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Supporting Information

Lysosomes Activating Chain Reactions against Cancer Cells with a pH-Switched Prodrug/Procatalyst Co-Delivery Nanosystem

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Experimental section

Materials

Cetyltrimethyl ammonium bromide (C_{16}TAB), tetraethyl orthosilicate (TEOS), iron(III) acetylacetonate ($Fe(acac)_3$), 1,2-hexadecanediol, oleylamine, oleic acid, phenyl ether, were obtained from Aladdin. 3-Aminopropyl triethoxysilane (APTES), succinic anhydride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDC), N-hydroxysuccinimide (NHS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,9-bis(3-aminopropyl)-2,4,8,10-tetraoxaspiro[5.5]undecane, dimethyl sulfoxide (DMSO), safranine, fluorescein isothiocyanate (FITC), paraformaldehyde (PFA) and artemisinin (ART) were purchased from Sigma-Aldrich. Potassium ferricyanide, potassium ferrocyanide were purchased from Sinopharm Chemical Reagent Co., Ltd. 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 4 nm Fe_3O_4 nanoparticles (Fe_3O_4 NPs)

Fe_3O_4 NPs were synthesized according to the previously reported method.[1,2] Typically, under the protection of nitrogen, $Fe(acac)_3$ (2 mM), 1,2-hexadecanediol (10 mM), oleylamine (6 mM), oleic acid (6 mM) and phenyl ether (20 mL) were mixed and magnetically stirred. Then, the mixture was heated to 200 °C for 45 min, followed by reflux at 265 °C for another 45 min. After that, the mixture was cooled to room temperature, followed by the adding of ethanol (60 mL). Then, the mixture was collected by centrifugation. After that, the product was dispersed into a solution containing hexane, oleic acid and oleylamine. After centrifugation and washes in ethanol for several times, the obtained 4 nm Fe_3O_4 NPs were dried under vacuum.

Synthesis of carboxylic acid modified Fe_3O_4 nanoparticles (Fe_3O_4-COOH)

Carboxylic acid modified Fe_3O_4 NPs were synthesized based on the reported method.[3] Firstly, amino-modified Fe_3O_4 NPs were synthesized. The as-prepared 4 nm Fe_3O_4 NPs (0.5 g) were dispersed in anhydrous toluene (40 mL) with magnetic stirring under the protection of nitrogen at ambient temperature for 24 h. Then, APTES (0.4 mL) was added and the mixture was heated to 60 °C for another 20 h. After centrifugation, the product was rinsed with ethanol and acetone, and dried under vacuum to obtain amino-modified Fe_3O_4 NPs. Afterwards, the as-prepared amino-modified Fe_3O_4 NPs (0.1 g) were suspended into acetone (10 mL) and stirred at ambient temperature for 4 h. Then, succinic anhydride acetone solution (1.5 M, 5 mL) was added dropwise and stirred at ambient temperature for another 24 h under the protection of nitrogen. The products were centrifugated, rinsed with ethanol and deionized water, and dried under vacuum to obtain carboxylic acid modified Fe_3O_4 NPs (defined as Fe_3O_4 NPs-COOH).

Synthesis of carboxylic acid modified hollow mesoporous silica nanoparticles (HMS-COOH)

Hollow mesoporous silica nanoparticles (HMS) were synthesized as reported.[4,5] The templates in HMS were then removed in acidic methanol solution (HCl/methanol mixture). To incorporate acetal linker onto the HMS, carboxylic acid groups were first introduced on the surface of mesoporous silica with an established method.[6] Briefly, HMS (1.5 g) were suspended in a mixture of dry toluene (10 mL) and APTES (0.50 g), and stirred at room temperature for 15 min to obtain amine-modified HMS (defined as HMS-NH_2). Then, the as-prepared HMS-NH_2 (0.5 g) were dispersed into DMSO solution containing succinic anhydride (80 mg) and triethylamine (80 mg), and stirred at 40 °C for 48 h. The products were collected to obtain carboxylic acid modified HMS (defined as HMS-COOH).

Synthesis of acetal linker modified hollow mesoporous silica nanoparticles (HMS-Acetal)

The acetal linker was grafted onto the HMS based on the reaction of 3,9-Bis(3-aminopropyl)-2,4,8,10-tetraoxaspiro[5.5]undecane with the carboxylic acid groups on the HMS-COOH.[7,8] Typically, the as-prepared HMS-COOH (100 mg) were suspended in a solution containing EDC (60 mg) and NHS (30 mg). Then, 3,9-Bis(3-

aminopropyl)-2,4,8,10-tetraoxaspiro[5.5]undecane (150 mg) was added and stirred at room temperature for 10 h. The products were collected and washed to obtain acetal linker containing HMS (defined as HMS-Acetal).

Synthesis of ART loaded and Fe_3O_4 capped hollow mesoporous silica nanocomposite (ART@HMS-Fe_3O_4)

HMS-Acetal (20 mg) were dispersed into ART acetone solution (0.4 mg mL^{-1}, 10 mL) and stirred in the dark for 24 h. Then, Fe_3O_4-COOH (10 mg) was added to the suspension, followed by addition of 20 mg EDC and 10 mg NHS. The reaction was carried on for another 4 h. The products were collected, washed and dried to obtain ART loaded and Fe_3O_4 capped hollow mesoporous silica nanocomposite (defined as ART@HMS-Fe_3O_4).

For comparison, Fe_3O_4 capped hollow mesoporous silica nanoparticle was also synthesized with a similar procedure. Briefly, the as-prepared HMS-Acetal (10 mg) was reacted with the Fe_3O_4-COOH (5 mg) in the presence of EDC (10 mg) and NHS (5 mg) for 5 h. The products were collected, washed and dried to obtain Fe_3O_4 capped hollow mesoporous silica nanoparticle (defined as HMS-Fe_3O_4).

Synthesis of FITC-Labeled ART@HMS (ART@HMS-FITC)

FITC was mixed with APTES in methanol under dark conditions to obtain FITC-APTES. Then, ART@HMS (50 mg) were added into the FITC-APTES solution (3 mL) and stirred in the dark for 12 h. The FITC-labeled ART@HMS (ART@HMS-FITC) were collected by centrifugation, washed with methanol, and freeze dried in the dark.

ART and Fe^{3+}/Fe^{2+} ions release from ART@HMS-Fe_3O_4

The ART and Fe^{3+}/Fe^{2+} ions release from ART@HMS-Fe_3O_4 was measured in PBS media with pH values of 7.4 and 5.0. A bag filter loaded with ART@HMS-Fe_3O_4 (100 mg) was immersed into the sink solution containing PBS release media (pH 7.4 or 5.0) and sodium lauryl sulfate (SLS, 0.1% w/v). The release systems were then maintained at ambient temperature. At each time point, the ART concentration in the PBS solution was monitored by UV-Vis spectrum measurement. All measurements were performed in triplicate.

The Fe^{3+}/Fe^{2+} ions release from the ART@HMS-Fe_3O_4 was monitored by bathophenanthroline (BPh) which forms pink-red $[Fe(BPh)_3]^{2+}$ complex with reduced iron (Fe^{2+} ions). The color can be measured using a UV-Vis spectrometer at 535 nm absorbance wavelength. Typically, ART@HMS-Fe_3O_4 (200 mg) was immersed into the PBS solutions (20 mL) with pH 7.4 and 5.0, respectively. After 24 h, the supernatant (0.5 mL) was withdrawn, filtered and then added to the glycine-HCl buffer solution (pH 3.6, 5 mL) containing BPh (50 μM), sodium dodecyl sulfate (SDS, 10 mM), hydroxylamine hydrochlorine (0.1 M) and sodium nitrate (50 mM).

Cell culture

Human breast cancer cell line ZR75-30 cells were purchased from the cell bank of the Chinese Academy of Sciences, and were cultured in RPMI-1640 medium (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humid atmosphere with 5.0% CO_2. All cell experiments were performed with cells in the logarithmic growth phase.

In vitro observation of cellular uptake of ART@HMS

ZR75-30 cells were plated in Petri dishes (Corning, NY) and cultured overnight. After incubation with ART@HMS-FITC (50 μg mL^{-1}) for 4 h, cells were washed and stained with DAPI (Cell Apoptosis DAPI Detection Kit, KeyGEM, 10% in methanol) for 15 min at 37 °C. After thorough washing with PBS, Cells were examined with an inverted fluorescence microscope (Olympus IX71, Japan).

Lysosome-tracker study of HMS-Fe_3O_4

ZR75-30 cells were plated in Petri dishes (Corning, NY) and cultured overnight. After incubation with HMS-Fe_3O_4-FITC (50 μg mL^{-1}) for 3 h, cells were washed and stained with Lyso-Tracker Red (50 nM in RPMI-1640 medium, Beyotime) for 30 min at 37 °C. After thorough washing with PBS, Cells were subjected to inverted fluorescence microscopy observation (Olympus IX71, Japan).

In vitro detection of Fe^{3+}/Fe^{2+} ions release from HMS-Fe_3O_4

In vitro Fe^{3+} ions release was determined by Fe^{3+}-sensitive Perls' staining. ZR75-30 cells were incubated with HMS-Fe_3O_4 for 12 h. Then cells were fixed with paraformaldehyde (4% w/v, in PBS) for 20 min at room temperature. After washing with PBS, cells were stained with Perls' working solution (4% potassium ferrocyanide and 4% HCl, 1:1 mixture) for 4 h at 37 °C. Then, cells were counterstained with safranine (0.5% w/v, in PBS) for 3 min. After wasing thoroughly in PBS, cells were subjected to inverted microscopy observation (Olympus IX71, Japan). The cells are stained red and the Fe^{3+}-positive cytosol is stained blue. Cells incubated with HMS NPs were used as the control.

In vitro Fe^{2+} ions release was determined by Fe^{2+}-sensitive Turnbull's blue staining. ZR75-30 cells were incubated with HMS-Fe_3O_4 for 12 h. After fixing with paraformaldehyde, cells were stained with 1% potassium ferricyanide and 1% HCl mixture for 4 h at 37 °C. Then cells were counterstained with safranine (0.5% w/v, in PBS) for 3 min. After final washes with PBS, cells were exposed to inverted microscopy observation. The cells are stained red and the Fe^{2+}-positive cytosol is stained blue. Cells incubated with HMS were used as the control.

In vitro cytotoxicity evaluation of ART@HMS-Fe_3O_4

MTT assay was performed to evaluate the *in vitro* toxicity of ART@HMS-Fe_3O_4 against ZR75-30 cancer cells. Besides, the cytotxicity of ART@HMS and HMS-Fe_3O_4 were also evaluated with a similar protocol. Cells incubated on fresh culture media were used as the control group. Data are represented as means ± SD (n = 6). ZR75-30 cells were plated into 96-well plates at a density of 5.0×10^4 cells per mL. After 24 h co-incubation, the growth media were replaced with RPMI-1640 media containing specified concentrations of ART@HMS, HMS-Fe_3O_4 or ART@HMS-Fe_3O_4. After incubation for 4 h or 24 h at 37 °C, the culture media were discarded and cells were washed twice with cold PBS. MTT assay was then processed with the standard protocol. The optical density was monitored by a microplate reader (Bio-Tek ELx800) at 570 nm. The cell viability value of the control group was normalized to be 1. The cytotoxicity was then quantified as the percentage of cell viability relative to the control group.

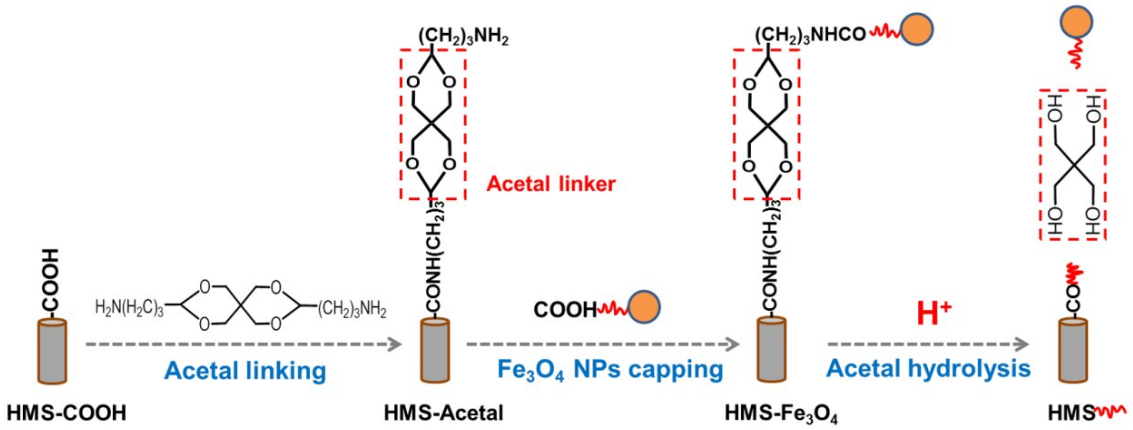
Characterization

Transmission electron microscopy (TEM) images were recorded on a JEM-2100F electron microscope. N_2 adsorption-desorption analysis was performed on a N_2 adsorption-desorption analyzer (Micrometitics Tristar 3000). Surface area and pore volume were determined by Barrette-Joynere-Halenda (BET) analysis. Powder X-ray diffraction (XRD) patterns were recorded on a Rigaku Ultima IV diffractometer. High performance liquid chromatography (HPLC) was performed with an AB-H column (Daicel Chemical Industries, Ltd. Japan). UV-Visible absorption spectra were obtained on a UV-3101 Shimadzu spectroscope. Quantity of the Fe element was performed by inductively coupled plasma atomic emission spectrometry (ICP-AES, Varian, USA). Fluorescence spectra were recorded on a HORIBA FluoroMax-4 fluorescence spectrophotometer.

Statistical analysis

Quantified data are expressed as means ± SDs of at least six independent experiments. Statistical analysis was performed by Student's *t*-test. $P < 0.05$ is considered as statistically significant.

Supplementary figures



Scheme S1 Illustration of the synthesis of HMS-Acetal and HMS-Fe_3O_4, and pH-responsive cleavage of the acetal linker in the HMS-Fe_3O_4.

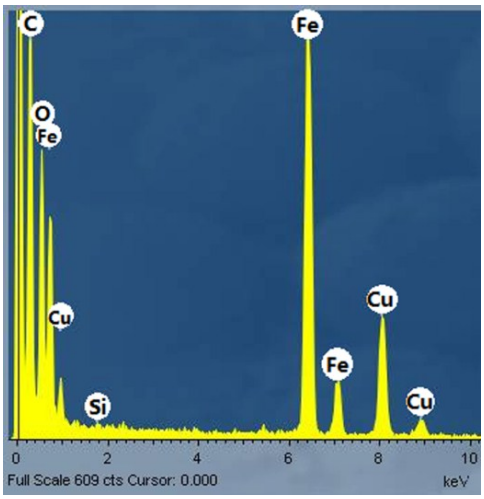


Fig. S1 EDS analysis of ART@HMS-Fe_3O_4.

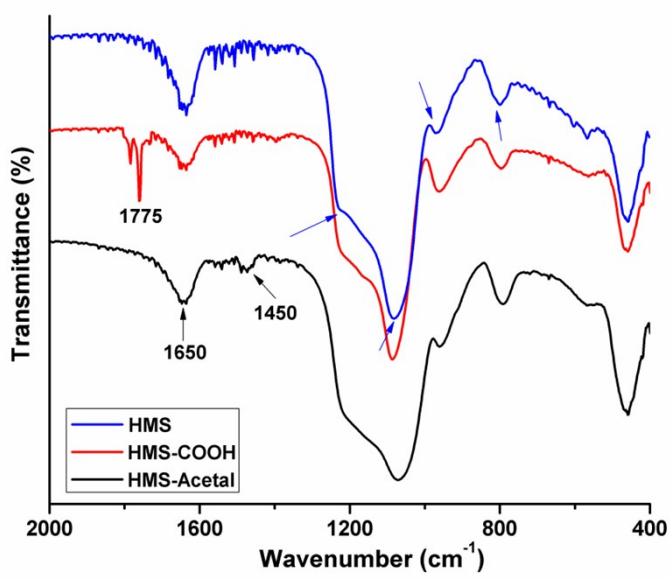


Fig. S2 FTIR spectra of HMS, HMS-COOH and HMS-Acetal.

Discussion:

As shown in Fig. S2, the as-prepared HMS showed characteristic bands of silica at 1225, 1080, 980 and 800 cm^{-1} (blue arrow), which were attributed to the asymmetric stretching vibrations of Si-O-Si at 1225 and 1080 cm^{-1}, the symmetric stretching vibration of Si-O-Si at 800 cm^{-1} and the stretching vibration of Si-OH at 980 cm^{-1}.[9] After the carboxylic acid modification, the HMS-COOH exhibited a strong characteristic peak around 1775 cm^{-1}, which can be assigned to the stretching vibration of carbonyl group ($\nu_{C=O}$) in carboxylic acid.[10] The successful preparation of HMS-Acetal was confirmed by the characteristic band at 1450 cm^{-1}, which was attributed to the stretching vibration of C-N bond (ν_{C-N}) in amides.[11] Furthermore, the stretching vibration of carbonyl group ($\nu_{C=O}$) in the amide at 1650 cm^{-1} was obviously strengthened, indicating the successful modification of acetal linker in HMS-Acetal.[10]

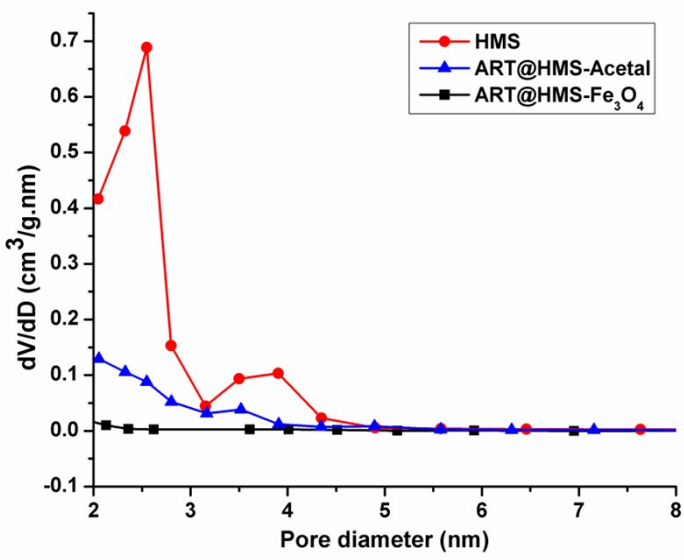


Fig. S3 BJH pore size distribution curves of the as-prepared HMS, ART@HMS-Acetal and ART@HMS-Fe_3O_4.

Table S1 Structure parameters of the samples.

Sample	S_{BET} [m^2 g^{-1}]	V_{Pore} [cm^3 g^{-1}]	D_{Pore} [nm]
HMS	987	0.71	2.6
ART@HMS-Acetal	413	0.39	2.0
ART@HMS-Fe_3O_4	92	0.11	-

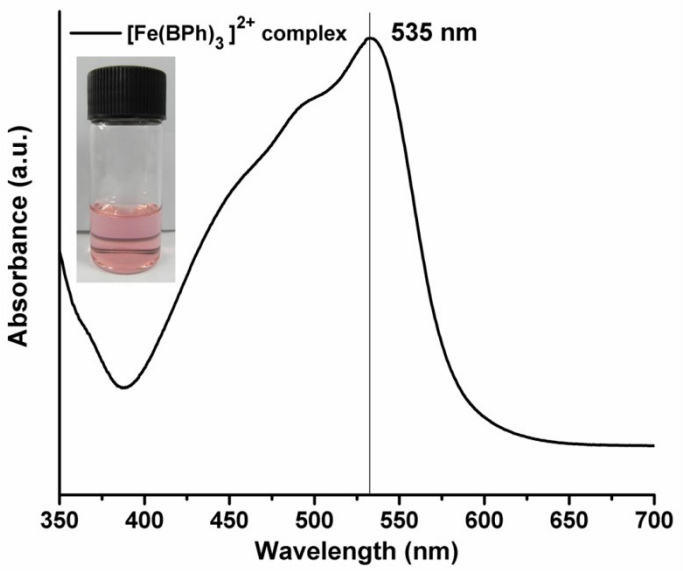


Fig. S4 UV-Vis absorption spectra of the $[Fe(BPh)_3]^{2+}$ complex.

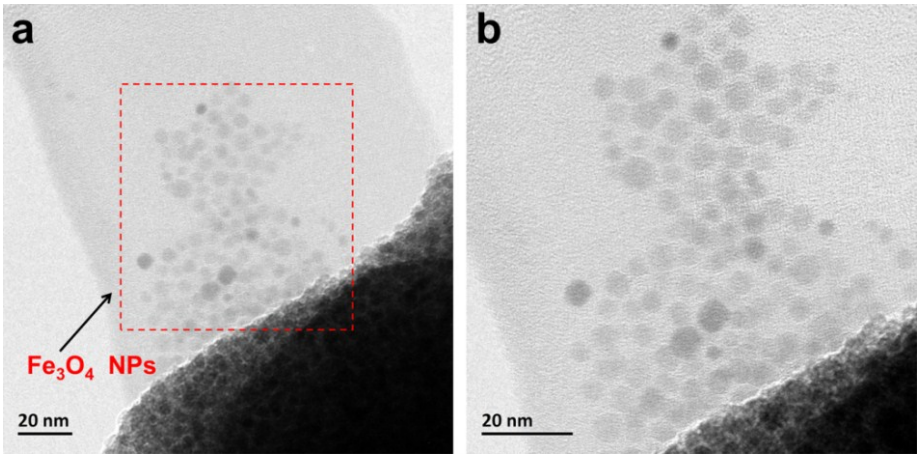


Fig. S5 TEM images of HMS-Fe_3O_4 after dispersing in PBS media (pH 5.0) for 12 h, which indicating that free Fe_3O_4 NPs were liberated form the nanosystem. Besides, the Fe_3O_4 NPs are intact with no iron ions leakage in the *in vitro* pH 5.0 PBS media.

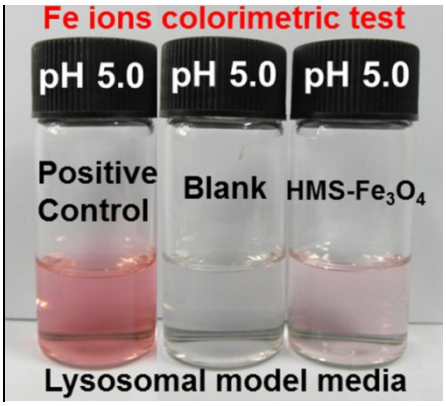
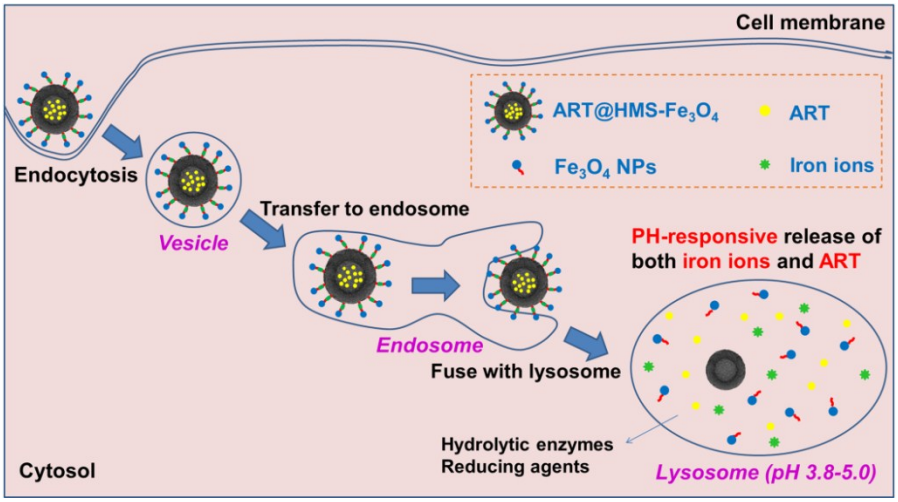


Fig. S6 Fe ions determination by BPh colorimetric method. $FeCl_3$ solution was used as the positive control. Lysosomal model medium (pH 5.0) was used as the blank control. The suspension of HMS-Fe_3O_4 in lysosomal model medium formed pink-red color after 24 h, demonstrating that free iron ions are liberated from the HMS-Fe_3O_4 in *in vitro* lysosomal model medium.



Scheme S2 Illustration for the uptake pathway of ART@HMS-Fe_3O_4 by cancer cells. The ART@HMS-Fe_3O_4 was speculated to be internalized *via* an endocytosis pathway where a depression on the cell membrane surface is initially generated, and then an endosome is separated to the lysosome compartment. The low pH as well as the hydrolytic enzymes and reducing agents in the lysosome help releasing both free ART and iron ions from the ART@HMS-Fe_3O_4.

References

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