Electronic Supplementary Information

A DNA-binding, albumin-targeting fusion protein promotes the cellular uptake and bioavailability of framework DNA nanostructure

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Materials and Methods

Materials

All the enzymes, protein markers and HSA (human serum albumin) were purchased from Thermo Fisher Scientific (Massachusetts, USA). Ampicillin, IPTG, DTT, kits for plasmid extraction and gel extraction in this work were purchased from Sangon Biotech Co., Ltd (Shanghai, China). DNA marker were purchased from TransGen Biotech Co., Ltd (Beijing, China). Reagents for cell culture were purchased from Gibco, such as FBS and DMEM. MSA (mouse serum albumin) was purchased from HuiJia Bio Co., Ltd (Xiamen, China). Oligonucleotides (S-1/2/3/4) for TDN assembly were purchased from Sangon Biotech Co., Ltd. Another oligonucleotide S-1-Cy5 was synthesized which had same sequence with ssTDN-1 except it was modified with Cy5 fluorescent group at 5' end.

Plasmids construction

Protein ABS was fused by two parts, a DNA binding domain Sso7d and an albumin binding domain ABD, a flexible linker $(GGGGS)_2$ was added between these two domains (table 1). This fused protein codon sequence was synthesized by company (Tsingke Biotech Co., Ltd, Shanghai, China), and inserted into a pet-32a derived vector which contained a 6His-tag for protein purification and a thrombin site for removing 6His-tag. The positive construction was confirmed by sequencing.

Table 1. Protein sequence

Protein name	Protein sequence
ABS	GSGGSLAEAKVLANRELDKYGVSDFYKRLINKAKTVEGVEALKLHILAAL PGGGGSGGGGSATVKFKYKGEEKQVDISKIKKVWRVGKMISFTYDEG GGKTGRGAVSEKDAPKELLQMLEKQKK

Note: sequence in blue color indicated albumin binding domain (ABD); sequence in red color indicated DNA binding domain (Sso7d); sequence in black color indicated flexible linker.

Protein expression and purification

Plasmid was transformed into the E. coli strain BL21(DE3) and selected by ampicillin. For protein expression, firstly, a single clone was cultured overnight in 10 ml LB medium, at 37 °C, 200 rpm. The

overnight culture was transferred into 1 L fresh LB medium with a 1:100 volume ratio for amplifying culture. All the LB culture contained 100 μg/mL ampicillin. While OD_{600} was reached to 0.6~0.8, IPTG (isopropyl β-D-1-thiogalactopyranoside) with a 0.3 mM final concentration was added into culture as an induced reagent for protein expression. At this time, cell culture temperature was decreased to 20 °C and further incubated for 16 hours. Subsequently, E. coli cells were collected by centrifugation at 4000 rpm, 20 min from culture. Cell pellets were resuspended in lysis buffer (20 mM Tris.HCl, pH 8.0), and lysed by sonication at 4 °C. The lysed cells were then centrifuged at 10000 rpm, 40 min for removing the precipitate. The target protein was purified by Ni-NTA affinity column with the elution buffer (20 mM Tris.HCl, 250 mM imidazole, pH 8.0) following the instructions from the manufacturer (QIAGEN, Germany). During this purification process, while target protein was binding with Ni-NTA, 8 M urea was added into column and incubated 2 h to denature target protein (ABS). Next, the washing and elution steps will be done. After affinity purification, target protein was dialyzed to 1×PBS buffer (pH 7.3), and thrombin enzyme was added for removing 6His-tag at the same time. Then, target protein was undergone FPLC purification (ÄKTA™pure, GE, American) to get high quality protein for the following experiments. The purity of protein was analyzed by SDS-PAGE and deeply its molecule wight was calculated by MALDI-TOF-MS. Target protein was done lyophilization for long time storage.

Circular Dichroism Spectroscopy

The secondary structure of target protein ABS was analyzed by Circular Dichroism (CD) spectra. The CD spectra were recorded on a Jasco J-810 spectropolarimeter equipped with a thermal controller. A 0.1 cm path length cuvette was used. The protein concentration was ~0.4 mg/mL in 1×PBS buffer (pH 7.3).

TDN assembly

The four oligonucleotides S-1/2/3/4 (or S-1-Cy5) were mixed at equimolar (1 μM) in TE/Mg^{2+} buffer (10 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, pH 8.0). The mixed solution was heated to 95 °C for 5 min and cooled to 4 °C in 30 min in a PCR machine. The assembled TDN was stored at 4 °C for further use. The quality of TDN nanostructure was analyzed by 8% PAGE or 1.5% agarose electrophoresis. The sequences of DNAs for TDN assembly were shown in table 2.

Table 2. Sequences of DNA strands for TDN assembly

Strand name	Stand sequence 5'-3'
S-1	ACATTCCTAAGTCTGAATTATTACAGCTTGCTACACTTGAAGAGCCGC CATAGTA
S-1-Cy5	Cy5ACATTCCTAAGTCTGAATTATTACAGCTTGCTACACTTGAAGAGCC GCCATAGTA
S-2	TATCACCAGGCAGTTGATTGTGTAGCAAGCTGTAATTTATGCGAGGGT CCAATAC
S-3	TCAACTGCCTGGTGATATTACGACACTACGTGGGAATTTACTATGGCG GCTCTTC
S-4	TTCAGACTTAGGAATGTTTTTCCCACGTAGTGTCGTTTGTATTGGACC CTCGCAT

Preparation of TDN and ABS complex

For cell culture or animal experiments, TDN-ABS complex was prepared by mixing ABS with TDN (or TDN-Cy5) in PBS buffer with a molar ratio of 20:1 for 1 hr at room temperature. For TDN-ABS-HSA

complex, HSA was added to preformed TDN-ABS complex with a molar ratio of 100:20:1 (HSA:ABS:TDN) and incubated for 1 hr before subsequent experiments. TDN and HSA incubated for 1hr with a mole ratio 1:100 for TDN-HSA group preparation, as the same with TDN+ABS complex incubating with HSA.

Binding affinity analysis

The binding affinity between ABS with TDN, HSA, MSA or BSA was firstly analyzed by native-page gel electrophoresis. In detail, ABS and the above ligand incubated with a predetermined molar ratio for 1 hr at room temperature, respectively, then analyzed by 8% PAGE (for ABS, TDN and HSA binding analysis) or 12% native-page (for the binding analysis of ABS and MSA or BSA). Furthermore, the Kd value between ABS and HSA or TDN were calculated by microscale thermophoresis (MST). Briefly, the amount of Cy5 labeled TDN or HSA must be consistent across samples and then increased the amount of the legend ABS. The Cy5 fluorescence intensity was detected and calculated for affinity analysis.

AFM imaging

5 μL of TDN with a 100 nM concentration was deposited onto freshly cleaved mica surface, and then 50 μL TE/Mg^{2+} buffer was added to the sample for liquid mode imaging. SNL-10 AFM probe was used for imaging.

Dynamic light scattering (DLS) measurement

The hydrodynamic size and zeta potential of TDN and TDN-ABS were determined by using Malvern Zetasizer Nano-ZS (Malvern Instruments). 100 μL of samples were used for each measurement. The hydrodynamic size and zeta potentials were averageg from three parallel measurements.

Stability analysis of TDN or TDN-ABS complex in cell medium

TDN or TDN-ABS were incubated in cell medium (containing FBS) at 37 °C for predetermined time (0.5, 1h, 2h, 4h, 6h or 8h) (TDN was labeled with Cy5, samples had finally equivalent Cy5 concentration: 200 nM; the volume ratio of sample and cell medium is 2:3, which is consistent with the following cellular experiment). Then, samples were subjected to 1.5% agarose gel electrophoresis assay.

Cell culture

Cells (HeLa cell or MCF-7 cell) were grown in DMEM medium containing 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin and cultured at 37 °C in humidified air containing 5% CO_2.

Confocal microscopic imaging

Cells were seeded at a density of 5×10^4 cells per well on specific plates for confocal microscopic imaging which had glass bottom and incubated at 37 °C for 24 h. For colocalization experiment, TDN was Cy5 and Cy3 labeled (Cy5 and Cy3 located at different vertex of TDN). Then MCF-7 cells were incubated with TDN or TDN-ABS for 4 hrs (samples had equivalent Cy5 and Cy3 concentration of 200 nM). After incubation, medium was removed and washed twice with PBS and the cells were stained with Hoechst (final concentration of 1 μg/mL) for 10 min. Subsequently, the cells were washed three times with PBS, and then fixed with 4% formaldehyde for 15 min at room temperature and the plates were rinsed with PBS three times, and observed under a laser scanning confocal microscope (Leica). The colocalization efficiency was analyzed by Image J software.

For cellular uptake experiment, the cells were incubated with TDN, TDN-ABS, TDN-HSA, or TDN-ABS-HSA samples (TDN was labeled with Cy5, samples had equivalent Cy5 concentration of 200 nM) for 4 hrs. The following steps were as the above.

Flow cytometry (FCM) analysis

Four groups were set for cellular uptake analysis, TDN, TDN-HSA, TDN-ABS, TDN-ABS-HSA. Samples preparation was following the above mothed. FCM was chosen to detect the fluorescence intensity of cells after incubation with samples. Cells were seeded at a density of 1×10^5 cells per well in 24-well plates with 1 mL cell culture medium and incubated at 37 °C overnight. Then the cells were treated with various samples (TDN was labeled with Cy5, samples had finally equivalent Cy5 concentration: 200 nM; the volume ratio of sample and cell medium is 2:3, which is consistent with the cellular experiment) for predetermined time (1h, 2h, 4h, or 8h). Subsequently, the medium was removed and the cells were washed three times with PBS buffer. Afterwards, the cells were digested with trypsin and collected throw centrifugation at 1500 rpm, 5 min, and also washed two times using PBS buffer. At last, 1×10^4 gated events were collected and the fluorescence intensity (Cy5) of collected cells were detected by FCM.

Cell Counting Kit-8 (CCK8)

CCK8 was used to quantify the proliferation of HeLa and MCF-7 cell. Cells in 100 μL were seeded in 96-well plates (2×10^3 cells per well) and cultured at 37 °C for 24 h. Then samples (TDN in all the samples had an equal concentration of 200 nM) were added to cells, incubated for 6 h. Afterwards, 10 μL CCK8 solution was added into per well and incubated for 2 h at 37 °C. The absorbance of supernatant was measured at 450 nm using a microplate reader (Biotek, USA).

Animals

The female Balb/c nude mice (6/8-week-old) were purchased from JSJ company (Shanghai, China). All the animals were carried out in compliance with the guidelines for the care and use of laboratory animals and were approved by the Animal Care Committee of Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital (RJ2020-0409).

Biodistribution and in vivo optical imaging

For biodistribution analysis, BALB/c nude mice were intravenously injected with 200 μL of 250 nM Cy5-ssDNA, Cy5-TDN, or Cy5-TDN-ABS. For tumor treatment analysis, tumor-implanted BALB/c nude mice were intravenously injected with 200 μL Cy5-TDN or Cy5-TDN-ABS (Cy5 concentration was 13 uM). The mice were anesthetized using isoflurane/O_2 (2% v/v), then they were imaged by an IVIS Lumina II in vivo imaging system (Caliper Life Sciences, USA) using 650 nm excitation wavelength and 700 nm emission wavelength at predetermined time points (1h, 4h, or 6h). After the image of the last time point was collected, the mice were dissected to obtain the main organs for optical imaging.

TDN retention time in blood

BALB/c mice were intravenously injected with 200 μL Cy5-TDN or Cy5-TDN-ABS (Cy5 concentration was 13 uM). Then, 50 μL blood was extracted from the mice at predetermined time points (oh=control, 1h, 2h, 4h, 8h), and Cy5 fluorescence intensity of the samples were detected using a microplate reader (Biotek, USA).

Statistical analysis

Statistical analysis was conducted by using one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test for multiple comparisons. A P value of less than 0.05 was considered to be statistically significant.

Supplementary figures

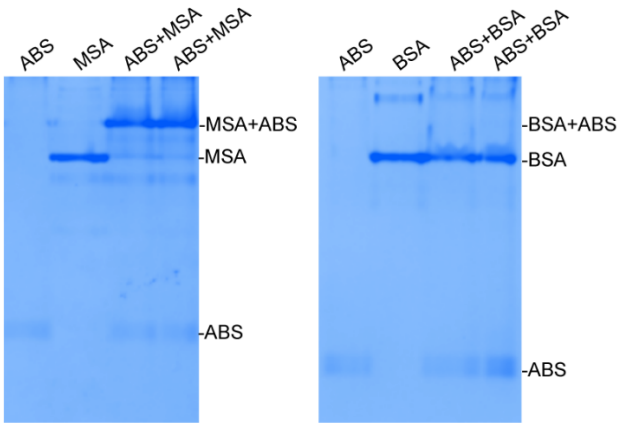

Figure S1. Binding assay of ABS to MSA or BSA. Molar ratio of MSA (BSA): ABS are 1:2 and 1:5.

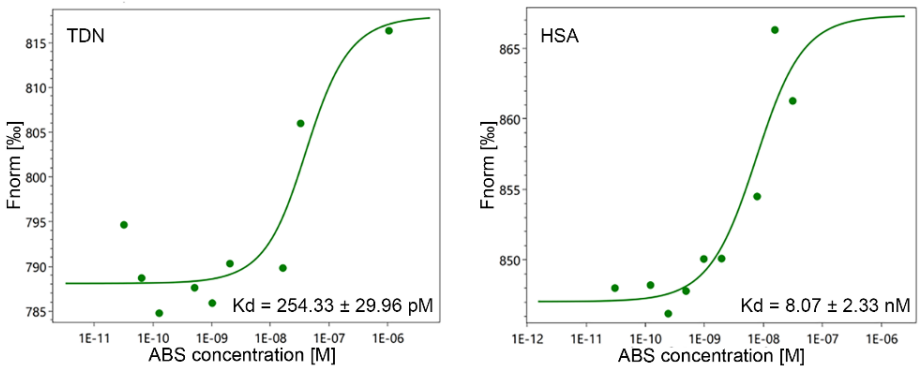

Figure S2. Binding affinity analysis of TDN-ABS and ABS-HSA.

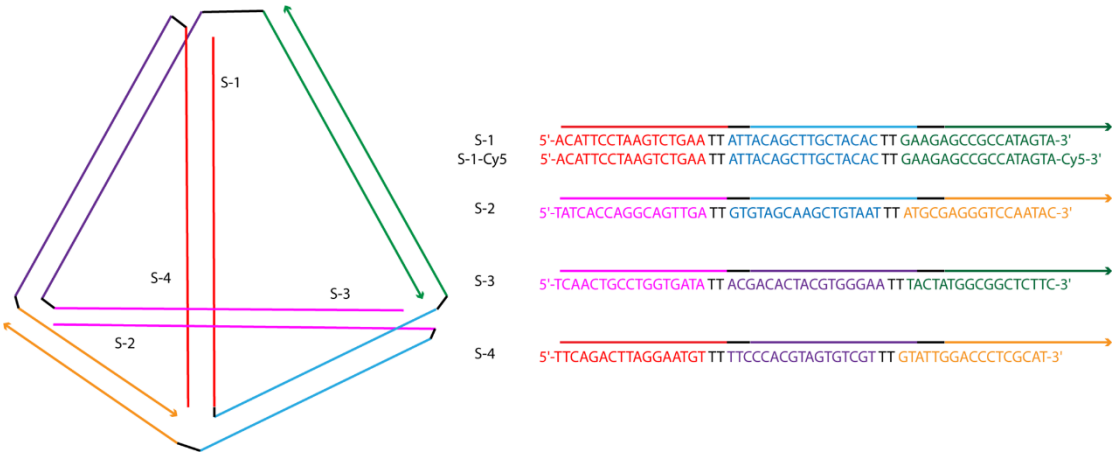

Figure S3. Strand diagram of TDN and sequences of DNA strands.

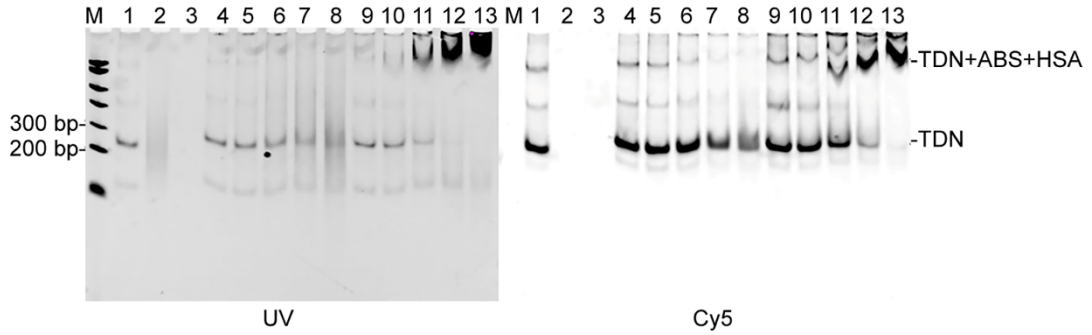

Figure S4. Binding affinity examination of TDN, ABS and HSA. M: DNA marker I; 1: TDN; 2; ABS; 3: HSA; 4-8: TDN-ABS complex with molar ratio of 1:1, 1:5, 1:10, 1:20, 1:40, respectively; 9-13: TDN-ABS (1:1, 1:5, 1:10, 1:20, 1:40, respectively) mixed with 100-fold of HSA. 8% PAGE, 90 V for 130 min.

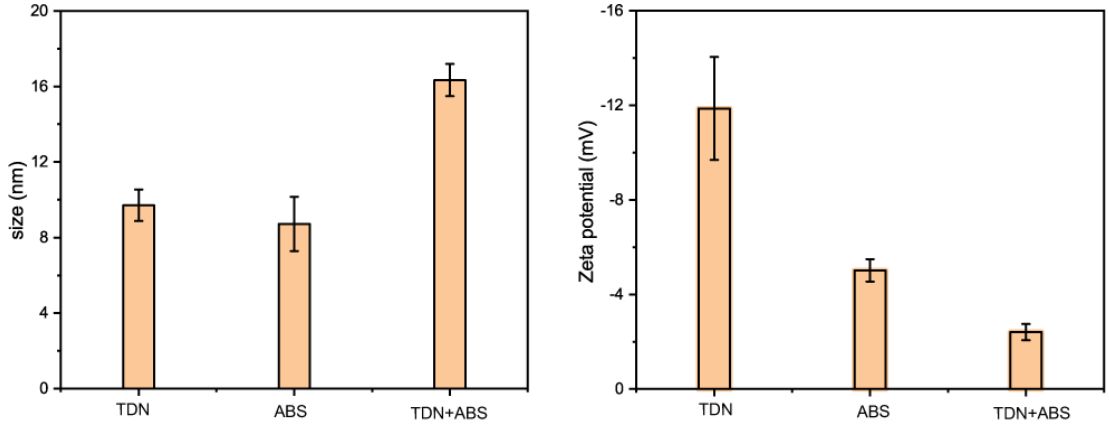

Figure S5. Size and Zeta potential of TDN, ABS, and TDN-ABS complex.

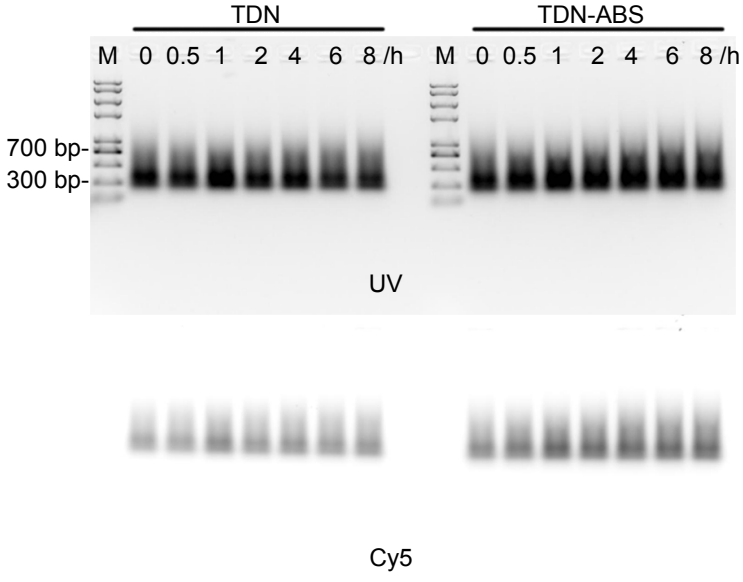

Figure S6. Stability analysis in FBS

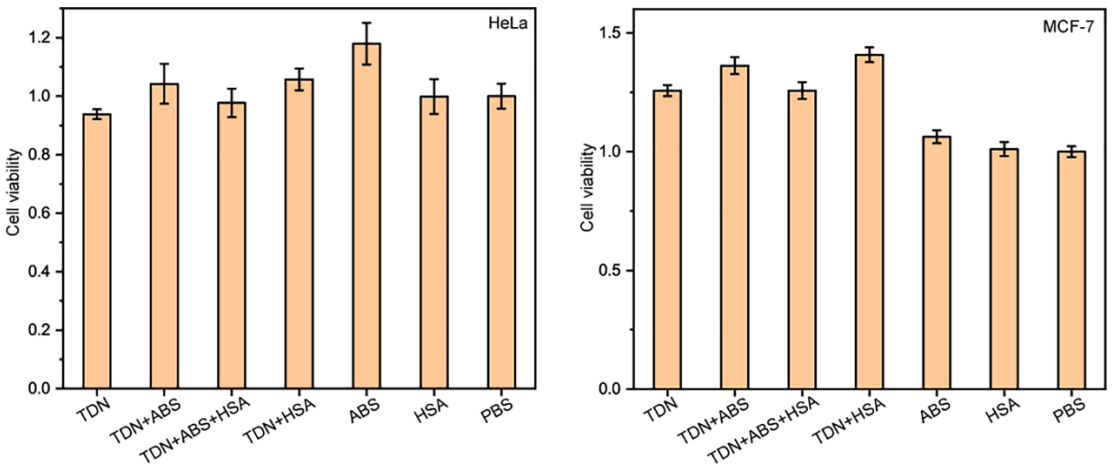

Figure S7. Cell viability of HeLa and MCF-7 cell with various treatments.

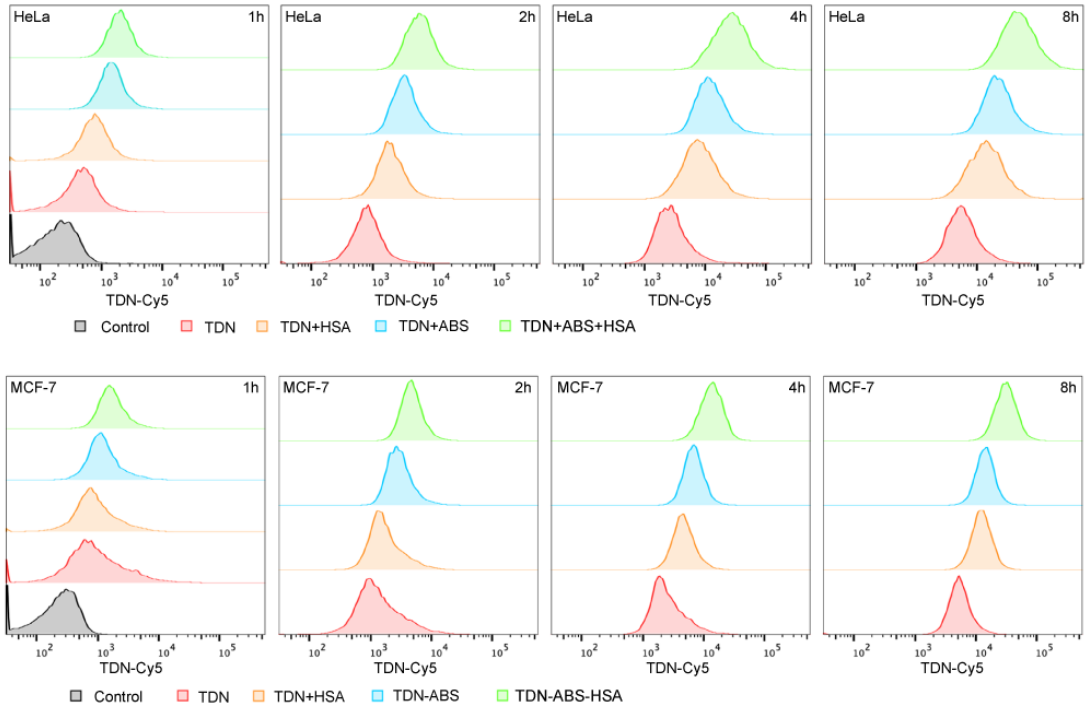

Figure S8. Cellular uptake of TDN, TDN-HSA, TDN-ABS, and TDN-ABS-HSA in HeLa and MCF-7 cells. The Cy5 fluoresce was recorded by flow cytometry at different time point (1h, 2h, 4h, and 8h).

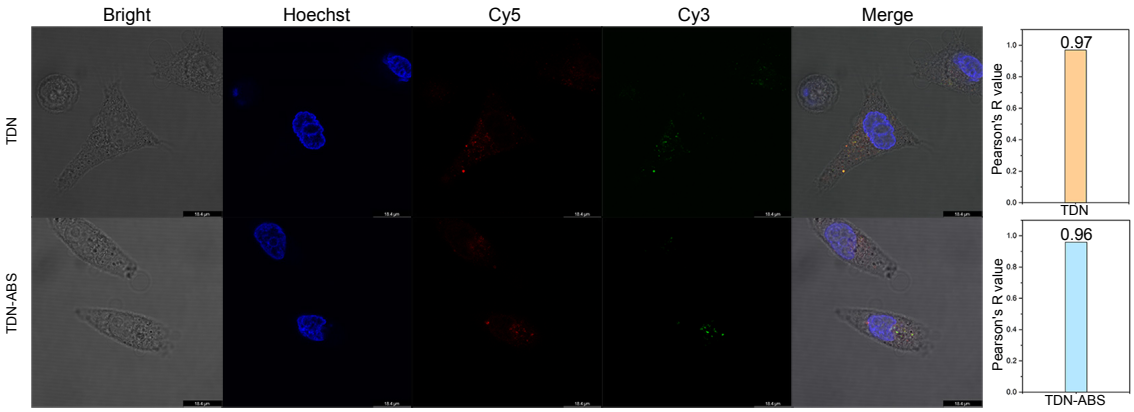

Figure S9. TDN cellular colocalization in MCF-7 cells.

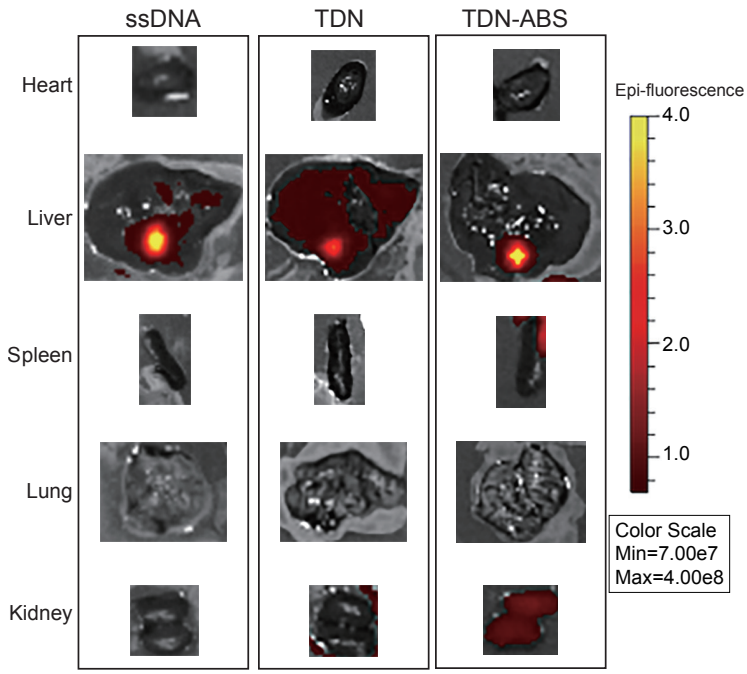

Figure S10. Organ distribution of various samples after 4 hours of i.v. injection in mice.