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Supplementary Information

Dually Responsive Mesoporous Silica Nanoparticles Regulated by Upper Critical Solution Temperature Polymers for Intracellular Drug Delivery

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Experimental Procedures

Chemicals

Acrylamide (AAm) with purity 99.9%, acrylonitrile (AN) with purity ≥99% containing 35-45 ppm monomethyl ester hydroquinone as inhibitor, 3-mercaptopropionic acid, azobisisobutyronitrile (98%), triethylamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), cystamine dihydrochloride, N-Hydroxysuccinimide (NHS), Rhodamine B (RB), L- Glutathione reduced (GSH) and Dithiothreitol (DTT) were all purchased from Sigma-Aldrich. Cetrimonium bromide (CTAB), (3-Isocyanatopropyl)triethoxysilane (95%), tetraethyl orthosilicate, hydrochloric acid (36.5-38.0%) and sodium hydroxide were from Thermo Fisher Scientific. Doxorubicin hydrochloride was purchased from Dalian Meilun Biotechnology Co., Ltd. CellTiter-Blue® Cell Viability Assay kit was from Promega. The phosphate buffer saline (PBS, 10 x) was from Life Technologies™ and diluted to standard concentration (1x) for use. Water was purified by a Milli-Q system.

Synthesis procedures

Synthesis of thermo-responsive polymers

For preparation of TRP1, acrylamide (2.88 g, 40.5 mmol), acrylonitrile (500 mg, 9.5 mmol), and azobisisobutyronitrile (39.2 mg, 0.24 mmol) were dissolved into 50 mL dimethyl sulfoxide (DMSO), then bubbled with N_2 for 30 min to remove the oxygen in the solution. The mixture was reacted at 60 ºC for 4 h. Then 1 mL solution of 3-mercaptopropionic acid (42.5 mg, 0.4 mmol) in DMSO was added into the solution. After 1 h, the reaction was ended by cooling in the ice bath and the polymer was precipitated in extensive methanol and dried under vacuum.

The preparation for TRP2 and TRP3 were the same as described above, except the amounts of acrylonitrile. The acrylonitrile used to synthesize TRP2 and TRP3 were 532mg and 552 mg, respectively.

The standard curve used to calibrate the copolymer composition was based on integrating ratios of the infrared vibration bands of carbonyl and nitrile group by measuring polyacrylamide and polyacrylonitrile mixtures in DMSO/H_2O (1/5, v/v) solution with FT-IR.

The upper critical solution temperature (UCST) of the polymers was determined using UV−Vis spectrophotometer (Hitachi U-2910 Spectrophotometer) with polymer concentration of 1% (wt%) by measuring transmittance in 670 nm over a temperature range of 20–70 °C. UCST was identified as the temperature at which the transmittance of the sample was 50%.

For characterization, ^{1}H-NMR spectra were collected in DMSO-d_6 at 25ºC on a Varian MR400 (Cobalt) spectrometer at the NMR Facility, University of Michigan. Chemical shifts were reported in ppm (TMS as internal standard). Fourier Transform Infrared Spectroscopy (FT-IR) was carried out on a Spectrum GX (Perkin-Elmer). Gel permeation chromatography was performed on a Shimadzu GPC system. Dynamic light scattering (DLS) was performed on a Delsa™ Nano C Particle Analyzer.

Synthesis of mesoporous silica nanoparticles

The mesoporous silica nanoparticles were synthesized according to the reported literature.[1] Cetrimonium bromide (CTAB, 3.00 g) and sodium hydroxide solution (2M, 10.5 mL) were dissolved into 1440 mL deionised water and the solution was held at 80 ºC for 1h. Then, tetraethyl orthosilicate (15 mL) was added dropwise into the mixture and subsequently reacted for 2 h. The final product **M1** with CTAB template was achieved by filtration, washed with extensive methanol and MiliQ-water, and dried at 80 ºC overnight.

To remove the CTAB in the channels of mesoporous silica nanoparticles, the above product **M1** (1.5 g) was uniformly dispersed with ultrasound into acid solution composed of 160 mL methanol and 9 mL concentrated hydrochloride. The mixture was filtrated after refluxed for 24 h and washed with extensive methanol and MiliQ-water. The white powder **M2** was achieved and dried at 80ºC for 24 h.

Surface modification of MSN with disulfide bond

The surface of **MSN** was first modified with isocyanate groups. **M2** (1.0 g) and (3-Isocyanatopropyl)triethoxysilane (2.5 g) were dispersed into 50 mL toluene. The mixture was fully dispersed by ultrasound and refluxed for 24 h. The product **M3** was collected by filtration and washed with extensive dichloromethane, acetone, MiliQ-water and methanol, and finally dried under vacuum.

M3 was further functionalized with cystamine to achieve disulfide bond linked amino groups on the surface of **MSN**. **M3** (1.0 g) and cystamine dihydrochloride (0.9 g, 4 mmol) were dispersed in N,N-Dimethylformamide (DMF, 40 mL) with 1 mL triethylamine. The mixture was reacted under room temperature for 24 h, then filtrated and washed with extensive methanol and MiliQ water. Light yellow powder **M4** was achieved after dried at 80 ºC.

Surface grafting of thermo-responsive polymers onto MSN

The thermo-responsive polymer, TRP2 (200 mg), was dissolved in 20 mL EDC/NHS water solution at 60ºC, in which the concentration of EDC and NHS corresponded to 0.4 mM and 0.1 mM.[2] **M4** (400 mg) was then added to the above mixture and reacted for 72 h at 60 ºC. The polymer grafted MSN (MSN-TRP2) was collected after centrifugation and washed with hot water three times to remove unreacted polymers.

Dynamic light scattering (DLS) was performed on a DelsaTM Nano C Particle Analyzer. Transmission electron microscopy images were collected from a JEOL 2010F AEM at the Michigan Center for Materials Characterization, University of Michigan.

Procedure for loading rhodamine B into MSN-TRP2

MSN-TRP2 (100 mg) was dispersed into 10 mL hot water (60 ºC) with Rhodamine B (10 mg). After stirring for 24 h, the solution was cooled down in an ice bath and centrifuged. The solid phase MSN-TRP2-RB was washed with extensive MillQ-water until the supernatant was clear, then dried through lyophilisation.

Procedure for loading Doxorubicin into MSN-TRP2

MSN-TRP2 (30 mg) was extensively dispersed into 10 mL hot water (60 ºC) using ultrasound. Doxorubicin hydrochloride (DOX, 3 mg) was dissolved with 1 mL DMSO and added dropwise to the MSN-TRP2 solution. After stirring at 60 ºC for 24 h, the mixture was cooled down in an ice bath and centrifuged. The solid phase MSN-TRP2-DOX was washed extensively with Milli-Q water and then dried through lyophilisation.

Stimuli-responsive drug release

For thermo-responsive release studies, MSN-TRP2-RB (10 mg) was dispersed in 5 mL PBS, and kept at 25ºC, 37ºC, and 42ºC, respectively, with magnetic stirring. 200 μL of supernatant was withdrawn after centrifugation at pre-determined time intervals. Equal amount of fresh PBS was added into the mixture. The released rhodamine B was quantified with fluorescence intensity of the supernatant with an excitation wavelength of 550 nm and emission wavelength of 580 nm.

For reductive response tests, dithiothreitol (5 mM) were added into the MSN-TRP2-RB solution (2 mg/mL), following the same procedures outlined for the thermo-response release study.

For cyclic controlled release tests, MSN-TRP2-RB (10 mg) was dispersed in 5 mL PBS, and kept at 25ºC for 2 h. The solid part was separated by centrifugation and washed with PBS for once. The supernatant was used to determine the released amount at 25ºC. The solid part was re-dispersed into 42 ºC buffer solution for another 1 h, then centrifuged and washed. The supernatant was used to test the released amount at 42ºC. Then, the collected solid was re-dispersed into 25ºC again for 2 h. The above process was repeated three times.

In vitro cell toxicity tests

Cell viability was assessed by exposing cells to MSN-TRP2 and MSN-TRP2-DOX particles with concentrations ranging from 0 μg/mL to 200 μg/mL for 24 h in 96-well plates (3000 cells/ well). The proliferation media consisted of 500 mL of DMEM, 50 mL of FBS, and 5 mL of PS. CellTiter-Blue® Cell Viability Assay Kit was used to quantify the cell viability. Fluorescence intensity was measured at an excitation wavelength of 570 nm and emission wavelength of 620 nm on a microplate reader. Cell viability was calculated by dividing the fluorescence intensity of the test sample by the fluorescence value of the blank control. Each sample was tested three times. Error bars represented the standard deviation of the results.

For the reductive response test, reduced glutathione (5 mM) was added to treat cells at 37 ºC and 42 ºC with MSN-TRP2 and MSN-TRP2-DOX particles. Then following the same procedures outlined for the thermal response test described above.

Confocal microscope imaging for particles in cellular uptake studies

Cellular uptake studies were performed in 96-well plates (3000 cells/well). Cells were exposed to 200 μg/mL of MSN-TRP2-DOX particles for 24 h at 37ºC and 42ºC. The proliferation media was discarded after incubation and the cells were washed with PBS for two times and further stabilized with dilute formaldehyde solution (10%). The cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for nucleus marking, then washed two times with PBS. The images were processed on a confocal microscope with DAPI channel (Ex: 405 nm, Em: 445 nm, HV: 150) and Texas Red channel (Ex: 561 nm, Em: 600 nm, HV: 110).

Intracellular delivery and release studies

Intracellular delivery and release of MSN-TRP2-DOX were studied with the following procedure. Two groups of cells were proliferated at 37ºC with media consisting of 500 mL of DMEM, 50 mL of FBS, and 5 mL of PS in 96-well plates (3000 cells/ well) by exposing cells to MSN-TRP2-DOX particles with concentrations 100 μg/mL or 200 μg/mL for 12 h. Then the cells were washed with PBS twice to remove MSN-TRP2-DOX particles which were not ingested, and the media was refreshed. Those two groups of cells were separately cultured at 37ºC and 42ºC for another 12 h. Cell viability was quantified using CellTiter-Blue® Cell Viability Assay Kit. Each sample was tested three times. Error bars represented the standard deviation of the results.

Laser scanning confocal microscope was used to study the cellular uptake of MSN-TRP2-DOX after cells were cultured at 37ºC for 12 h.

Besides, the release and cell toxicity results were collected from an automatic microplate reader (Varioskan Flash, Thermo Scientific). Fluorescence imaging was performed with an inverted fluorescence microscope (Nikon Eclipse Ti-E 2000).

Supplementary data

FTIR and ^{1}H-NMR spectrum of TRP2

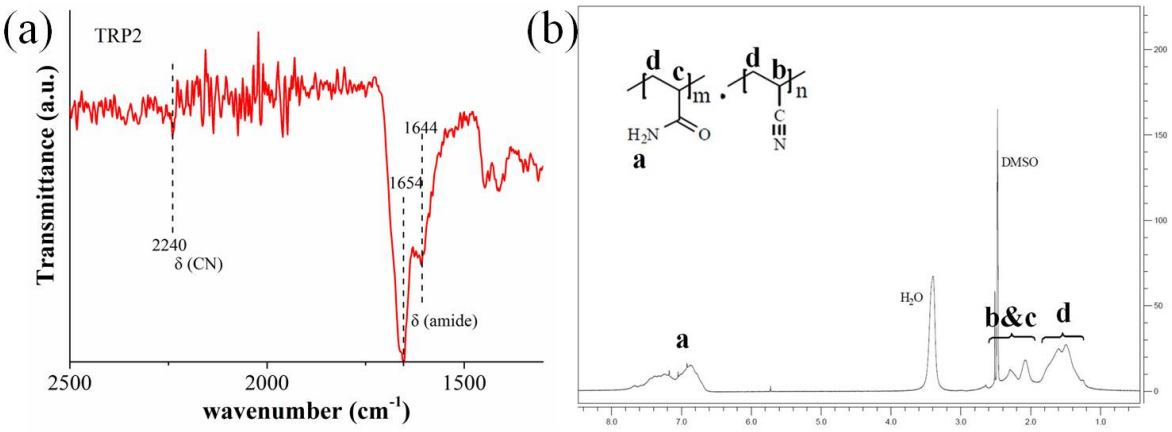

Figure S1. FTIR spectrum of TRP2 (a) and ^{1}H-NMR spectrum of TRP2 in DMSO-d_6

^{1}H NMR (400 MHz, DMSO-d_6, δ): 1.2-1.8 (polymer backbone, -CH_2-), 2.0-2.7 (polymer backbone, -CH-$CONH_2$ and -CH-CN), 6.6-7.6 (-NH_2).
TRP1 and TRP3 have similar spectrum of FTIR and ^{1}H-NMR as TRP2.

Determination of copolymer composition

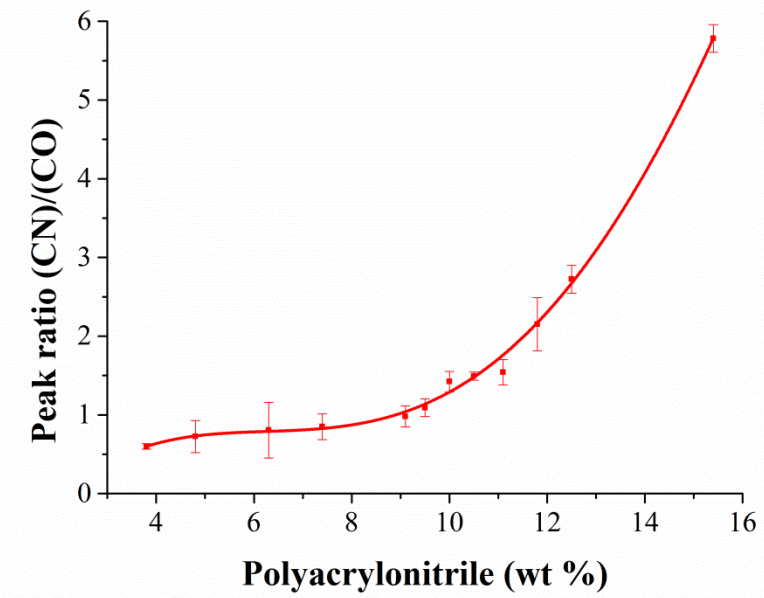

Figure S2. Calibration curve used for the determination of the copolymer compositions of TRP1, TRP2 and TRP3.

The hydrogen atoms connected to the carbon atom next to carbonyl and nitrile group (Figure S1b, **b** and **c**) have overlapped spectrum, which makes it difficult to determine the ratio of those two units. Thus, the ratios between the two segments in the copolymer were semi-quantified by integrating ratios of infrared vibration bands of the carbonyl and nitrile group using FTIR spectra according to above standard curve.

Dynamic light scattering results of polymer TRP1

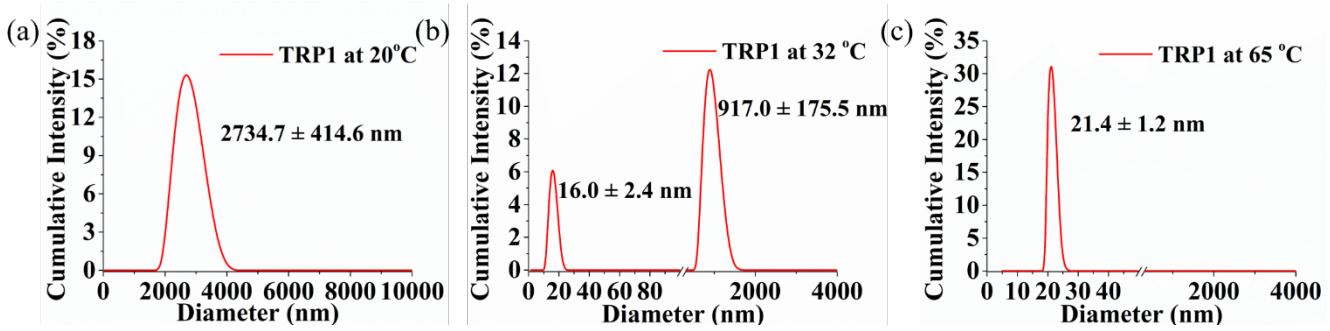

Figure S3. Dynamic light scattering results of polymer TRP1 (1 wt%) at different temperatures in PBS. (a) TRP1 solution at 20°C; (b) TRP1 solution at 32°C; (c) TRP1 solution at 65°C.

The dynamic light scattering results of TRP1 at different temperatures showed similar change tendency as TRP2 shown in the text of this paper (Figure 2).

Dynamic light scattering results of polymer TRP3

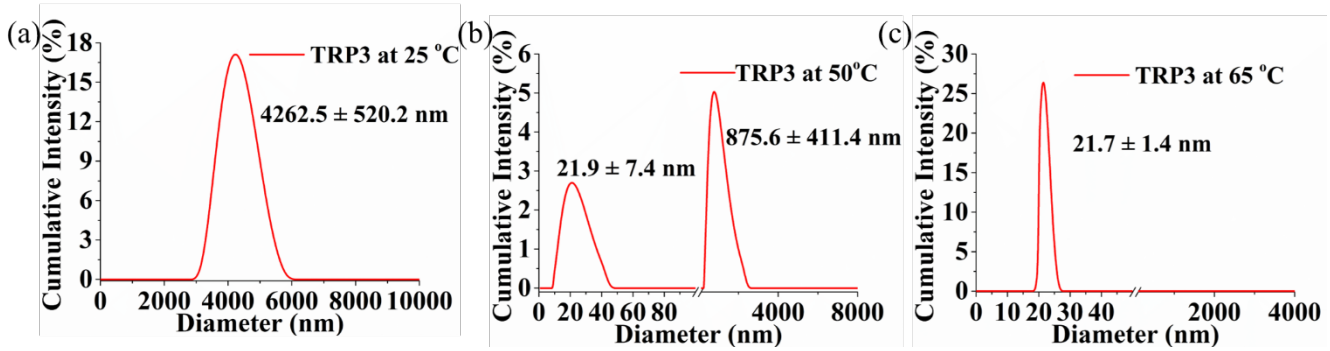

Figure S4. Dynamic light scattering results of polymer TRP3 (1 wt%) at different temperatures in PBS. (a) TRP3 solution at 25°C; (b) TRP3 solution at 50°C; (c) TRP3 solution at 65°C.

The dynamic light scattering results of TRP3 at different temperatures showed similar change tendency as TRP2 shown in the text of this paper (Figure 2).

Polymer morphology analysis in aqueous solution

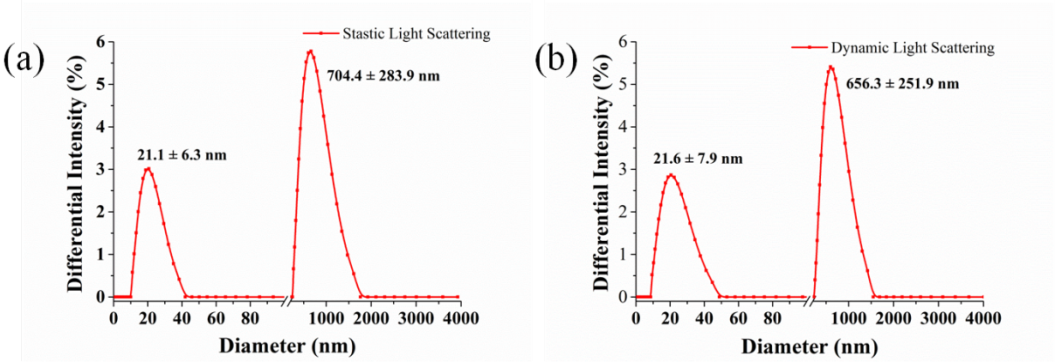

Figure S5. Size distribution of TRP2 under 42°C with static light scattering and dynamic light scattering on Zetasizer Nano ZSP (a) Gyration diameter D_g and (b) Hydrodynamic diameter D_h

The morphology of polymer chains in the aqueous solution could be verified through measuring the gyration diameter (D_g) and hydrodynamic diameter (D_h). Typical value of D_g/D_h is 1.55 for linear flexible chain coils, and around 0.774 for spheres or micelles. Herein, the ratio of D_g/D_h was around 1 for both smaller and larger particles, indicating that the TRP2 in the solution was twisted chains, rather than unimolecular polymer chains or micelles.

FTIR spectrum of MSN, TRP2 and MSN-TRP2

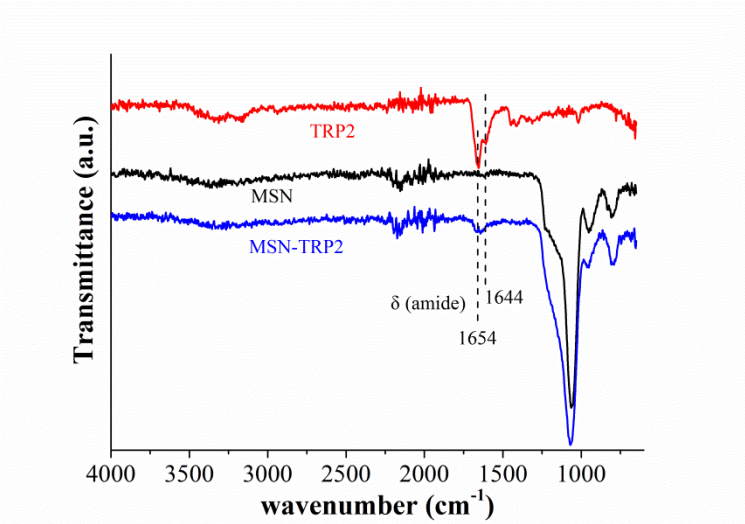

Figure S6. FT-IR spectrum of MSN, TRP2 and MSN-TRP2.

Amide bond vibration around 1650 cm^{-1} is the characteristic peak for TRP2. The FTIR spectrum of MSN-TRP2 included both the characteristic peaks of amide bond of TRP2 and the silica-oxygen characteristic peak of MSN around 1050 cm^{-1}, which illustrate the successful conjugation of TRP2 to MSN.

Zeta potential analysis of MSN, MSN-NH_2 and MSN-TRP2

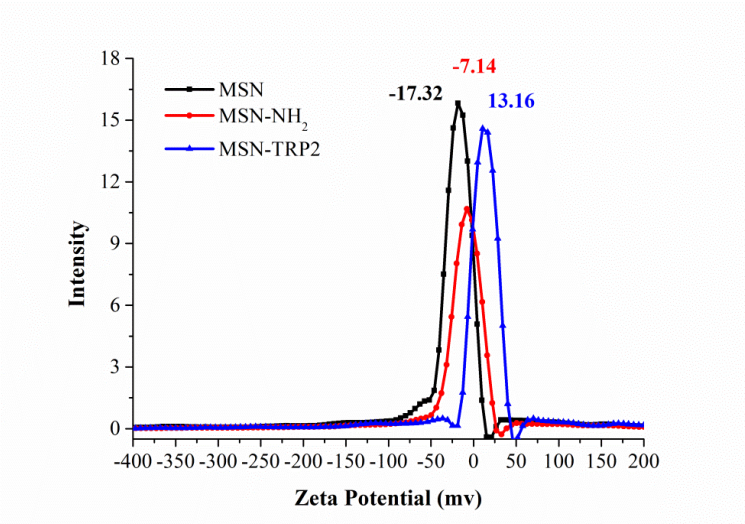

Figure S7. Zeta potential changes of MSN, MSN-NH_2 and MSN-TRP2 in PBS (0.1 wt %).

Surface modification on mesoporous silica nanoparticles could result in changes of surface charge, which was monitored with Delsa™ Nano C Particle Analyzer and showed as Zeta potential. The results in Figure S7 showed that the MSN presented negative charge on the surface and amino group modification partially neutralized the negative charge. Further conjugation with TRP2, which contains many of amide groups, made the surface of MSN-TRP2 positive with zeta potential around 13.16 mV.

Morphology changes of MSN-TRP2 with addition of DTT in solution at 37°C and 42°C

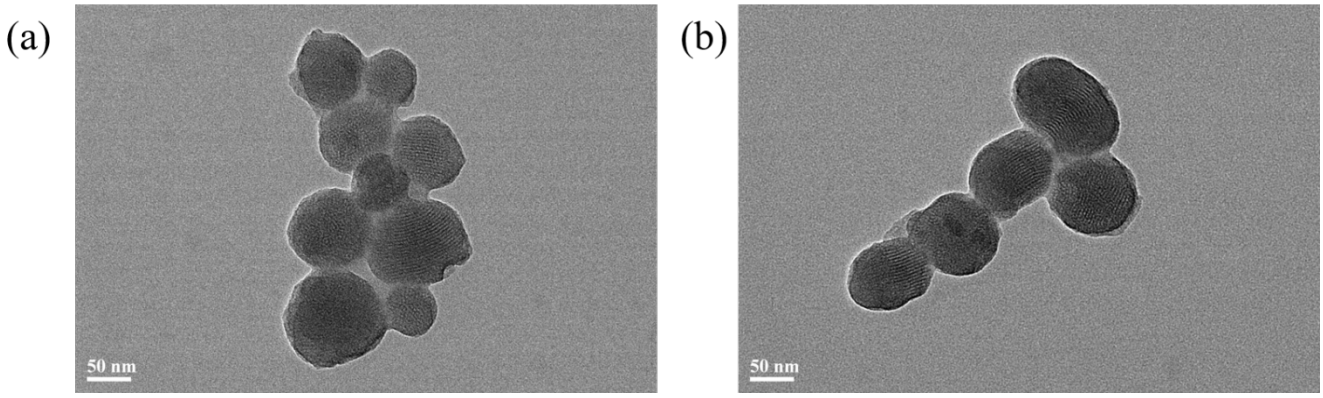

Figure S8. Transmission electron microscopy (TEM) images of MSN-TRP2 after reacted with DTT at 37°C (a) and 42°C (b) for 2 h.

The TEM images showed that there was no obvious change in MSN-TRP2 morphology after reacted with DTT at 37°C, compared with Figure 2b (not treated with DTT). The surface of those nanoparticles reacted with DTT at 42°C showed less polymer coating on the MSN. The difference could be due to the hindrance of a relatively more hydrophobic surface to DTT penetration at 37°C, and a more hydrophilic surface for DTT penetration and more disulfide bond breakage at 42°C.

Confocal imaging of cellular uptake for MSN-TRP2-DOX

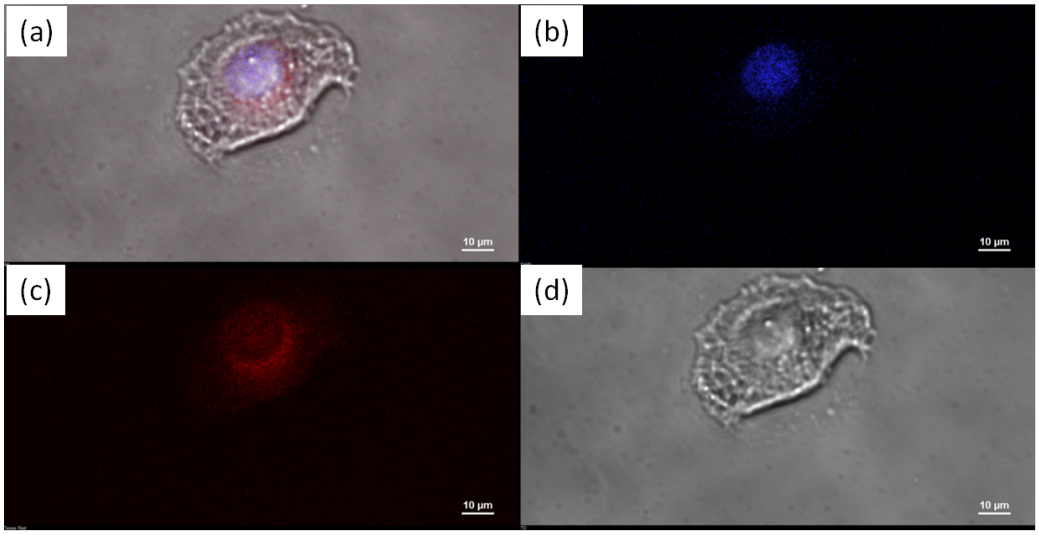

Figure S9. Confocal images of cellular uptake of MSN-TRP2-DOX after cultured at 37ºC for 12 h. (a) Merged images; (b) DAPI; (c) MSN-TRP2-DOX; (d) Bright field.

Cellular uptake test at 37ºC after 12 hours showed that the particles MSN-TRP2-DOX could enter the breast cancer cells, which provide the possibility for intracellular delivery and triggered release.

References

[1] M. Chen, C. Huang, C. He, W. Zhu,Y. Xu, and Y. Lu, *Chemical Communications*, **2012**,*48*, 9522-9524.
[2] W.Yang, H. Xue, W.Li, J. Zhang and S. Jiang, *Langmuir*, **2009**, *25*, 11911–11916.