Electronic Supplementary Information

DNA nanotriangle-scaffolded multivalent activatable aptamer probe with ultralow background and robust stability for cancer theranostics

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Material and Methods

Chemicals and Materials. All DNA probes used in this study were custom-designed and then synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). Sequences of the oligos are listed in Table S1. Dulbecco's phosphate buffered saline (PBS) (with or without $MgCl_2$ and $CaCl_2$), Hoechst 33342 and SYBR Gold were purchased from Thermo Fisher Scientific (MA, USA). Streptavidin (SA), bovine serum albumin (BSA) and yeast tRNA were obtained from Sigma-Aldrich (MO, USA). CellTiter 96 Cell Proliferation Assay was purchased from Promega (WI, USA). Doxorubicin hydrochloride was obtained from Dingguo reagent company (Beijing, China). All other reagents were of the highest grade available and obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Deionized water was obtained by the Milli-Q ultrapure water system. Binding buffer was prepared by adding 4.5 mg/mL glucose, 1 mg/mL BSA and 0.1 mg/mL yeast tRNA into PBS (with $MgCl_2$ and $CaCl_2$). 1×TMS buffer was composed of 40 mM Tris, 10 mM $MgCl_2$ and 100 mM NaCl. The pH of the 1×TMS buffer was adjusted to 8 using glacial acetic acid.

Cells. CCRF-CEM cells (T lymphoblast, human acute lymphoblastic leukemia) and L02 cells (human hepatocyte cell line) used in this study were obtained from Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Ramos cells (B lymphocyte, human Burkitt's lymphoma) and U266 cells (B lymphocyte, human myeloma, plasmacytoma) were purchased from the Cancer Institute & Hospital of Chinese Academy of Medical Sciences (Beijing, China). Cells were cultured in RPMI 1640 medium supplemented with 13% fetal bovine serum (FBS, heat inactivated) and 100 IU/mL penicillin−streptomycin. All cells were maintained at 37 °C in a 5% wt/vol CO_2 atmosphere. The cell density was determined using a hemocytometer, and this was performed prior to any cell experiment.

Animals. Male athymic BALB/c (Balb/C-nu) mice were obtained from Hunan SJA Laboratory Animal Co. Ltd. They were 4–6 weeks old at the start of each experiment and weighed 20-25 g. All animal operations were in accord with institutional animal use and care regulations, according to protocol No. SCXK (Xiang) 2013-0001, approved by the Laboratory Animal Center of Hunan Province.

Preparation of NTri-SAAP. The nanotriangular DNA scaffold (NTri) was prepared by mixing

equimolar amounts of four scaffold clip strands (a, b, c, d) in 1×TMS buffer. The solution was heated to 95 °C for 4 min and rapidly cooled to 4 °C, with an additional incubation at room temperature for 2 h to generate NTri. Cy5-Apt-L and BHQ3-S were mixed at a molar ratio of 1:2 and then annealed to prepare SAAP in the same way. The NTri-SAAP was achieved by incubating SAAP with streptavidin at room temperature for 20 min, and then adding NTri to react for another 20 min. The molar ratio of SAAP, streptavidin and NTri in the NTri-SAAP system was 3:3:1. Note that the preparation of NTri-Control, which was assembled from Control, streptavidin and NTri adopted the same approach.

Characterization of NTri-SAAP. The NTri was characterized by native polyacrylamide gel electrophoresis (PAGE) (10%, acrylamide/bisacrylamide 29:1) at 80 V in 1×TMS buffer for 150 min on ice. Formation of the resultant NTri-SAAP was confirmed by PAGE gel (6%, 29:1 acrylamide/bisacrylamide). All the samples were stained with SYBR Gold, and then analyzed with Tanon 2500R (China).

For AFM analysis, DNA samples were diluted with PBS buffer to a final concentration of 25 nM and then deposited on freshly cleaved mica with Ni^{2+} (25 nM) pretreatment for 10 min. Excess samples were washed three times with deionized water and dried for imaging. AFM imaging was performed in air in a tapping mode on a Bioscope system AFM (Brucker, USA) with Tap300Al-G probe (BudgetSensors, Bulgaria).

The fluorescence spectra of NTri-SAAP or NTri-Cy5-Apt-L were monitored by an F-7000 fluorescence spectrophotometer (Hitachi, Japan; Ex = 620 nm, Em =640-740 nm).

Dox Loading and Release Investigation. Dox solution (2 μM) was incubated with various amounts of prepared NTri-SAAP, NTri or SAAP in PBS at room temperature for 30 min. The drug loading capacity was quantified by recording the fluorescence of Dox with an F-7000 fluorescence spectrophotometer (Hitachi, Japan; Ex = 485 nm, Em = 520-740 nm).

To investigate the drug release of NTri-SAAP-Dox and SAAP-Dox, NTri-SAAP or SAAP and Dox (mole rations of 1:10) were firstly mixed to prepare DNA-Dox complexes as described above. Afterwards, the DNA-Dox complexes were transferred into PBS buffer, PBS buffer containing DNase I (1.33 U/mL), 10% FBS (un-inactivated) and cell lysate respectively, with a final concentration of 0.1 μM. Then fluorescence of Dox was measured over time at 37 °C using an F-7000 fluorescence spectrophotometer.

Flow Cytometry Assays. Generally, DNA probes were incubated with 1.5×10^5 cells in 150 μL of PBS with $MgCl_2$ and $CaCl_2$ (unless otherwise noted) at 20 °C for 60 min in the dark and then immediately determined with a Gallios cytometer (Beckman Coulter, USA) by counting 10,000 events.

To investigate the sensitivity of this strategy, samples with varying cell numbers ranging from 0 to 2.9×10^5 in 150 μL of binding buffer were determined after incubation at 20 °C for 60 min. Each sample was detected with flow cytometer at high rate for 120 s. Every group was repeated for 3 times. For all the probes used, the Cy5 labeled units was maintained to be a consistent concentration of 15 nM.

The binding affinity of NTri-SAAP or SAAP was determined by incubating CCRF-CEM cells with varying concentrations of the corresponding probes in a 200 μL volume of PBS (with $MgCl_2$ and $CaCl_2$ supplied) at 20 °C for 60 min. Cells were then washed twice with 200 μL of PBS, after which the cells were suspended in 0.2 mL of PBS and subjected to flow-cytometric analysis. NTri-Control and monomer Control were used as negative controls to determine nonspecific binding, respectively. All of the experiments for binding assay were repeated three times. The mean fluorescence intensity of CCRF-CEM cells labeled by the aptamer probe was used to calculate for specific binding by subtracting the mean fluorescence intensity of nonspecific binding from the control probe. The equilibrium dissociation constant (K_d) of the aptamer probe-cell interaction was obtained by fitting the dependence of fluorescence intensity of specific binding on the concentration of the aptamer probe to the equation $Y=B_{max}X/(K_d+X)$, using SigmaPlot.

Laser Scanning Confocal Microscopy Imaging. Fluorescent images were acquired on a FV500 confocal microscope (Olympus, Japan) with a 100× oil immersion objective. Excitation wavelength and emission filters were described as follows. Hoechst 33342: Ex=405 nm, Em=430–460 nm bandpass; Dox: Ex=488 nm, Em=505 nm long-pass; Cy5: Ex=633nm, Em=660 nm long-pass. To investigate the specifically binding of NTri-SAAP to target CCRF-CEM cells, 1×10^5/mL of CCRF-CEM or Ramos cells were incubated with DNA probes (250 nM) at 20 °C for 60 min, and then resuspended in PBS for microscopic observation after twice wash. Co-localization of Dox and NTri-SAAP was studied by incubating CCRF-CEM cells with DNA probes or free Dox (Dox: 2 μM; NTri-SAAP: 0.2 μM)

for 6 h at 37 °C in a 5% CO_2 atmosphere. After staining with Hoechst 33342 for 15 min, cells were visualized by confocal microscope after washing for twice.

***In Vitro* Cytotoxicity Assays.** The cell viability was evaluated using CellTiter 96 Cell Proliferation Assay. In brief, cells (5×10^4 cells per well) were treated with NTri-SAAP (0.2 μM), free Dox (2 μM), NTri-Control-Dox and NTri-SAAP-Dox (Dox: 2 μM; NTri-SAAP: 0.2 μM) in FBS-free medium (37 °C, 5% CO_2) for 2 h, respectively. Then, 80% of the supernatant was removed and replenish fresh medium (13% FBS) for further cell growth (48 h). Then, MTS reagent (20 μL) was added to each well and incubated for 1–2 h at 37 °C. The absorbance (490 nm) was recorded using a Bio-RAD (Benchmark, USA). Cell viability was calculated as described by the manufacturer.

***In Vivo* Fluorescence Imaging.** The mouse xenograft tumor model was established by s.c. injecting approximately 5×10^6 cancer cells into the back of BAL/c nude mice. Tumors were then allowed to grow for 3−4 weeks to 1−2 cm in diameter. Before imaging, BALB/c nude mice, with or without tumors, were anesthetized to be motionless with anesthetic. After 0.35 nmol DNA probes was injected intravenously *via* tail vein, time-lapse fluorescence imaging was taken by an IVIS Lumina II *in vivo* imaging system (Caliper Life Sicence, USA).

***In Vivo* Anti-tumor Efficacy Evaluation.** The anti-tumor efficacy of NTri-SAAP*-Dox was evaluated using CCRF-CEM tumor-bearing BALB/c nude mice. When the tumors exhibited a volume of ~100 mm^3, these mice were randomly divided into four groups and i.v. injected with NTri-SAAP*-Dox, Dox, NTri-SAAP* and PBS, respectively. The dosage of Dox in the NTri-SAAP*-Dox group and the Dox group was both 2 mg/kg. The amount of NTri-SAAP* in the NTri-SAAP* group (0.2 mg/kg) was given equivalently to that used for the NTri-SAAP*-Dox group. The control group received an equal amount of PBS injection. Drugs were injected through tail veins every other day, and the tumor length and width for each mouse were measured with calipers every other day. The tumor volume was calculated using the following equation:

$$\text{Tumor volume}=\text{Length}\times\text{Width}^2/2$$

After 13 days from drugs administration, the mice were sacrificed.

Table S1. All of the oligonucleotides used in this work[a]

Probe	Name	Sequence (5'-3')
NTri (FAM-NTri)	a	**biotin-**TTTAAGTCTCTACGTCAGCGTTAGACTGAA TAGGC
	b	**biotin-**TTTGCCTATCGAGTCATCTGTCTACTGAGC CACTG
	c	**biotin-**TTTCAGTGTCTATCATGGCAAGGTCCTACG GACTT
	d	GATGACTCGTTTCAGTCTAACGCTGACGTAGTCGTAG GACCTTGCCATGATAGTGCTCAGTAGACA
	FAM-d	**FAM-**GATGACTCGTTTCAGTCTAACGCTGACGTAGTCG TAGGACCTTGCCATGATAGTGCTCAGTAGACA
SAAP	Cy5-Apt-L	*TACTGTACGGTTAGAT***-Cy5-**CTGCCTGCTCT**-biotin-** *ATCTAACTGCTGCGCCGCCGGGAAAA*
	BHQ3-S	GAGCAGGCAG-**BHQ3**
Control	Cy5-Control	*ATGTGCACGGTTAGAT***-Cy5-**CTGCCTGCTCT**-biotin-** *ATCTAACTGCGTATCAGGTAAGACTC*
	BHQ3-S	GAGCAGGCAG-**BHQ3**
SAAP*	Cy5-Apt-L*	*TACTGTACGGTTAGAT***-Cy5-**CTGCCTGCGTTCT**-biotin-** *ATCTAACTGCTGCGCCGCCGGGAAAA*
	BHQ3-S*	GAACGCAGGCAG-**BHQ3**
Control*	Cy5-Control*	*ATGTGCACGGTTAGAT***-Cy5-**CTGCCTGCGTTCT**-biotin-** *ATCTAACTGCGTATCAGGTAAGACTC*
	BHQ3-S*	GAACGCAGGCAG-**BHQ3**
	cDNA	CAGTTAGATGAGCAGGCAG

[a] In all the oligonucleotides, a, b, c, and d (or FAM-d) are used for nanotriangular DNA scaffold (NTri or FAM-NTri) formation. The corresponding hybridization regions of d with a, b, or c are shown in different colors. In the sequences of Cy5-Apt-L (or Cy5-Apt-L*), aptamer fragments are shown in italic, and the hybridization regions with BHQ3-S (or BHQ3-S*) are shown underlined.

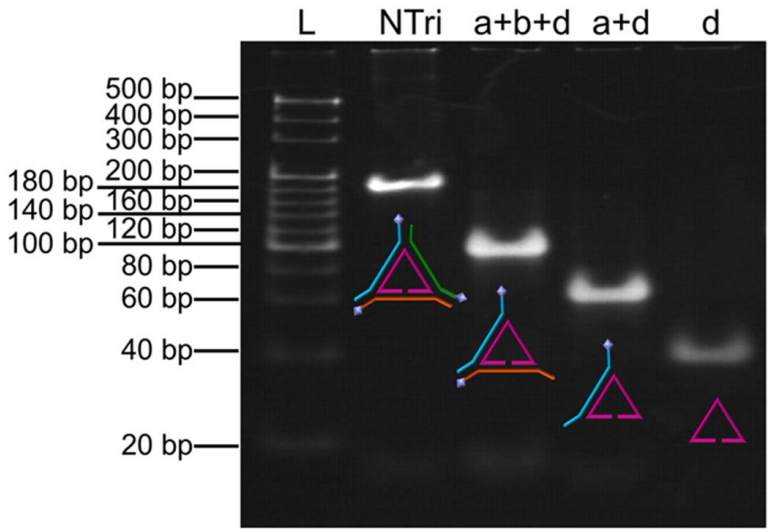

Figure S1. Native PAGE (10%, 1×TMS) analysis of the stepwise assembly of DNA NTri scaffold from an inner strand (d) and three Bio-modified exterior strands (a, b, and c). Lane "L" indicates a DNA ladder.

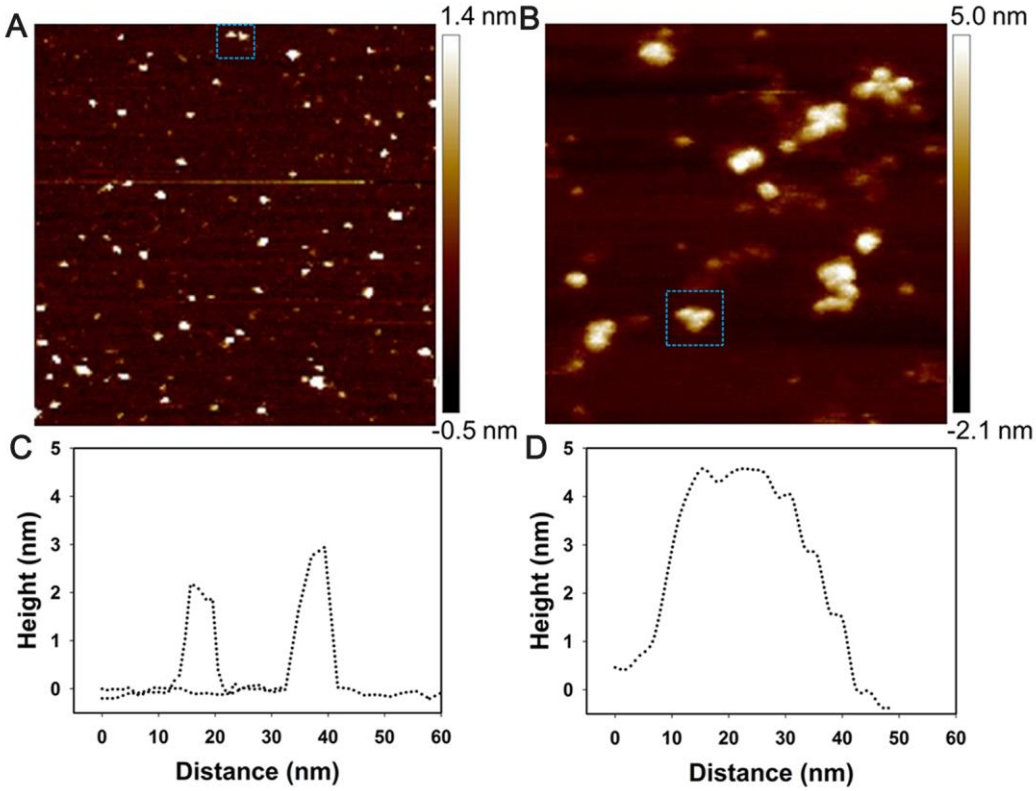

Figure S2. AFM images of (A) NTri and (B) NTri-SAAP. The sizes of NTri and NTri-SAAP marked in blue boxes are shown in (C) and (D), which are calculated as ~9×2 nm and ~32×5 nm (distance×height), respectively. (The AFM images were acquired on a scale of 300 nm×300 nm.)

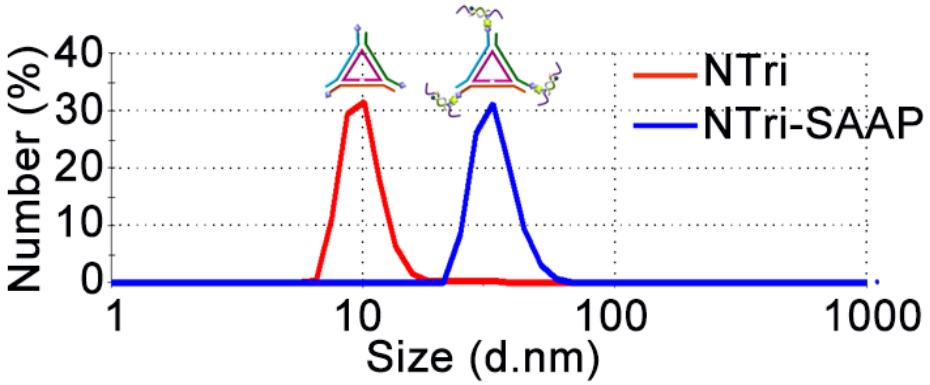

Figure S3. Dynamic light scattering (DLS) analysis of the sizes of NTri and NTri-SAAP, showing the mean diameters at 10.2 nm and 33.7 nm, respectively.

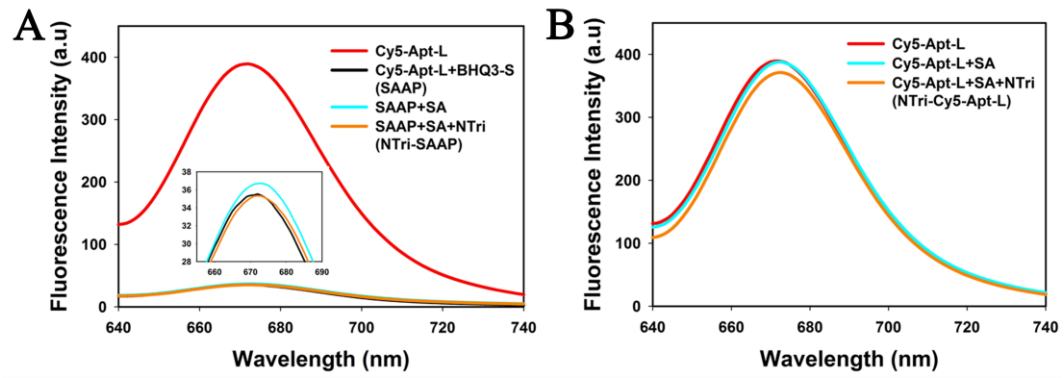

Figure S4. Fluorescence spectra measurements of (A) NTri-SAAP and (B) NTri-Cy5-Apt-L during their self-assembly processes, indicating little influence of streptavidin (SA) or NTri on Cy5 fluorescence.

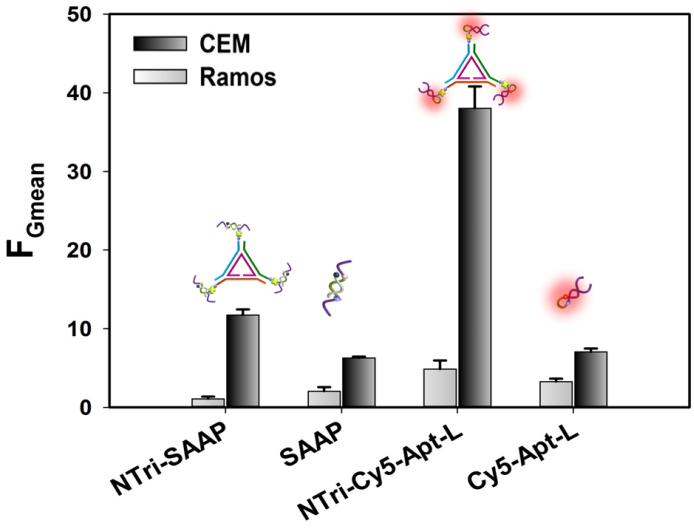

Figure S5. The histogram of the fluorescence intensities of CEM cells or Ramos cells after incubation with different probes in PBS with Mg^{2+} supplied, corresponding to the results in Figure 1B.

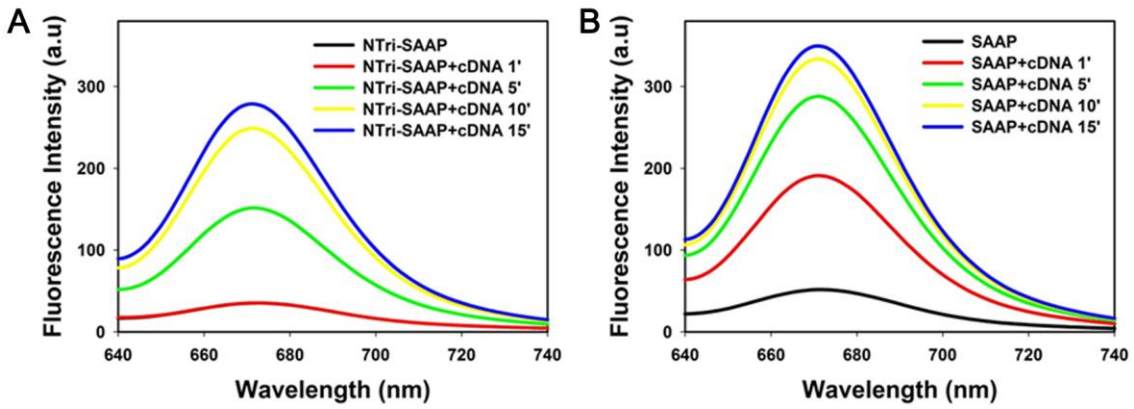

Figure S6. Time-lapse fluorescence recovery of (A) NTri-SAAP and (B) SAAP monomer, after addition of cDNA molecules acting as the simulated target to break the structure of SAAP.

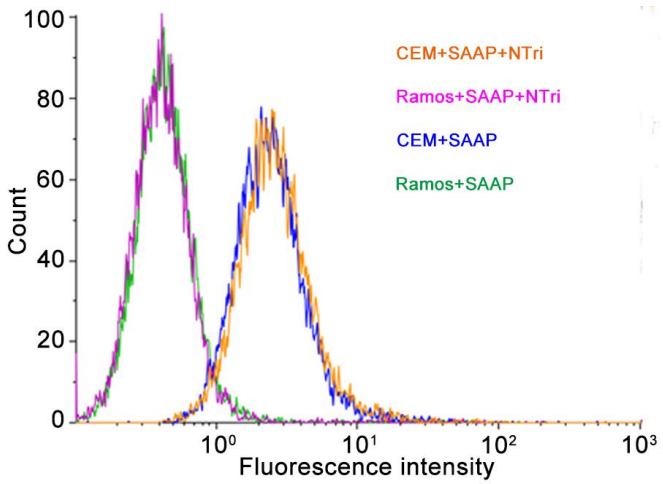

Figure S7. Flow cytometry assays of CEM or Ramos cells, incubated with the SAAP monomer or the mixture of SAAP and NTri (without streptavidin conjugation).

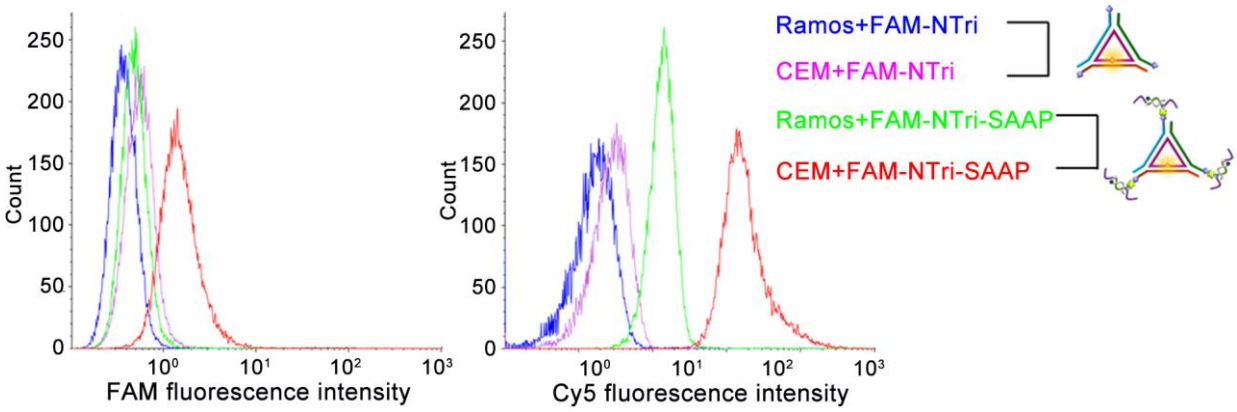

Figure S8. Flow cytometry assays of CEM or Ramos cells after incubation with FAM-NTri and FAM-NTri-SAAP, respectively, revealing the selective binding of multivalent NTri-SAAP system to target cells as a whole unit. The fluorescence of FAM indicated the location of NTri, while Cy5 fluorescence indicated the location of SAAP.

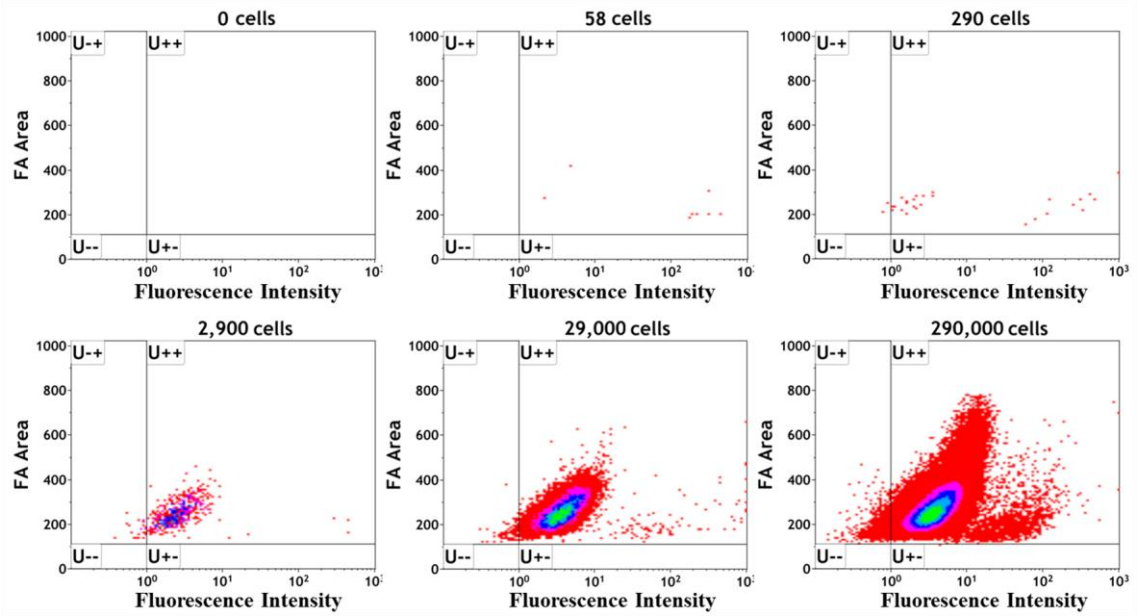

Figure S9. Flow cytometry assays of CEM cells with different cell amounts in 150 μL binding buffer using the NTri-SAAP strategy.

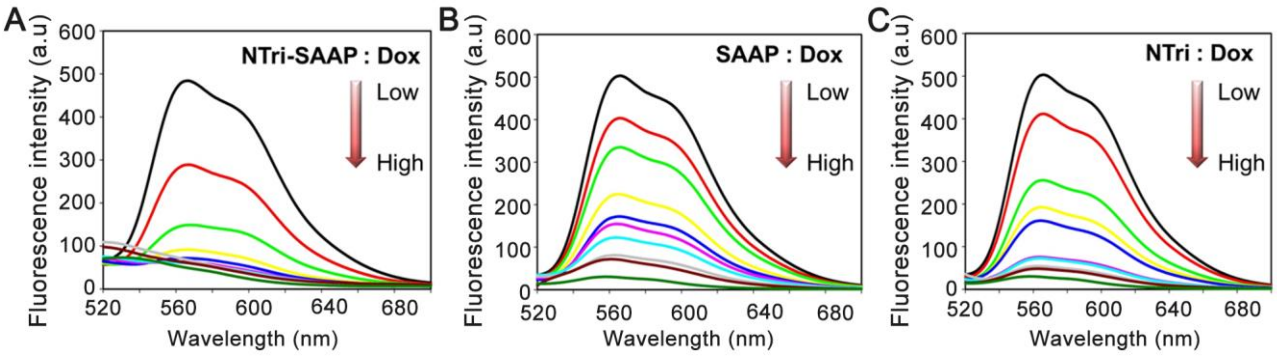

Figure S10. Drug loading feasibility investigation of NTri-SAAP. Fluorescence spectra of doxorubicin (Dox) solution (1 μM) after mixed with (A) NTri-SAAP, (B) SAAP or (C) NTri at different SAAP-to-drug molar ratios (0, 0.02, 0.06, 0.10, 0.14, 0.20, 0.26, 0.40, 0.6, PBS). The amount of SAAP in (A) and (B) was kept equivalent, which was the triple dose of NTri in (C).

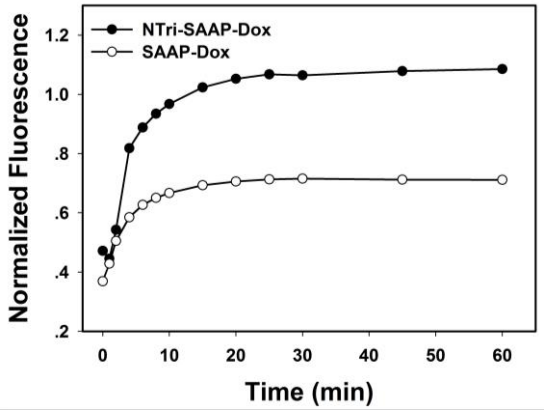

Figure S11. Time-lapse fluorescence monitoring of Dox released from NTri-SAAP or SAAP in PBS buffer containing DNase I at 37 °C.

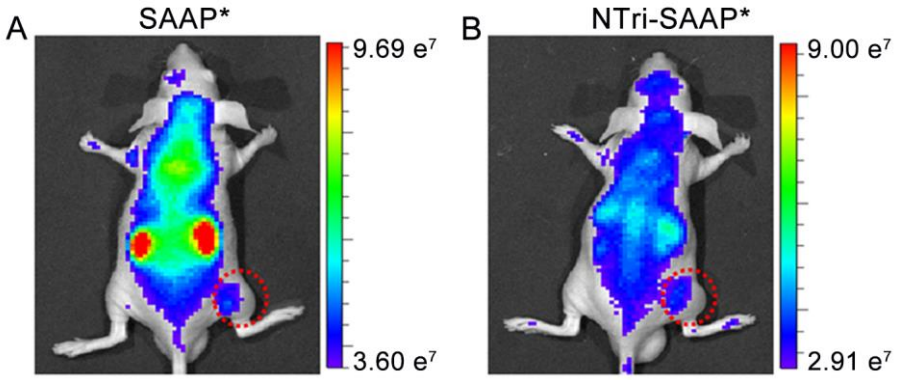

Figure S12. Fluorescence images of the CEM tumor-bearing mice injected with (A) SAAP* or (B) NTri-SAAP* for 60 min in Figure 6, readjusted by deducting more background signals to reveal clear tumor figures. (Radiant efficiency was used as calibrated unit.)

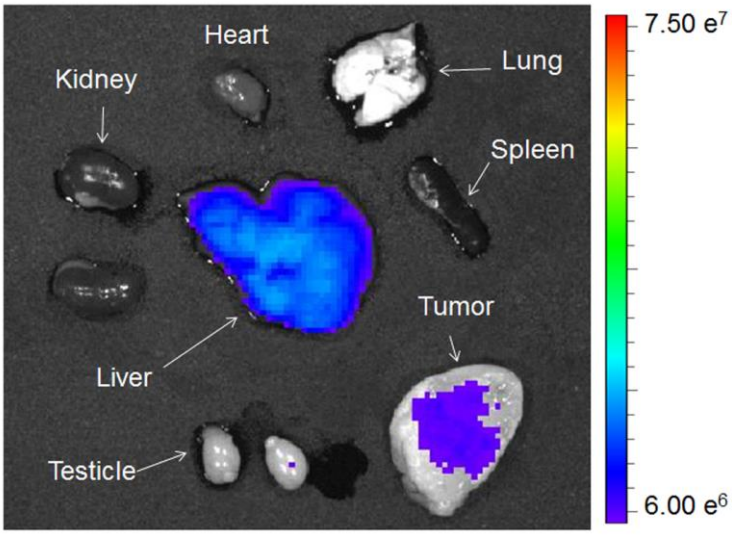

Figure S13. *Ex vivo* fluorescence imaging of the tumor and major organs (heart, liver, spleen, lung, kidneys and testicles) resected at 8 h after intravenous injection of NTri-SAAP* into a CEM tumor-bearing mouse. (Radiant efficiency was used as calibrated unit.)

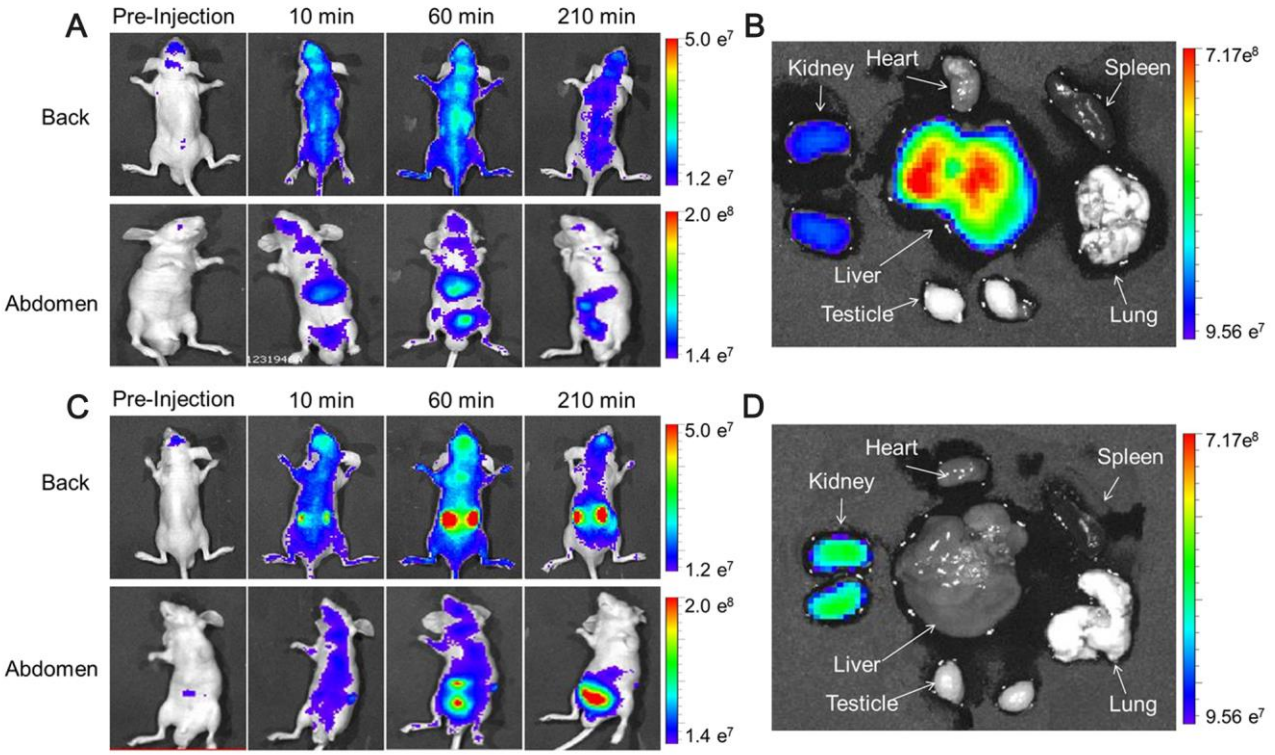

Figure S14. Biodistribution investigation of NTri-SAAP* and SAAP* inside normal mice. *In vivo* time-lapse fluorescence imaging of nude mice after intravenous injection of (A) NTri-SAAP* and (C) SAAP*, through both back and abdomen imaging for continuous 210 min. *Ex vivo* fluorescence imaging of the major organs (heart, liver, spleen, lung, kidneys and testicles) resected at 4 h after intravenous injection of (B) NTri-SAAP* and (D) SAAP*, respectively. (Radiant efficiency was used as calibrated unit.)

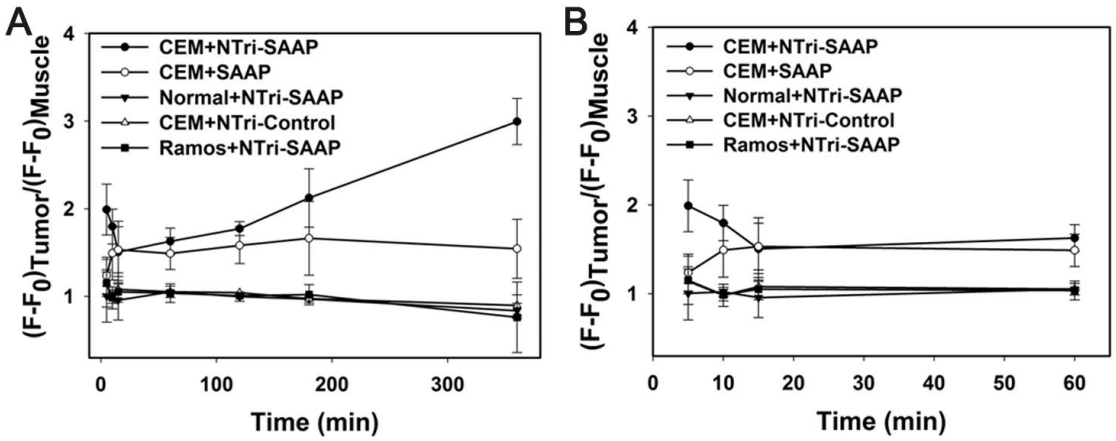

Figure S15. Plot of the ratios of the enhanced fluorescence intensity in tumor sites (red circles in Figure 6) to that in muscle sites (pink circles in Figure 6) as a function of time (F, the fluorescence intensity at a specified post-injection time point; F_0, the fluorescence intensity at the pre-injection time point). (A) The plot of the entire 6 h-imaging process; (B) The plot of the first 1 h-imaging process in (A).