Dual responsive mesoporous silica nanoparticles for targeted co-delivery of hydrophobic and hydrophilic anticancer drugs to tumor cells

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Supporting Information

1. Materials

Doxorubicin hydrochloride (DOX.HCl), Methotrexate (MTX), glutathione (GSH), 3mercaptopropyltrimethoxysilane (MPTMS), 3-(trimethoxysilyl)propyl methacrylate (TMSPMA), 2-aminoethanethiol hydrochloride, aldrithiol-2,fluoresceinisothiocyanate (FITC), generation 2 PAMAM dendrimer(PAMAM-G2), hyaluronic acid (HA, M_w = 10kDa), tetraethyl orthosilicate (TEOS), *N*-cetyltrimethylammonium bromide (CTAB) and ammonium hydroxidewere purchased from Sigma-Aldrich, USA. All the chemicals were analytical grade and used without further treatment.

2. Synthesis of fluorescein isothiocyanate functionalized mesoporous silica nanosphere through disulfide linkage (MSN-S-S-FITC)

Mesoporous silica nanospheres (MSNs) were prepared by the classical CTABtemplated, base-catalyzed sol-gel method^{1, 2}. 1.12 g CTAB was dissolved in 1000 mL deionized water. With magnetic stirring, 52.8 mL ammonium hydroxide (29 wt% NH₃ in water) was added to approximately adjust the pH value to 11. Then 5.8 mL TEOS were added with rapid stirring and the resulting solution was heated up to 50°C. After 2 h, the mixture was aged overnight, then centrifuged and washed thoroughly with distilled water and ethanol. The surfactant templates were then removed by extraction using acidic methanol (9 mL of HCl/400 mL of methanol, 36 h) at 70 °C, which were further centrifuged, washed several times with ethanol and dried under vacuum for 20 h.

The as-synthesized MSNs were dispersed in 50 mL anhydrous ethanol, and then 3-(trimethoxysilyl)propyl methacrylate (1 mL, 50% in ethanol) was added into the suspension and stirred for 8 h. The resultant solid was filtered, washed with ethanol, and then dried under vacuum to get thiol-MSN.

The purified thiol-MSN (1.00 g) was treated with a methanol solution (60.00 mL) of 2-(pyridyldisulfanyl)ethylamine (PDEA) (9.12 \times 10⁻⁴mol), which was synthesized via a literature procedure^{3, 4} at room temperature for 24 h under vigorous stirring to undergo the desired disulfide bond exchange reaction. The resulting amino groups functionalized mesoporous silica nanosphere through disulfide linkage (MSN-S-S-NH₂) were filtered and washed with methanol and dried in air.

Furthermore, the MSN-S-S- NH_2 was then dispersed in 50 mL absolute ethanol containing 0.1 g fluorescein isothiocyanate (FITC) under vigorous stirring. The suspension was stirred at room temperature for 2 h before filtration and complete

washing with copious ethanol by a fritted funnel. To ensure that the FITC physically adsorbed on MSNs was removed completely, the washing procedure was repeated until there is no visible color in the eluant. The resulting MSN-S-S-FITC particles were allowed to dry at room temperature overnight.

3. Drug loading and fabrication of MSN-dendrimer-HA^{1, 5, 6}

To load hydrophobic drug molecules (Methotrexate, MTX) into the pores of these particles, the MSN-S-S-FITC were soaked in a concentrated solution containing MTX. Typically, the MSN-S-S-FITC (50 mg) was stirred in a solution containing MTX (25 mg) and DMSO (3 mL). After 24 h, the mixture was centrifuged and the supernatant was removed. By using UV/Vis spectroscopy, the absorption measurements of the original solution and the supernatant were compared to determine the amount of MTX that was loaded inside the MSN-S-S-FITC. The drug-loaded MSNs were dried under vacuum to remove trace DMSO and then sonicated and washed twice with PBS (pH 7.4) solution to remove MTX that was adsorbed on the surface and not inside the pores. The MTX loaded MSN-S-S-FITC (0.15 g) was dispersed in anhydrous ethanol (3.00 mL). A second generation (G2) polyamidoamine dendrimer (PAMAM, 2.00 mL, 0.11 mmol) was then added to the suspension. The amino groups of the G2-PAMAM were allowed to react with the carboxyl groups in FITC presented on the surface of MSN for 20 h at room temperature to yield the G2-PAMAM-capped MSNs (MSN-S-Sdendrimer) with MTX loading. The resulting nanoparticles was centrifuged and washed thoroughly with ethanol, methanol and acetone and dried under high vacuum.

To load the hydrophilic drug molecules (Doxorubicin hydrochloride, DOX.HCl) into

the PAMAM dendrimer and introduce the hyaluronic acid (HA) shell on the surface of MSN-S-S-dendrimer as smart cap and tumor targeting agent, 1 g MTX loaded MSN-S-S-dendrimer was dispersed in 50 mL water, and then 0.5 g DOX.HCl was added. The mixture was stirred at room temperature for 24 h. After that, HA (10 mL, 5 % in water) was added into the suspension and stirred for another 8 h. The resultant solid was centrifuged, washed with water, and then dried under vacuum to get the DOX.HCl and MTX co-loaded MSN-dendrimer-HA.

4. Stimulated drug release

In the pH and GSH triggered drug release experiment, a certain amount of DOX.HCl and MTX co-loaded MSN-dendrimer-HA powder was dispersed in 50 mL of 3 different type of PBS buffer (i: pH 7.4in the absence of GSH, ii: pH 5.5 in the absence of GSH and iii: pH 5.5 in the presence of GSH) at 25 °C. Subsequently, 2 mL of supernatant was taken periodically from the suspension at 25 °C followed by centrifugation (15 000 rpm, 20 min). The release of DOX.HCl and MTX from the pores to the buffer solution was determined by UV-Vis spectroscopy (Ab at 540 nm and 350 nm, respectively).

5. Confocal microscopy analysis of tumor targeting and selective drug co-delivery

To observe the selective tumor targeting drug co-delivery of the DOX.HCl and MTX co-loaded MSN-dendrimer-HA, SCC cells (cancer cells) and HaCaT cells (normal cells) were employed. The cells were seeded at 2.5×10^4 per well onto 24-well plates containing glass cover slips, and were cultured in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Gibico, USA) and penicillin-streptomycin (100 U/mL and 100 µg/mL, Gibico, USA),

and incubated at 37 °C in 5% CO₂. On the following day, pure MSN-dendrimer-HA, DOX.HCl/MTX loaded MSN-dendrimer-HA containing 0.5 µg/mLDOX.HCl/MTX and/or 0.5 µg/mL free DOX.HCl/MTX mixture were added to the cells, respectively. After 1 h, 3 h, 6 h and 12 h, the cells were rinsed with PBS, fixed with 4% paraformaldehyde for 20 min, permeabilized in 0.1% Triton X-100 for 5 min, stained with 4′, 6-diamidino-2-phenylindole (DAPI, Life Technologies, USA). Afterwards, the cells were rinsed, mounted and the fluorescence was observed under a fluorescence microscope (Olympus BX51, Olympus, Japan).

6. In vitro cytotoxicity analysis

SCC cells and HaCaT cells were seeded at 3×10^3 per well in 96-well plate for 24h before treatment, respectively. The cells were exposed to free DOX.HCl/MTX mixture and equivalent DOX.HCl/MTX loaded MSN-dendrimer-HA with concentration range from 0.005 to 100µg/mL for 6h, 12h, 24h, 48h and 72h. Cell viability was measured by using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) according to the manufacture's protocol. The absorbance of the wells was read at 570nm by using Varioskan Flash multimode reader (Thermo Fisher Scientific, USA).

7.Characterization

Transmission electron microscopy (TEM) images were recorded on a Philips CM200 transmission electron microscope operated at 200 kV. For the TEM observation, samples were obtained by dropping 5 μ L of solution onto carbon-coated copper grids.

All the TEM images were visualized without staining. The infrared (IR) spectra were measured by AVATAR 320 FT-IR using KBr pellets. The ultraviolet-visible (UV-Vis) spectra were measured with dilute aqueous solution in a 2 mm thick quartz cell using a HitachiU-2910 spectrophotometer. All pH value measurements were carried out on a Sartorius BECKMAN F 34 pH meter. The zeta potentials were measured by a DelsaTM Nano C particle analyzer (Beckman Coulter, USA) running Delsa Nano software and using 4mW He-Ne laser operating at a wavelength of 633nm and avalanche photodiode (APD) detector. *In vitro*and intracellular release was respectively monitored by ultraviolet-visible spectroscopy and confocal fluorescence microscopy using anOlympus BX51 microscope equipped with a fluorescent lamp; ex = 488 nm, em = 560 nm for DOX and ex = 488 nm, em = 520 nm for FITC



Figure S1. Dynamic Light Scattering(DLS) of fluorescein isothiocyanate (FITC) modified mesoporous silica nanoparticles through disulfide bonds(MSN-S-S-FITC, black curve), generation 2 PAMAM dendrimer fabricated MSN-S-S-FITC (MSN-S-S-dendrimer, red curve)and hyaluronic acid (HA) functionalized MSN-S-S-dendrimer (MSN-dendrimer-HA, blue curve).



Figure S2. The fourier transform infrared spectroscopy (FTIR) of amino groups functionalized mesoporous silica nanoparticles through disulfide bond (MSN-S-S-NH₂, black curve), fluorescein isothiocyanate (FITC) modified MSN-S-S-NH₂ (MSN-S-S-FITC, red curve), generation 2 PAMAM dendrimer fabricated MSN-S-S-FITC (MSN-S-S-dendrimer, blue curve) and hyaluronic acid (HA) functionalized MSN-S-S-dendrimer (MSN-dendrimer-HA, purple curve).



Figure S3. a) N_2 adsorption desorption isotherms and b) pore-size distribution of fluorescein isothiocyanate (FITC) modified mesoporous silica nanoparticles through disulfide bonds(MSN-S-S-FITC, black curve), generation 2 PAMAM dendrimer fabricated MSN-S-S-FITC (MSN-S-S-dendrimer, red curve) and hyaluronic acid (HA) functionalized MSN-S-S-dendrimer (MSN-dendrimer-HA, blue curve). The

adsorption-desorption isotherm showing significant reduction of the pore volume and diameter to virtually 0 nm after dendrimer and HA covering.



Figure S4. a) UV-Vis spectra of DOX.HCl loaded MSN-dendrimer-HA in PBS at pH7.4 (black curve), MTX loaded MSN-dendrimer-HA at pH 7.4 (red curve) and DOX.HCl and MTX co-loaded MSN-dendrimer-HA at pH 7.4 (blue curve). b) UV-Vis spectra of PBS with glutathione (GSH) at pH 5.5(black curve), MTX in PBS with GSH at pH 5.5(red curve) and DOX.HCl in PBS with GSH at pH 5.5 (blue curve)



Figure S5. TGA curve of MSN-S-S-NH2 (black curve), MTX loaded MSN-S-S-NH2 (red curve), MTX loaded MSN-S-S-dendrimer (blue curve), MTX/DOX loaded MSN-S-S-dendrimer (magenta curve) and MTX/DOX loaded MSN-dendrimer-HA (dark yellow curve).



Figure S6. a) Time-dependent kinetic curve of DOX.HCl loaded MSNs-dendrimer-HA nanocarriers aqueous solution (pH 5.5) represented by the fluorescence intensity of DOX. b) The concentration calibration curve of DOX. c) Time-dependent kinetic curve of DOX.HCl loaded MSNs-dendrimer-HA nanocarriers aqueous solution (pH 5.5) represented by release percentage calculated from a and b.



Figure S7. Confocal microscope images of SCC cells after 2 h co-culture with MSNdendrimer-HA focusing on various positions in Z axis. The positions in Z axis decrease 1.284 μ m each step with a total distance of 15.408 μ m from a to g (larger than the thickness of cell).



Figure S8. Confocal fluorescent microscope images of HaCaT cells (normal cell) after incubation with free doxorubicin hydrochloride (DOX.HCl) for 1 h, 6 h and 12 h.



Figure S9. Viability of tumour cells (SCC) incubated with the increasing amounts of DOX.HCl/MTX in the form of DOX.HCl/MTX co-loaded MSN-dendrimer-HA (black curve) and/or free DOX.HCl/MTX mixture (red curve).



Figure S10. Viability of tumour cells (SCC) incubated with the increasing amounts of drug loaded MSN vehicles in the form of DOX.HCl/MTX co-loaded MSN-dendrimer-HA (black curve), DOX.HCl loaded MSN-dendrimer-HA (red curve) and MTX loaded MSN-dendrimer-HA (blue curve).



Figure S11. Cell viability of SCC cells (a, tumor cells) and HaCaT cells (b, normal cells) exposed to different concentrations of MSNs (sample 1), PAMAM dendrimer (sample 2), HA (sample 3) and MSN-dendrimer-HA (sample 4), as determined by the MTT assay. Materials at the indicated concentrations were incubated with cells for 48 hours. For each sample, columns represent 100 mg mL⁻¹, 50 mg mL⁻¹, 25 mg mL⁻¹, 12.5 mg mL⁻¹, 6.25 mg mL⁻¹ respectively from left to right.

Table 1. MTX and DOX Loading Efficiency of MSNs-dendrimer-HA

MTX/DOX/MSNs-dendrimer-HA	Loading	efficiency	Loading	efficiency
	(MTX)		(DOX)	
0.3/0.2/1	88%		86%	
0.2/0.1/1	94%		95%	
0.1/0.05/1	90%		91%	
0.05/0.025/1	85%		83%	

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