Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Tumor suppression via diverting intracellular sialylation with multifunctional nanoparticles

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Experimental section

Materials. Mesoporous silica nanoparticles (MSNs) with an average size of 80 nm and pore volume of 1.4 mL/g were obtained from Nanjing XFNANO Materials Tech. Co., Ltd (Nanjing, China). (3-Aminopropyl) triethoxysilane (APTES, ≥ 98%), 4-mercaptophenylboronic acid (MPBA), cytidine-5'-monophospho-N-acetylneuraminic acid sodium salt (CMP-Sia), alpha-2,3-sialyltransferase (ST3Gal) expressed in *E. coli* BL21 from *Pasteurella multocid*, alpha-2,6-sialyltransferase (ST6Gal) expressed in *E. coli* BL21 from *Photobacterium damsela*, cytidine 5'-monophosphate disodium salt (CMP), and uridine 5'-diphosphate disodium salt hydrate (UDP) were obtained from Sigma-Aldrich Inc. (USA). Galactose PEG 35K succinimidyl carboxymethyl ester (Gal-PEG-NHS) was obtained from Beijing JenKem Tech. Co., LTD (Beijing, China). Folic acid PEG 10K succinimidyl carboxymethyl ester (FA-PEG-NHS) and succinimidyl carboxymethyl ester PEG 20K succinimidyl carboxymethyl ester (NHS-PEG-NHS) were obtained from Shanghai Peng Sheng Biotech. Co., Ltd (Shanghai, China). All DNA sequences were customized from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China). Their sequences were listed as follows (5'-3'):

DNA1: FAM-GTCGTGGGTCT-NH_2;

DNA2: AGACCCACGAC-BHQ1;

DNA1': Cy5-GTCGTGGGTCT-NH_2;

DNA2': AGACCCACGAC-BHQ2;

DNA3: Maleimide-ACCCACGAC-Cy5;

DNA3': Maleimide-ACCCACGAC.

Cell Light™ Lysosomes-RFP and Golgi-RFP, BacMam 2.0 were obtained from Thermo Fisher Scientific (USA). 4',6-Diamidino-2-phenylindole (DAPI) was obtained from Titan Technology Co., Ltd. (Shanghai, China). FITC conjugated Sambucus nigra agglutinin (F-SNA) was provided by Vector Laboratories (USA). MCF-7 cells were from KeyGen Biotech. Co. Ltd. (Nanjing, China). Phosphate buffer saline (PBS) contained 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, and 1.41 mM KH_2PO_4. The pH of PBS was adjusted by addition of HCl or NaOH. Tris EDTA buffer (TE, pH 8.0) contained 10 mM Tris-HCl and 1 mM EDTA. For hybridization of DNA, 100 mM NaCl and 10 mM $MgCl_2$ were added in TE. All other reagents were of analytical grade. All aqueous solutions were prepared using ultrapure water (≥ 18 MΩ/cm, Milli-Q, Millipore).

Apparatus. The UV-vis absorption spectra were recorded on an UV-3600 UV-vis-NIR spectrophotometer (Shimadzu, Japan). Fluores-cence spectra were recorded on an F-7000 fluorescence spectrophotometer (Hitachi, Japan). The gel electrophoresis was performed on an electrophoresis analyser (Bio-Rad, USA) and imaged on a Bio-Rad ChemDoc XRS (Bio-Rad, USA). The zeta potential and dynamic light scattering analyses were performed on a Brookhaven 90Plus Particle Size Analyzer (Brookhaven, USA). Transmission electron microscopic (TEM) images were acquired on a JEM-2100 transmission electron microscope (JEOL Ltd., Japan). BET analysis were performed on an ASAP 2460 automatic BET surface area and pore size analyzer (Micromeritics, USA). Fourier Transform infrared (FTIR) spectra were acquired on a Nicolet iS50 FTIR spectropho-tometer (Nicolet iS50, USA). Confocal fluorescence imaging of cells was performed on a SP8 STED 3X confocal laser scan-ning microscope (CLSM) (Leica, Germany). Flow cytometric analysis was performed on a FACSAria II flow cytometer

(Beckman-Coulter, USA). The numbers of cells were determined using a Countess II FL automated cell counter (Life Tech-nologies, USA). CCK8 assays were performed on a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, USA). *In vivo* imaging experiments were performed on a PerkinElmer Caliper IVIS Lumina XR III *in vivo* imag-ing system (Waltham, Massachusetts, USA).

Calculation of the average molar mass of MSNs. As the density of amorphous SiO_2 has been reported as 2.2 g/cm^3, the volume of 1 g amorphous SiO_2 could be calculated as 0.45 mL. Since the pore volume of the used MSNs was 1.4 mL/g, the total volume of 1 g MSNs could be regarded as the sum of amorphous SiO_2 and pore, which was 1.85 mL. Thus the average density of the used MSNs could be calculated as 0.54 g/cm^3. Combining with average size of the used MSNs (80 nm), the average mass of each MSN could be calculated to be 1.45×10^{-16} g. Thus the average molar mass of the used MSNs was determined to be 8.73×10^7g/mol.

Preparation of multifunctional MSNs (MSNs/ssDNA@FA/Gal/dsDNA, MFMSNs). The dsDNA and dsDNA' probes were firstly prepared by incubating 10 μM of DNA1 or DNA1' with 10 μM of DNA2 or DNA2' in TE (containing 100 mM NaCl and 10 mM $MgCl_2$) with a total volume of 2 mL at 37 °C overnight, respectively. The ssDNA and ssDNA' probes were obtained respectively by incubating 100 μM of DNA3 or DNA3' with 1 mM of MPBA in TE (containing 1% DMSO) with a total volume of 0.5 mL at room temperature overnight, which were then purified by ultrafiltration with 3kD ultrafilter for three times and dispersed in 0.5 mL pH 7.4 PBS.

The amino MSNs were then prepared by hydroxylating 500 mg of MSNs with 10 mL of piranha solution (containing 7 mL of H_2SO_4 and 3 mL 30% H_2O_2) under stirring overnight at room temperature, and incubating the MSNs with 10 mL of APTES solution (1% in ethanol) under stirring at 36 °C overnight. After washed with ethanol and water for three times respectively, the amino MSNs were dispersed in TE with a final concentration of 100 nM.

Afterward, 10 nM of amino MSNs was incubated with the mixture of 50 μM Gal-PEG-NHS, FA-PEG-NHS, and NHS-PEG-NHS in TE with a total volume of 2 mL under stirring overnight at room temperature, and the obtained FA, Gal and NHS co-functionalized MSNs (MSNs@FA/Gal/NHS) were separated by centrifugation and washed with TE for three times. For tumor suppression, the FA and Gal co-functionalized MSNs (MSNs@FA/Gal) were prepared by incubating 10 nM of amino MSNs with the mixture of 50 μM Gal-PEG-NHS and FA-PEG-NHS in TE with a total volume of 2 mL under stirring overnight at room temperature, which were separated by centrifugation and washed with pH 7.4 PBS for three times. The obtained MSNs@FA/Gal were dispersed in 2 mL pH 7.4 PBS and stored at 4 °C. Besides, the only FA functionalized MSNs (MSNs@FA) and FA-free MSNs (MSNs@Gal/NHS) were respectively prepared with the same procedure without addition of Gal-PEG-NHS and NHS-PEG-NHS or FA-PEG-NHS, which were used as the negative controls.

For cell imaging, 10 nM of MSNs@FA/Gal/NHS was further incubated with 5 μM of dsDNA probe in TE with a total volume of 2 mL under stirring overnight to obtain FA, Gal and dsDNA co-functionalized MSNs (MSNs@FA/Gal/dsDNA), which were separated by centrifugation and washed thrice with PBS under stirring for 30 min. The complete cleaning of unconjugated dsDNA probe was verified with UV-vis measurement of the supernatant. 10 nM of MSNs@FA/Gal/dsDNA was then incubated with 100 μM of ssDNA probe with a total volume of 0.5 mL in PBS under stirring overnight at room temperature to obtain MSNs/ssDNA@FA/Gal/dsDNA (MFMSNs). The FA-free and PBA-free MFMSNs were respectively prepared by using MSNs@Gal/NHS instead of MSNs@FA/Gal/NHS and DNA3 instead of ssDNA probe with the same procedure. Prior to use, the MFMSNs were separated by centrifugation, and dispersed

in PBS to obtain different dispersions.

For *in vivo* imaging, MSNs/ssDNA'@FA/Gal/dsDNA' (MFMSNs') were prepared with the same procedure by replacing dsDNA and ssDNA probes with dsDNA' and ssDNA' probes, respectively.

***In vitro* performance of MFMSNs.** To test the release ability of ssDNA probe from the MFMSNs, 10 nM of MFMSNs was incubated at room temperature for different times. After removing the MFMSNs by centrifugation, the supernatants were subjected to measure the Cy5 fluorescence.

To test the *in vitro* responses of MFMSNs or PBA-free MFMSNs to sialyltransferases, 10 nM of MFMSNs or PBA-free MFMSNs were incubated with 100 μM of CMP-Sia and 5 mU of ST3Gal or ST6Gal in PBS with pH 5, 6, 7 or 7.4 at 37 ^{o}C for different times. The mixtures were directly subjected to measure the recovered FAM fluorescence.

Imaging of intracellular diverted sialylation. MCF-7 and MCF-10A cells were cultured at 37 ^{o}C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 μg/mL) and streptomycin (100 μg/mL) in a humidified atmosphere containing 5% CO_2. 1 × 10^4 MCF-7 cells were then seeded on 10 mm single-well confocal dishes and cultured overnight. After washing with PBS for three times, the cells were incubated with 1-6 nM of MFMSNs at 37 ^{o}C for 0.5-5 h, which were subjected to perform CLSM imaging. For verification experiments, 1 × 10^4 MCF-7 or MCF-10A cells were incubated with 5 nM of MFMSNs, FA-free MFMSNs or PBA-free MFMSNs and analyzed with same procedure.

For nucleus colocalization analysis, MCF-7 cells treated with 5 nM MFMSN for 4 h were incubated with 10 μg/mL of DAPI in PBS for 10 min, and washed with PBS twice to perform CLSM imaging. The lysosomes and Golgi colocalization analyses were performed by incubating MCF-7 cells with Cell LightTM lysosomes-RFP or Golgi-RFP, BacMam 2.0 (1 × 10^6 particles/mL) at 37 ^{o}C overnight, and then 5 nM of MFMSNs at 37 ^{o}C for 4 h to perform CLSM imaging. The colocalization analysis was performed with Fiji (SciJava).

To inhibit the intracellular sialylation, MCF-7 cells were firstly incubated with 1, 2 or 3 mM of CMP or UDP in PBS at 37 ^{o}C for 18 h. Afterward, the cells were washed with PBS twice and then incubated with 5 nM of MFMSNs at 37 ^{o}C for 4 h to perform CLSM imaging and analysis with Leica Application Suite X (Leica).

Analysis of cell surface sialic acid. 1 × 10^4 MCF-7 cells were incubated with different concentrations of MSNs@FA/Gal (0-5 nM) at 37 ^{o}C for 5 h, and F-SNA (0.1 μM in PBS) at room temperature for 1 h. After washing with PBS twice, the cells were subjected to perform CLSM imaging.

For flow cytometric analysis, 5 × 10^4 MCF-7 cells seeded on six-wells plates were incubated with 0-5 nM of MSNs@FA/Gal at 37 ^{o}C for 5 h, and then F-SNA (0.1 μM in PBS) at room temperature for 1 h. After the cells were washed with PBS twice and then treated with trypsin at 37 ^{o}C for 1.5 min, they were washed with PBS by centrifugation (142 g, 3 min), and then dispersed in 1 mL PBS (5 × 10^4 cells/mL) for flow cytometric analysis with cell population abundance counted at 1 × 10^4. The data were analyzed with CytExpert 2.4 (Beckman-Coulter).

Cell proliferation assay. After MCF-7 cells were cultured in 6-well plates (1 × 10^5 cells/well) for 12 h, 1-5 nM of MSN@FA and MSN@FA/Gal were added into each well and incubated for 5 h, respectively. Then the cells were repeatedly washed with PBS to perform CCK8 assays following the instruction procedure.

***In vivo* imaging of tumor bearing mice.** Pathogen-free BALB/c nude mice (5-6 week old, female) were obtained from Simcere Biomedical Co., Ltd (Nanjing, China). All animal experiments were preformed

according to the Laboratory Animal Management of Jiangsu Province published by Department of Science and Technology of Jiangsu Province, and the license number is SYXK 2017-0015. All mice had free access to rodent chow and water. To establish a MCF-7 tumor xenograft mouse model, 1×10^6 MCF-7 cells were inoculated subcutaneously into the selected position of the mice. The tumor volumes were calculated using formula $V = (L \times W^2)/2$, where W and L are respectively the width and length of the tumor. After the tumor volumes rose to 400 mm^3, these mice were intratumorally injected with 50 μL of MFMSNs' (5 nM, ~436 μg/mL), and imaged at 1, 2, 3, 4, 5, 6, 12, 24 and 48 h after injection.

Imaging of intracellular diverted sialylation. MCF-7 and MCF-10A cells were cultured at 37 ^oC in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 μg/mL) and streptomycin (100 μg/mL) in a humidified atmosphere containing 5% CO_2. 1×10^4 MCF-7 cells were then seeded on 10 mm single-well confocal dishes and cultured overnight. After washing with PBS for three times, the cells were incubated with 1-6 nM of MFMSNs at 37 ^oC for 0.5-5 h, which were subjected to perform CLSM imaging. For verification experiments, 1×10^4 MCF-7 or MCF-10A cells were incubated with 5 nM of MFMSNs, FA-free MFMSNs or PBA-free MFMSNs and analyzed with same procedure.

Suppression of tumor growth. Mice with the tumor volume risen to 80 mm^3 were weighed and stochastically divided into 3 groups to intratumorally inject with (1) saline, (2) 50 μL of MSNs@FA (5 nM, ~436 μg/mL) and (3) 50 μL of MSNs@FA/Gal (5 nM, ~436 μg/mL), respectively. The injections were repeated every 2 days, while the tumor sizes and weights were recorded every day. At Day 15, all the mice were euthanized to take the representative photos of the tumors, and collect the tumors and organs for histopathological analysis with optical microscope (Olympus BX51, Japan). The histopathological analysis was performed by fixing the tumors and organs in 4% paraformaldehyde solution and then embedding them in paraffin blocks to get the sliced sections of 5-μm thickness, which were stained with hematoxylin and eosin.

Supporting Figures

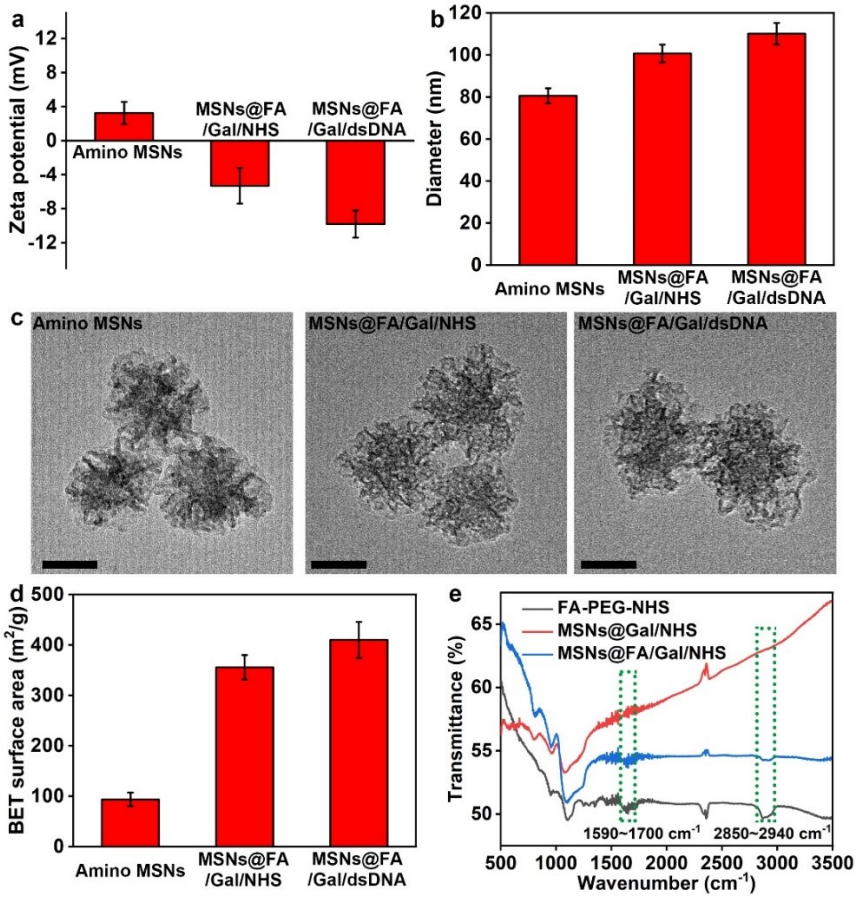

Fig. S1. Characterization of step-by-step binding. (a) Zeta potentials, (b) dynamic light scattering analysis, (c) TEM images and (d) BET analysis of amino MSNs, MSNs@FA/Gal/NHS, and MSNs@FA/Gal/dsDNA. (e) FTIR spectra of FA-PEG-NHS, MSNs@Gal/NHS and MSNs@FA/Gal/NHS. Scale bar: 50 nm. Error bars represent ± S.D. (n=3).

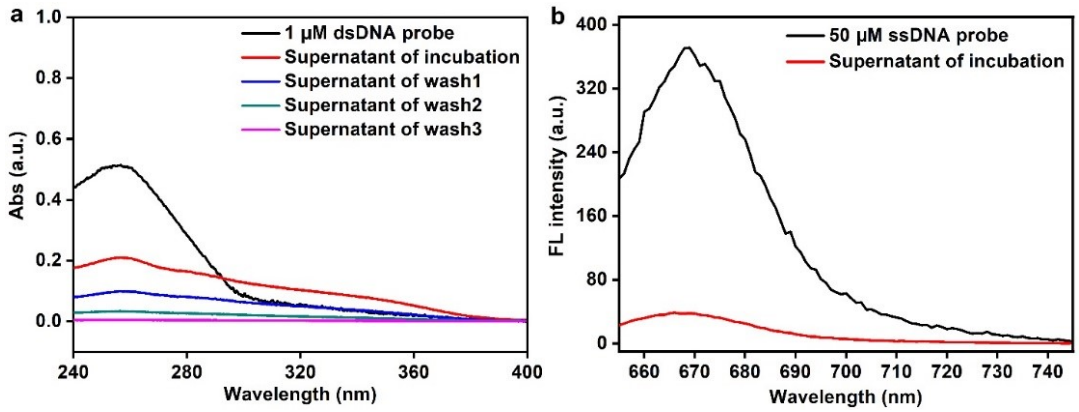

Fig. S2. Modification of MSNs@FA/Gal/NHS with DNA probes. (a) UV-Vis absorption spectra of 1.0 μM dsDNA probe, supernatant of the incubation solution of MSNs@FA/Gal/NHS with dsDNA probe, and supernatants after washing the obtained MSNs@FA/Gal/dsDNA with PBS for 1, 2, and 3 times. (b) Fluorescence spectra of 50 μM ssDNA probe and supernatant of the incubation solution of MSNs@FA/Gal/dsDNA with ssDNA probe.

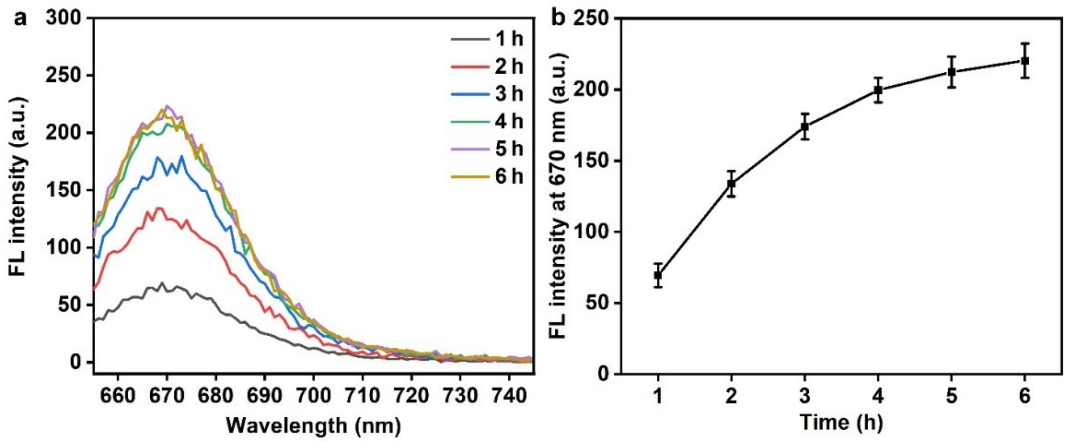

Fig. S3. Release of ssDNA probe. (a) Fluorescence spectra of the supernatants of 10 nM MFMSN solutions after kept at room temperature for 1 to 6 hours. (b) Fluorescence intensities at 670 nm from (a). Error bars represent ± S.D. (n=3).

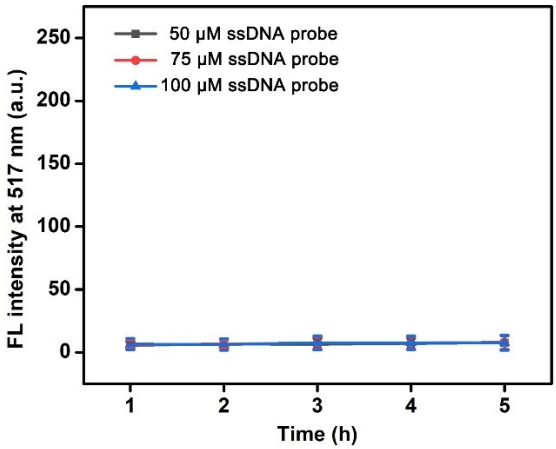

Fig. S4. Incubation of MFMSNs with high concentrations of ssDNA probes. Fluorescence spectra of 10 nM MFMSNs incubated with 50, 75 or 100 μM of ssDNA probes for 1-5 h at pH 7.4. Error bars represent ± S.D. (n=3).

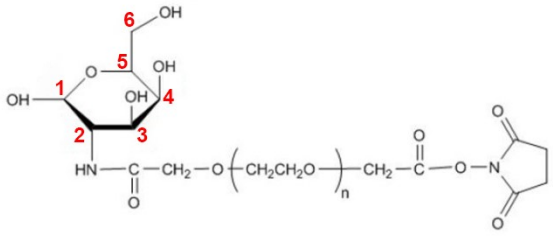

Fig. S5. Structure of Gal-PEG-NHS used in this work.

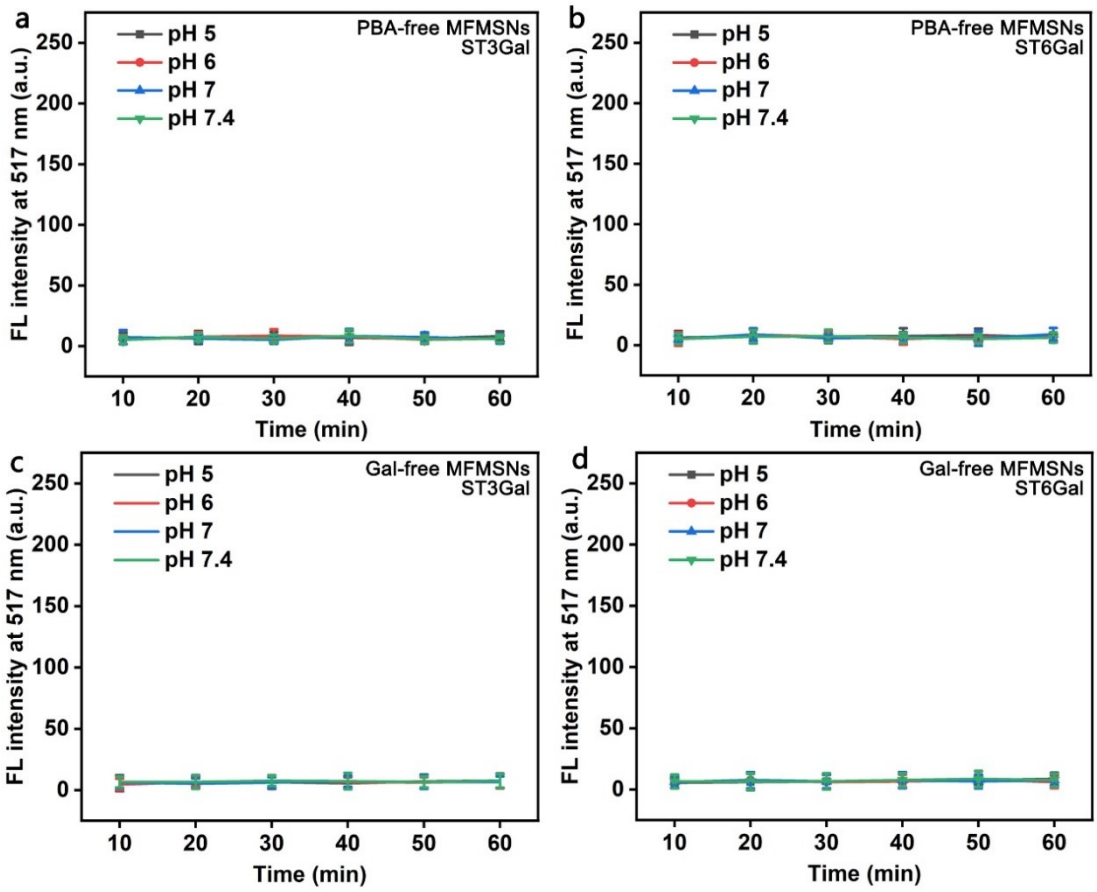

Fig. S6. *In vitro* response of PBA-free and Gal-free MFMSNs. Fluorescence spectra of 10 nM PBA-free (a, b) or Gal-free (c, d) MFMSNs incubated with 5 mU of ST3Gal (a, c) or ST6Gal (b, d) and 100 μM of CMP-Sia in PBS (pH 7.4) for 10-60 min at pH 5, 6, 7 and 7.4. Error bars repre-sent ± S.D. (n=3).

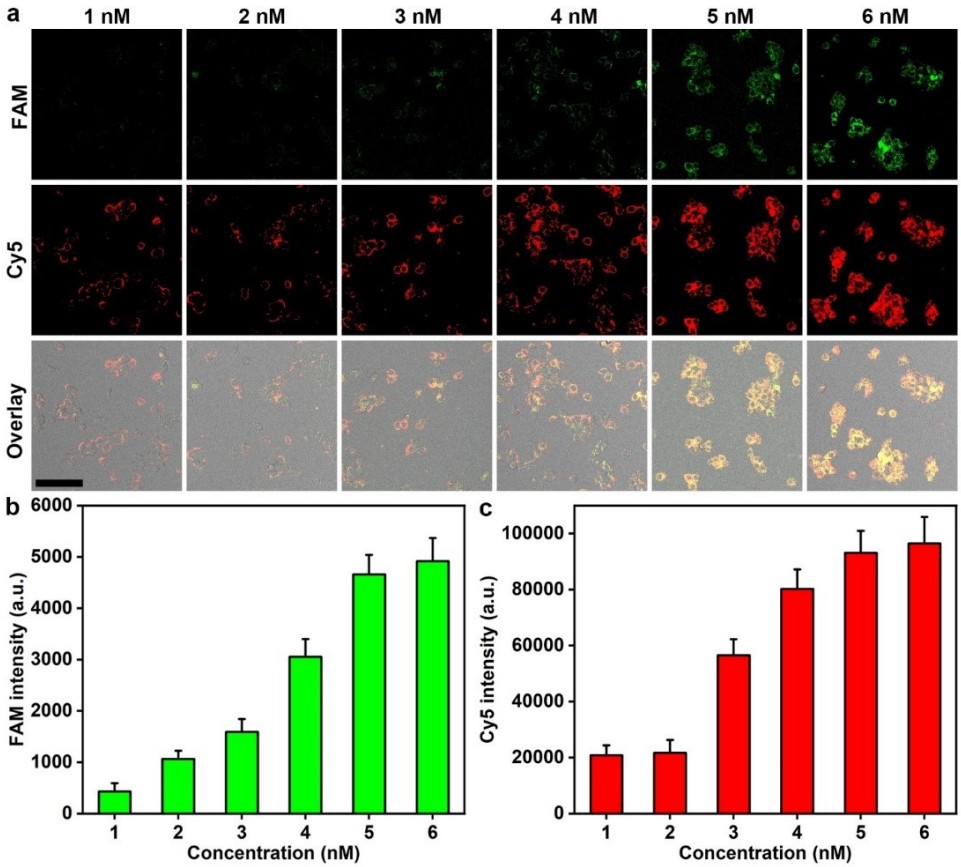

Fig. S7. Optimization of MFMSN concentration for cell imaging. (a) CLSM images and (b, c) corresponding FAM and Cy5 fluorescence intensities of MCF-7 cells incubated with 1-6 nM MFMSNs at 37 °C for 5 h. Scale bar: 100 μm. Error bars represent ± S.D. (n=3).

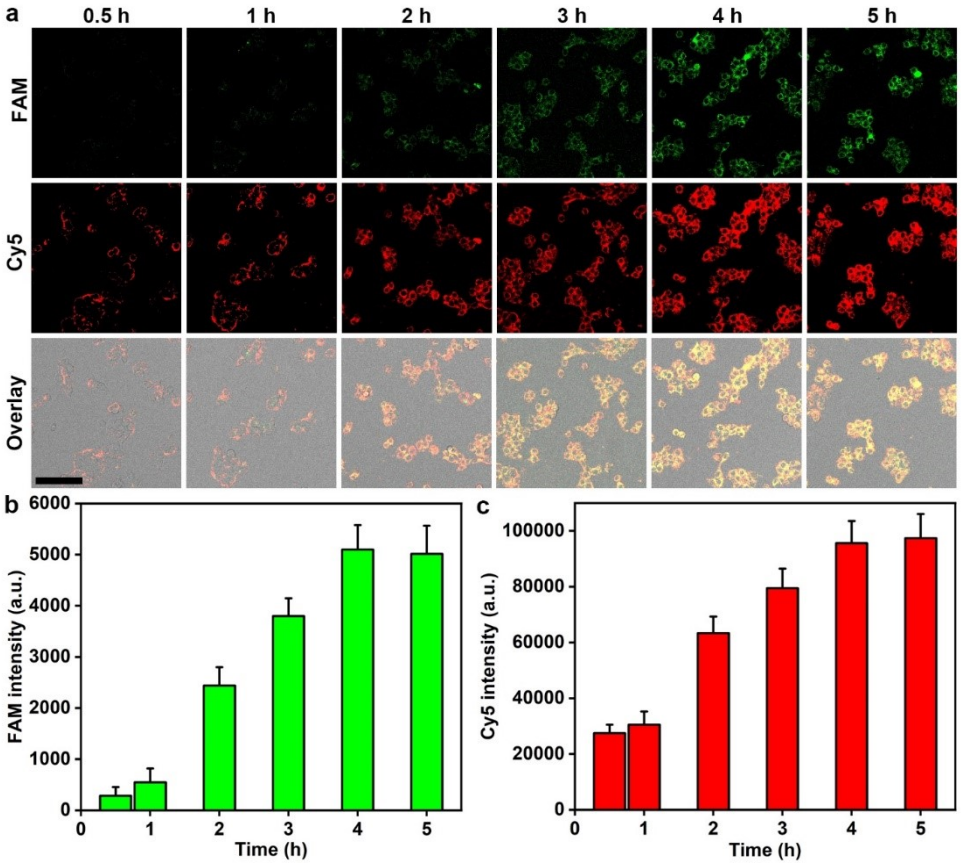

Fig. S8. Optimization of incubation time for cell imaging. (a) CLSM images and (b, c) corresponding FAM and Cy5 fluorescence intensities of MCF-7 cells incubated with 5 nM MFMSNs at 37 °C for 1-5 h. Scale bar: 100 μm. Error bars represent ± S.D. (n=3).

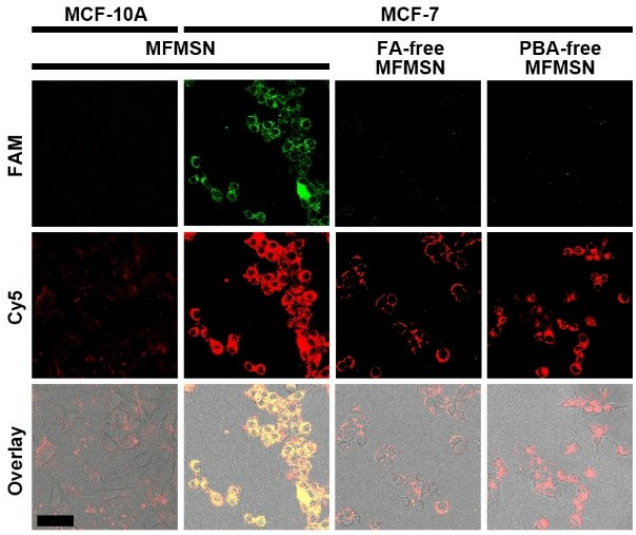

Fig. S9. Intracellular response of FA-free and PBA-free MFMSNs. CLSM images of MCF-10A or MCF-7 cells incubated with 5 nM MFMSNs, FA-free MFMSNs, and PBA-free MFMSNs at 37 °C for 4 h, respectively. Scale bar: 50 μm.

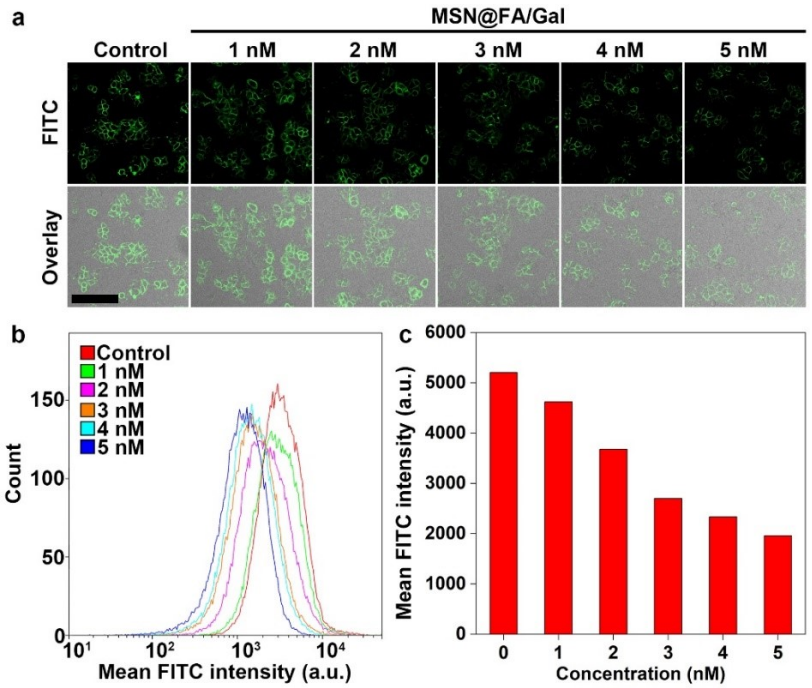

Fig. S10. Variation of SA expression on cell surface. (a) CLSM images and (b) flow cytometric analysis of MCF-7 cells treated with 1-5 nM MSN@FA/Gal at 37 °C for 4 h and then incubated with 0.1 μM F-SNA at room temperature for 1 h. Scale bar, 150 μm. (c) Mean FITC fluorescence intensities from (b).

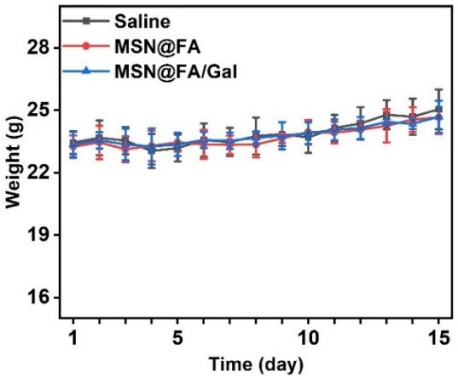

Fig. S11. Variation of the body weight of the treated mice. Body weight of mice injected successively with saline, MSN@FA and MSN@FA/Gal in an interval of two days. Error bars represent ± S.D. (n=5).

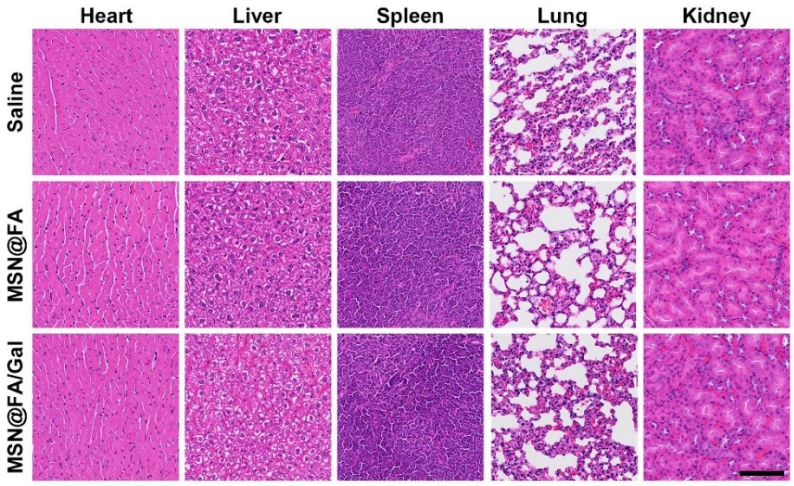

Fig. S12. Histological observations of the normal tissues. Normal tissues of mice treated with 7 successive injections of saline, MSN@FA and MSN@FA/Gal in an interval of two days. Scale bar, 100 μm.