Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

In vivo Dual Fluorescence Imaging of Mucin 1 and Its Glycoform in Tumor Cells

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Experimental Section

Materials and instruments

All chemicals and reagents were commercially available and used as received without further purification. All DNA sequences, RNA sequences and sialidase were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). CdSe@ZnS QDs were purchased from Wuhan Jiayuan Quantumdots Co., Ltd (Wuhan, China). MUC1 mAbs and IgG secondary antibodies (PV-6002) were purchased from Beijing Golden Bridge Biotechnology (Beijing, China). 20 mm glass-bottomed Petri dishes (for confocal microscopy) were purchased from Nest Biotechnology Co., Ltd. (Wuxi, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffer solution (PBS, pH = 7.4), Bovine serum albumin (BSA), penicillin and streptomycin were obtained from Hyclone. The ddH_2O used throughout the study was obtained with a Milli-Q® water purification system. The MCF-7 and HepG2 cell lines were obtained from the China Center for Type Culture Collection (Shanghai, China).

Transmission electron microscopy (TEM) images were obtained with a JEOL JEM-2100 microscope at an acceleration voltage of 200 kV. One drop of particle suspension in water was placed onto a 200-mesh copper grid and dried at room temperature. The hydrodynamic particle size and zeta potential were measured with a Malvern ZetaSizer Nano series Nano-ZS (Malvern, UK). Measurements were performed at 37 °C in ddH_2O, PBS and DMEM medium. X-ray powder diffraction (XRD) analysis were measured with a Bruker D8 X-ray Powder Diffractometer (German Bruker AXS Co., Ltd.). Recording of the data was done in the 2θ-range of 5–90° with a step size of

0.0001°.The ultraviolet-visible (UV-Vis) spectra were obtained with a UNICO 2100 PC UV-Vis spectrophotometer and processed with OriginLab software. Fluorescence measurements were performed in a Molecular Devices SpectraMax M5. Confocal images were taken by using a LSM 700 confocal scanning microscope (Zeiss, Oberkochen, Germany). *In vivo* images were obtained with the In Vivo Imaging System (IVIS) Spectrum.

Methods

Synthesis of gold nanostars (GNSs)

GNSs were synthesized by a seed-mediated growth approach. All glassware was cleaned in freshly prepared aqua regia, rinsed thoroughly with ddH_2O and oven-dried prior to use. The seed solution was prepared by adding 1 mL of 1% (w/v) trisodium citrate to 20 mL of a boiling solution of 1 mM $HAuCl_4$. The solution was kept under boiling for 15 min (a red-wine color appeared) and stored at 4 °C for future use. The GNSs was synthesized as follows: 1 mL of the above seed solution was added to 100 mL of 0.25 mM $HAuCl_4$ solution (with 100 µL of 1 M HCl) at room temperature under moderate stirring. Subsequently, 1 mL of 2 mM $AgNO_3$ and 0.5 mL of 100 mM ascorbic acid were simultaneously added. The solution was stirred for 30 s as its color rapidly turned from light red to greenish-black. The obtained GNSs were centrifuged at 4000 rpm for 10 min twice. The solution was redispersed in ddH_2O, filtered by a 0.22 µm Millipore membrane and then stored in dark at 4 °C before use. The quantitation of

GNSs was performed using an approximate extinction coefficient of 20.1×10^8 $mol^{-1}cm^{-1}$ for GNSs with λ_{max} = 711 nm. Typical TEM images of GNSs were shown in **Fig. S2A**.

Preparation of GNSs- and QDs-nanoprobes

GNS-2: Conjugation of DNA1 (5'-AGGCAATCGATATAGTCACGGACTAGGCCCAGCGTCTTTTTT-3'-SH) to GNSs was performed as follows. Typically, 5 μL of 100 nM thiol-modified DNA1 was added to 1 mL of 5 nM GNSs solution with a molar ratio of 100:1. After 12 h of incubation at room temperature protected from light, the hybridization solution was centrifuged at 4500 rpm for 10 min twice to remove any excess DNA1. The DNA1 modified GNSs (termed as GNS-2) were resuspended in 1 mL ddH_2O and stored at 4 °C before use.

GNS-3: 25 μL of 20 nM Sia aptamer (Cy5-5'-GACGCUGGGCCUAGUCCGUGACUAUAUCGAUUGCCUUUUCAGCUUGGGUC-3') was added to 1 mL of GNS-2 solution with a molar ratio of 100:1. The hybridization solution was reacted for 1 h at 60 °C water bath and then cooled to room temperature for 10 h protected from light. Then, the hybridization solution was centrifuged at 4500 rpm for 10 min twice to remove the nonconjugated Sia aptamer. The GNS-3 were resuspended in 1 mL ddH_2O and stored at 4 °C before use.

GNS-4: 5 μL of 100 nM DNA2 (5'-GACGCAAGTCTGAAA-3') was added to 1 mL of GNS-3 solution with a molar ratio of 100:1. The hybridization solution was reacted for 1 h at 60 °C water bath and then cooled to room temperature for 10 h protected from light. Then, the hybridization solution was centrifuged at 4500 rpm for 10 min twice to

remove excess DNA2. The GNS-4 were resuspended in 1 mL ddH_2O and stored at 4 °C before use.

Sia-GNSs: 2 μL of 10 mg/mL SH-PEG (molecular weight 5,000) was added to 1 mL of GNS-4 solution with a molar ratio of 3000:1 and kept at 60 °C water bath for 1 h protected from light. The mixture was centrifuged at 4500 rpm for 10 min twice to remove the nonspecific SH-PEG adsorption. The Sia-GNSs were redispersed in 1 mL ddH_2O and stored at 4 °C before use.

QD-2: 80 μL of 2 μM carboxyl CdSe@ZnS QDs (excitation wavelength 385 nm, emission wavelength 620 nm) in PBS were activated in the presence of 6 μL of 10 mg/mL 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) and 4 μL of 10 mg/mL N-Hydroxysuccinimide (NHS) for 1 h. After that, 160 μL of 100 μM 5'-NH_2 modified MUC1 aptamer (NH_2-5'-C_6-GCAGTTGATCCTTTGGATACCCTGG-3') in PBS was added to the mixture in a 100:1 molar ratio and the solution was incubated under gentle stirring for 3-4 h at room temperature protected from light. Then the conjugates were ultrafiltrated with Millipore Amicon Ultra centrifugal filter unit (30 kDa molecular-weight cut-off) for 10 min at 13,000 rpm three times to remove non-specifically bound MUC1 aptamer. The final QD-2 were resuspended in 80 μL PBS and stored at 4 °C before use.

MUC1-QDs: 80 μL of 2 μM QD-2 in PBS were activated with 6 μL of 10 mg/mL EDC in PBS and 4 μL of 10 mg/mL NHS in PBS for 1 h. Afterwards, 45 μL of 10 mg/mL NH_2-PEG (molecular weight 2,000) were added to the reaction mixture and incubated under gentle stirring for 3 h at room temperature protected from light. After

the incubation, the conjugates were ultrafiltrated with Millipore Amicon Ultra centrifugal filter unit (30 kDa molecular-weight cut-off) for 10 min at 13,000 rpm three exchanges with PBS. The final MUC1-QDs were resuspended in 80 μL PBS and stored at 4 °C before use.

Quantitation of DNA1, Sia aptamer, DNA2 on GNSs and MUC1 aptamer on QDs: The general procedure is as following: The absorbance (A) of supernatant at the 650 nm was measured by UV-visible spectroscopy and used to determine the molar (n_1) of the unconjugated nanoprobes. ($n_1 = cV$. $c = A/b\varepsilon_{650}$ (b = 1 cm, $\varepsilon_{650} = 0.25 \times 10^6\ M^{-1}cm^{-1}$)). The average amount of conjugated fragment on each nanoprobe could be calculated as $(n - n_1)/n$ (n was the total molar of input nanoprobes).

In vitro Cytotoxicity

MCF-7 and HepG2 cells were seeded in DMEM medium in a 96-well plate, with six wells for each concentration of the detecting nanoprobes. The cells were incubated at 37 °C and in 5% CO_2 for 12 h. Medium was removed and cells were washed three times with PBS. Different concentrations of Sia-GNSs and MUC1-QDs (0-30 nM) were then individually or both added to the 96-well plate and incubated for 24 h. The culture medium was replaced with 100 μL of fresh Opti-MEM (Life Technologies) and the cells were treated with 10 μL of Cell Counting Kit-8 (CCK-8, Beyotime) for 4 h. The absorbance (A) of each well was measured on a microplate reader at 450 nm and the relative cell viability (%) was calculated as $(A_{test}/A_{control}) \times 100$.

Hemolysis test

Hemolysis occurred when water was combined to red blood cells. Therefore, the determination of hemolytic toxicity of nanomaterials was crucial. In this experiment, the detecting mixture of Sia-GNSs and MUC1-QDs nanoprobes was dissolved in DMEM medium without phenol red, and the concentration was 1-50 nM. The positive control was sterilization deionized water, and the negative control was DMEM medium without phenol red. 250 μL of detecting mixture nanoprobes DMEM solution with different concentration gradients was added to the 24-well plate, and 250 μL of fresh sheep blood red blood cell solution was added. In addition, the PBS solution was added as the negative control while the water was added as the positive control. After gently mixed and incubated at 37 °C for 2 h, solutions were centrifugated at 10, 000 rpm for 20 min. Then, supernatants were transferred to a 96-well plate and read by a microplate reader for the absorbance value at 540 nm. Finally, the hemolysis rates in different samples were calculated using the formula below:

$$\text{Hemolysis ratio (\%)} = (A_s - A_n) / (A_p - A_n) \times 100.$$

where, A_s stands for the absorbance of the sample, A_n stands for the absorbance of the negative control, and A_p stands for the absorbance of the positive control.

Detection of MUC1 and its sialylation in vitro

Cell lines and incubation conditions: human breast adenocarcinoma cells MCF-7 (MUC1 positive) and hepatocellular carcinoma cells HepG2 (MUC1 negative) cells were cultured in DMEM medium (with 10% FBS and 1% penicillin/streptomycin) at 37 °C under a 5% CO_2 atmosphere. The cell numbers were counted with a Petroff-Hausser cell counter (USA).

Confocal laser-scanning microscopy (CLSM): MCF-7 and HepG2 cells were incubated with detecting mixture of Sia-GNSs and MUC1-QDs nanoprobes (5 nM) for 2, 4, 6, 8 and 12 h, respectively. After that, the fluorescence of the nanoprobes was excited with NIR light (980 nm laser, 5 mW for 2 min), followed by incubation for 30 min. The fluorescence images were recorded using CLSM.

CLSM of cell lines

2×10^5 cells/dish were cultured on 20 mm glass-bottomed Petri dishes with DMEM medium for 12 h. Medium was removed and cells were washed three times with PBS. The cells were randomized into six groups (n = 3). Group 1: MCF-7 cells, without NIR illumination; Group 2: MCF-7 cells, with NIR illumination; Group 3: MCF-7 cells, with NIR illumination, add sialidase; Group 4: MCF-7 cells, with NIR illumination, add MUC1 mAbs; Group 5: HepG2 cells, without NIR illumination; Group 6: HepG2 cells, with NIR illumination. MCF-7 cells were co-incubated with 10 μL 5 unites of sialidase for 1 h (Group 3) or MUC1 mAbs (1:100 dilution) for 12 h (Group 4) in 500 μL DMEM medium, and were then washed three times with PBS. Next, detecting mixture of Sia-GNSs and MUC1-QDs nanoprobes (5 nM) in 500 μL fresh DMEM medium were added to each dish and incubated for 6 h at 37 °C and in 5% CO_2. After that, the fluorescence of the nanoprobes was excited with or without NIR light, followed by incubation for 30 min. Then the cells were washed three times with PBS and fixed for 15 min in 0.5 mL of 4% paraformaldehyde (PFA). For cell membrane labeling, the cells were incubated with DiO before fixed with PFA for 15 min. After washing with PBS three times, the cells were soaked in DAPI solution. Confocal images of the treated

cells were obtained with confocal fluorescence microscopy (63 × oil objective). The DAPI signal was obtained by CLSM under 405 nm excitation and collected at 420-460 nm. The QDs signal was obtained by CLSM under 448 nm excitation and collected at 600-640 nm. The FRET-induced Cy5 signal was obtained by CLSM under 448 nm (excitation wavelength of QDs) and collected at 660-700 nm. The Cy5 signal was obtained by CLSM under 650 nm (excitation wavelength of normal Cy5) and collected at 660-700 nm. The fluorescence signal of DiO was obtained by CLSM under 488 nm excitation and collected at 485-525 nm. The emission intensity of QDs, FRET-induced Cy5 and Cy5 were collected and processed by Leica SP5 software of CLSM.

CLSM of tumor histological sections

Tumor sections were de-paraffinized with xylene, alcohol and PBS. And then incubated in 1 mM citrate buffer (pH 7.4) for 10 min in boiling water and kept warm for 10 min. Then the sections were incubated with 3% H_2O_2 for 10 min to inactivate the endogenous peroxidases. After washed by PBS for three times, the sections were incubated with detecting mixture of Sia-GNSs and MUC1-QDs nanoprobes (5 nM) for 6 h in wet box in room temperature. After that, the fluorescence of the nanoprobes was excited with NIR light, followed by incubation for 30 min. Washing with PBS three times, the tissue sections were soaked in DAPI solution and monitored live under CLSM (20 × objective).

Construction of MCF-7 tumor-bearing nude mice models

All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Jiangnan University (JN.

No20190430b0480815[88]). Five-week-old female BALB/c nude mice (average weight 15-18 g) were maintained under standard housing conditions. MCF-7 cells (5×10^6 cells/100 μL PBS) were slowly subcutaneously implanted into the right flanks of mice. When the tumor volume reached about 60 mm^3, calculated by the formula volume = 0.5 × (tumor length) × $(\text{tumor width})^2$, nude mice models were prepared for the following experiments.

Fluorescence imaging of living and frozen sections imaging

MCF-7 tumor-bearing nude mice were randomized into three groups (n = 6), tail intravenous injection of detecting mixture of Sia-GNSs and MUC1-QDs nanoprobes (30 nM) and illumination with NIR (Group I), tail intravenous injection of detecting mixture of Sia-GNSs and MUC1-QDs nanoprobes (30 nM) and illumination without NIR (Group II), and tail intravenous injection of saline and illumination with NIR (Group III). Finally, the fluorescence of QDs and FRET-induced Cy5 at assigned time points (2, 4, 6 and 12 h) were tracked by IVIS Spectrum. The QDs signal was obtained by IVIS Spectrum under 448 nm excitation and collected at 600-640 nm. The FRET-induced Cy5 signal was obtained by IVIS Spectrum under 448 nm (excitation wavelength of QDs) and collected at 660-700 nm. The excreted tumors and main organs (heart, liver, spleen, lung and kidney) were also imaged.

Hematoxylin and eosin (H&E) analysis

The tumors isolated from nude mice and clinical breast cancer patients were used for histological investigation. The collected tissues were soaked in 4% paraformaldehyde for 2 days and then dehydrated in gradient ethanol. After embedded in paraffin, the

specimens were cut into 4 μm thick sections and mounted on glass slides. The sections were de-paraffinized, hydrated and stained with H&E according to a standard protocol for microscopic observation (magnification: 200×).

Immunohistochemistry (IHC) analysis

IHC studies were carried out as follows: tumor sections were de-paraffinized with xylene, alcohol and PBS. And then incubated in 10 mM citrate buffer (pH 7.4) for 10 min in boiling water and kept warm for 10 min. Then the sections were incubated with 3% H_2O_2 for 10 min to inactivate the endogenous peroxidases. After that, the sections were incubated with MUC1 mAbs (1:400 dilution in 5% BSA) overnight at 4 °C. After washed with PBS, the tumor sections were incubated for another 1 h with antimouse IgG secondary antibodies (PV-6002). The peroxidase substrate DAB was then used to visualize the immunoreactivity on the tissue sections. Hematoxylin was used to counterstain the nuclei. All stained tissue sections were investigated and imaged by using microscope at magnification 200×.

Clinical Samples

Breast cancer cohorts, comprising 15 human primary breast cancer tissues and their matched adiacent noncancerous (NCTs) were obtained from the Affiliated Hospital of Jiangnan University (LS2019011). All patients' data were obtained with informed consent and the project was approved by the Clinical Research Ethics Committee of the participating institutions. The study was conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS).

Supplemental Figures

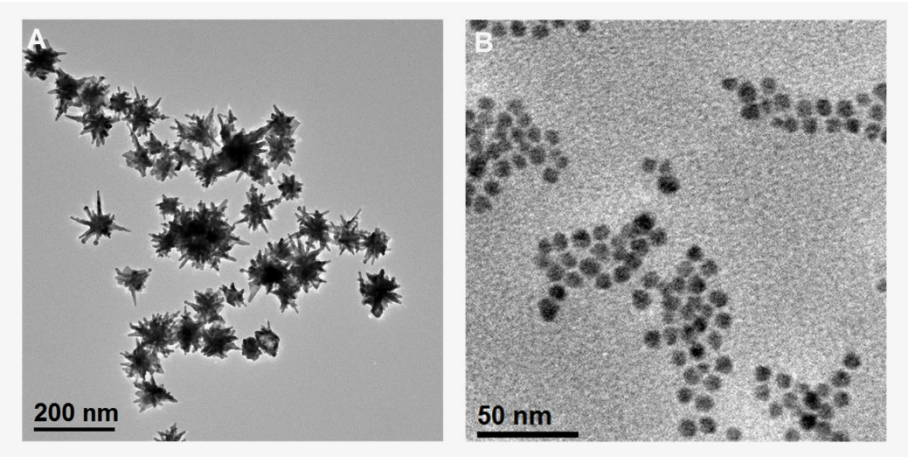

Fig. S1. TEM images of (**A**) GNSs and (**B**) QDs nanoparticles.

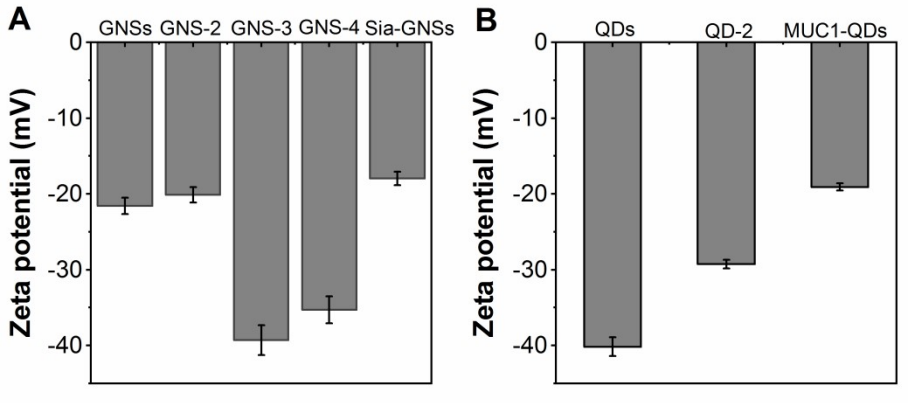

Fig. S2. Zeta potential of (**A**) GNSs-nanoprobes and (**B**) QDs-nanoprobes.

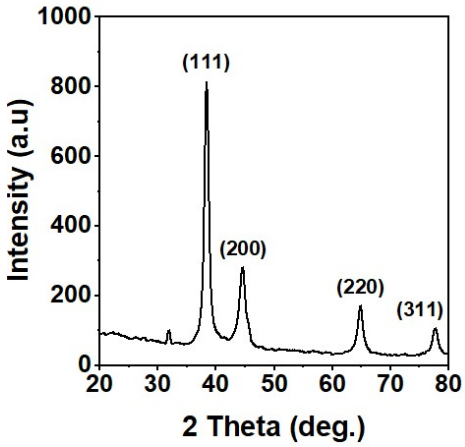

Fig. S3. X-ray diffraction pattern of GNSs nanoparticles.

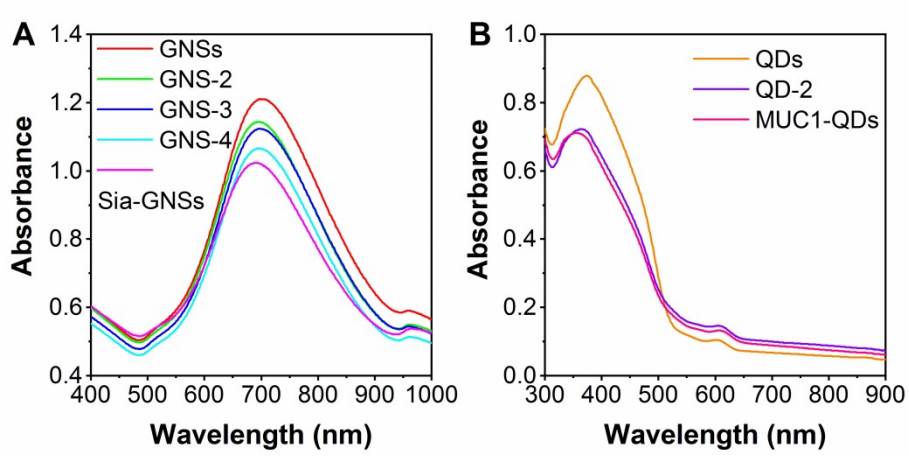

Fig. S4. UV-Vis absorption spectra of (**A**) GNSs-nanoprobes and (**B**) QDs-nanoprobes.

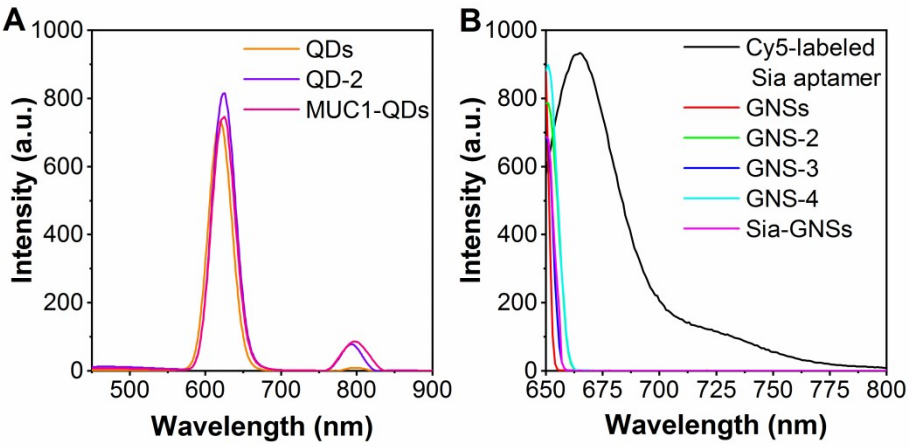

Fig. S5. Fluorescence spectra of (**A**) QDs-nanoprobes and (**B**) Cy5-labeled Sia aptamer and GNSs-nanoprobes.

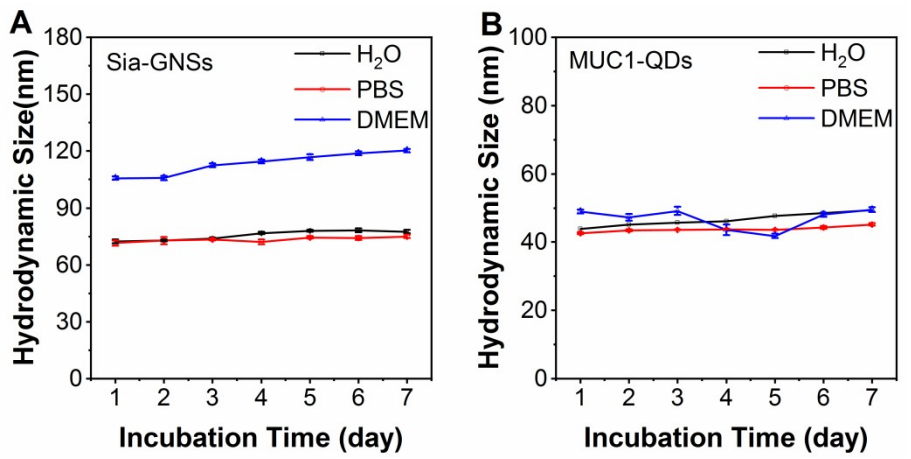

Fig. S6. Long term hydrodynamic size stability of (**A**) Sia-GNSs and (**B**) MUC1-QDs nanoprobes incubating in water, PBS (pH = 7.4), or DEME medium containing 10% FBS. Error bars are mean ± SD (n = 3).

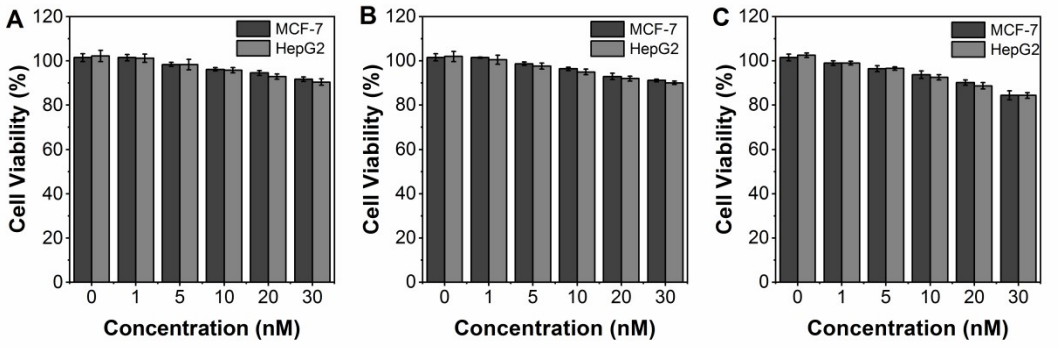

Fig. S7. The cell viability of MCF-7 and HepG2 cells incubated with (**A**) Sia-GNSs, (**B**) Sia-GNSs and (**C**) detecting mixture of Sia-GNSs and MUC1-QDs nanoprobes (molar ratio 1:1) at various concentrations for 24 h. Error bars are mean ± SD (n = 3).

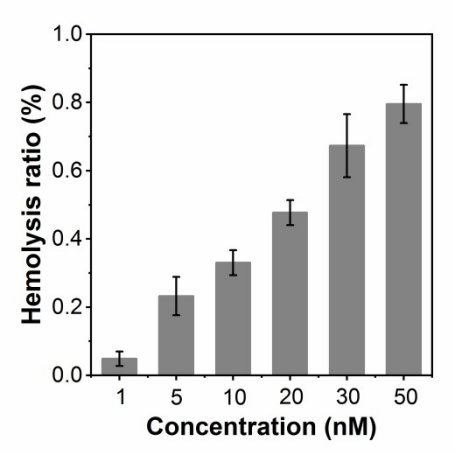

Fig. S8. Hemolysis test results of detecting mixture of Sia-GNSs and MUC1-QDs nanoprobes at different concentrations, where the hemolysis values of the positive control (water) and negative control (PBS) were 1 and 0, respectively. Error bars are mean ± SD (n = 3).

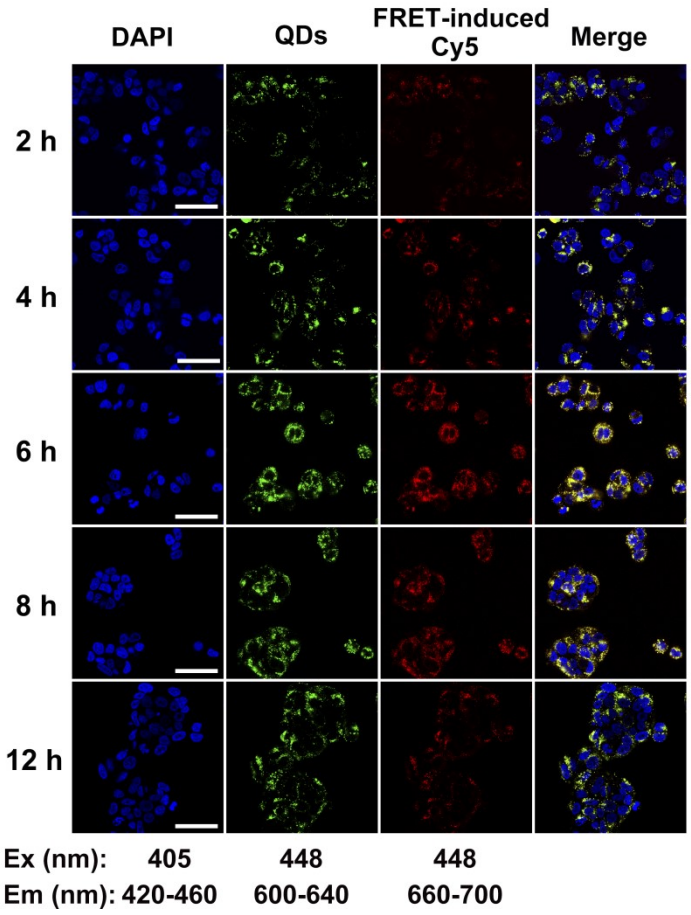

Fig. S9. Imaging of MUC1 and its sialylation in MCF-7 cells (MUC1 positive) with NIR illumination. Confocal images of MCF-7 cells after incubation with detecting mixture of Sia-GNSs and MUC1-QDs (5 nM) for different periods of time: 2, 4, 6, 8 and 12 h (n= 3). NIR laser (980 nm, 5 mW/cm^2 for 2 min) was used for activation. Blue: DAPI (stain the nuclei, excitation: 405 nm, emission: 420-460 nm); Green: QDs (excitation: 448 nm, emission: 600-640 nm); Red: FRET-induced Cy5 (excitation: 448 nm (excitation wavelength of QDs), emission: 660-700 nm). Scale bars: 50 μm.

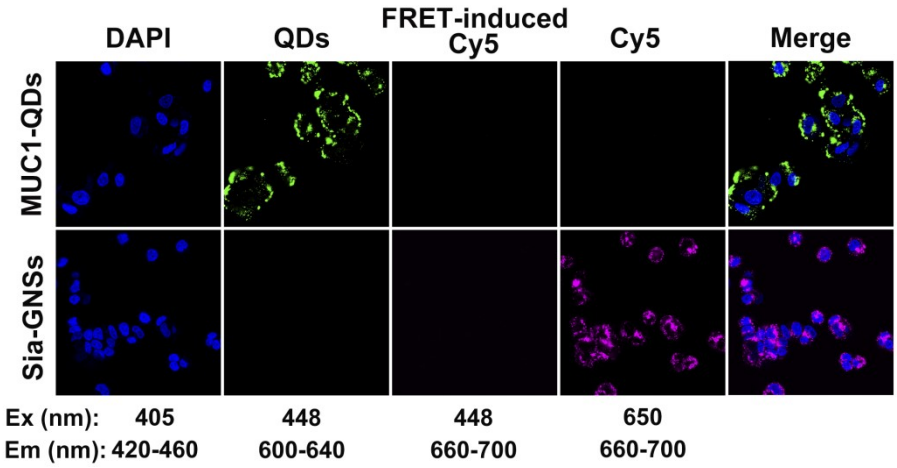

Fig. S10. Imaging of MUC1 in MCF-7 cells with NIR illumination. Confocal images of MCF-7 cells after incubation with respective Sia-GNSs (5 nM) or MUC1-QDs (5 nM) for 6 h (n = 3). NIR laser (980 nm, 5 mW/cm^2 for 2 min) was used for activation. Blue: DAPI (excitation: 405 nm, emission: 420-460 nm); Green: QDs (excitation: 448 nm, emission: 600-640 nm); Red: FRET-induced Cy5 (excitation: 448 nm (excitation wavelength of QDs), emission: 660-700 nm). Rose: Cy5 (excitation: 650 nm (excitation wavelength of normal Cy5), emission: 660-700 nm). Scale bars: 50 μm.

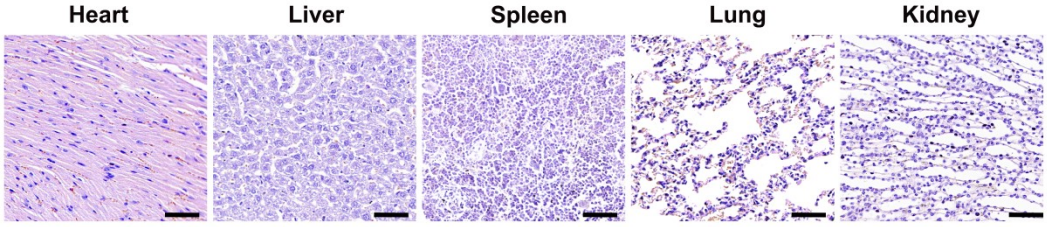

Fig. S11. Histologic assessments of major organs with H&E staining in the subcutaneous MCF-7 bearing mice (n = 6). microscope magnification 200 ×. Scale bars: 100 μm.

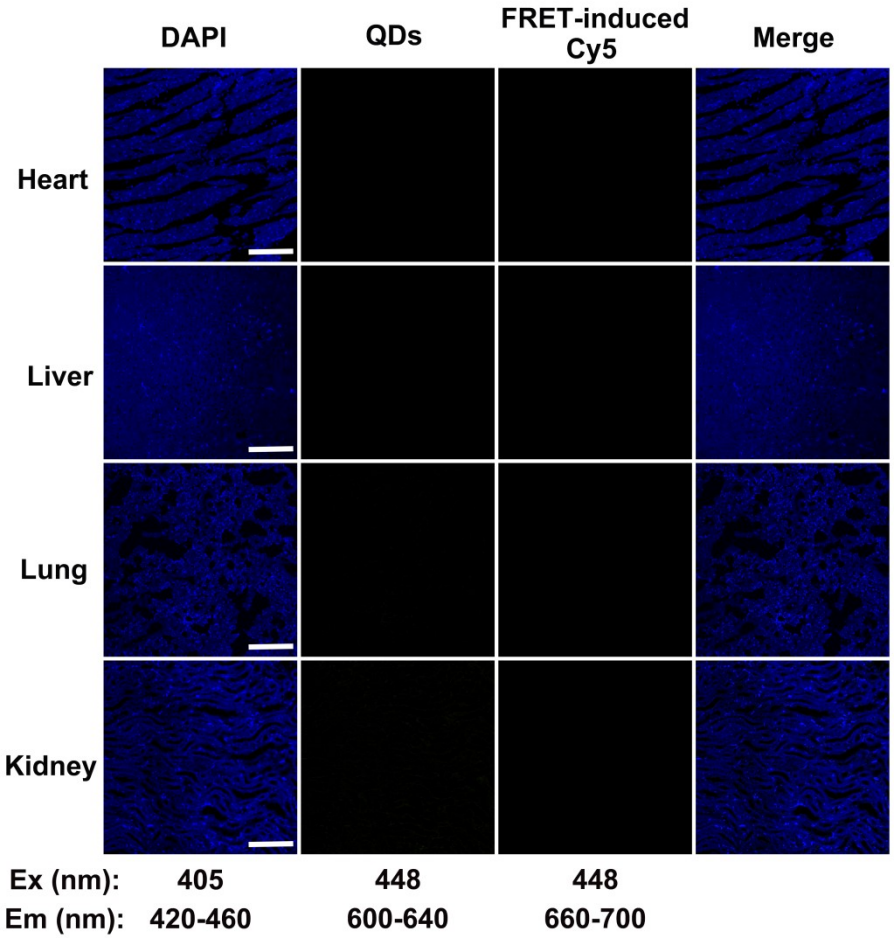

Fig. S12. Confocal images of heart, liver, lung and kidney sections after incubation with detecting mixture of Sia-GNSs and MUC1-QDs (5 nM) for 6 h (n = 6). NIR laser (980 nm, 5 mW/cm^2 for 2 min) was used for activation. Blue: DAPI (excitation: 405 nm, emission: 420-460 nm); Green: QDs (excitation: 448 nm, emission: 600-640 nm); Red: FRET-induced Cy5 (excitation: 448 nm (excitation wavelength of QDs), emission: 660-700 nm). Scale bars: 150 μm.