Electronic Supplementary Information (ESI)

Two-photon fluorescence imaging of sialylated glycans in vivo based on a sialic acid imprinted conjugated polymer nanoprobe

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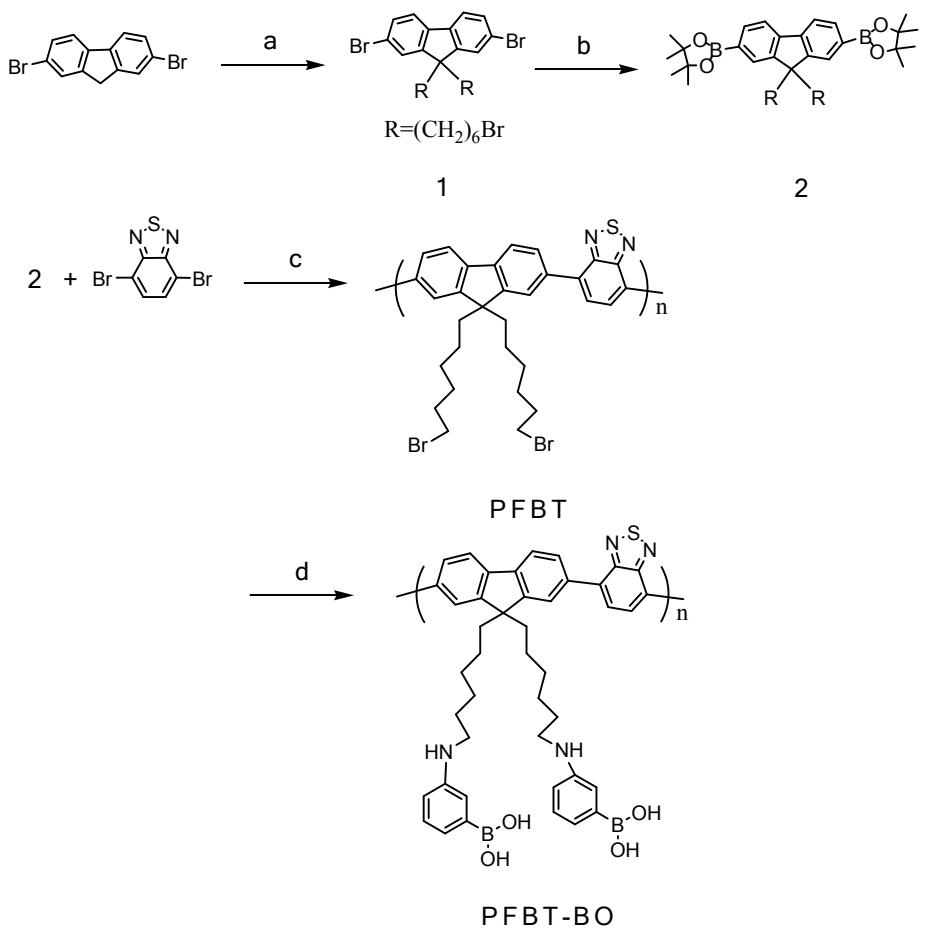
Materials. All chemicals were available commercially and the solvents were purified by conventional methods before use. 2,7-Dibromofluorene, Hexamethylene dibromide, were purchased from Zhengzhou alfa chemical engineering Co., Ltd. Tetrabutylammonium bromide, bis(pinacolato)diboron, 3-Aminobenzeneboronic acid, Tetrakis(triphenylphosphine)palladium (0), 4,7-Dibromo-2,1,3-BenZzothiadiazole, 1,1′-Bis(diphenylphosphino)ferrocene]dichloropalladium(II), N-acetylneuraminicacid, Alizarin Red S were purchased from Sun Chemical Technology (Shanghai) Co., Ltd. Hemoglobin from bovine blood (BHb), Lecithos (Lec, biological source: from egg yolk), Lysozyme (Lys), Pepsin (Pep), Horseradish Peroxidase (HRP), Albumin (Alb) were purchased from Shanghai Lan ji biological technology Co., Ltd.

Instruments. ^{1}H NMR spectra were recorded with Bruker NMR spectrometers at 300 MHz and JOEL JNM-ECA600.The mass spectra were obtained by Bruker maXis ultra high resolution TOF MS system. The fluorescence spectra measurements were performed using FLS-920 Edinburgh fluorescence spectrometer. The fluorescent images were measured on a Leica TCS SP5, confocal lasers canning microscope with an objective lens (×40). The excitation wavelength was 488 nm and 405 nm (5 mW).

Synthesis of polymer monomer



Scheme S1. Synthesis of polymer monomer. The synthesis of 1, 2, PFBT and PFBT-BO; Reaction conditions: a) 1,6-dibromohexane, Bu_4NBr, KOH, 75 °C, 25 min; b) $Pd(dppf)Cl_2$, KOAc, dioxane, 85 °C, 12 h; c) $Pd(PPh_3)_4$, K_2CO_3, toluene/H_2O, 90 °C, 24 h; d) DMF, triethylamine, 3-Aminobenzeneboronic acid, 70 °C, 6 h.

Compound 1 and 2 was prepared according to previously published experimental procedures.[S1] Compound 2 (0.744 g, 1 mmol), 4,7-Dibromo-2,1,3-BenZzothiadiazole (0.293 g, 1 mmol) and K_2CO_3 (3.317 g, 24 mmol) $Pd(PPh_3)_4$ (1 mol%) were dissolved in a mixture of degassed toluene and degassed 20 % aqueous tetraethylammonium hydroxide in a 50 mL two-necked round-bottomed flask avoided light under argon. The solution was flushed with argon for 15 min. The mixture then heated at 85 ˚C

with vigorously stirred under an argon atmosphere. After 24 hours, cooling to room temperature, the mixture was poured into methanol. The precipitated crude material was collected by filtration through a funnel. After washing with methanol for 5 times to remove oligomers and catalyst residues, the resulting polymer was dissolved in chloroform. The solution was filtered with a 0.20 μm PTFE filter, concentrated and precipitated from methanol to yield the neutral precursor polymer. ^{1}H NMR ($CDCl_3$, 400 MHz), δ (ppm): 8.609-7.712 (m, 9H, benzothiadiazole ring, fluorene ring), 3.297 (br, 4H, -CH_2Br), 2.166-1.550 (m, 8H, H-alkyl), 1.413-0.949 (m, 16H, H-alkyl). GPC (THF, polystyrene standard): Mn = 10.5 kg mol^{-1}, PDI = 2.5. ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.36, 153.23, 150.31, 149.21, 142.63, 140.17, 139.88, 135.58, 135.42, 133.75, 132.99, 130.47, 128.68, 127.83, 127.42, 127.25, 127.03, 126.82, 125.42, 123.13, 123.00, 120.36, 119.15, 118.35, 54.35, 54.19, 39.10, 32.97, 31.63, 28.68, 28.10, 28.03, 26.47, 23.97, 22.81, 22.60.

Preparation of MIP and NIP. The MIP and NIP were prepared through a modified nano-precipitation method. The typical steps are as follows: the solution of polymer monomer PFBT-BO (0.0045 g) in 1.0 mL of THF is rapidly added to 8.0 mL 1.0 mmol/L SA solution (in which proper amount of ammonia was added to make the pH of solution is 8.0) under continue ultrasonification. After sonication for additional 2 min, THF was evaporated under nitrogen atmosphere. Then the solution is heated to 110 °C for 2 h, until the concentration of the solution was 1.0 mg/mL and stewed for overnight. The non-imprinted polymer (NIP) was prepared using the same procedure

but without addition of model target. The fluorescence quantum yield for the MIP-based nanoprobe was 0.062.

Removal of the template SA. To remove the template SA, the obtained MIP-based nanoprobe was washed with acid solution, and doubly distilled water in order. The MIP particles were collected based on centrifugalization. At last, the fluorescent nanoparticles was suspended in water as a stock solution stored at 4 °C.

MTT Assay. RAW.264.7 cells (10^6 cell mL^{-1}) and MCF-10 cells were dispersed within replicate 96-well microtiter plates to a total volume of 200 μL $well^{-1}$. Plates were maintained at 37 °C in a 5% CO_2/95% air incubator for 24 h. Then RAW.264.7 cells were incubated for 12 h upon different probe concentrations of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} mg/mL. MTT solution (5 mg/mL, PBS) was then added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a Triturus microplate reader.

Cell culture.[S2] RAW.264.7, MCF-10 cells were cultured in DMEM (4T1 were cultured in 1640) containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 °C (w/v) in a 5% CO_2/95% air incubator MCO-15AC (Sanyo, Tokyo, Japan). The concentrations of counted cells were adjusted to 1×10^6 cells mL^{-1} for confocal imaging in high-glucose DMEM (or in 1640) (4.5 g of glucose/L) supplemented with 10% fetal bovine serum (FBS), $NaHCO_3$ (2 ng/L), and 1%

antibiotics (penicillin/streptomycin, 100 U/mL). Cultures were maintained at 37 °C under a humidified atmosphere containing 5% CO_2.

Mice culture and mouse mammary carcinoma model.[S3] All 8-10-week-old KM mice were purchased from Shandong University Laboratory Animal Center. All the animal experiments were carried out in accordance with the relevant laws and guidelines issued by the Ethical Committee of Shandong University. The mice were anesthetized with 4% chloral hydrate (3 mL/kg) by intraperitoneal injection. The mouse mammary carcinoma model was produced by injecting 4T1 cells in forelimb armpit. After ten days, a suitable tumor model was obtained. The mice were then imaged by using a Leica TCS MP5 in vivo imaging system.

In Fig. 3, the authors show two-photon fluorescence images in the abdomen of mice with the nanoprobes. The experiment was performed in normal mice. Three times was repeated for this experiment. The mouse mammary carcinoma model was produced by injecting 4T1 cells in forelimb armpit. After ten days, a suitable tumor model was obtained.[S4] Then, the nanoprobe was injected in tumor (Fig. 4a) and normal (Fig. 4c) tissues of the mice, respectively. In Fig. 4, the authors show two-photon fluorescence images of normal and tumor tissues in mice. The sialidase was injected into tumor tissue of mice, after 30 min treated with sialidase, the mice were imaged by using in vivo imaging system. Furthermore, the output of fluorescence intensity from the fluorescence image by the using a Leica TCS MP5 software.

Confocal imaging. Fluorescent images were acquired on a Leica TCS SP5 confocal laser-scanning microscope with an objective lens (×40). About 10-80 cells were plated in confocal microscopy experiments. The excitation wavelength was 405 nm (5 mW), and 488 nm (15 mW) respectively. Following incubation, the cells were washed three times with DMEM or 1640 without FBS and imaged.

Sample collection and pretreatment. The human serum samples were collected from healthy volunteers (The samples were provided by blood center of Shandong province, China). The human serum samples was placed at room temperature for 2 hours, and then centrifuged at 1000 rpm for 20 min, the supernatant was separated, finally all the samples were diluted to a 100-fold solution for this experiment.

Fluorescence response of the nanoprobe. The dynamic response of the nanoprobe with target SA was investigated. As shown in Fig. S1, the fluorescence intensity of the probe increased evidently in the presence of SA, and the nanoprobe displayed instantaneous response. The changes of fluorescence spectra of nanoprobe with SA in various concentrations were monitored (Fig. S2). The fluorescence intensity of nanoprobe increases almost linearly with the concentration of SA in the range of 0–10.0 μM and 10.0–50.0 μM as displayed in the Fig. S2 and a correlation coefficient of 0.9939 and 0.9977, respectively. Accordingly, the detection limit of SA was estimated to be 1.62 μM (n= 11 and S/N = 3). Good linear correlation was obtained, which exhibited that nanoprobe have the ability to qualitatively and quantitatively determine

the level of SA. The imprinting factor (IF), which is the ratio of K_{MIP} and K_{NIP} (K_{MIP} and K_{NIP} are the linear slopes in Fig. S3a), was used to evaluate the selectivity of the nanoprobe. Under the optimal conditions, the IF value (K_{MIP}/K_{NIP}) was 3.93 and 12.40, respectively, which showed the selectively recognition performance of the nanoprobe.

Specificity of the nanoprobe. The specificity of the nanoprobe was further investigated (Fig. S3, S4 and S5). Various protein and small molecules were selected to test its fluorescence responses with nanoprobe and the results were shown in Fig. S3b and Fig. S4. The nanoprobe exhibited specific recognition of the target SA in the presence of proteins and small molecules, thus clearly demonstrating that the nanoprobe displayed selectivity for the target SA. Thus, the nanoprobe has potential as a fluorescence tool for monitoring sialylated glycans in cells and in vivo. In addition, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in RAW.264.7 cells was performed (Fig. S6, S7, S8 and S9) and the results showed less cytotoxicity of the nanoprobe.

Reference

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(S4) P. Li, L. Liu, H. Xiao, W. Zhang, L. Wang and B. Tang, *J. Am. Chem. Soc.*, 2016, **138**, 2893-2896.

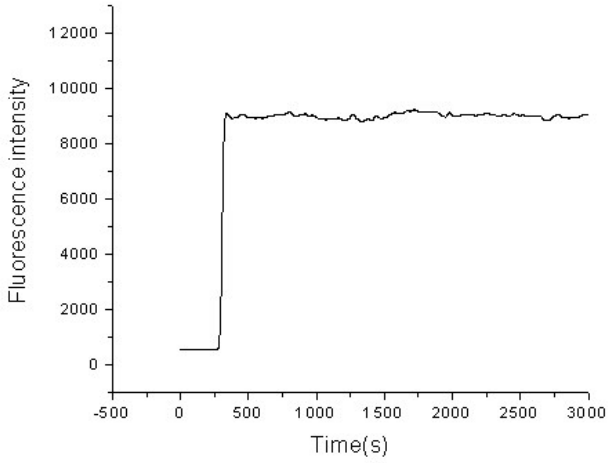

Fig. S1 Dynamic response of the compound CPs/ARS with target SA. Time course of compound CPs/ARS as measured by a spectrofluorometer. The concentration of the MIP-based nanoprobe was 35 mg/L, and the concentration of SA was 100 μM. 0.015 M PBS, pH =7.4, λ_{ex} = 470 nm.

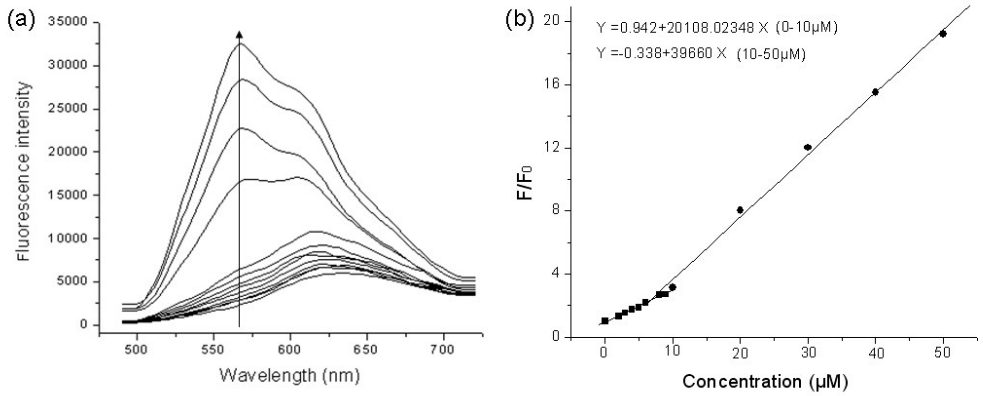

Fig. S2 Fluorescence emission spectra of (a) MIP-based nanoprobe (35 mg/L) with addition of indicated concentration of target SA solution. A linear correlation between the fluorescence intensity and SA concentrations. 0.015 M PBS, pH =7.4, λ_{ex} = 470 nm.

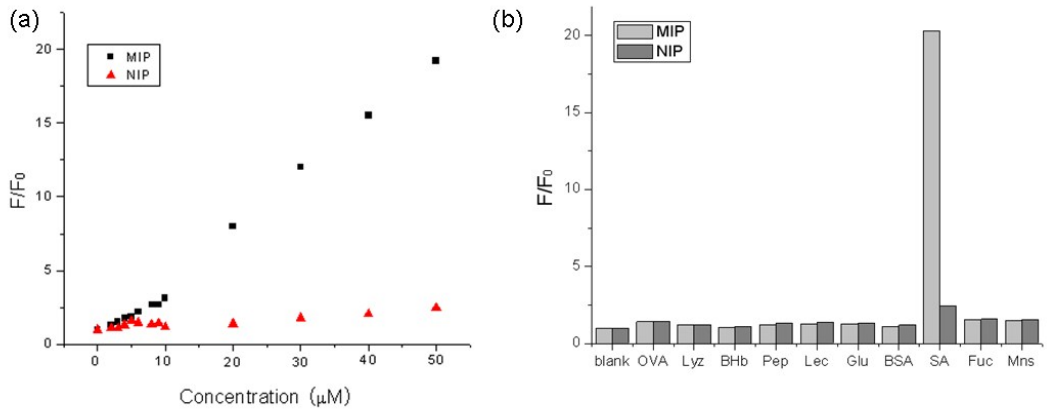

Fig. S3 Fluorescence response of the MIP-based nanoprobe and NIP nanoparticles with addition of indicated concentration of target SA solution (a); Fluorescence responses of MIP-based probe and NIP nanoparticles to SA and other different proteins. The concentration of the MIP and NIP-based nanoparticles was 35 mg/L; The concentration of SA was 0.015 mg/L, and the concentration of other competing molecules was 0.15 mg/L. 0.015 M PBS, pH =7.4, λ_{ex} = 470 nm. Ovalbumin (OVA), Lysozyme (Lyz), Hemoglobin from bovine blood (BHb), Pepsin (pep), Lecithin (Lec), Glucose (Glu), Albumin from bovine serum (BSA), Fucose (Fuc), Mannose (Mns).

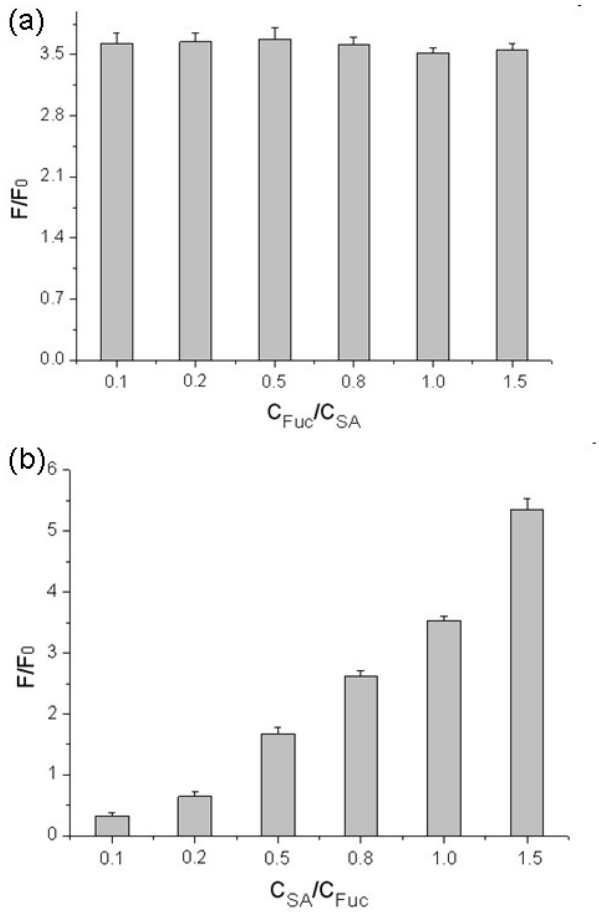

Fig. S4 Effect of the competitive molecules fucose (Fuc) on the binding of template SA. (a) Binding was done by fixing the concentration of SA (10 μM) and increasing the concentration of Fuc. (b) Binding was done by fixing the concentration of Fuc (10 μM) and increasing the concentration of SA. The concentration of the MIP-based nanoparticles was 35 mg/L. The error bars were from three parallel experiments.

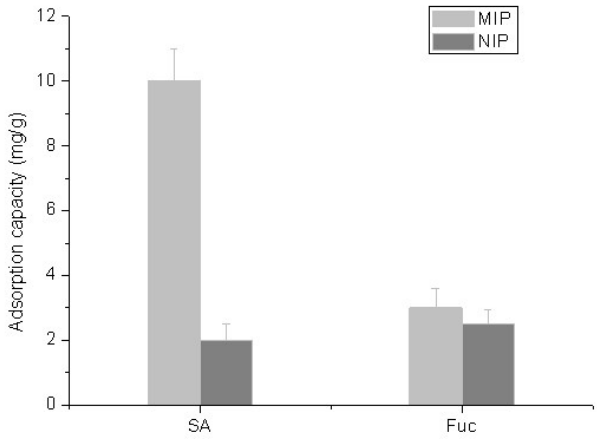

Fig. S5 The adsorption capacity of SA or Fuc binding by the MIP or NIP. Experiment was conducted by the addition of 20 mg of MIP or NIP in 0.2 mg/mL SA or Fuc solution at room temperature. The results were obtained from three parallel experiments.

The adsorption capacity (Q, expressed in units of mg/g) of the SA or Fuc bound to the MIP or NIP is calculated by

$$Q = (C_0 - C_t)\, V / W$$

where C_0 and C_t (mg/ml) are the initial concentration and the residual concentration of the SA or Fuc, respectively, V (ml) is the volume of the initial solution, and W (g) is the weight of the MIP or NIP.

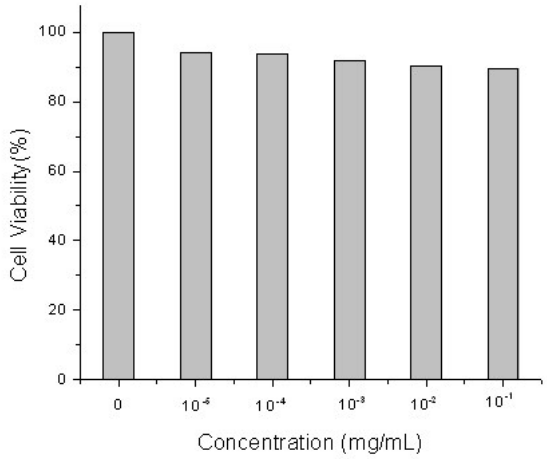

Fig. S6 Viability of RAW.264.7 cells in the presence of nanoprobe as measured by using MTT assay. The cells were incubated with nanoprobe for 12 h.

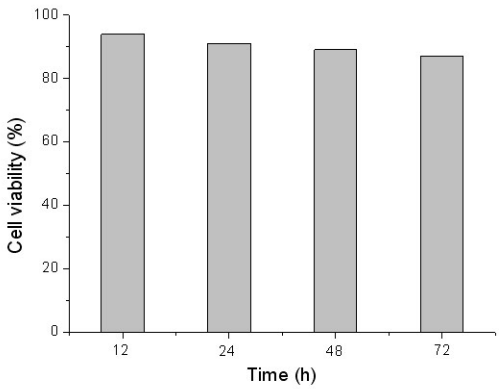

Fig. S7 Viability of RAW.264.7 cells in the presence of nanoprobe (10^{-5} mg/mL) as measured by using MTT assay. The cells were incubated with nanoprobe for 12 h, 24 h, 48 h, and 72 h, respectively.

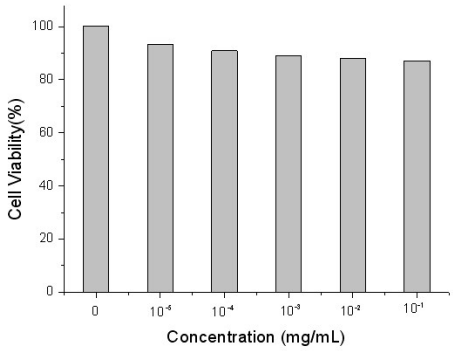

Fig. S8 Viability of MCF-10 cells in the presence of nanoprobe as measured by using MTT assay. The cells were incubated with nanoprobe for 12 h.

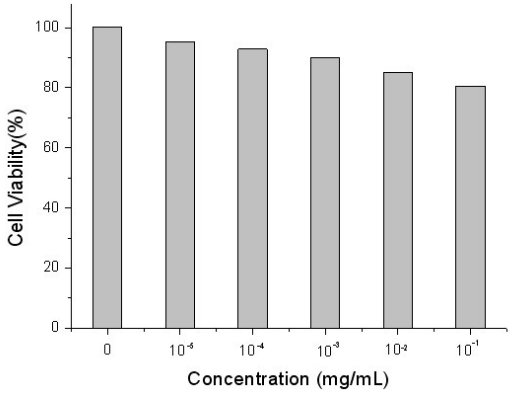

Fig. S9 Viability of RAW.264.7 cells in the presence of ARS as measured by using MTT assay. The cells were incubated with ARS for 12 h.

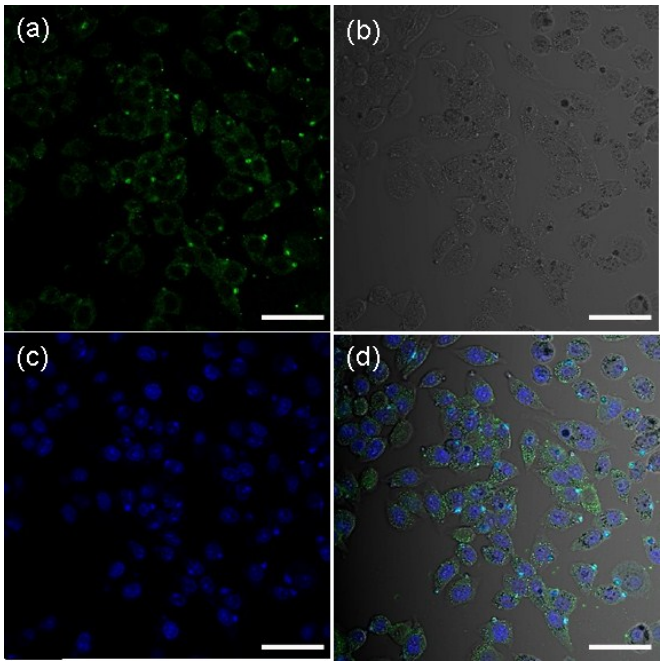

Fig. S10 The one-photon fluorescence images of SA in MCF-10 cells with MIP-based nanoprobe. (a) nanoprobe-loaded cells; (b) bright field confocal microscopy images of the cell; (c) nuclear staining with Hoechst (d) merged image of (a) and (2). The concentration of the MIP-based nanoparticles was 35 mg/L. The scale bars was 25 μm.

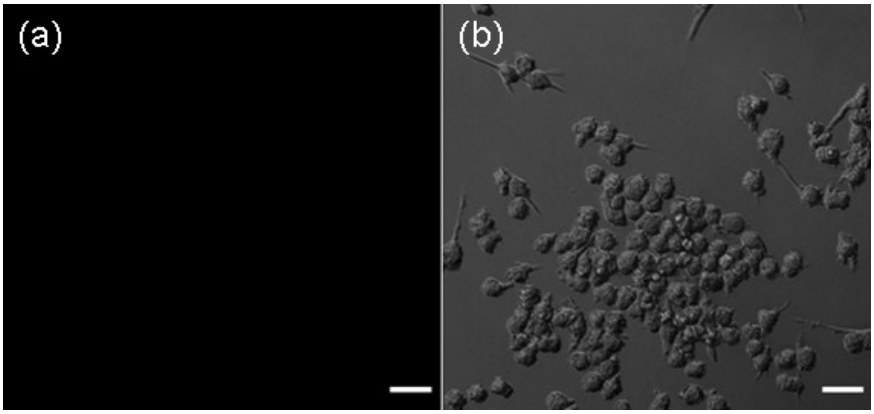

Fig. S11 The two-photon fluorescence images of SA in Raw264.7 cells with NIP-based nanoparticles. (a) nanoparticles-loaded cells; (b) bright field confocal microscopy images of the cell. The concentration of the NIP-based nanoparticles was 35 mg/L. The scale bars was 25 μm.

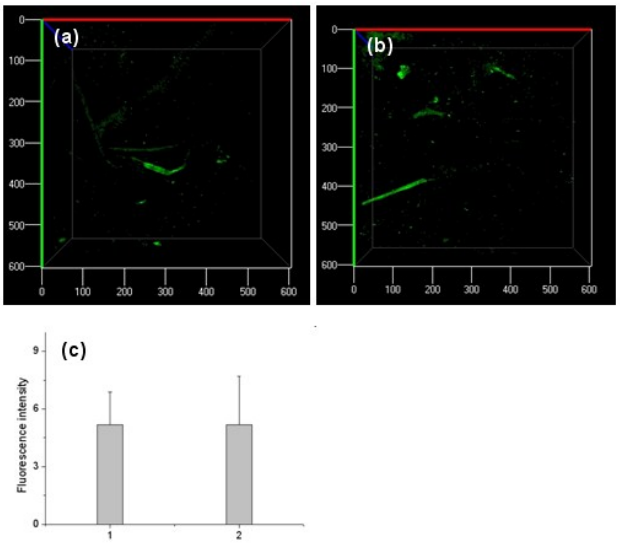

Fig. S12 (a) The 3D distribution of sialylated glycans in normal tissues of mice. (b) The 3D distribution of sialylated glycans in normal tissues of mice after injection of PBS. (c) Fluorescence intensity output from the fluorescent images (panels a−b), (1) and (2) was connected with (a) and (b), respectively, the statistics of the Fig. S9c was performed by selecting three representative regions and output the fluorescence intensity. And the error bars were calculated from three parallel experiments.

Table S1. Results for determination of SA in human serum samples (0.015 M PBS, pH =7.4)

Samples	Sialic acid concentration (×mg/L)		
	Spiked	Measured[a]	Amount detected %[a]
1	30.90	32.445±0.03	105.08±3.32
2	30.90	30.186±0.02	97.69±2.08
3	30.90	29.469±0.03	95.37±3.44

[a] The mean ± the standard deviation for three experiments is given.