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SUPPORTING INFORMATION

Engineered thermo-sensitive nanohybrid for accurate temperature sensing at single-cell level and biologically controlled thermal therapy

Fangyuan Dong,[1] Tingting Zheng,[1] Rongrong Zhu,[2] Shilong Wang,[2] Yang Tian[1]*

[1]*Shanghai Key Laboratory of Green Chemistry and Chemical Processes, Department of Chemistry, School of Chemistry and Molecular Engineering, East China Normal University, Dongchuan Road 500, Shanghai 200241, China*

[2]*School of Life Science, Tongji University, Siping Road 1239, Shanghai 200092, China*

**E-mail: ytian@chem.ecnu.edu.cn*

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Methods

Chemicals and materials

N-vinylimidazole (Monomer 1) (99%) was obtained from Aladdin (China) and distilled before use. Paraformaldehyde, 3-Methacryloxypropyltrimethoxysilane, N, N' -Methylenebisacrylamide (MBA), 3-mercaptopropyltrimethoxysilane (MPTES), tetraethyoxysilane (TEOS), 4-Benzylamino-7-nitro-2, 1,3-benzoxadiazole (BBD), and N, N-Methylenebisacrylamide were obtained from Aladdin (China) and used as received. 2, 2'-Azobis [2-(2-imidazolin-2-yl) propane] dihydrochloride (VA-044) was obtained from Wako Chemicals (Japan). Cetyltrimethylammonium bromide (CTAB) was purchased from Fluka (Japan). 3-(trimethoxylsilyl) propyl methacrylate (MPS) and Potassium Persulfate (KPS) were obtained from Macklin (China). Sodium Hydroxide AR (NaOH), sodium carbonate (Na_2CO_3), formaldehyde (HCHO), methanol, and ethanol were obtained from General-Reagent (China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and Annexin V-FITC apoptosis detection kit were purchased from Nanjing Keygen Biotech. Co., Ltd (China). Carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone (FCCP), Phorbol 12,13-dibutyrate (PDBu) was purchased from Sigma-Aldrich (USA). (S)-10-Hydroxycamptothecin (HCPT) was obtained from Selleck Company (USA). The solutions for the cell culture including phosphate buffered saline (PBS) solution; Dulbecco's modified eagle media/F12 (DMEM/F12) and 0.25% trypsin (with EDTA) were obtained from Hyclone Laboratories Company (USA). The penicillin (100 μg mL^{-1}) and streptomycin (100 μg mL^{-1}) were purchased from Gibco Life Technologies Company (USA). The fetal bovine serum was obtained from ScienceCell Research Laboratories Company (USA).

Instruments and apparatus

The morphology of the as-prepared materials was characterized by transmission electron microscope (TEM, JEOL JEM-2100F, Japan). Dynamic light scattering (DLS) measurement was obtained using a particle size analyzer (Nano ZS, Malvern Instruments Ltd., UK). Nitrogen sorption isotherm (BET assay) was obtained using a pore analyzer (Tristar 3000, Micromeritics, American). X-ray diffraction (XRD) pattern was recorded on an X-ray diffractometer (D8 advance, Bruker, Germany). XPS spectrum was recorded on X-Ray Photoelectron Spectrometer (XPS, AXIS Ultra DLD, Japan). The NMR spectrum was obtained using an NMR spectrometer (AC-80, BrukerBioSpin, Germany). Mass spectrum was obtained using an ESI time-of-flight mass spectrometer (micrOTOF II, Bruker, Germany). Transmittance and absorbance spectra were recorded using a UV-Vis spectrophotometer (Hitachi 5300, Hitachi High-Technologies Co., Japan). The fluorescence spectrum was conducted on a fluorescence spectrophotometer (F-4600, Hitachi High-Technologies Co., Japan) equipped with a peltier temperature controller (PTC-2, PGENERAL, China). Photoluminescence lifetime was measured by a homebuilt time-correlated single photon counting (TCSPC) system (4 ps per channel and 12500 channels) with electronics (PicoHarp 300 from Picoquant, Germany) and a super-continuum pulsed fiber laser (SC400-pp-4 from Fianium, UK) as the excitation source (405 nm).

Preparation of CD-MSN-MPS

According to the process outlined in a previous report [20], (1-Hexadecyl) trimethylammonium bromide (CTAB, 0.2 g) was dissolved in 96 mL purified water at 80°C under intensive stirring for 0.5 h. Next, 0.35 mL of 2 M NaOH, 1.4 mL of TEOS, and 0.15 mL of MPTPS were added drop-wise, 1.5 mL of

ethylacetate was then instantly added into the mixture, and the resulting solution was stirred for another 2 h. The products were collected by centrifugation and washed several times with ethanol and water before being re-dispersed in methanol. The powder was then calcined in a muffle furnace at 400°C for 2 h in the open air to obtain luminescent CD-MSN. The resulting CD-MSN nanoparticles were functionalized by saline MPS. First, 50 mg of CD-MSN nanoparticles were dispersed in 20 mL anhydrous ethanol at 80°C. Then, 0.1 mL MPS were added into the above solution. After 6 h, the products were collected by centrifugation and washed several times with ethanol and water to remove excessive MPS. Then, the suspension was extracted for 3 h with 20 μL saturated hydrogen chloride (HCl) at 60°C to remove the template CTAB. The as-prepared product was denoted as CD-MSN-MPS.

Synthesis of UPVH and CD-MSN@UP

Poly (N-vinylimidazole-co-1-vinyl-2-(hydroxymethyl)imidazole) with a UCST was designed and synthesized for modification of CD-MSN-MPS. As the starting point, 1-vinyl-2-(hydroxymethyl)imidazole (Monomer 1), was synthesized according to a previously reported procedure[19]. Paraformaldehyde (7.2 g), N-vinylimidazole (15 g), and Na_2CO_3 (0.3 g，2 wt%) were mixed with purified water (50 mL) and the suspension was heated to 130°C for 18 h. The products were purified through fractional distillation in a vacuum (1 mbar, 140°C) to yield 8.8 g (yield 44%) of 1-vinyl-2-(hydroxymethyl)imidazole (Monomer 2). 2.49 g of Monomer 2 and 2.07 g of Monomer 1 were dissolved in 10 mL pure water and flushed with pure nitrogen for 30 min before 0.13 g of initiator VA-044 was added. Then, the solution was polymerized for 48 h at 50°C. The solution was subsequently dialyzed (1 kDa) for 3 days against distilled water and then collected by centrifugation for further characterization of UCST behaviour. The as-prepared UCST polymer is denoted as UPVH hereafter. The as-prepared polymer with critical temperature (T_c) of 38°C is denoted as UP38.

Finally, the copolymer chains were grafted onto the surface of CD-MSN-MPS nanoparticles by seeded precipitation polymerization. Two monomers were copolymerized with the feed ratio Monomer 1: Monomer 2=0.95:1 using CD-MSN-MPS as the seed, KPS as the initiator, and MBA as the cross-linker. 50 mg of CD-MSN-MPS nanoparticles, 284.4 mg of Monomer 1, 215.6 mg of Monomer 2, 25 mg of MBA, 15 mg of KPS, and 50 mL of deionized water were added into a 250 mL two-neck flask equipped with a reflux condenser. The reaction was carried out for 5 h at 55°C under a nitrogen flow. The generated microspheres were collected by centrifugation and then washed with deionized water. The microspheres were then dispersed into a small amount of pure water and dialyzed for 48 h (50 kDa). The as-prepared microsphere using the polymer with T_c of 38°C was denoted as CD-MSN@UP38.

Dye and drug loading procedures

Water-sensitive fluorescent BBD was employed as an indicator dye for probing the temperature. For loading the dye (or anti-cancer drug), 20 mg BBD was first dissolved in 10 mL anhydrous ethanol, or 50 mg HCPT was dissolved in 20 mL trichloromethane. Next, 100 mg CD-MSN-MPS was added and the suspension was stirred for 24 h at room temperature under darkness. Then, the dispersion was centrifuged at 12,000 rpm for 10 min to collect the BBD-loaded or HCPT-loaded microspheres. Finally, the as-prepared microspheres were dried at 40°C under a vacuum overnight. The as-prepared CD-MSN@UP38 loaded with BBD and loaded with both BBD and HCPT were denoted as CD-MSN@BUP38 and CD-MSN@BHUP38, respectively.

The concentration of the remaining BBD solution was determined by fluorescence spectrophotometry (λ_{ex}=405 nm, λ_{em}=530 nm). The dye loading capacity was calculated as follows:

Encapsulation capacity (wt%) = ((fluorescence intensity of BBD in solution after loading)/(fluorescence intensity of feed BBD)) × 20 /100 × 100%. The drug loading capacity was estimated by comparing the UV absorbance of HCPT (290 nm) in supernatant before and after loading using the following equation: Encapsulation capacity (%) = ((UV absorbance of HCPT in solution after loading)/(UV absorbance of feed HCPT)) × 50 /100 × 100%.

Cell culture

HeLa and SH-SY5Y cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. HeLa cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's medium/F12 supplemented with 10% foetal bovine serum (Sciencell, California, USA), 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. On the night before the imaging experiments, all cells were seeded on 35-mm Petri dishes with 20 mm bottom wells. On the following day, the cells were incubated in fresh PBS containing 0.1 μM PDBu for 20 min, before the nanoparticle dispersions were added at a concentration of 100 μg mL^{-1}. After a 1-h incubation, the PBS buffer was replaced to remove the excessive particles that did not enter the cells.

In vitro cytotoxicity and apoptosis assay

SH-SY5Y cells were used to determine cytotoxicity and biocompatibility of the as-prepared hybrid probe CD-MSN@BUP38. In a typical MTT assay, cells were cultured in 96-well plates with 1 × 10^5 cells per well in the culture media (DMEM with 10% foetal bovine serum and 100 μg mL^{-1} penicillin/streptomycin). After incubation at 37°C in 5% CO_2 / 95% air for 24 h, the culture media was removed and the cells were incubated in culture medium containing the as-prepared CD-MSN@UP38 probes with different concentrations (1, 2, 4, 6, 10, 20, 40, 60, 80 or 100 μg mL^{-1}). Fresh PBS-containing media was used as the negative control. After another 24-h or 48-h incubation, 20 μL MTT (5 mg mL^{-1}) was added into every well and incubated at 28° C for another 4 h in the dark. After removing the medium, 150 μL DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in an ELx800 reader (BioTek Instruments, Inc, Winooski, VT). Cell viability values were determined according to the following formulae: cell viability (%) = absorbance of the experimental group/ absorbance of the blank control group × 100%.

The percentage of apoptotic cells was determined using an Annexin V-FITC apoptosis detection kit (Key-GEN Biotech) according to the standard protocol. In an apoptosis assay, SH-SY5Y cells were incubated with CD-MSN@UP38 probes at concentrations of 1, 2, 4, 6, 10, 20, 40, 60, or 100 μg mL^{-1} for 48 h. After removing the culture media, the cells were harvested using EDTA-free trypsin and collected by centrifugation. After washing with PBS, the cell pellets were suspended in 500 μL binding buffer and incubated with 5 μL FITC-Annexin V and 5 μL propidium iodide solution for 30 min in the dark. A Becton-Dickinson flow cytometer was used to evaluate cell apoptosis by monitoring the fluorescence of FL-1 (530 nm) and FL-2 (585 nm) at an excitation wavelength of 480 nm.

Confocal fluorescence imaging and fluorescence lifetime imaging

Confocal fluorescence imaging, including XY-scan and spectrum-scan, was performed with a Leica TCS-SP8 confocal scanning microscope using a 63x glycerol immersion objective (NA 1.4). Excitation was carried out with a 405 nm diode laser. Emissions were collected simultaneously from the two channel scan units: 425-480 nm (CD channel, F_{green}) and 485-700 nm (BBD channel, F_{red}). Heating of CD-MSN@UP loaded cells was performed using a thermostatic water bath. An increase in intracellular

temperature was induced by FCCP, a classical uncoupler of oxidative phosphorylation in mitochondria that can cause heat production by respiration. The fluorescence signal was measured at the indicated time points (0, 5, 10, 15, 25, and 35 min) after the addition of 10 μM FCCP. Confocal image processing was performed using the Leica LAS-X software. The merged images of the BBD channel and CD channel were generated by the Leica LAS-X software.

Fluorescence lifetime imaging was conducted on a TCS-SP8 confocal laser-scanning microscope (Leica) equipped with a multiple-photon laser (PDL 800-B, PicoQuant, Berlin, Germany) and TCSPC module SyPhotime-64 (Becker & Hickl, Berlin, Germany) for FLIM analysis. The obtained fluorescence decay curve in each pixel was fitted with a double exponential function to obtain the average lifetime. Histograms of the fluorescence lifetime in whole living cells were also obtained using SyPhotime-64 software (Becker & Hickl). The temperature of the culture medium was maintained at 28.0°C during the introduction of CD-MSN@UP38.

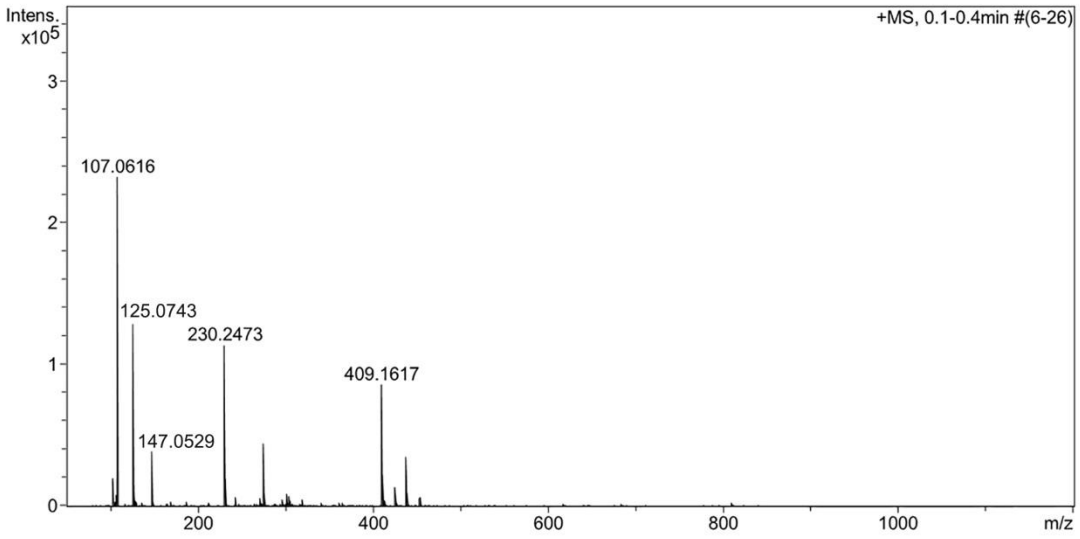
Animal experiments and drug release

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tongji University. Six-week-old female BALB/c mice, with an average weight of 20 ± 2 g, were purchased from the Laboratory Animal Center of the Chinese Academy of Science, and housed in the pathogen-free animal facility of Tongji University. SH-SY5Y cells were selected as the cancer model and HCPT was used as the anti-cancer drug. Before the animal experiments, MTT assays were conducted according to the above-mentioned procedure (Section: In vitro cytotoxicity and apoptosis assay) to assess the antitumour effects of HCPT against SH-SY5Y cells. SH-SY5Y cells at a density of 2×10^7 cells/mouse were subcutaneously injected into flanks of the nude mice two weeks before the animal tests. Mice were randomly divided into four groups consisting of 4 mice in each group on Day 1. Mice of Group 4 were administered an intraperitoneal injection of CD-MSN@BHUP38 for distribution imaging. Mice of Group 1, 2 and 3 received intraperitoneal injections of PBS (100 μL), 1 mg mL^{-1} HCPT (100 μL), and 6 mg mL^{-1} CD-MSN@HUP38 (100 μL) (dose: 5 mg HCPT/kg of body weight), respectively.

In vivo fluorescence imaging of Group 4 mice was conducted at the indicated time points after drug injection (0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, and 48 h) using a Berthold technologies LB 983 NC100 imaging system (λ_{ex} 475 nm, λ_{em} 560 nm). Thereafter, the mice were sacrificed and the tumour, liver, spleen, kidney, lung, and heart were harvested for isolated organ imaging to estimate the tissue distribution of nanoparticles.

After treatment for 14 days, all the mice were sacrificed by cervical dislocation for necropsy. Tumours were removed and weighed to test the drugs' effects on tumour growth. Major organs including the heart, liver, spleen, lung, and kidney as well as the tumours were harvested, fixed in 10% neutral buffered formalin, processed routinely into paraffin, sectioned at 4 μm, stained with H&E and examined under a BX51 optical microscope.

a)



b)

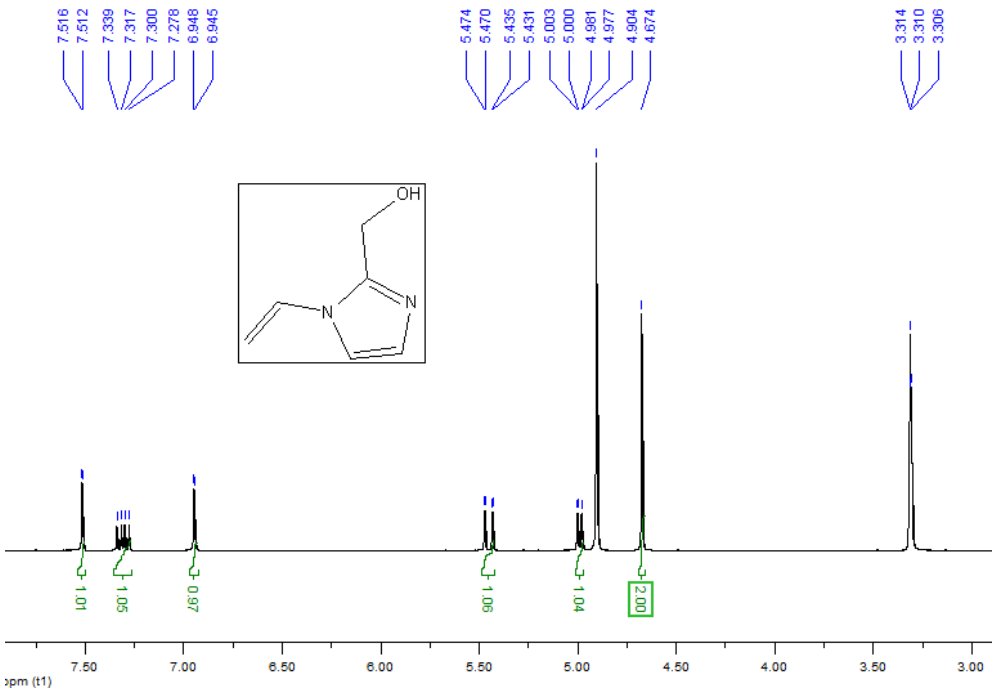


Figure S1. a) MS spectrum of 1-vinyl-2-(hydroxymethyl)imidazole (Monomer 1). MS *m/z* = 124 [M (2)], 230, 123, 107, 95, 81, 68. b)^{1}H NMR spectrum of 1-vinyl-2-(hydroxymethyl)imidazole (Monomer 1). ^{1}H NMR (400Hz,MeOD): δ (ppm) = 7.514 (d, 1H, CH-CH, J=1.6Hz); 7.309 (dd, 1H, CH-CH_2, J=15.6Hz, 9.2Hz); 6.947 (d,1H, CH-CH, J=1.6Hz); 5.463 (dd,1H,CH_2 –H, J=15.6Hz, 1.6Hz); 4.990 (dd, 1H, CH-CH_2, J=9.2Hz, 1.6Hz); 4.644 (s, 2H,CH_2 –H).

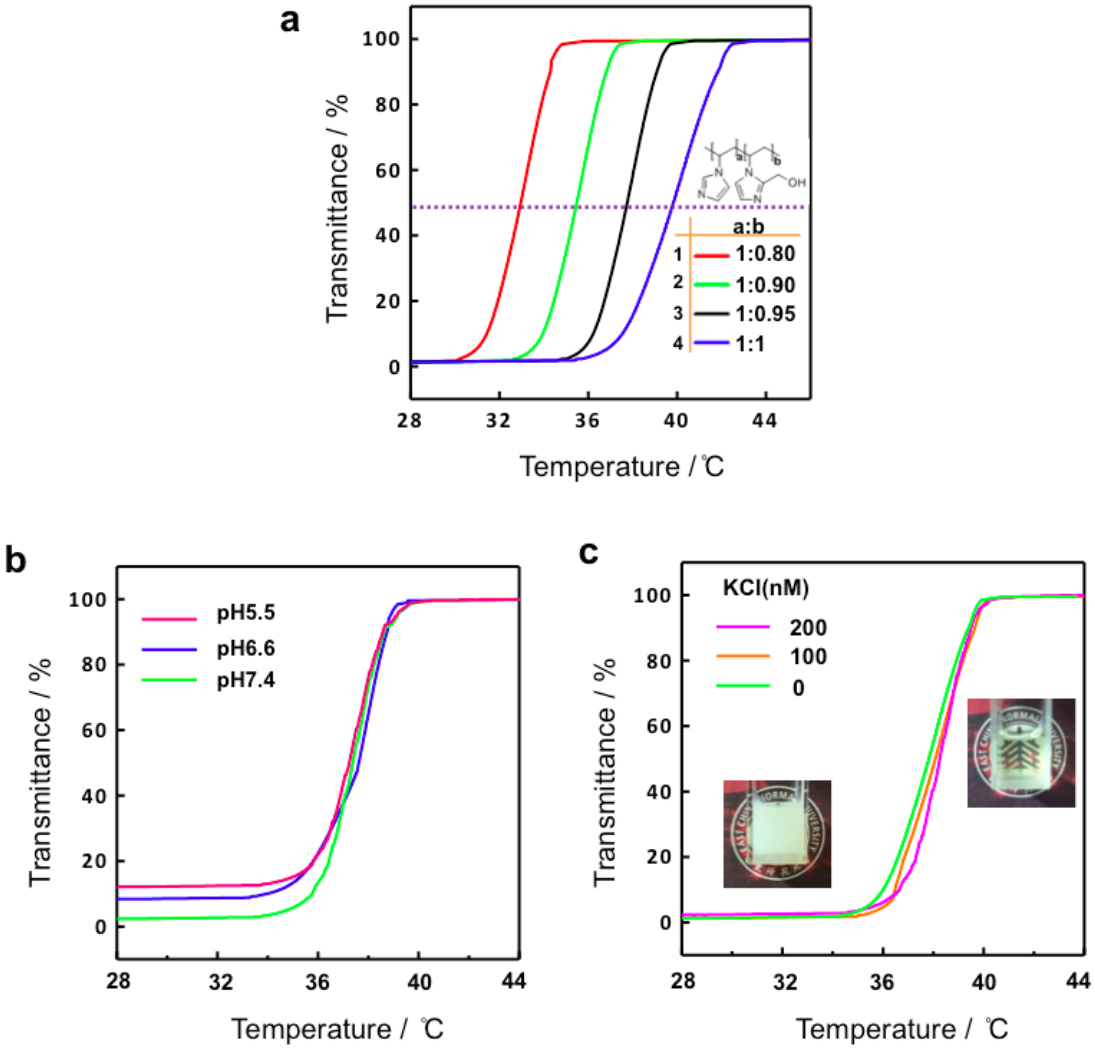


Figure S2. a) Transmittance curves upon heating of copolymer 1, 2, 3, 4 (with different feed ratio) in pure water (pH=7.4) at a heating rate of 0.2 °C min^{-1}. b) Transmittance curves upon heating of 1wt% solutions of copolymer UP38 (1:0.95 / Monomer 2: Monomer 1) towards environmental pH change at a heating rate of 0.2 °C min^{-1}.c) Transmittance curves upon heating of 1wt% solutions of copolymer UP38 (1:0.95 / Monomer 2: Monomer 1) towards environmental ion strength change at a heating rate of 0.2 °C min^{-1}.

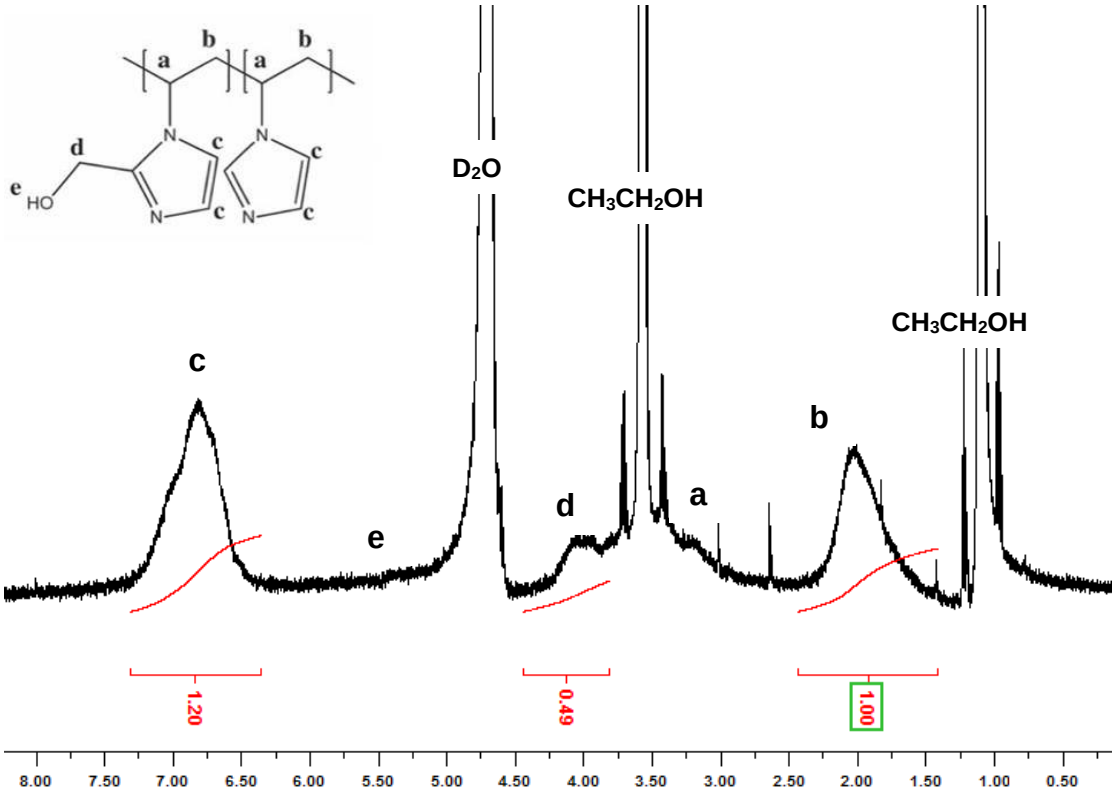


Figure S3. ^{1}H NMR spectrum of polymer UP38 (monomer feed ratio of 1: 0.95 / Monomer 2: Monomer 1) after purification. Characteristic signals for the methylene group (d) and OH group (e) of monomer 1 are assigned. Peak *a* and *d* were used for determination of copolymer composition through peak integration. The calculation equation goes like: molar ratio (Monomer 2: Monomer 1) =(Peak area of b-Peak area of d)/ Peak area of d, which in this case was 1:0.96. The signals for methylene group and methyl group appeared in the spectrum belong to ethanol since small amount of ethanol were added to improve the solubility of copolymers crystal in D_2O.

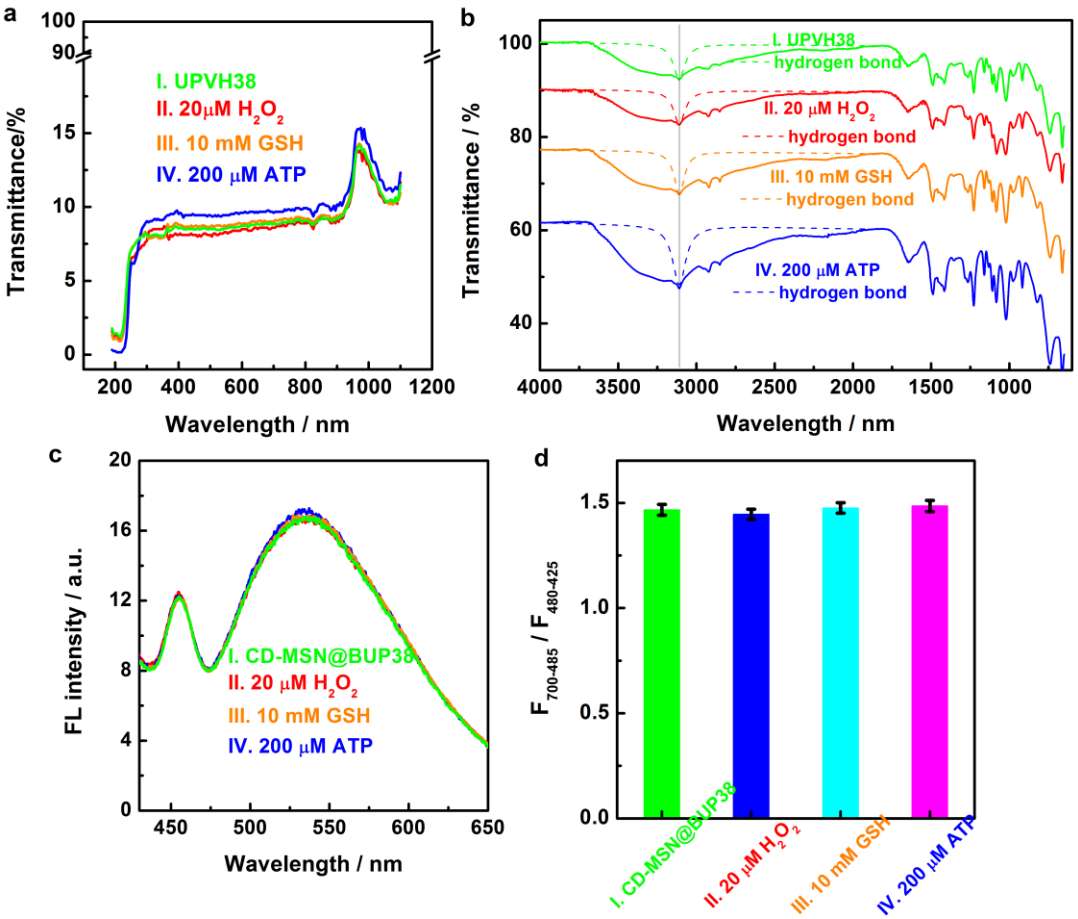


Figure S4. a) Transmittance curves of copolymer UPVH38 in I) pure water, and upon the addition of II) 20 μM H_2O_2, III) 10 mM GSH and IV) 200 μM ATP. b) IR spectroscopy of hydrogen bond of copolymer UPVH38 in I) pure water, and upon the addition of II) 20 μM H_2O_2, III) 10 mM GSH and IV) 200 μM ATP. c) Fluorescence spectra of UPVH38 in I) pure water, and upon the addition of II) 20 μM H_2O_2, III) 10 mM GSH and IV) 200 μM ATP. Excitation: 405 nm. d) Plot of $F_{700\text{-}485}/F_{480\text{-}425}$ of UPVH38 in I) pure water, and upon the addition of II) 20 μM H_2O_2, III) 10 mM GSH and IV) 200 μM ATP (Error bars: S.D., n = 3).

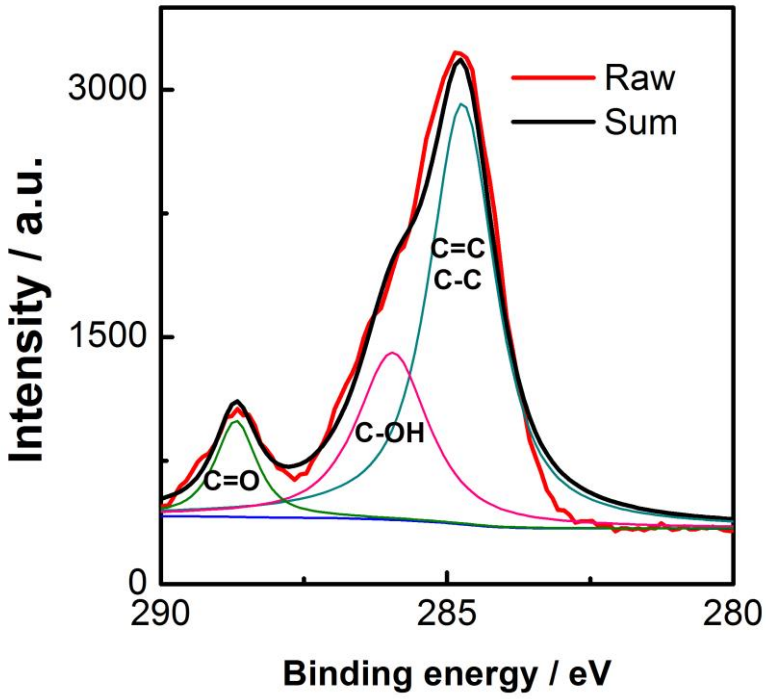


Figure S5. X-ray photon spectroscopy (XPS) of CD-MSN.

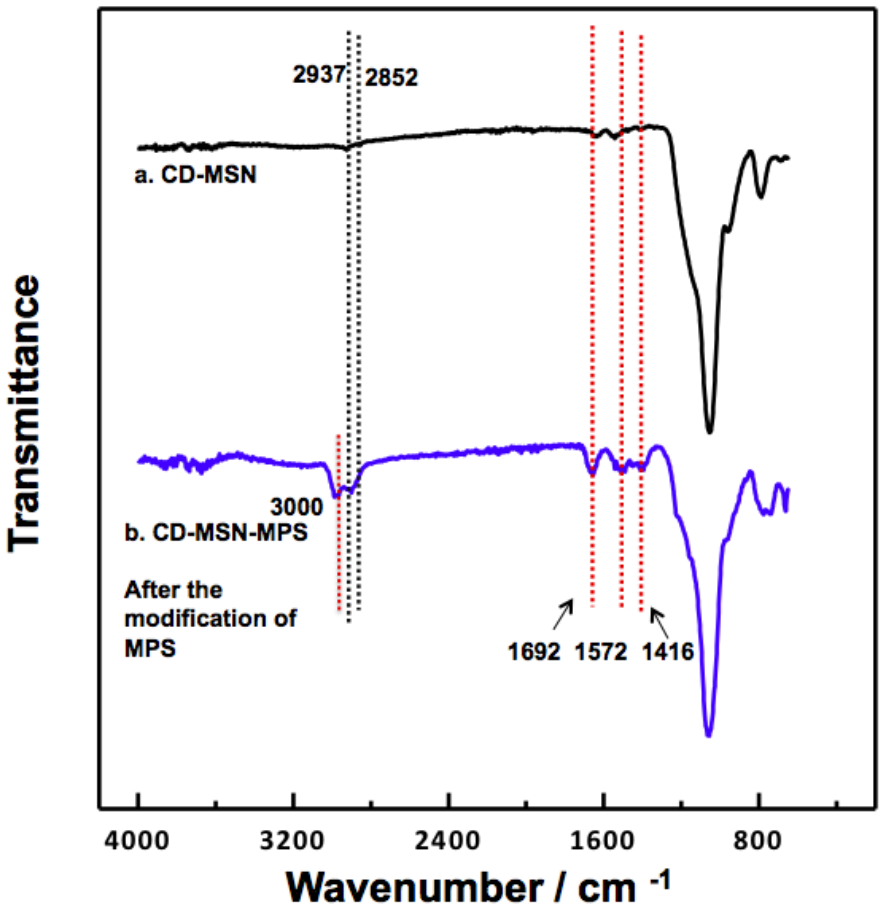


Figure S6. FT-IR spectra of a) CD-MSN and b) CD-MSN-MPS (red lines show distinct characteristic bands of MPS).

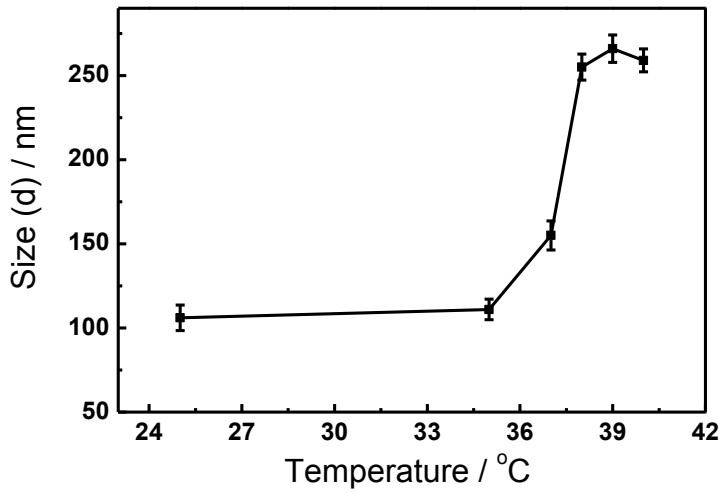


Figure S7.Hydrodynamic diameters of CD-MSN@BUP38 at different temperatures (25°C, 35°C, 37°C, 38°C, 39°C, 40°C) measured by DLS method. (Error bars: S.D., n=3).

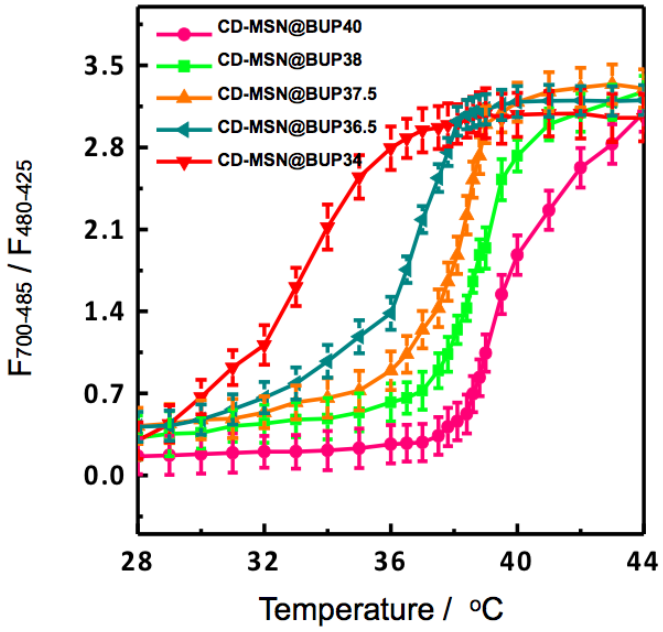


Figure S8.Thermometry measure results of CD-MSN@BUP prepared with different monomer feed ratios: CD-MSN@BUP34 (1:0.80 / Monomer 2: Monomer 1), CD-MSN@BUP36.5 (1:0.85 / Monomer 2: Monomer 1), CD-MSN@BUP37.5 (1:0.90 / Monomer 2:Monomer 1), CD-MSN@BUP38 (1:0.95 / Monomer 2: Monomer 1), CD-MSN@BUP40 (1:1 / Monomer 2:Monomer 1). (Error bars: S.D., n=3).

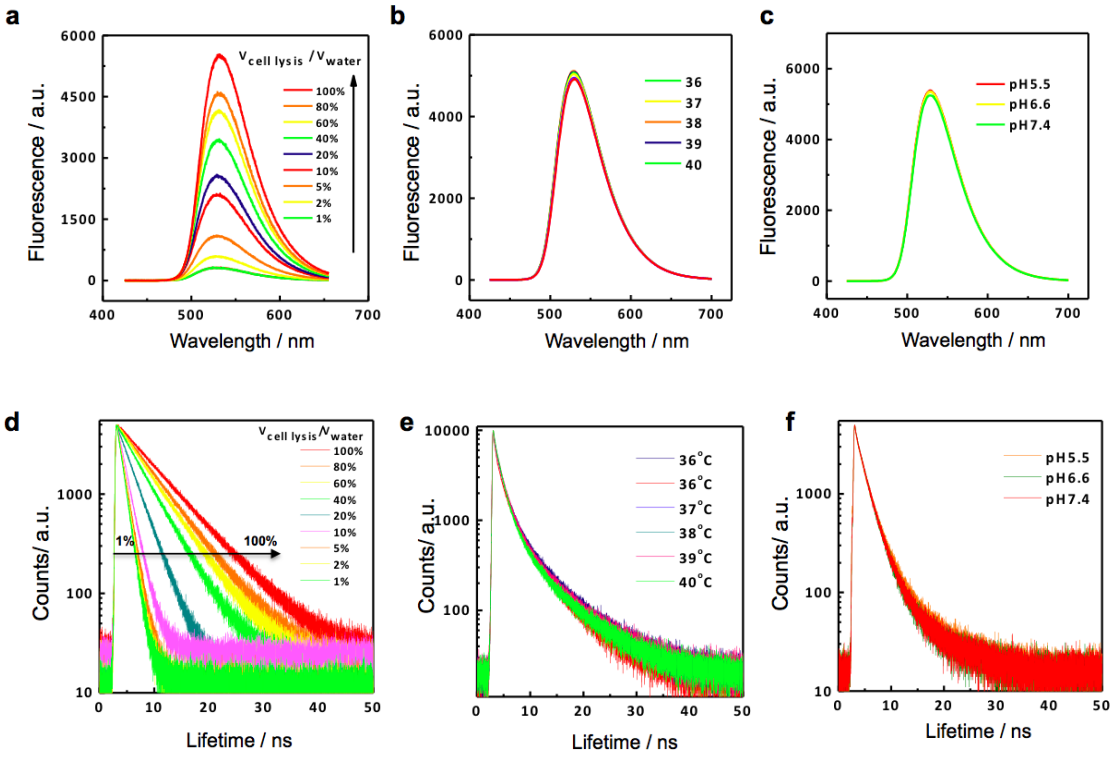


Figure S9.**Fluorescence properties of dye BBD:** a) Fluorescence spectra of BBD in different mixed solvents. b) Fluorescence spectra of BBD measured under different temperatures in SHSY-5Y cell lysis solution. c) Fluorescence spectra of BBD measured at different pH environment in SHSY-5Y cell lysis solution. d) Fluorescence decay curves of BBD in different mixed solvents. e) Fluorescence decay curves of BBD measured under different temperatures in SHSY-5Y cell lysis solution. f) Fluorescence decay curves of BBD measured at different pH environment in 50% SHSY-5Y cell lysis solution.

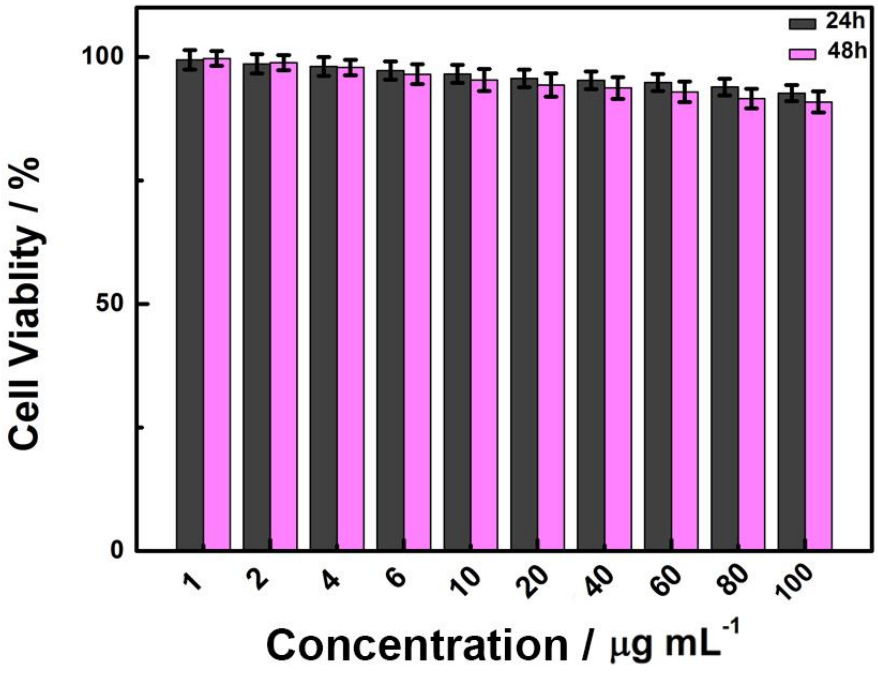


Figure S10. MTT assay results of SHSY-5Y cell incubated with different concentrations (1, 2, 4, 6, 10, 20, 40, 60, 80, 100 μg mL^{-1}) of CD-MSN@BUP38 for 24 and 48 h. (Error bars: S.D., n=5).

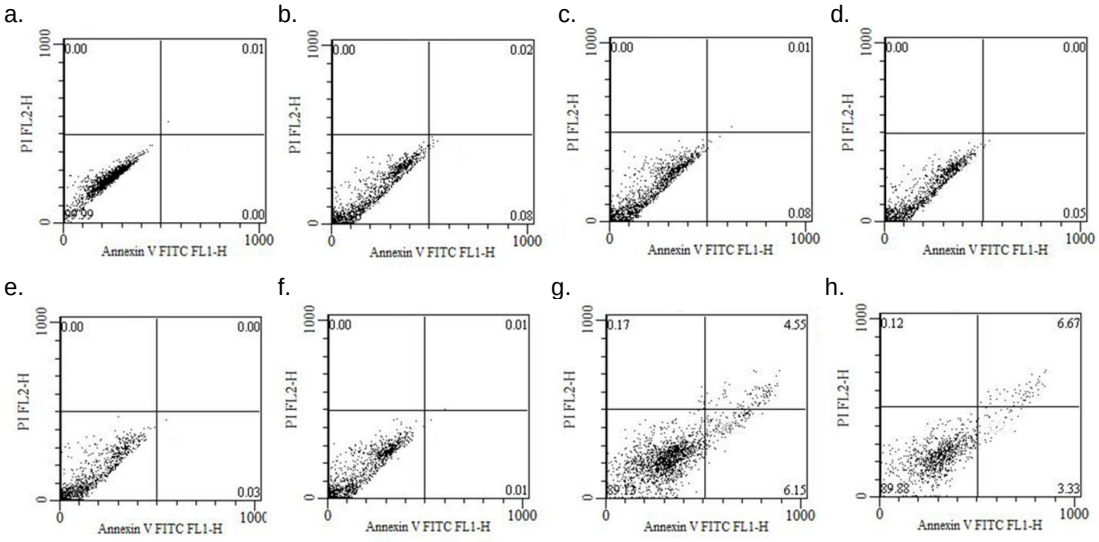


Figure S11. Cell apoptosis assay of SH-SY5Y cell after incubated with different concentrations: a) 1, b) 2, c) 6, d) 10,e) 20, f) 40, g) 60, h) 100μg mL^{-1} of CD-MSN@BUP38 for 24 h

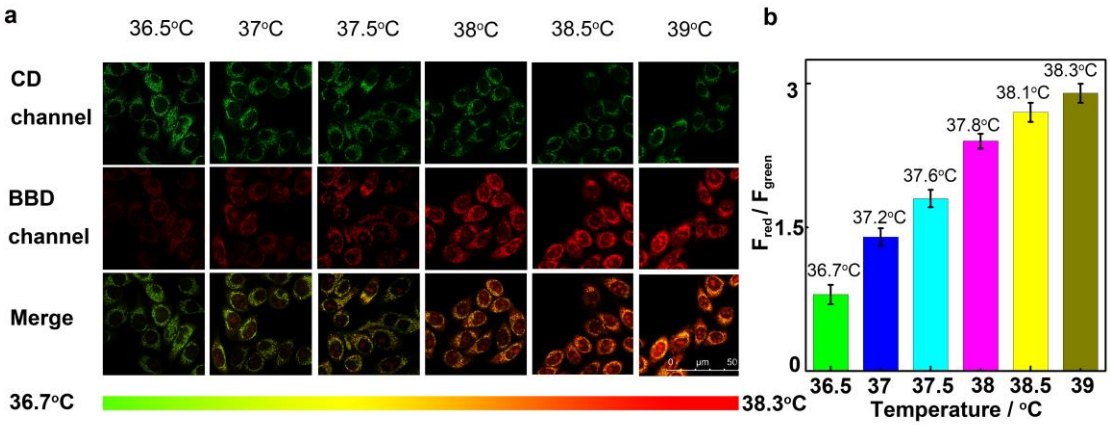


Figure S12. **Intracellular thermometry of HeLa cells using fluorescent thermometer CD-MSN@UP38.** a) Confocal fluorescence images of live Hela cells labeled with CD-MSN@BUP38 under different ambient temperatures at 36.5, 37, 37.5, 38, 38.5, and 39°C. b) The total fluorescence ratios between two channels (green channel: 425-480 nm, red channel: 485-700 nm) as a function of temperature. Data were obtained from Figure S11a (Error bars: S.D., n=40).

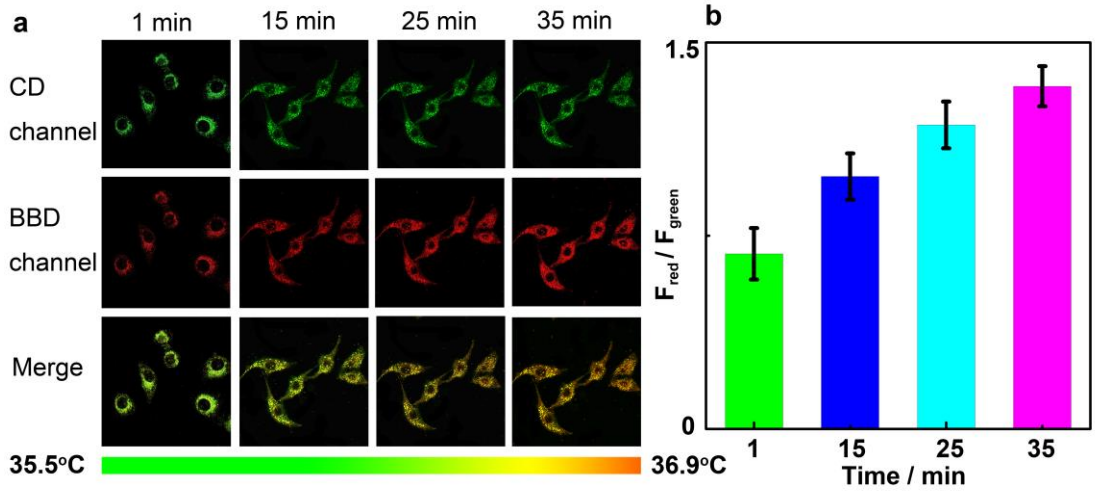


Figure S13. a) Confocal fluorescence images of live HeLa cells labeled with CD-MSN@BUP38 after stimulated by FCCP for 1, 15, 25 and 35 min. b) The total fluorescence ratios between CD channel and BBD channel as a function of temperature. Data were obtained from Figure S12a (Error bars: S.D., n=40).

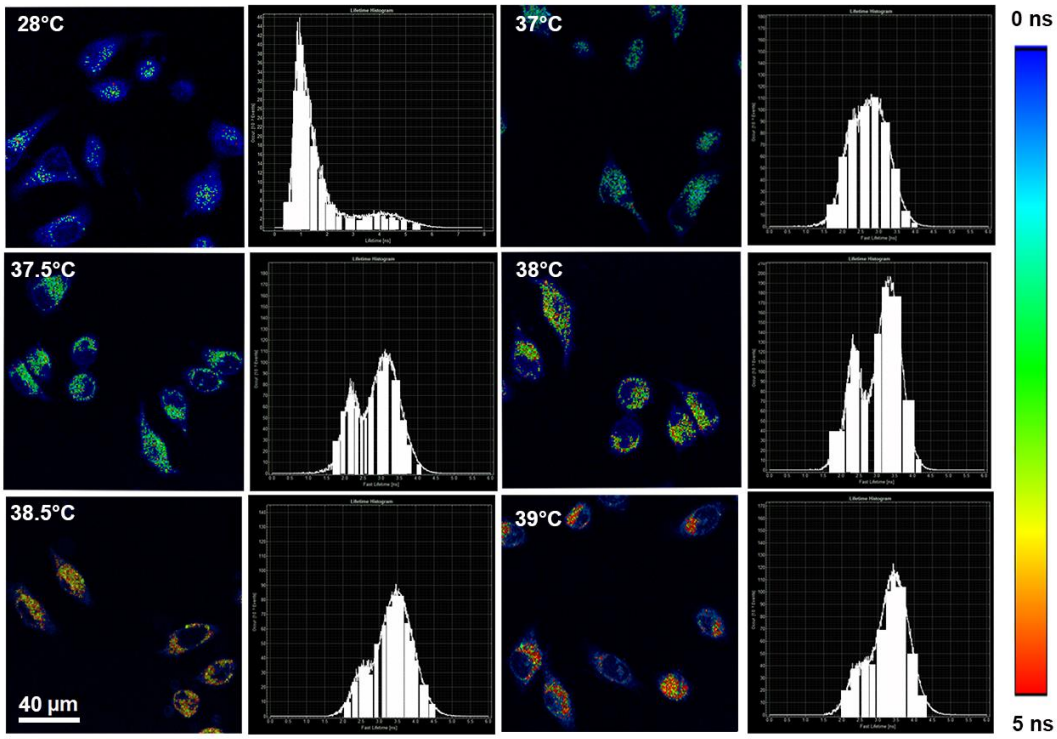


Figure S14. Fluorescence lifetime images Hela cells labeled with CD-MSN@BUP38 and the histograms of fluorescence lifetime in whole living cells. The temperatures were the culture medium temperature. Color bar represents the average lifetime calculated from the decay curves.

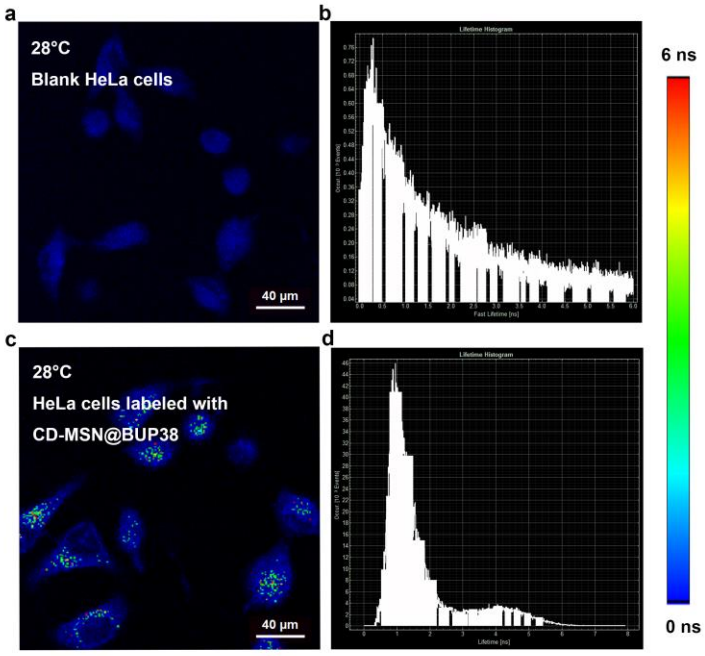


Figure S15. a, c) Fluorescence lifetime imaging of HeLa cells (a) before and (c) after labeled with CD-MSN@BUP38 at room temperature. b, d) Histograms of the fluorescence lifetime in Hela cells (b) before and (d) after labeled with CD-MSN@BUP38 at room temperature.

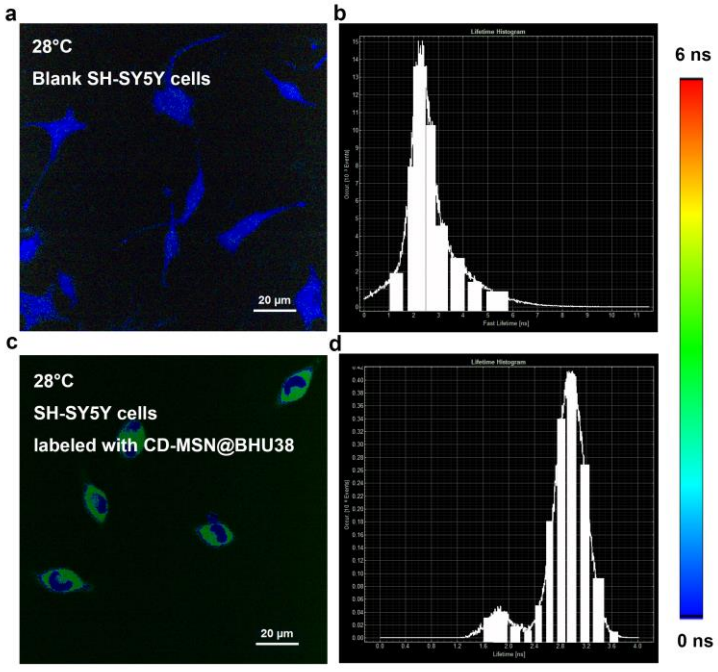


Figure S16. a, c) Fluorescence lifetime imaging of SH-SY5Y cells (a) before and (c) after labeled with CD-MSN@BUP38 at room temperature. b, d) Histograms of the fluorescence lifetime in SH-SY5Y cells (b) before and (d) after labeled with CD-MSN@BUP38 at room temperature.

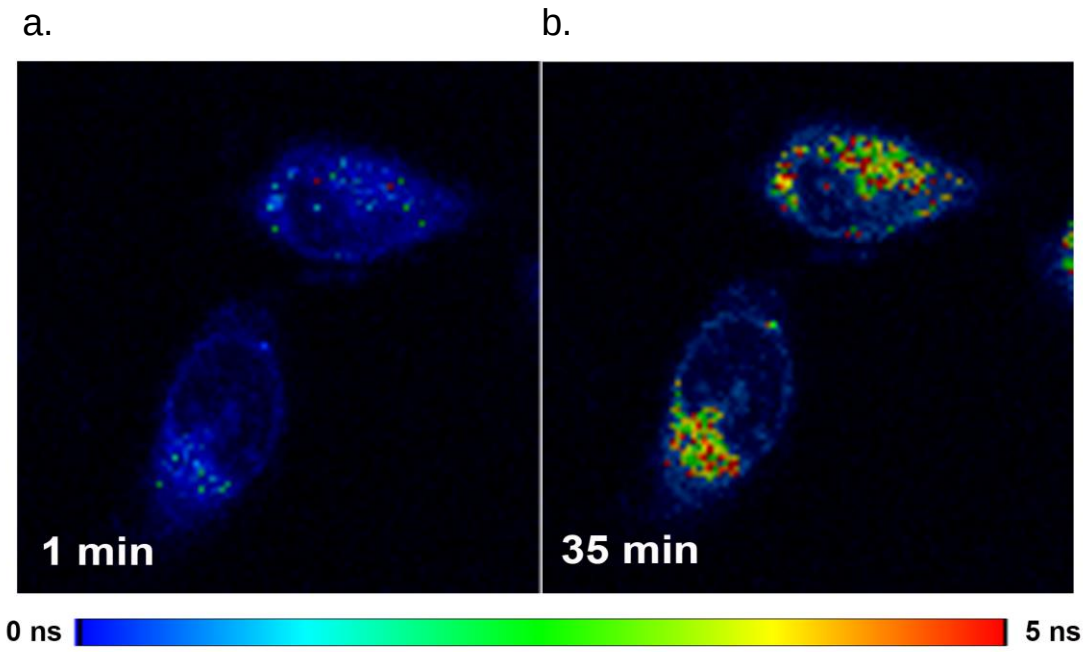


Figure S17. a, b) Fluorescence lifetime imaging of SH-SY5Y cells labeled with CD-MSN@BUP38 after FCCP treatment for a) 1 minute and b) 35 minutes at room temperature.

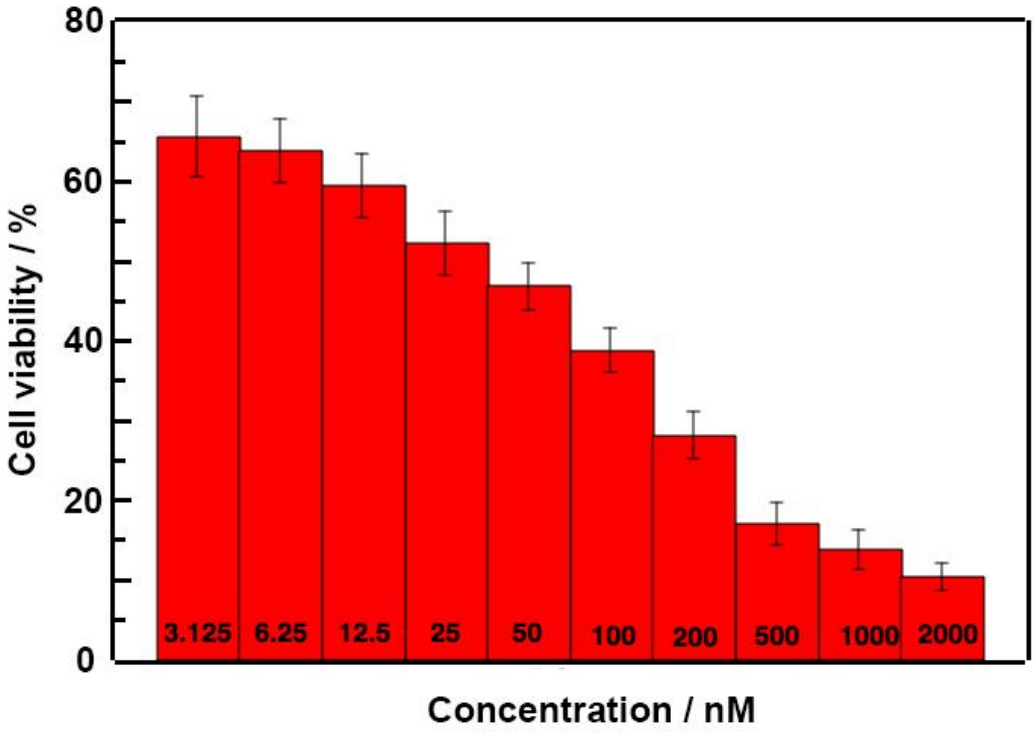


Figure S18. Cell viability assay of SHSY-5Y cells after treatment with different concentrations (3.125, 6.25, 12.5, 25, 50, 100, 200, 500, 1000, 2000 nM) of HCPT loaded CD-MSN@UP38 for 24 h.

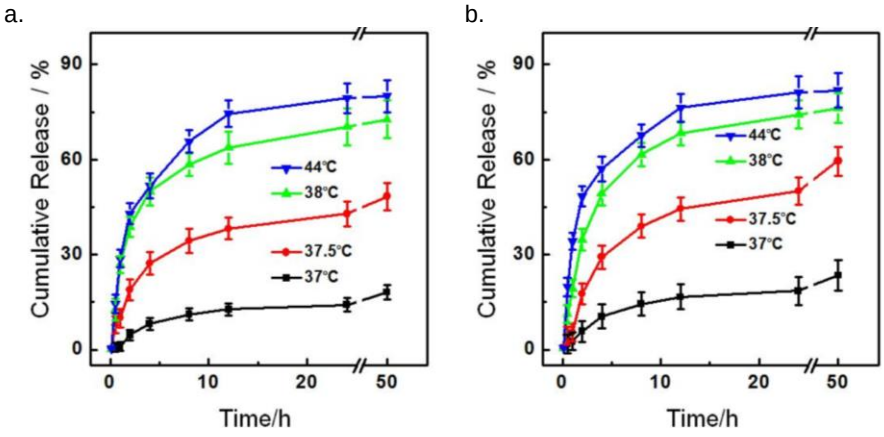


Figure S19. Cumulative HCPT release profiles of CD-MSN@HUP38 at (a) pH 6.5 and (b) pH 5.5 in 150 nM KCl solution at 37, 37.5, 38, 40 ºC.

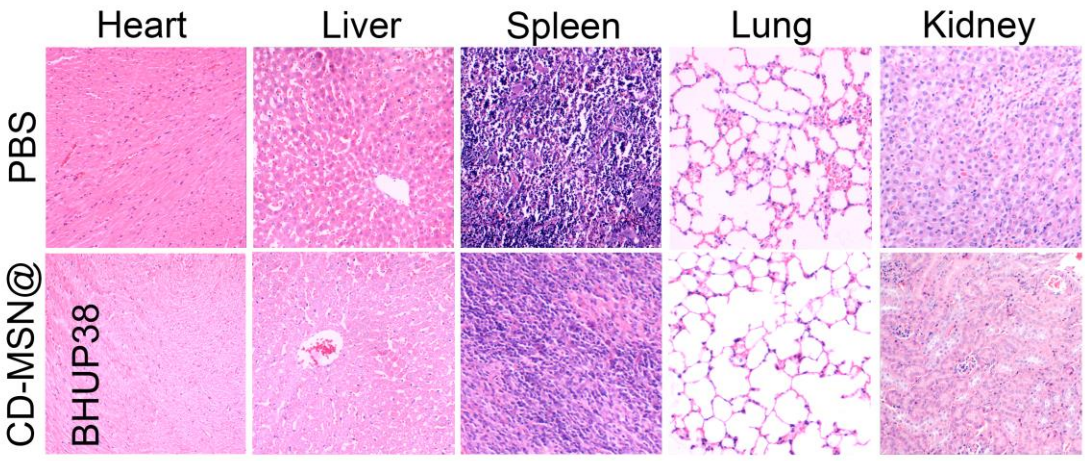


Figure S20. Representative HE-stained sections of heart, liver, spleen, lung, and kidney harvested from mice in PBS group and CD-MSN@BHUP38 group after anti-cancer treatment for 14 days.