Supporting Information

Lysosome-Controlled Efficient ROS Overproduction against Cancer Cells with High pH-Responsive Catalytic Nanosystem

*Jingke Fu, Yiran Shao, Liyao Wang and Yingchun Zhu**

Key Lab of Inorganic Coating Materials, Shanghai Institute of Ceramics, Chinese Academy of Sciences, Shanghai 200050, China.

* To whom correspondence should be addressed:
Tel.: 86-21-52412632; Fax: 86-21-52412632; E-mail: yzhu@mail.sic.ac.cn.

Experimental section

Materials

Cetyltrimethyl ammonium bromide (C_{16}TAB), tetraethyl orthosilicate (TEOS), Fe_3O_4 NPs (*ca.* 20 nm) and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Aladdin. Titanium potassium oxalate ($K_2TiO(C_2O_4)_2 \cdot 2H_2O$), hydrogen peroxide ($H_2O_2$, 30%) and potassium ferricyanide were purchased from Sinopharm Chemical Reagent Co., Ltd. Potassium ferrate (K_2FeO_4), 3-aminopropyltriethoxysilane (APTES), fluorescein isothiocyanate (FITC), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were from Sigma. Phosphate buffered saline (PBS, pH 7.4), RPMI-1640 medium, trypsin and fetal bovine serum (FBS) were obtained from Gibco. All chemicals were used as received without further purification. Milli-Q water was used in all experiments.

Synthesis of FeOx-MSNs

Firstly, mono-dispersed mesoporous silica nanoparticles (MSNs) were prepared according to the literature procedure.[1] The obtained C_{16}TAB-containing product was referred as MSNs-C. Afterwards, FeO_x species were *in situ* introduced into the mesopores of MSNs by using K_2FeO_4 as the oxidant and iron precursor. Briefly, the MSNs-C (0.5 g) was suspended into PBS (pH~12, 50 mL) under ultrasonic treatment. Then K_2FeO_4 (0.005, 0.01 or 0.02 mol L^{-1}) was added into the suspension. After stiring for 12 h in the dark, the solid was collected by centrifugation, washed with water and enthanol, and free dried. Finally, the dry solid was calcined in air and in mixed H_2/Ar atmosphere to obtain FeO_x-MSNs-*n*, where *n* is the initial K_2FeO_4 content (mol L^{-1}) in the reaction solution.

Catalytic Decomposition of H_2O_2

Hydrogen peroxide (H_2O_2, 5 mM in PBS with pH 7.4 or 5.0) was mixed with FeO_x-MSNs or commercially available Fe_3O_4 NPs (*ca.* 20 nm) in sealed flasks. The flasks were kept at ambient temperature and left incubated in a shaking table in the dark. At specific time-points, aliquots of the mixture were withdrawn and filtrated. The H_2O_2 concentration was then measured by spectrophotometer. Briefly, the filtrated sample was diluted and mixed with titanium potassium oxalate solution (50 μM, 0.5 mL) under acidic condition. The UV-Vis absorbance of the mixture

was recorded at 400 nm after 10 min. The H_2O_2 concentration was determined according to the standard curves (H_2O_2 concentration: 25-40 μg mL^{-1}, λ_{max} = 400 nm, r = 0.9997).

Catalytic Oxidation of TMB

FeO_x-MSNs were added to the H_2O_2 solution (530 mM in PBS with pH 7.4 or 5.0) in the presence of 3,3',5,5'-tetramethylbenzidine (TMB, 816 μM). The mixture was incubated at 37 °C for 30 min. The reaction was then stopped by adding H_2SO_4 (0.5 M, 200 μL). H_2O_2 solution (530 mM in PBS with pH 7.4 or 5.0) in the presence of TMB (816 μM) without samples was used as the control.

TPA-Na for •OH Detection

To explore the catalytic decomposition of H_2O_2 by FeO_x-MSNs, sodium terephthalate (TPA-Na), which is a well-known probe for •OH detection, was added to the H_2O_2 solution (5 mM in PBS with pH 7.4 or 5.0) with or without FeO_x-MSNs. After a given time-course, the sample was withdrawn, filtrated, and measured by fluorescence spectrometer (FluoroMax-4, HORIBA, USA).

Cell Culture

Human breast carcinoma ZR75-30 cells were purchased from cell bank of Chinese Academy of Sciences, and cultured in RPMI-1640 medium (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 ℃ in a humid atmosphere with 5.0% CO_2.

Fluorescence Labeling of FeO_x-MSNs with FITC

FITC (10 mg) was mixed with APTES (400 μL) in ethanol under dark condition for 24 h to obtain FITC-APTES. Then, FeO_x-MSNs (50 mg) were dispersed in absolute ethanol and sonicated for 30 min, followed by adding FITC-APTES (3 mL). After stirring for 24 h in the dark, the FITC-conjugated nanoparticles (FeO_x-MSNs-FITC) were collected, washed with ethanol for several times, and freeze dried in the dark.

Fluorescence Microscopy Observation of Intracellular Uptake of FeO_x-MSNs

ZR75-30 cells were plated in Petri dishes (Corning, NY) at 1.0×10^4 cells mL^{-1} and cultured overnight at 37 ℃. After incubation with FeO_x-MSNs-FITC (50 μg mL^{-1}) for 4 h, cells were washed with cold PBS (pH 7.4) followed by staining with DAPI (Cell Apoptosis DAPI Detection Kit, KeyGEM, 10% in methanol) for 15 min at 37 ℃. Cells were washed twice with PBS and then examined with an inverted fluorescence microscope (Olympus IX71, Japan).

Detection of Intracellular Fe^{2+} by Turnbull's Blue Stain

ZR75-30 cells were incubated with FeO_x-MSNs for 12 h. After washing twice with PBS, cells were stained with 1% potassium ferricyanide and 1% HCl mixture for 4 h at 37 ℃. Then cells were counterstained with safranine (0.5% w/v, in PBS) for 3 min. After final washes with PBS, cells were exposed to fluorescence microscopy observation. Cells are stained red and the Fe^{2+}-positive cytosol is stained blue. Cells incubated with MSNs were used as the control.

Cell Proliferation Assays by MTT Protocol

MTT assay was performed to evaluate the *in vitro* cytotoxicity of FeO_x-MSNs and/or H_2O_2. ZR75-30 cells were seeded in 96-well plates at 5.0×10^4 cells per mL and incubated at 37 ℃ for 24 h. Then the culture media were replaced with serum-free media containing various concentrations of FeO_x-MSNs or H_2O_2 for 4 h or 24 h. At the end of the incubation, the culture media were removed and cells were washed twice with cold PBS. Then, 20 μL of MTT

solutions (5 mg mL^{-1} in PBS) were added to each well and co-incubated for another 4 h at 37 ℃. Finally, the media were replaced with dimethyl sulfoxide (DMSO, 150 μL per well) and gently shook for 5 min. The absorbance at 570 nm was monitored by a microplate reader (Bio-Tek ELx800). The cytotoxicity was quantified as the percentage of cell viability compared to the negative control group. Each data point is represented as means ± SD of six independent experiments.

Detection and Quantification of Intracellular ROS by Fluorescence Microscopy and Fluorescence Spectrometer

A stock solution of DCFH-DA (1 mM in DMSO) was prepared and stored in -20 ℃. ZR75-30 cells growing on petri dishes were exposed to serum-free media with FeO_x-MSNs and/or H_2O_2 for 6 h at 37 ℃. Then the culture media were replaced with DCFH-DA (10 μM) for 30 min at 37 °C. After washing carefully with PBS twice, the fluorescence was observed by fluorescence microscopy (Olympus IX71, Japan). Besides, cells were collected by trypsin and analyzed by fluorescence spectrophotometer (HORIBA FluoroMax-4, France) with an excitation wavelength of 488 nm.

Characterization

The morphology of the samples was recorded by transmission electron microscopy (TEM) on a JEM-2100F electron microscope. The microstructures were measured on a Micrometitics Tristar 3000 N_2 adsorption-desorption analyzer. Barrette-Joynere-Halenda (BET) analysis was performed to determine the surface area and pore volume. Powder X-ray diffraction (XRD) pattern was recorded on a Rigaku Ultima IV diffractometer. UV-Visible absorbance spectra were tested on a UV-3101 Shimadzu spectroscope. Quantity of the Fe element was performed by inductively coupled plasma atomic emission spectrometry (ICP-AES, Varian, USA). UV-Visible diffuse reflection spectra (DRS) were recorded on a Hitachi U-4100 Spectrophotometer. X-ray photoelectron spectroscopy (XPS) was obtained on an Axis Ultra DLD spectrometer (Kratos, UK).

Statistical Analysis

Quantified data are presented as means ± SDs (n = 6). Significance of differences was analyzed by Student's *t*-test. $P < 0.05$ is considered as significant.

Supplementary Figures

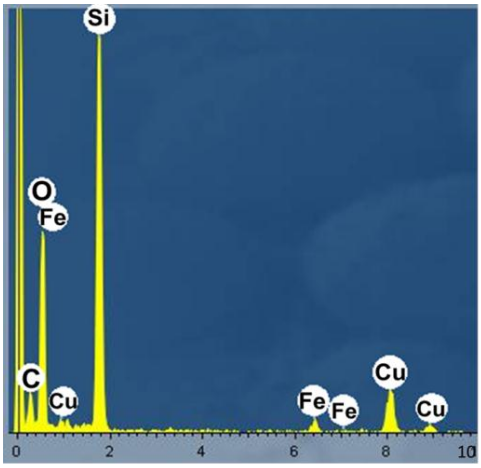
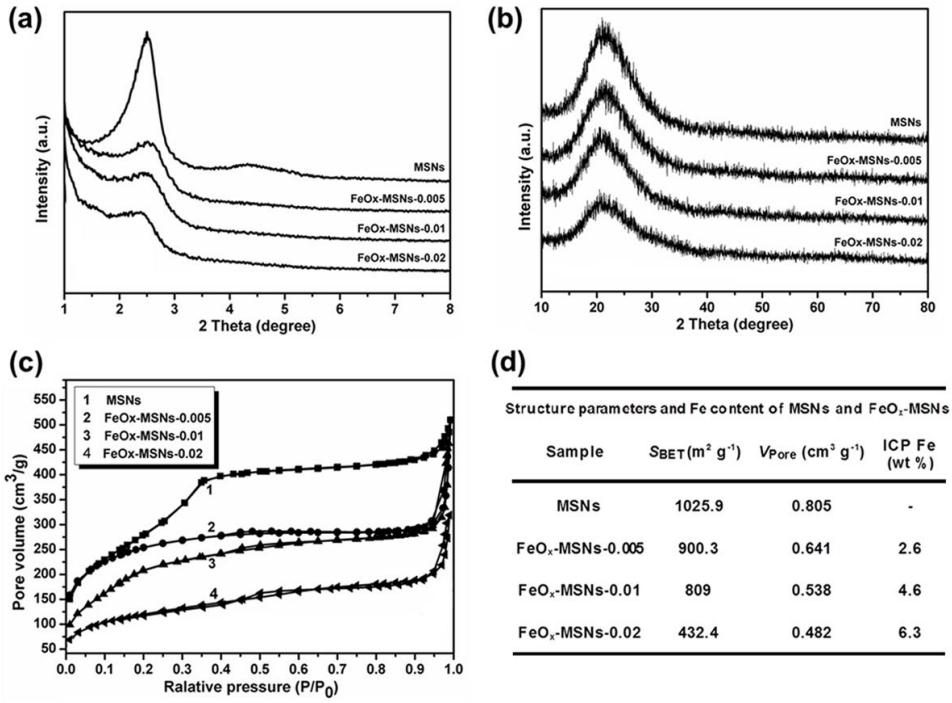


Fig. S1 EDS element analysis of FeOx-MSNs-0.005.



Sample	S_{BET} (m^2 g^{-1})	V_{Pore} (cm^3 g^{-1})	ICP Fe (wt %)
MSNs	1025.9	0.805	-
FeO_x-MSNs-0.005	900.3	0.641	2.6
FeO_x-MSNs-0.01	809	0.538	4.6
FeO_x-MSNs-0.02	432.4	0.482	6.3

Fig. S2 (a, b) Small-angle and wide-angle XRD patterns of MSNs and FeO_x-MSNs-*n*. (c) N_2 adsorption-desorption isotherms of MSNs and FeO_x-MSNs-*n*. (d) Structure parameters and Fe content of MSNs and FeO_x-MSNs-*n*.

Discussion: The small-angle XRD patterns (Fig. S2a) show that all the samples remain well-defined mesostructure while the intensity of the diffraction peaks decreases as the content of FeO_x species increases. It was speculated that the introduction of FeO_x species in the pores led to partial collapse of the orderly mesopores and the reduction of Bragg diffraction intensity. The wide-angle XRD patterns (Fig. S2b) show no obvious diffraction peaks of iron oxides in all samples, indicating that the FeO_x species in the nanocomposite are amorphous and no large iron oxides crystals are present on the surface of the FeO_x-MSNs-*n*.

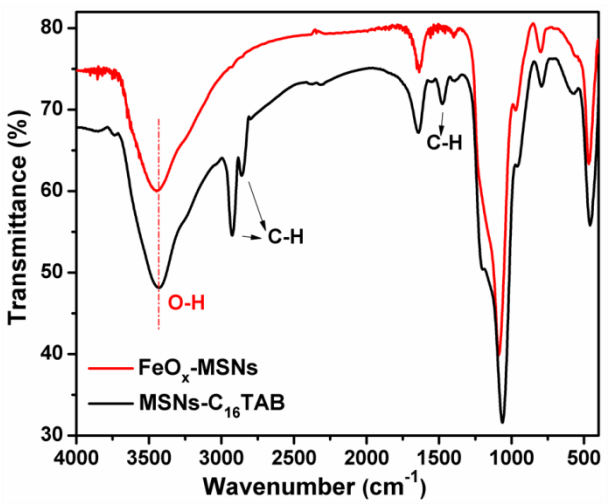


Fig. S3 FTIR spectra of MSNs with surfactants (MSNs-C) and FeO_x-MSNs. The C-H stretching vibrations at 2850-2900 cm^{-1} and C-H bending/deformation vibrations at 1240-1500 cm^{-1} are clearly observed in MSNs-C, but not in FeO_x-MSNs, indicating the remove of surfactants in the FeO_x-MSNs. The strong O-H vibration around 3400 cm^{-1} in the FeO_x-MSNs implys the high hydroxylation (-OH) level in the as-prepared FeO_x-MSNs.

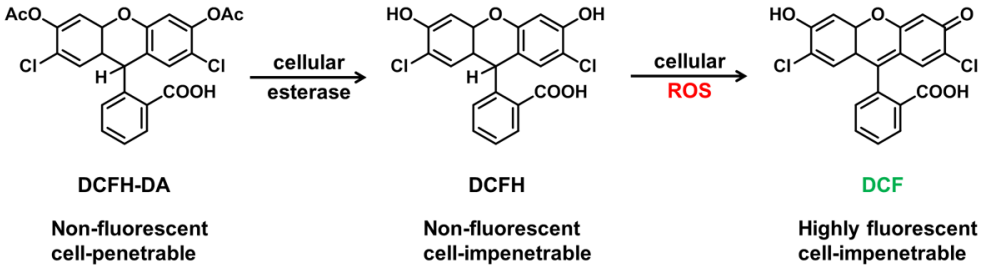


Fig. S4 The reaction of non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and ROS leads to fluorescent 2',7'-dichlorofluorescein (DCF). DCFH-DA is non-fluorescent but can penetrate into cells where it is converted into non-fluorescent dichlorofluorescin (DCFH) by cellular esterases, and is further oxidized to highly fluorescent, cell-impenetrable DCF by intracellular ROS. The DCF fluorescence intensity indicates the overall ROS levels in cells.

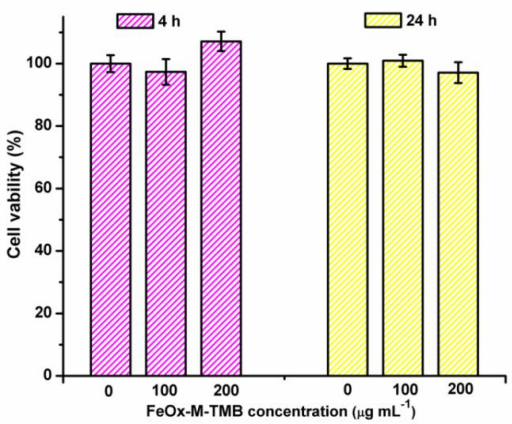


Fig. S5 4 h and 24 h *in vitro* cytotoxicity of FeO_x-MSNs-TMB toward ZR75-30 cells.

References

1 Q. J. He, J. L. Shi, F. Chen, M. Zhu and L. X. Zhang, *Biomaterials*, 2010, **31**, 3335-3346.