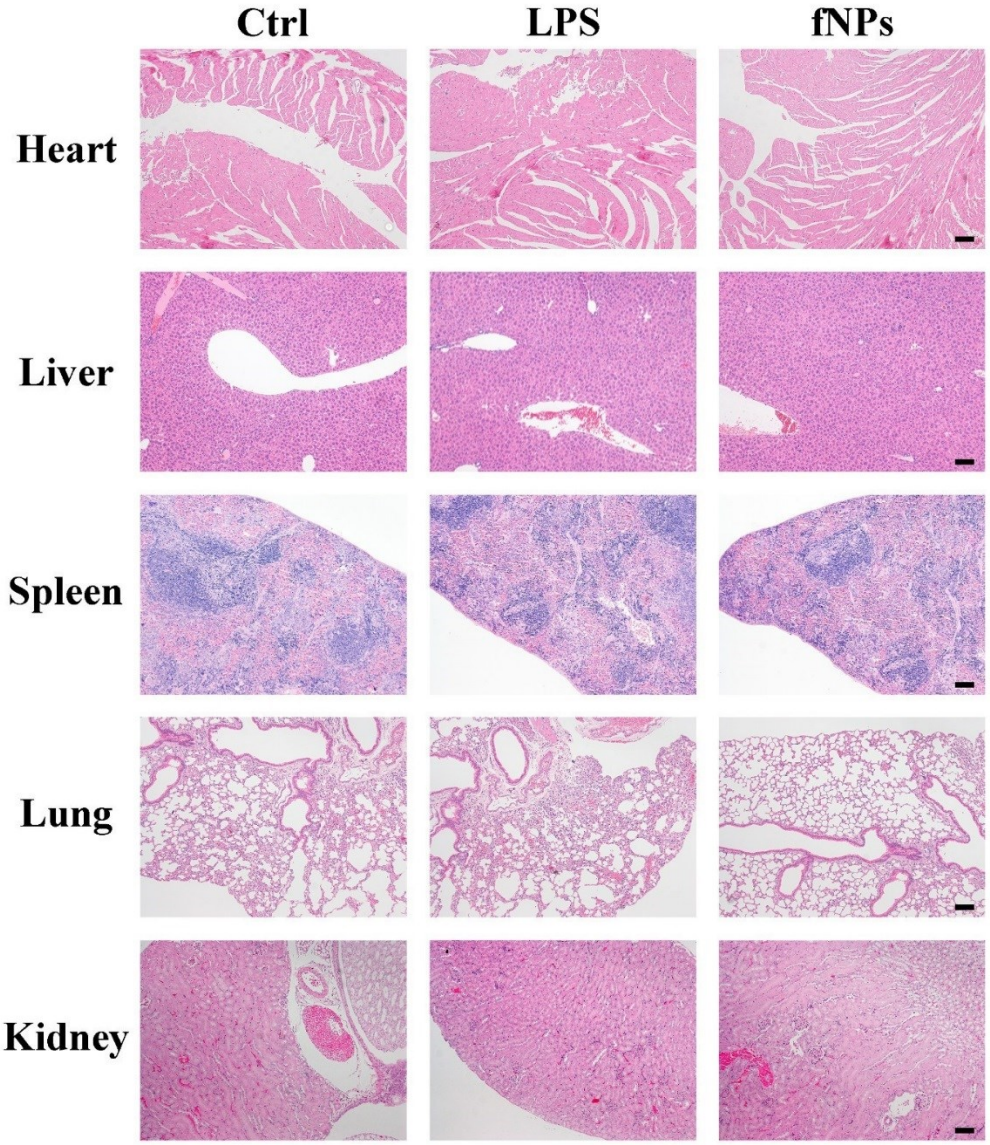
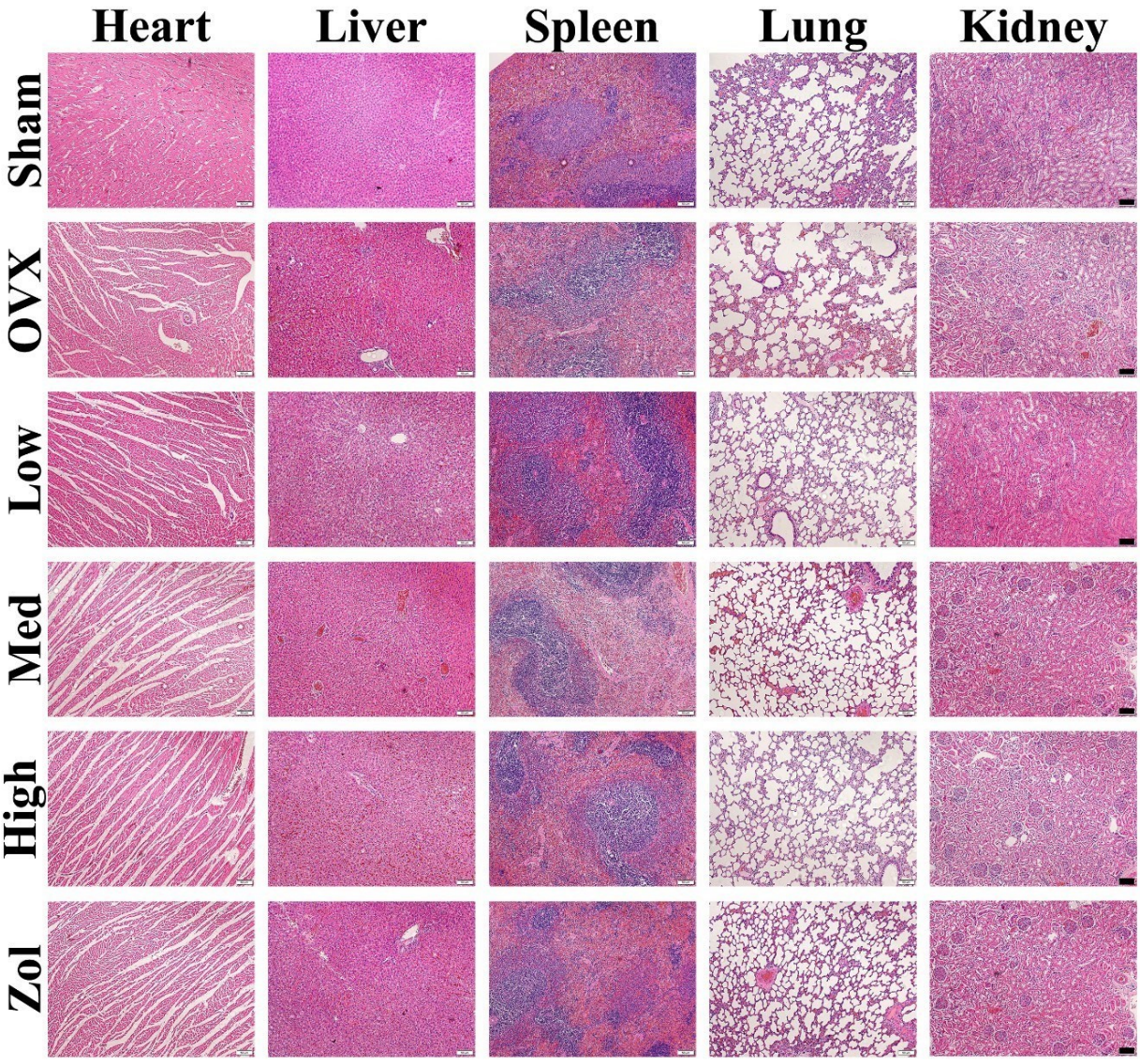


Figure S8:



Supporting Information

Modulated Podosome Patterning in Osteoclasts by Fullerenol Nanoparticles Disturbs the Bone Resorption for Osteoporosis Treatment

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Materials and Methods

Synthesis and characterization of fNPs

fNPs were synthesized by an alkaline reaction with NaOH and 40% tetrabutylammonium hydroxide (TBAH) as catalysts, and purified with liquid phase isoelectric focusing separation as described in our recent work. [1, 2] Briefly, a solution of fullerene in toluene was added with an aqueous solution containing NaOH and 40% TBAH as catalysts. The mixture was stirred at room temperature for up to 24 h. The colour of the solution changed from violet to colourless, while a brown sludge precipitated on the bottom of the beaker. The aqueous phase was separated and evaporated under vacuum to obtain a crude product. The crude product was then washed by methanol and passed through a Sephadex G 25 column and eluted with distilled water.

Particle morphology and size was study by atomic force microscopy (Agilent 5500, USA). The particles were dissolved in phosphate buffered saline containing 10% FBS to evaluate the hydrodynamic size and Zeta potential using a dynamic light scattering spectrophotometer (Brookhaven NanoBrooK Omni, USA) three times for each case.

Bone marrow osteoblasts culture

4-week-old Sprague-Dawley rats were sacrificed and immersed in 75% alcohol for 5 minutes. We extracted primary bone marrow cells from tibiae and femora by bone marrow lavage. Bone marrow derived mesenchymal stem cells (BMMSCs) were isolated after adherent culture for 12-24 h, and cultured at the condition of 37^{o}C with 5% CO_2 in DMEM-high glucose medium supplied with 10% FBS, and the second generation of BMMSCs with the density of 10^6 cells/cm^2 were differentiated into osteoblasts with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 0.1 mM dexamethasone. For fNPs groups, fNPs with various concentrations (1 μg/ml, 10 μg/ml, 50 μg/ml) were added into the culture medium and incubated for 24 h.

Evaluation of OB activity

After continuous culture for 14 days, OBs were fixed with 70% ethanol for 10 min, and then stained with 0.5% alizarin red S in H_2O, pH 4.0, for 5 min at room temperature. After staining, cultures were washed three times with H_2O. To quantify matrix mineralization, the alizarin red S–stained cultures were incubated with 100 mM cetylpyridinium chloride for 1 h to solubilize and release calcium-bound alizarin red into solution. Data are reported as biological replicates at least three independent experiments.

For ALP activity assay, ALP in cell supernatant was tested with an ALP Kit (BC2145, Solarbio, Beijing, China). Data are reported as biological replicates at least three independent experiments.

Bone marrow monocytes (BMMs) culture

For osteoclast differentiation analyses *in vitro*, we isolated primary bone marrow cells from tibiae and femora of 3-4 weeks old C57/BL6 mice as previously described. [3] Briefly, after centrifuged and the precipitated cells were incubated in red blood cell lysis buffer (Beyotime, Haimen, China) for 10 min at room temperature. Then primary bone marrow cells were washed with sterile PBS and collected by centrifugation. Primary bone marrow cells (1×105 cells per cm^2) were plated onto culture dish suspended in culture medium (α-MEM containing 10% heat-inactivated fetal bovine serum, 1% penicillin, and 1% streptomycin) supplemented with 50 ng ml-1 M-CSF (R&D Systems, Minneapolis, MN, USA) for 3 days to obtain BMMs.

Cell viability assay

BMMSCs and BMMs were seeded on a 96-well plate at a density of 5x10^3 cells per well in DMEM-high glucose medium and α-MEM containing 10% FBS, respectively. For BMMs culture, 30 ng/ml M-CSF was added into the culture medium. After culture overnight, various concentrations of fNPs (0, 25, 50, 100 μg/ml) were added into the medium and culture for 24 h. Then the cell viability was detected with a Cell Counting Kit-8 (DOJINDO, Japan). Data are reported as biological replicates at least three independent experiments.

TRAP staining.

BMMs were cultured on 96-well plates (5×10^3 cells per well) in α-MEM containing 10% FBS and 1% Penicillin-streptomycin solution with 30 ng ml^{-1} M-CSF and 50 ng ml^{-1} RANKL (R&D Systems, Minneapolis, MN, USA) for 4 days to generate mature multinucleated osteoclasts. The cells were fixed in 3.7% paraformaldehyde for 10 min and then stained using the TRAP staining kit (387A-1KT, Sigma–Aldrich, St Louis, MO, USA) following the manufacturer's instructions and incubated at 37 °C for 1 h in the dark. The TRAP-positive cells were observed under a light microscope (IX71; Olympus, Tokyo, Japan); and

multinucleated (>3 nuclei) cells were counted as osteoclasts. Data are reported as biological replicates at least three independent experiments.

Bone resorption assay

BMMs (2×10^4 cells per well) were cultured in Corning Osteo Assay Surface 24-well plates coated with calcium phosphate substrate. After the cells are attached, the medium was removed and complete inducing culture medium with or without 50 μg/mL fNPs was added. Culture medium was changed every two days. After 6 days, 10% sodium hypochlorite were added to remove cells and rinsed three times with water. Pictures were captured using light microscope after the well dry. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the percentage of the resorbed bone surface area. Data are reported as biological replicates at least three independent experiments.

Podosome and actin ring staining assay

BMMs (2×10^4 cells per dish) were seeded in glass-bottom dishes (Nest, #801002, Wuxi, China) and incubated in the presence of complete inducing medium with or without 50 μg/mL fNPs for different time. After 1, 2, 3 and 4 days, the cells were washed twice with PBS, fixed in 4% paraformaldehyde for 20 min, and permeabilized with 0.1% Triton X-100 for 15 min at room temperature, respectively. After three washes with PBS, the actin rings were stained with Rhodamine-conjugated Phalloidin (Cytoskeleton, Inc., Denver, CO, USA), and the cell nuclei were stained with 40,6-diamidine-20-phenylindole dihydrochloride (DAPI; Sigma–Aldrich, St Louis, MO, USA) for 20 min. Cells was visualized by confocal laser scanning microscopy (Nikon Ti-E imaging system, Tokyo, Japan).

Cell counting

A cell containing more than 50 individual podosome dots (without cluster) would be counted as cells with podosomes. A cell producing cluster only and producing at least one cluster would be counted as cells with cluster. A cell both containing more than 50 individual podosome dots and cluster would be counted as cells with podosomes and cluster. A cell with peripheral ring would be counted as cells with belt.[4]

Western Blotting

BMMs cells were seeded in cell culture dishes (430165, 35 mm × 10 mm, Corning, USA) for 24 h before the treatment by fNPs (50 μg/mL) or not (control) at day 3 and day 4, respectively. Cells were lysed with RAPI lysis buffer complemented with a protease inhibitor cocktail (P8340, Sigma, USA). After suspending with SDS loading buffer and heating at 95°C for 10 min, 10 μl samples were load onto SDS-PAGE gel and electrotransfer to polyvinylidene fluoride (PVDF) membrane. The PVDF membrane were blocked with TBST supplemented with 5% non-fat dry milk at room temperature for 1 h, and then incubated with primary antibodies at indicated concentrations at 4°C overnight. Primary antibody used were fascin (#ab116772, abcam), 1:10000, α-actinin (#ab108198, abcam), 1:1000, Arp 2 (#ab128934, abcam), 1:1000, cofilin (#ab42824, abcam), 1:2000, β-actin (#TA-08, ZSGB-BIO), 1:1000. After incubation with HRP-linked goat anti-mouse (#7072, CST) or anti-rabbit (#7071, CST) secondary antibody for 1 h at room temperature, PVDF membrane were visualized with ECL chemoluminescence kit (#32106, Thermo Fisher) and imaged using TANON 5200 Multi (TANON, Beijing, China).

Bone erosion assay

All the *in vivo* experiments were performed according to the National Institutes of Health Regulations for the care and use of animals. 5-week-old female BALB/c mice were randomly divided into 3 groups of 7 mice each (Ctrl, LPS, and fNPs group). LPS and fNPs groups were intraperitoneal injected with 5 mg/kg body weight LPS at day 1 and day 4. PBS was injected with the same volume into control group. The therapy was administered with 7 mg/kg b.w. fNPs or same volume of PBS via intravenous injection on every other day for up to 8 d. All mice were sacrificed, and the femurs were fixed in 4% PFA for at least 48 h at room temperature. All samples were scanned with a micro-CT (Quantum GX, PerkinElmer) to analyse the bone-associated parameters. The major organs and decalcifying femurs were further analysed for H&E staining and TRAP staining.

Treatment of osteoporosis

All the *in vivo* experiments were performed according to the National Institutes of Health Regulations for the care and use of animals. 12 weeks old Sprague–Dawley (SD) female rats were used in this study. Five rats received a SHAM-operation exposure without removing the ovaries as SHAM group. Ovariectomy was performed on the other 25 female rats under 2% pentobarbital sodium solution anaesthesia (600 μg/kg; i.p.). All rats were bred for one months after surgery to allow for the development of osteoporosis. One months later, ovariectomized mice were divided into 5 groups of 5 animals each. Then, various treatments were performed with intraperitoneal injection. The SHAM group and the OVX group were administered with saline, which was used as vehicle. The ZOL group were injected with 10 mg of ZOL solution, LOW

group, MID group and HIGH group were injected with fNPs (2.5, 5.0 or 10.0 mg/kg body weight) in 1 ml of saline, respectively. All treatments were performed three times a week. At the end of the study (2 months), all animals were sacrificed and collected major organs and femurs for histological study. All samples were fixed in 4% PFA for at least 48 h at room temperature.

Micro-CT analysis

The femurs were scanned with a micro-CT (Quantum GX, PerkinElmer) to analyse the bone-associated parameters. After image acquisition, all samples were binarized using the same parameters. The following morphometric analysis was carried out using a direct 3D approach: (i) [BV/TV], bone volume fraction, the percentage of the bone tissue volume, indicates the amount of bone; (ii) [BS/TV], bone surface area tissue volume ratio, also known as bone surface area density, can indirectly reflect the amount of bone; (iii) Tb.N, trabecular number; (iv) Tb.Sp, trabecular spacing; (v) Tb.Th, trabecular thickness. Tb.N, Tb.Sp, Tb.Th are all the main indexes to evaluate the spatial morphological structure of bone trabecular.

Histological staining

After fixing with 4% paraformaldehyde (PFA) for 24 h, femur bones continued to be immobilized with 80% ethanol. Then femurs were soaked in a new decalcified solution (phosphate buffer supplied with 12% EDTA) every 2 days for over 4 weeks. After decalcification, femurs were cut into 10 μm section. The slices were staining with haematoxylin and eosin (G1120, Solarbio, Beijing, China), TRAP (387-1KT, Sigma, St Louis, MO, USA), and ALP (G1480, Solarbio, Beijing, China) according to their instructions. For BMP-2 immunohistochemical staining, BMP-2 Rabbit polyclonal antibody (AF0075, Beyotime, Shanghai, China), diluted in a 1:100 ratio, was used to evaluate the activity of osteoblasts.

Nanoindentation *ex vivo*

Nanoindentation experiments were used to evaluate the mechanical properties at the tissue level. The indentations were carried out using Agilent G200 Nanoindenter (Agilent Technologies Inc., Chandler, AZ) at room temperature (24 °C) equipped with a Berkovich diamond tip as described, previously. [5] Briefly, prior to nanoindentation testing, femur samples were mounted and set in low temperature curing epoxy resin and then ground through silicon carbide grinders followed by diamond polishing on cortical surface. Ten indentations were produced on each sample (n=6/group), where neighbouring indents were spaced more than 10 μm apart with a Continuous Stiffness Measurement (CSM) system. The hardness, indentation modulus material properties at the tissue level were determined according to previous. Indentations were made up to a depth of 1000 nm with a loading/unloading rate of 40 mN/min to eliminate surface roughness interference. Bone was assumed to be isotropic with a Poisson ratio of 0.3.

Three-point bending test ex vivo

Three femurs from various groups performed three-point bending test. Three-point bending strength was measured with a constant span length of 20 mm. The press head as well as the support points were rounded to avoid shear load and cutting. The femurs were positioned horizontally with the anterior surface upwards, centred on the supports. The pressing force was directed vertically to the midshaft of the bone. Each femur was compressed with a constant speed of 0.2 mm/min until fracture. The load-time curve was converted into a load-displacement curve. Breaking force *F* was defined as bending load at fracture. Bending stress σ and elastic modulus *E* were calculated according to the following formulas.

$$\sigma = FLc/4I$$
$$E = FL^3/d48I$$

Where *F* is the applied load, *L* is the span of the support points (20 mm), *c* is the half-diameter of midshaft in the load direction, *d* is displacement, and *I* is the second moment of area.

Statistics analysis

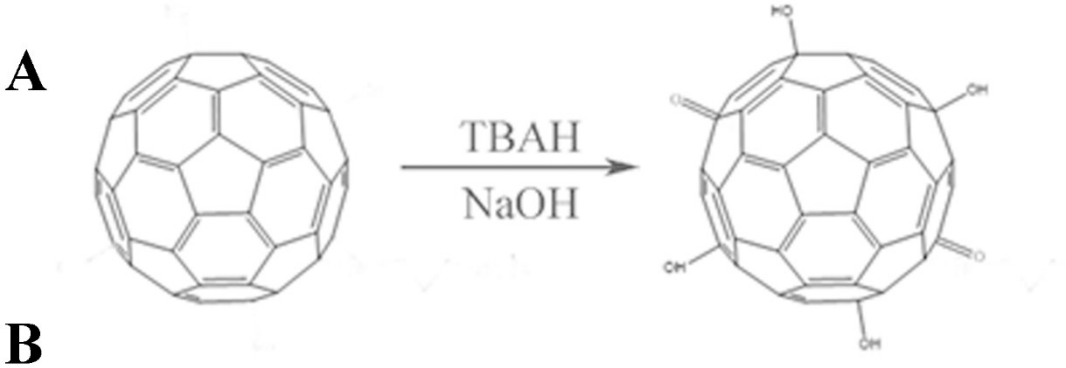
All results were calculated as mean ± SD. Data are reported as biological replicates at least three independent experiments. Data were carried out with the IBM SPSS statistics (Chicago, USA). Differences between groups were analysed using analysis of variance (ANOVA). Statistical significance thresholds were set at *p<0.05; **p<0.01; ***p<0.001.

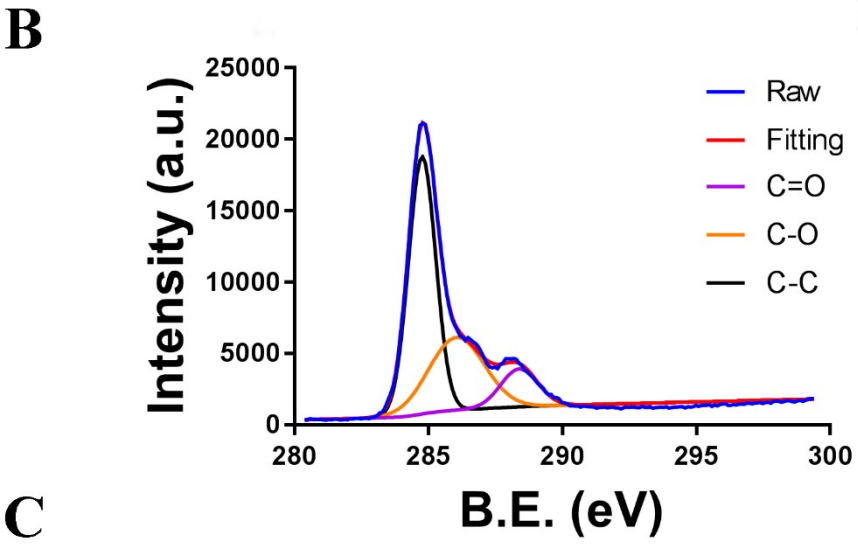
Reference：

1. J. Li, M. Zhang, B. Sun, G. Xing, Y. Song, H. Guo, Y. Chang, Y. Ge and Y. Zhao, Carbon, 2012, 50, 460-469.
2. Y. Qin, K. Chen, W. Gu, X. Dong, R. Lei, Y. Chang, X. Bai, S. Xia, L. Zeng, J. Zhang, S. Ma, J. Li, S. Li and G. Xing, Journal of Nanobiotechnology, 2018, 16, 54.
3. W. Gu, K. Chen, X. Zhao, H. Geng, J. Li, Y. Qin, X. Bai, Y.-N. Chang, S. Xia, J. Zhang, S. Ma, Z. Wu, G. Xing and G. Xing, Small (Weinheim an der Bergstrasse, Germany), 2018, DOI: 10.1002/smll.201802549, e1802549-e1802549.

4. R. Eves, R. Oldham, L. Jia and A. Mak, Cancers, 2015, 7, 96-111.
5. D. Pienkowski, C. Wood and H. Malluche, Osteoporosis International, 2019, 30, 277-285.

Figure s1 the schematic of synthetic process of fullerenol. A solution of fullerene in toluene was added with an aqueous solution containing NaOH and 40% TBAH as catalysts. After stirring at room temperature for up to 24 h. The aqueous phase was separated and evaporated under vacuum to obtain a crude product. The crude product was then washed by methanol and passed through a Sephadex G 25 column and eluted with distilled water. Fullerenols are water-soluble derivatives of fullerenes modified with high hydroxylation. Based on the data of Synchrotron radiation X-ray photoelectron spectroscopy (SR-XPS), functional groups on fullerenol surface include 32.49% hydroxyl (C-O) group and 11.28% carbonyl (C=O) group.





C

Peak	Position	B. E. (eV)	Ratio (%)
C=O	288.385	4583.06	11.28
C-O	286.052	13201.41	32.49
C-C	284.764	22853.83	56.24

Figure s2 ALP, an enzyme specific expressed by OBs, activity was quantified analysis, showing litter effect of fNPs on osteogenic differentiation.

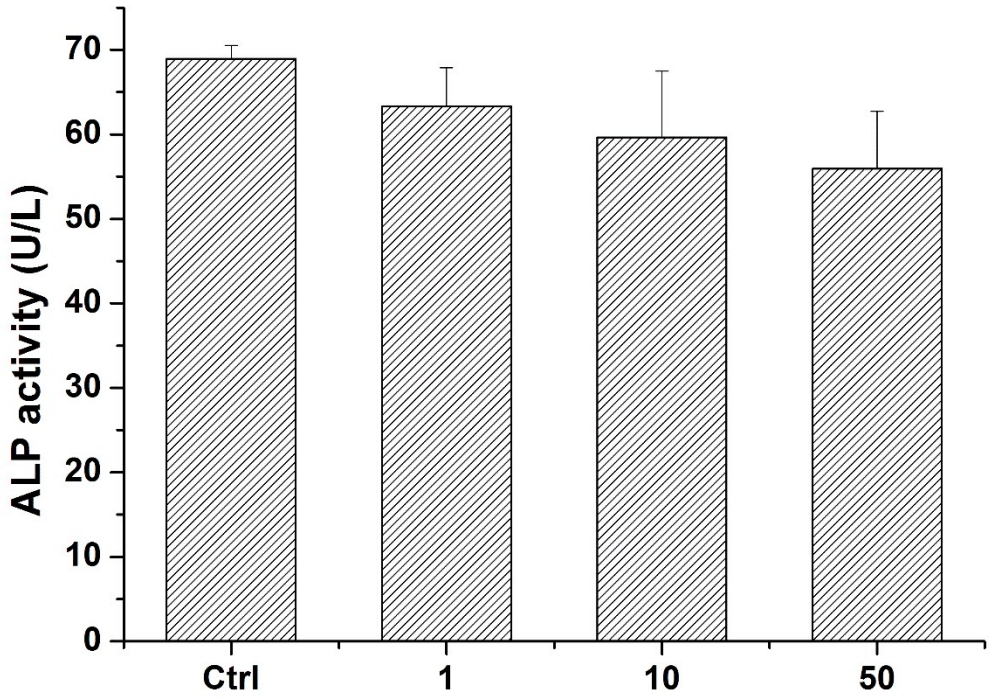


Figure s3 pre-osteoclasts treated with or without fNPs were stained with TRAP, scale bar, 20 μm.

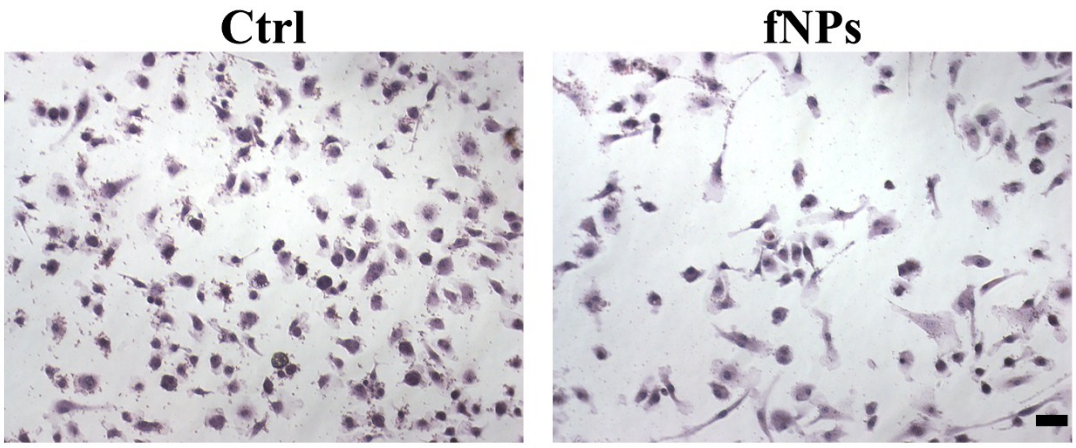


Figure s4 the expression of NFATc1 in pre-osteoclasts with or without treatment of fNPs for 2 days.

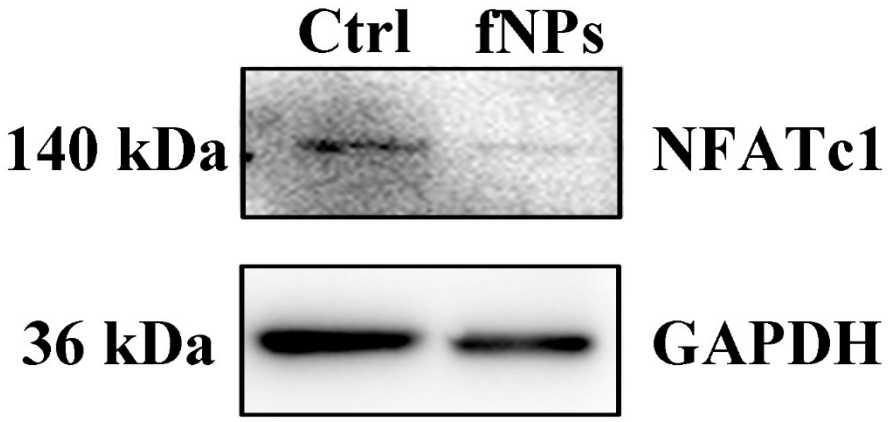


Figure s5 H&E staining of femur bones from the LPS-induced bone erosion mice. Scale bar, 50 μm.

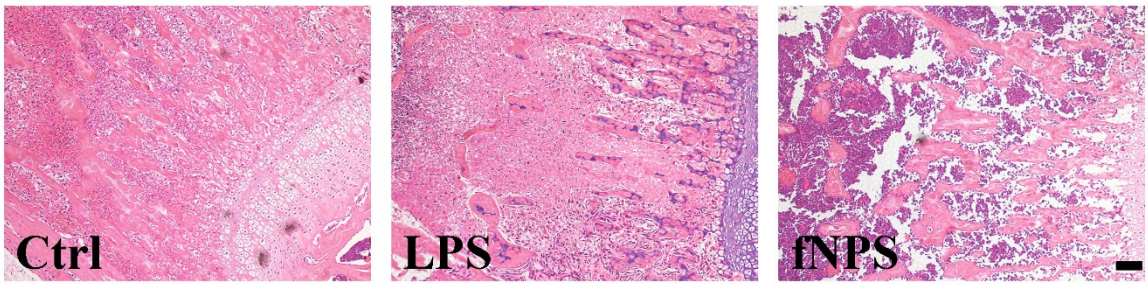


Figure s6 H&E staining of major organs from the LPS-induced bone erosion mice. Scale bar, 50 μm.

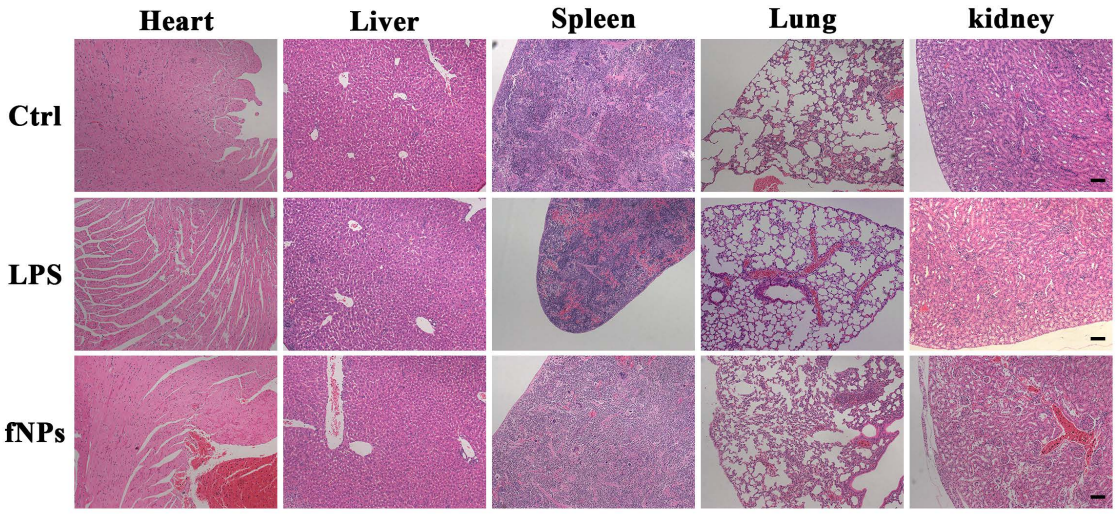


Figure s7 ALP staining of femur bone from ovariectomy-induced osteoporosis rats with different treatments. Scale bar, 50 μm.

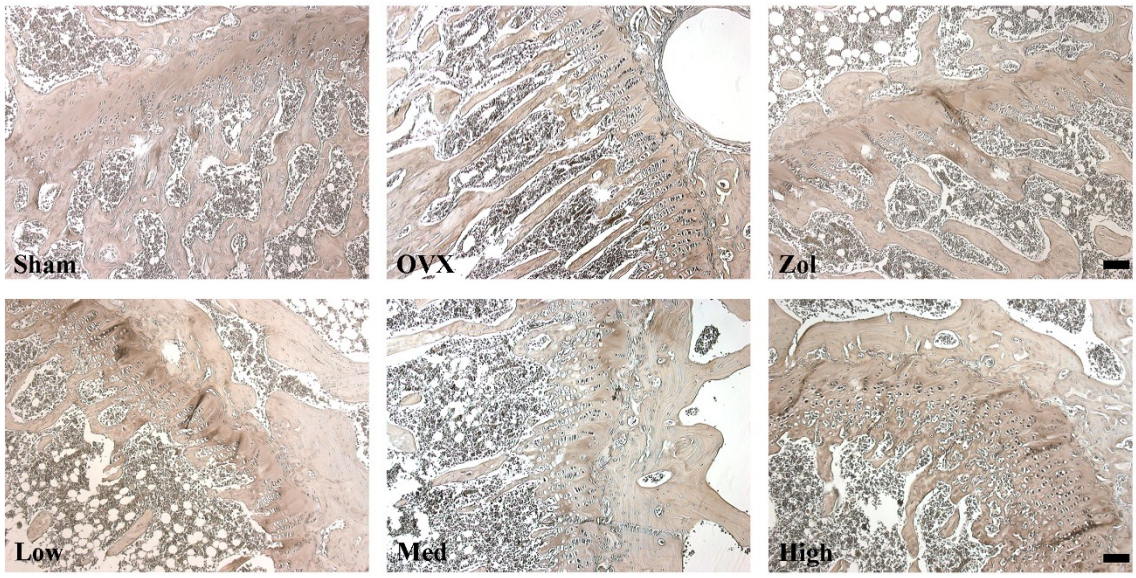


Figure s8 H&E staining of major organs from ovariectomy-induced osteoporosis rats with different treatments. Scale bar, 50 μm.

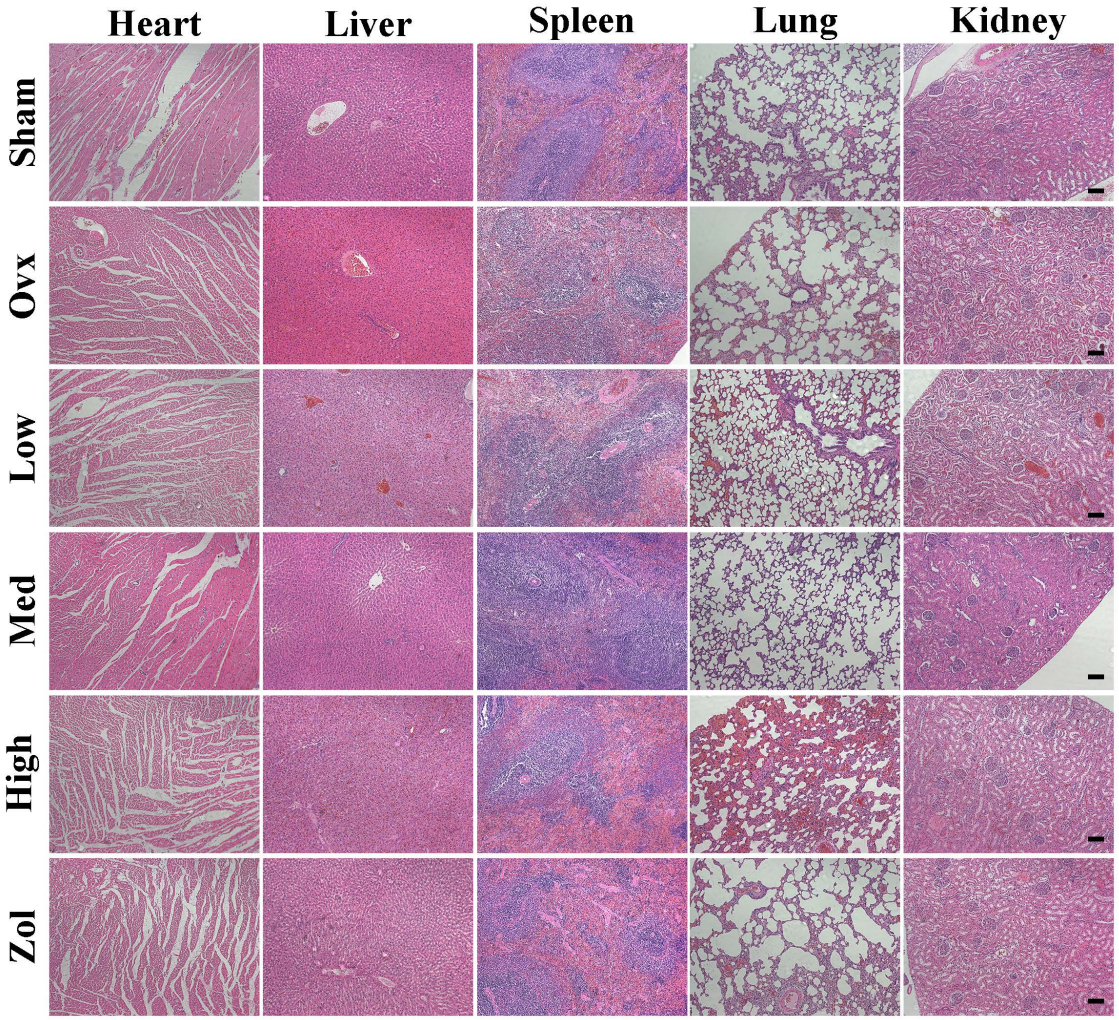


Figure s9 The bending stress from three-point bending test. Compared with Ovx, treatments of fNPs and Zol increased the bending stress significantly.