SUPPLEMENTARY DATA

A photochemically covalent lock stabilizes aptamer conformation and strengthens its performance for biomedicine

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F.Zhou, P.Wang, and J.Chen contributed equally to this work. Z.Zhu, Y.Li, S.Wu, S.Wang, Y.Sima, and T.Fu discussed and analyzed data. W.Tan gave his constructive advice. Z.Zhao conceived the idea, supervised experiments, analyzed data and wrote the manuscript.

Reagents and materials

8-methoxypsoralen was purchased from Sigma-Aldrich. HPLC-purified DNA sequences used in this study (Supplementary Table S1) were provided by Sangon Biotech (Shanghai, China). 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris, 99.9%) was purchased from Beyotime Co. (Jiangsu, China). Magnesium chloride hexahydrate ($MgCl_2$•$6H_2O$, AR), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, AR, 98%), and boric acid (H_3BO_3, AR, 99.5%) were purchased from Aladdin Industrial Co. (Shanghai, China). N,N,N',N'-tetramethylethylenediamine (TEMED), 30% acrylamide, ammonium persulfate (APS, 98%), and agarose were purchased from Beijing Dingguo Changsheng Biotechnology Co.. Exonuclease I (Exo I) with 10× Exo I buffer (670 mM Glycine-KOH, pH 9.5, containing 10 mM DTT and 67 mM $MgCl_2$) were purchased from Takara Biotechnology Co. (Dalian, China). Single-stranded DNA binding protein (SSB) was purchased from Promega. All reagents were received and used without further purification. Nanosep® Centrifugal tubes with OmegaTM membrane were purchased from Pall (China) Co., Ltd.

Instruments

All solutions used in the experiments were prepared with ultrapure water (resistance > 18 MΩ cm) generated through a Millipore Milli-Q ultrapure water system (Billerica, MA, USA). The amount of DNA was determined by measuring the maximum absorbance with a UV-2460 UV-vis spectrometer (Shimadzu, Japan). All fluorescence measurements were performed on a Fluoromax-4 spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, NJ).

Cell lines and cell culture

CCRF-CEM, K562, HCT116, HEK293, and RAW264.7 cell lines were purchased from ATCC. The CCRF-CEM cells, K562 cells, HCT116 cells were cultured in RPMI-1640 cell medium supplemented by 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (PS). HEK293 cells and RAW264.7 cells were grown in DMEM cell medium supplemented with 10% FBS and 1% PS. The cells were grown at 37 °C in atmosphere with 5% CO2 and 95% humidity. Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS, Gibco) was used to wash cells.

Buffers

Washing buffer was prepared by adding 2.25 g and 2.5 mL $MgCl_2$ (1 M) into 500 mL DPBS. Binding buffer was prepared by adding 50 mg yeast tRNA and 500 mg BSA into 500 mL washing buffer.

Molecular dynamic simulation of the aptamer-protein interaction

The PTK7 structure IG3-4 structure was predicted by using AlphaFold (https://www.uniprot.org/uniprot/Q13308). The DNA secondary structure with the minimum free energy of the aptamer was predicted by mfold web server (http://mfold.rna.albany.edu/?=mfold). The predicted 2D structure was used as a starting point to model and visualize the 3D structure of aptamer. U bases were replaced with T bases, and the hydroxyl group on the ribose was removed to form the deoxynucleotide structure. To simulate aptamer-protein complexes, the AMBER FF99SB force field was utilized to simulate the protein structure. Firstly, these complexes were restrained by a harmonic potential of the form $k(\Delta x)^2$ where the force constant k was 200 $kcal/mol^{-1}Å^{-2}$. Then, the water molecules and counter ions were optimized by using the steepest descent and conjugate gradient for 5000 steps separately. After that, the entire systems without any constraint were optimized by using the first step method, following by an annealing simulation process under a weak restraint (k=100 $kcal/mol^{-1}Å^{-2}$). The complex systems were heated from 0 to 298 K gradually over 500 ps in the NVT ensemble (i.e., N, V and T represents the number of particles, the volume and the temperature of the system, respectively, and their product was a constant). After this heating phase,

a 10 ns MD simulation was performed under 1 atm with a constant temperature at 298 K and a constant pressure maintained by isotropic position scaling algorithm with a relaxation time of 2 ps. Based on 3000 snapshots extracted from the last 8 ns trajectory, the final average structure was obtained.

Production and purification of PTK7 protein constructs

The information of PTK7 was obtained from the website (https://www.uniprot.org/uniprot/Q13308). The gene of PTK7 protein constructs spanning residues 219-410 (PTK7 Ig3-4) were produced by PCR amplification using the full-length cDNA coding for human PTK7. Then the gene of PTK7 Ig3-4 was cloned into a modified pFastbac expression vector encoding a signal peptide and a N-terminal hexa-histidine tag. PTK7 Ig3-4 constructs were produced by baculovirus insect expression systems as previously described[S1]. Expression media containing the PTK7 Ig3-4 constructs was harvested at 3 days post-transfection. The PTK7 Ig3-4 constructs were purified from the media by the batch binding method to Ni Sepharose excel (GE Healthcare Life Sciences). This resin was subsequently washed with buffer containing 500 mM NaCl and 25 mM Tris-HCl (pH 8.0). Non-specifically bound proteins were eluted with 25 mM imidazole in the same buffer. The purified proteins were subsequently eluted using an imidazole gradient up to 500 mM imidazole and analyzed by SDS-PAGE. Fractions containing the relevant protein were pooled and further purified by size-exclusion chromatography (Superdex 75, GE Healthcare Life Sciences) in 200 mM NaCl and 25 mM Tris-HCl (pH 8.0), anion-exchange (Mono Q™ 5/50 GL, GE Healthcare Life Sciences), and size-exclusion chromatography (Superdex 75, GE Healthcare Life Sciences).

Stability analysis of aptamers in exonuclease solution and serum

Fluorescein amidate (FAM)-labeled aptamer (1 μM) was incubated with Exo I (0.5 U/μL) or RPMI 1640 with 50% FBS at 37 °C in a volume of 100 μL. At designated time points, samples were heated at 95 °C for 5 min to denature Exo I or FBS, and subsequently stored at -20 °C until all samples were collected. Samples were then thawed on ice for polyacrylamide gel electrophoresis or flow cytometry.

Gel electrophoresis assay

Polyacrylamide gel electrophoresis (PAGE) was utilized to analyze their integrity after aptamers were treated with Exo I (0.5 U/μL) or 50% FBS. DNA samples (10 μL) were mixed with 2 μL 6 × loading buffer and then loaded into 15% polyacrylamide gel in electrophoresis buffer (9 mM Tris, pH 8.0, containing 9 mM boric acid and 1 mM EDTA). After electrophoresis, the gels were analyzed with a molecular imager (BIO-RAD).

Western blot analysis

CCRF-CEM, K562, HCT116 and HEK293 cells were placed in a 25 cm^2 flask and cultured for 24 h, respectively. The medium was removed, and the cells were washed twice with cold DPBS (1 mL each). After that, the cells were treated with lysate (Beyotime Co. Jiangsu, China) containing protease inhibitors (no. B14001, Biotool) and lysed for 30 min on ice. The lysates were gathered and centrifuged for 15 min to collect the supernatant (10000 × g). The protein concentration of supernatant was determined by a NanoDrop 2000/2000c (Thermo). All proteins were separated by SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was divided into two parts and then treated with PTK7 antibodies and tubulin antibodies (Cell Signaling Technology, dilution 1:1000) for 12 h at 4 °C, respectively, followed by incubating with a secondary antibody for 2 h at room temperature. Finally, the PTK7 was visualized on ChemiDoc XRS^+ with Image Lab software (Bio-RAD).

Confocal laser scanning microscopy imaging

For the selectivity assay of various aptamers, 3 x 10^5 CCRF-CEM or K562 cells were incubated with FAM-labeled Lib, Sgc8, Sgc8m or PCCL-Sgc8m (100 nM) for 1 h at 37 °C, respectively. Then cells were washed twice by DPBS and subjected to imaging by confocal microscopy. The data were analyzed with NIS-element Viewer 4.50.

For cellular internalization assay, 3 x 10^5 HCT116 adherent cells were also seeded in 35-mm confocal dish and incubated overnight for adherence. The cells were incubated with Cy5-labeled Lib, Sgc8, Sgc8m or PCCL-Sgc8m (100 nM) for 1 h at 37 °C, Then the cells were fixed with 4% formaldehyde and 10 μg/mL DAPI were added for nuclear staining. After incubation, cells were washed thrice with DPBS, and subjected to imaging by confocal microscopy. The data were analyzed with NIS-element Viewer 4.50.

Synthesis of CA4-PEG3-azide, Sgc8m-CA4 and PCCL-Sgc8m-CA4

CA4-PEG_3-azide was synthesized according to our previous report[S3]. Combretastatin A4 (CA4, 0.1 mmol), azide-PEG3-COOH (0.2 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl, 0.2 mmol), and 4-dimethylaminopyridine (DMAP, 0.2 mmol) were dissolved in 5 mL dichloromethane, and allowed to stir overnight under N_2 at room temperature. The mixture was then concentrated under vacuum, and CA4-PEG_3-azide was purified by flash chromatography.

To synthesize Sgc8m-CA4 conjugations, the mixture of DBCO-modified Sgc8m (1 OD) and CA4-PEG_3-azide (1.5-fold equivalent) was added to 200 μL DPBS and then shaken at 300 x g for 8 hours at 37 °C. Then Sgc8m-CA4 conjugations were directly purified by C-18 column by reverse phase high-performance liquid chromatography (HPLC, Agilent 1260 Infinity) equipped with UV and fluorescence detection systems. Sgc8m-CA4 conjugations was purified by the following program: 5% acetonitrile (ACN) and 95% 0.1 M TEAA at 1.0 mL/min over 4 min, and then 10 to 65% ACN at 1.0 mL/min over 30 min, followed by 95% ACN at 1.0 mL/min over 5 min.

To prepare PCCL-Sgc8m-CA4 conjugations, Sgc8m-CA4 (2 nmol) was dissolved in 180 μL DPBS containing 5 mM $MgCl_2$, and mixed with 20-fold equivalent 8-methoxypsoralen dissolved in 20 μL DMSO. The mixture was exposed to 365 nm UV-A illumination at 3.9 mW/cm^2 for 15 min on ice. The DNA sample was then precipitated by adding 20 μL NaCl (3 M) and 500 μL cold ethanol, followed by standing at -20 °C overnight. After centrifugation for 30 min (4 °C, 12000 rpm), the white pellet was dissolved in 0.1 M TEAA, and further purified on a HPLC. The gradient elution procedure for PCCL-Sgc8m-CA4 purification was the same as that for Sgc8m-CA4 conjugations. The PCCL-Sgc8m-CA4 conjugation was evidenced by ESI-MS, which was performed Sangon Biotech (Shanghai, China).

Cell apoptosis analysis by flow cytometry

Cell apoptosis analysis was performed according to the manufacturer's protocol (Annexin V-FITC kit, Beyotime). Briefly, 1 x 10^5 HCT116 cells were seeded in a 12-well culture plate and incubated over for adherence. After removing the culture medium, cells were separately incubated with 200 nM of CA4, CA4-PEG_3-azide, Sgc8m-CA4 and PCCL-Sgc8m-CA4 for 6 h. After two washes with DPBS, the cells were allowed to grow for another 42 h. The cells were detached with trypsin, centrifuged at 300 g for 5 min, washed twice with ice-cold DPBS, and re-suspended in 200 μL binding buffer. Thereafter, 5 μL of Annexin V-FITC and 10 μL of PI were added, and the mixtures were incubated for 15 min in the dark. The stained cells were analyzed by a BD FACSVerse™ system, and the data were analyzed with FlowJo_V10.

Cytotoxicity assay

HCT116 and HEK293 cells were seeded in a 96-well plate (5000 cells/well) and then incubated overnight for adherence. After removing the culture medium, cells were incubated with 100 μL RPMI-1640 medium containing

different concentration of free CA4, CA4-PEG_3-azide, Sgc8m-CA4 or PCCL-Sgc8m-CA4 for 6 h and then washed with DPBS, followed by incubation until to 72 h. After washing with DPBS, cells were incubated with 100 μL fresh complete cell medium containing 10% CCK-8. After incubation for about 1 h, cell viability was determined by measuring absorbance at 450 nm using a Synergy 2 Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT).

In vivo and ex vivo fluorescence imaging of tumor

Four- to six-week-old BALB/c nude mice were received a subcutaneous injection of 1 x 10^7 HCT116 cells at the right axilla. Tumors were then allowed to grow to 300-400 mm^3 (volume = 0.5 × length × $width^2$). Tumor-bearing BALB/c nude mice were anesthetized to be motionless with both tranquilizer and anesthetic before 4 nmol of Cy5-labeled Sgc8, Sgc8m, PCCL-Sgc8m or library was injected via the tail vein (n =3). At the designated time points, whole-body in vivo fluorescence images of these mice were recorded with by an in vivo imaging system (IVIS) Lumina II (Caliper LifeSicence, USA). After 6 h, the mice were sacrificed, tumors and main organs were collected. The ex vivo fluorescence of tumors and main organs were examined with an IVIS Lumina II.

For analyzing the penetration depth of various aptamers in tumors, the tumor from mice treated with Cy5-labeled Sgc8, Sgc8m or PCCL-Sgc8m were collected after ex vivo imaging. The tumor tissues were transferred to molds, embedded in OCT (optimal cutting temperature compound), and stored at -80°C until sectioning. The tumor tissues were consecutively cut into 10-μm-thick sections in z-axis with a cryostat. After staining with DAPI (10 μg/mL) for 10 min, these slices were mounted and scanned by the digital slice scanning system (Pannoramic MIDI, 3DHISTECH Ltd., Hungary).

For analyzing the red fluorescence signals from Cy5-labeled aptamers around tumor blood vessels, the preparation of cryosections was performed according to our previous work[S2]. Briefly, the cryosections of tumor tissue were dried at room temperature for 10 min and then washing by DPBS for 5 min × 3 times to eliminate OCT. Next, the sections were blocked in 5 % BSA for 2 h, followed by incubation with anti-CD31 rabbit polyclonal antibody (1:250, Servicebio Co.) at 4 °C for overnight. After washing with DPBS, the sections were incubated with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:100, Servicebio Co) and then washed with DPBS. Finally, the sections were stained by DAPI (Servicebio Co) for 10 min and subjected to imaging using the digital slice scanning system (Pannoramic MIDI, 3DHISTECH Ltd., Hungary).

Antitumor analysis in vivo

When the tumor volume of the BALB/c nude mice reached around 100 mm^3 (volume = 0.5 × length × $width^2$), PBS, CA4, Sgc8m-CA4, and PCCL-Sgc8m-CA4 (the equivalent CA4 dose of 4 mg/kg) were i.v. injected to HCT116 tumor-bearing mice once every 2 days for six times in total. CA4 was dissolved by PBS containing 5% DMSO and 20% PEG, and other materials were dissolved by PBS only. Tumor size and body weight were measured continuously. At day 22, the mice were sacrificed, the heart, liver, spleen, lung, kidney and tumor were taken out for preparing paraffin tissue sections. After antigen retrieval, these organ and tumor tissue slices were further stained with hematoxylin and eosin (H&E) staining.

TUNEL analysis of tissue slices were performed with the TUNEL kit (Servicebio Co, Lot: G1501). Briefly, the retrieved tumor tissues were incubated with TDT enzyme, FITC-labeled dUTP and equilibration buffer at 1:5:50 ratio in a flat box for 2 h at 37 °C. To avoid the volatilization of samples, the tissue slices were kept in a moist environment. After washing thrice with DPBS, these slices were then incubated with 10 μg/mL DAPI solution at room temperature and kept in dark for 10 min. After staining, these tissue slices were washed with DPBS, and sealed with glycerin.

These slices with H&E staining or TUNEL staining were imaged with a digital slice scanning system (Pannoramic MIDI, 3DHISTECH Ltd., Hungary). For statistical analysis, Graphpad Prism 8.0 (https://www.graphpad.com) was used to show differences between two groups (Student's t test) or among three or more groups (One-way ANOVA or Two-way ANOVA).

Supplementary tables

Table S1. DNA sequences used in this study.

Name	Detailed sequence information (5'→3')	Destination
Sgc8	ATCTAA CTG CTGCGCCGCCGGGAAAATACTGTA CGG TTAGA	Binding
FAM-Sgc8	ATCTAA CTG CTGCGCCGCCGGGAAAAT(FAM)ACTGTA CGG TTAGA	CLSM analysis and flow cytometric analysis
Cy5-Sgc8	ATCTAA CTG CTGCGCCGCCGGGAAAAT(Cy5)ACTGTA CGG TTAGA	CLSM analysis, flow cytometric and *in vivo* fluorescence imaging analysis
Sgc8m	ATATATATCTGC TGCGCCGCCGGGAAAATACTGTA CGGATATATAT	Binding, Crosslinking, Stability & Melting temperature analysis
FAM-Sgc8m	ATATATATCTG CTGCGCCGCCGGGAAAAT(FAM)ACTGTA CGG ATATATAT	CLSM analysis and flow cytometric analysis
Cy5-Sgc8m	ATATATATCTG CTGCGCCGCCGGGAAAAT(Cy5)ACTGTA CGG ATATATAT	CLSM analysis, flow cytometric and *in vivo* fluorescence imaging analysis
DBCO-Sgc8m	ATATATATCTG CTGCGCCGCCGGGAAAAT(DBCO)ACTGTA CGG ATATATAT	Drug linking
Sgc8m1	TTTTTTTTCTG CTGCGCCGCCGGGAAAATACTGTA CGG AAAAAAAA	Crosslinking control
Sgc8m2	AAAAAAAACTG CTGCGCCGCCGGGAAAATACTGTA CGG TTTTTTTT	Crosslinking control
library (Lib)	ATCTAACTGATTATTATTATTATTATTATTATTCGGTTAGA	Binding control
FAM-Lib	ATCTAACTGATTATTATTATTATTATTAT(FAM)TATTCGGTTAGA	CLSM analysis and flow cytometric analysis
Cy5-Lib	ATCTAACTGATTATTATTATTATTATTAT(Cy5)TATTCGGTTAGA	CLSM analysis, flow cytometric and *in vivo* fluorescence imaging analysis
Sgc8m with inverted T	AT*ATATATCTG CTGCGCCGCCGGGAAAATACTGTA CGG ATATATA T*	Stability analysis
Sgc8m-4mA	AT*A*TATAT CTGCTGCGCCGCCGGGAAAATACTGTACGG ATATATA*T*	Stability, Crosslink & Melting temperature analysis
Sgc8m-4PS	AT*A* TATAT CTGCTGCGCCGCCGGGAAAATACTGTACGG ATAT AT*A* T	Stability, Crosslink & Melting temperature analysis
Sgc8m-5FU	AT*AT*AT*AT*CTGC TGCGCCGCCGGGAAAATACTGTA CGGAT*AT*AT*AT*	Crosslink & Melting temperature analysis
SL1	A TCA GGC TGG ATG GTA GCT CGG TCG GGG TGG GTG GGT TGG CAA GTC TGA T	Crosslink & Melting temperature analysis
SL1m	ATATATAT GGC TGG ATG GTA GCT CGG TCG GGG TGG GTG GGT TGG CAA GTC ATATATAT	Crosslink & Melting temperature analysis
TCO1	ACC AAA CAC AGA TGC AAC CTG ACT TCT AAC GTC ATT TGG TG	Binding & Stability analysis
TCO1m	TATATATA AACACAGATGCAACCTGACTTCTAACGTCATT TATAT ATA	Binding, Crosslinking, Stability & Melting temperature analysis
HG1-9	GGATAGGGATTCTGTTGGTCGGCTGGTTGGTATCC	Binding & Stability analysis
HG1m	TATATATA GGGATTCTGTTGGTCGGCTGGTTGG TATATATA	Binding, Crosslinking, Stability & Melting temperature analysis

[N*] meant that the nucleotide was inverted T, fluorouracil, or modified with phosphorothioate or 2'-O-methylation.

Table S2. Microscale thermophoresis analysis of the thermodynamic parameters of the interaction of Sgc8, Sgc8m and PCCL-Sgc8m to PTK7 Ig3-4 in DPBS containing 5 mM $MgCl_2$ or 1 mM $MgCl_2$.

	Sgc8		**Sgc8m**		**PCCL-Sgc8m**	
	DPBS		DPBS		DPBS	
	5 mM Mg^{2+}	1mM Mg^{2+}	5 mM Mg^{2+}	1 mM Mg^{2+}	5 mM Mg^{2+}	1 mM Mg^{2+}
K_d(25°)	907	1010	1920	2350	945	1040
K_d(37°)	2040	2860	5550	5910	3220	4150
ΔH°(cal/mol)	-14573.3	-19095.8	-18430.9	-19376.3	-21810.5	-22983.8
ΔS°(cal/mol)	-21.2813	-36.6411	-35.6434	-38.9724	-45.7393	-49.9752
$\Delta G^\circ_{25^\circ}$ (cal/mol)	-8228.32	-8171.3	-7803.84	-7756.65	-8173.33	-8083.7
$\Delta G^\circ_{37^\circ}$ (cal/mol)	-7972.9	-7731.56	-7376.1	-7289.01	-7624.46	-7483.99

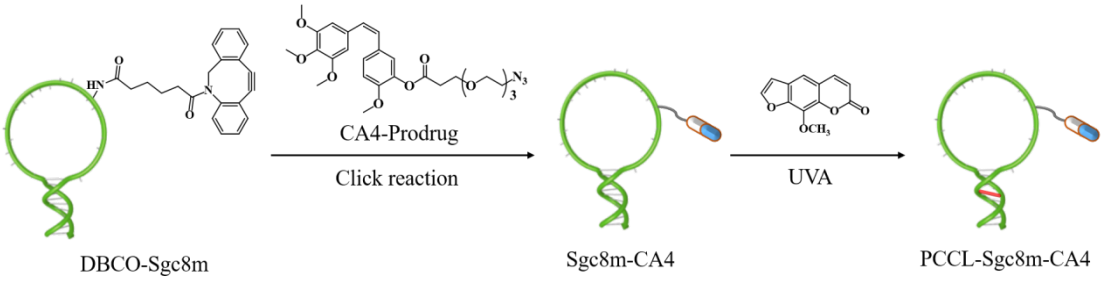
Table S3. Surface plasmon resonance (SPR) analysis of the association rate constant (k_{on}), the dissociation rate constant (k_{off}) and the association constant (K_d) of Sgc8, Sgc8m and PCCL-Sgc8m with PTK7 Ig3-4.

Analyte	k_{on} (1/Ms)	k_{off} (1/s)	K_d (M)
Sgc8	7.31e+5	2.15e-1	2.95e-7
Sgc8m	1.21e+5	1.73e-1	1.43e-6
PCCL-Sgc8m	1.57e+6	6.17e-1	3.94e-7

Table S4. The residues of aptamers and PTK7 Ig3-4 obtained from molecular dynamics simulations.

The binding interface of PCCL-Sgc8m-PTK7 Ig 3-4		The binding interface of Sgc8m-PTK7 Ig 3-4	
Aptamer residues	PTK7 Ig 3-4 residues	Aptamer residues	PTK7 Ig 3-4 residues
G-11	ASP-324	C-12	ARG-400
C-18	ARG-362	G-16	GLN-399
C-18	SER-353	C-17	ARG-362
A-27	THR-279	C-18	ALA-394
T-29	ARG-276	C-18	ARG-362
A-30	ARG-276	A-27	THR-279
C-31	ARG-292	T-29	ARG-276
C-31	ARG-294	C-31	ARG-294
C-31	ARG-277	T-32	ARG-294
T-32	ARG-292	G-33	TYR-239
T-32	TYR-239		
G-33	TYR-239		

Supplementary figures



Scheme S1. Illustrating of preparation of PCCL-aptamer-CA4 conjugation.

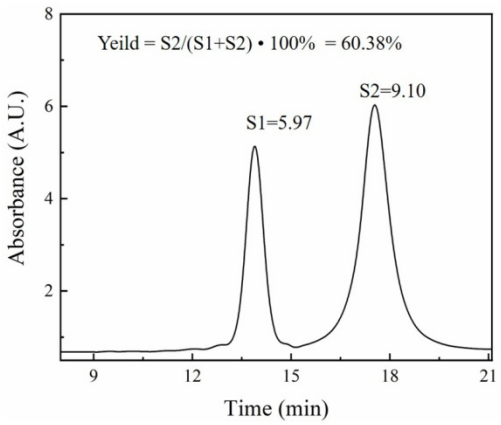


Figure S1. HPLC Purification profile of the product of photochemically covalent locked aptamer PCCL-Sgc8m.

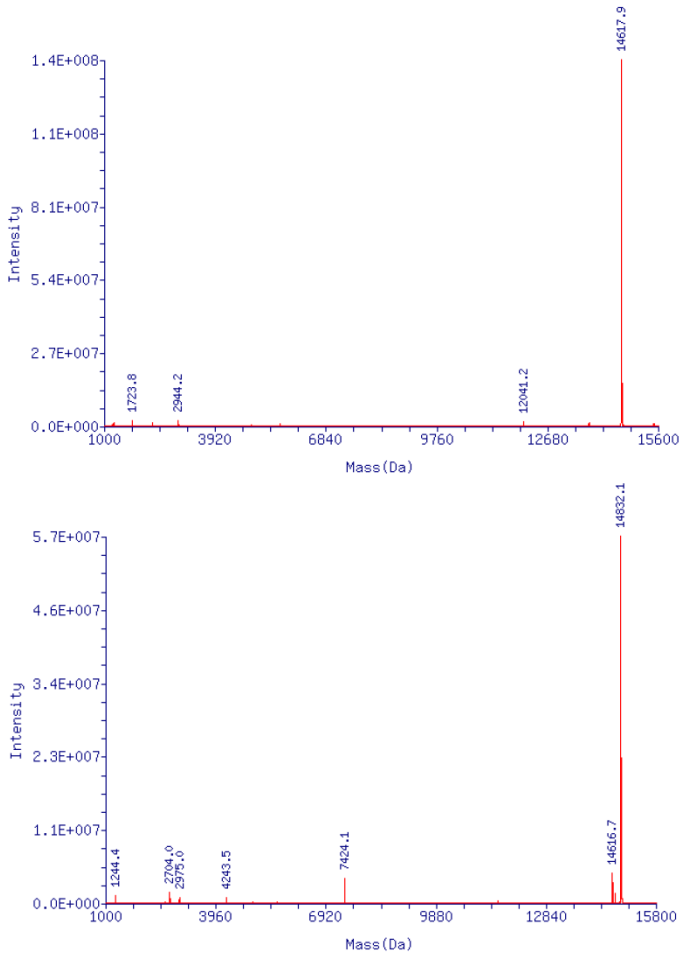


Figure S2. ESI-MS analysis of Sgc8m (top) and PCCL-Sgc8m (bottom). Calculated molecular weight of Sgc8m:14619.1, Found: 14617.9; Calculated molecular weight of PCCL-Sgc8m: 14835.3, Found: 14832.1.

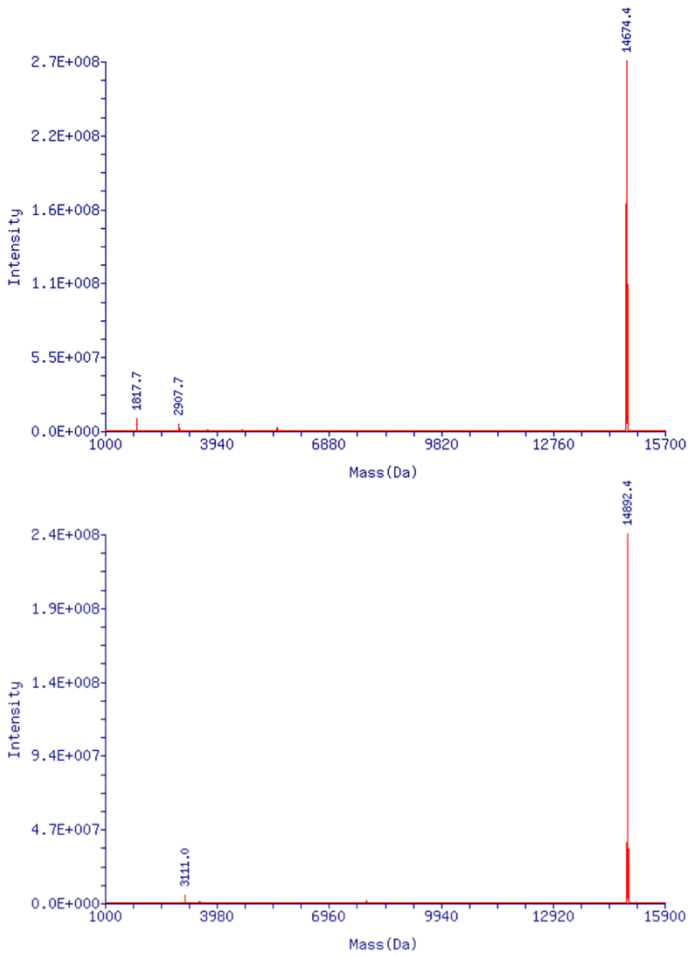


Figure S3. ESI-MS analysis of TCO1m (top) and PCCL-TCO1m (bottom). Calculated molecular weight of TCO1m:14675.6, Found: 14674.4; Calculated molecular weight of PCCL-TCO1m: 14891.8, Found: 14892.4.

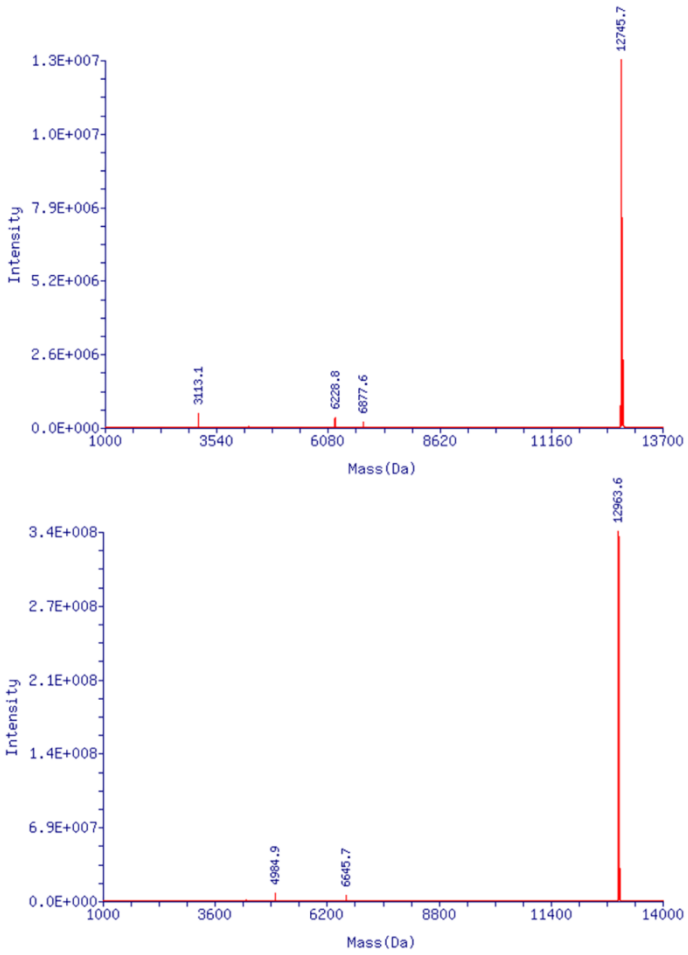


Figure S4. ESI-MS analysis of HG1m (top) and PCCL-HG1m (bottom). Calculated molecular weight of HG1m:12746.3, Found: 12745.7; Calculated molecular weight of PCCL-HG1m: 12962.5, Found: 12963.6.

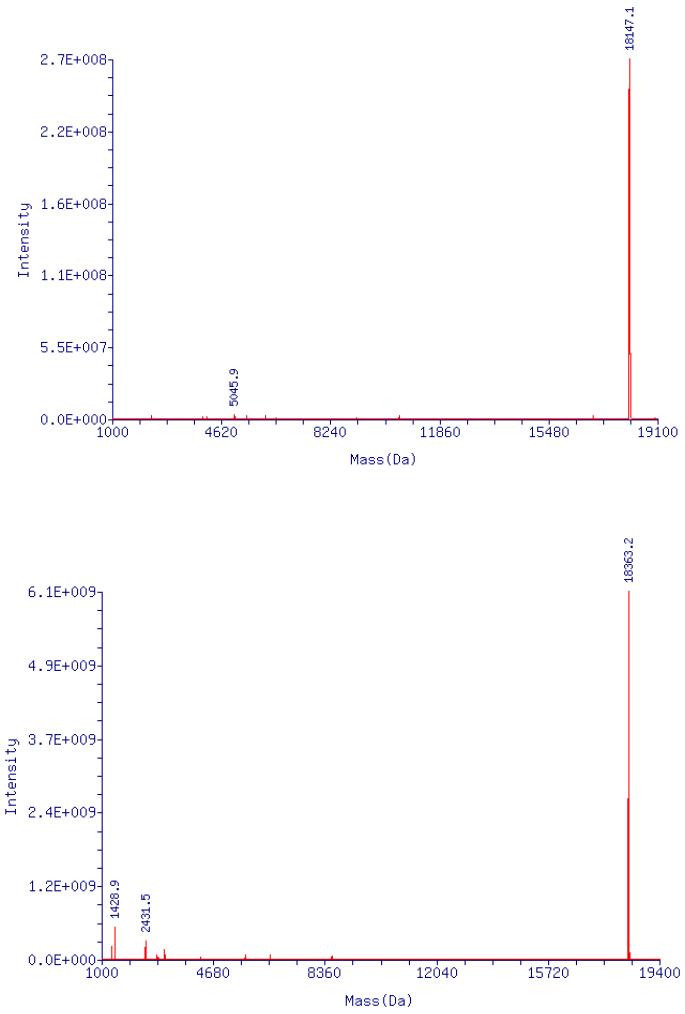


Figure S5. ESI-MS analysis of SL1m (top) and PCCL-SL1m (bottom). Calculated molecular weight of SL1m: 18149.75, Found: 18147.1; Calculated molecular weight of PCCL-SL1m: 18365.94, Found: 18363.2.

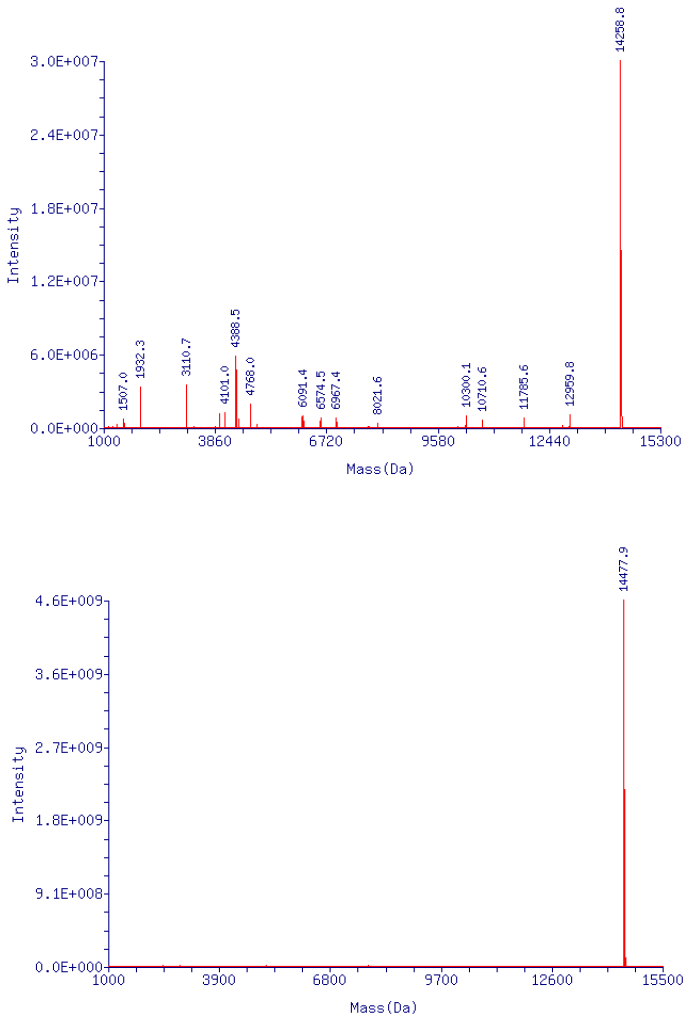


Figure S6. ESI-MS analysis of Sgc8m-4mA (top) and PCCL-Sgc8m-4mA (bottom). Calculated molecular weight of Sgc8m-4mA: 14264.3, Found: 14258.8; Calculated molecular weight of PCCL-Sgc8m-4mA: 14480.49, Found:14477.9.

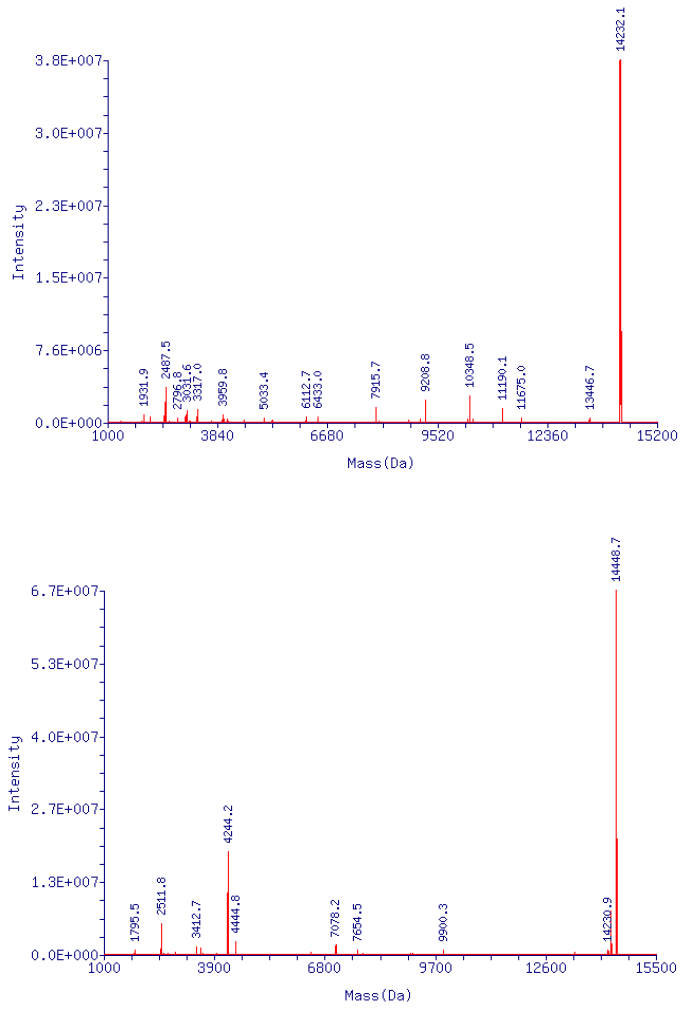


Figure S7. ESI-MS analysis of Sgc8m-4PS (top) and PCCL-Sgc8m-4PS (bottom). Calculated molecular weight of Sgc8m-4PS: 14236.49, Found: 14232.1; Calculated molecular weight of PCCL-Sgc8m-4PS: 14452.68, Found:14448.7.

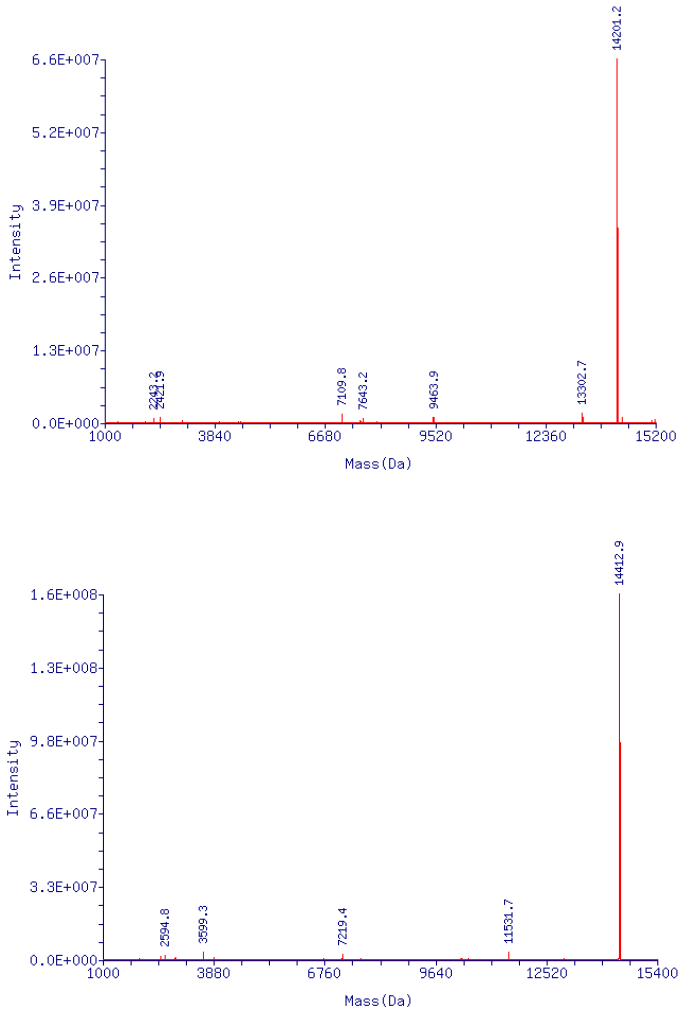
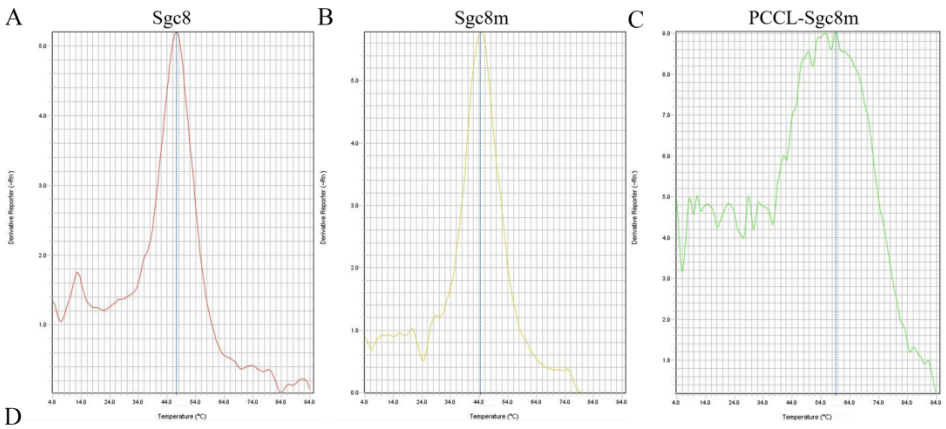


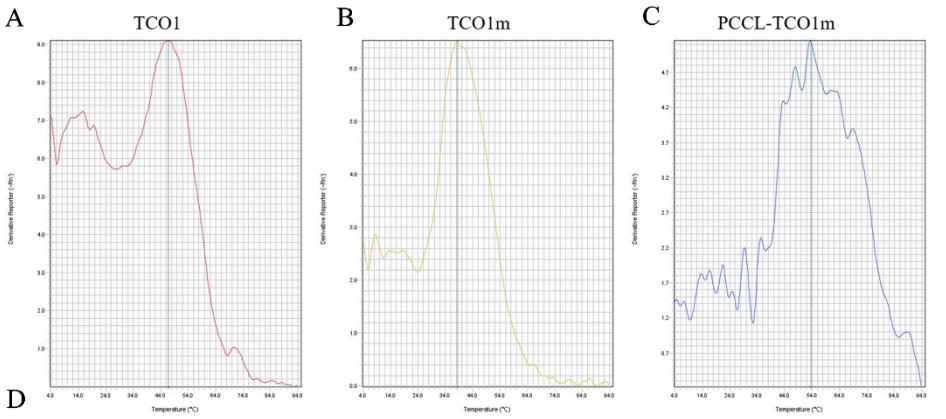
Figure S8. ESI-MS analysis of Sgc8m-5FU (top) and PCCL-Sgc8m-5FU (bottom). Calculated molecular weight of Sgc8m-5FU: 14203.48, Found: 14201.2; Calculated molecular weight of PCCL-Sgc8m-5FU: 14419.67, Found:14412.9.



D

	Sgc8	Sgc8m	PCCL-Sgc8m
Tm (°C)	48.17±0.85	44.64±0.3	59.93±0.86

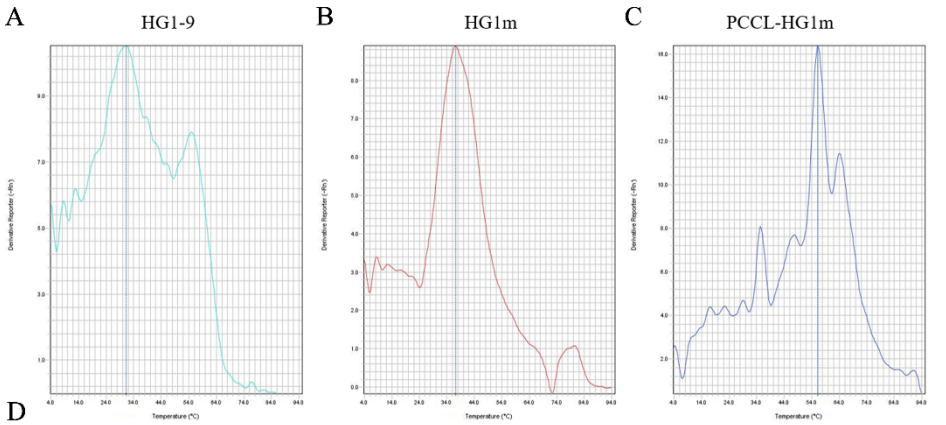
Figure S9. Melting temperature analysis of aptamer Sgc8 (A), Sgc8m (B), PCCL-Sgc8m (C), and the melting temperature values of aptamers (D).



D

	TCO1	TCO1m	PCCL-TCO1m
Tm (°C)	46.89 ± 0.81	38.34 ± 0.26	53.53 ± 0.57

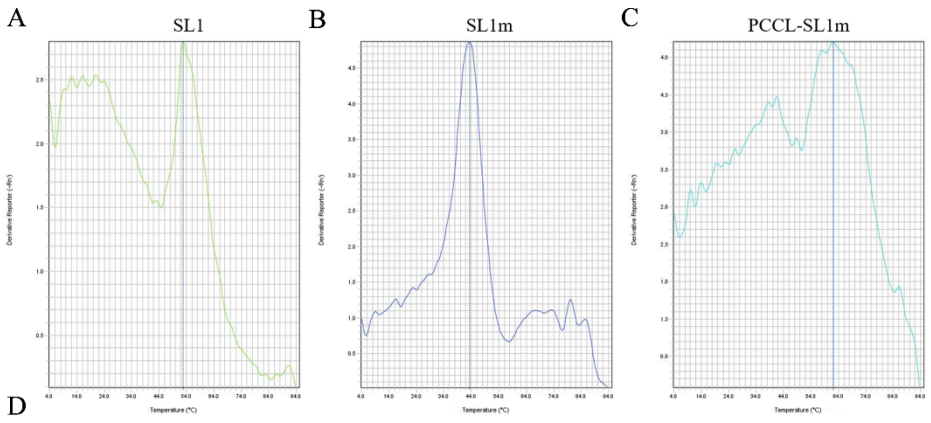
Figure S10. Melting temperature analysis of aptamer TCO1 (A), TCO1m (B), PCCL-TCO1m (C), and the melting temperature values of aptamers (D).



D

	HG1-9	HG1m	PCCL-HG1m
Tm (°C)	31.3 ± 0.93	37.13 ± 0.13	56.28 ± 0.35

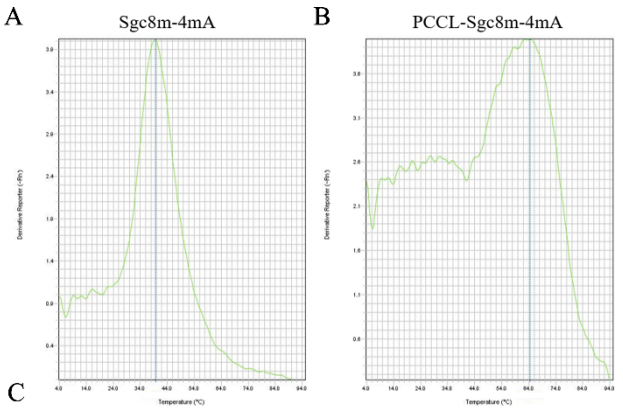
Figure S11. Melting temperature analysis of aptamer HG1-9 (A), HG1m (B), PCCL-HG1m (C), and the melting temperature values of aptamers (D).



D

	SL1	SL1m	PCCL-SL1m
Tm (℃)	52.75±0.64	43.37±0.32	62.3±0.79

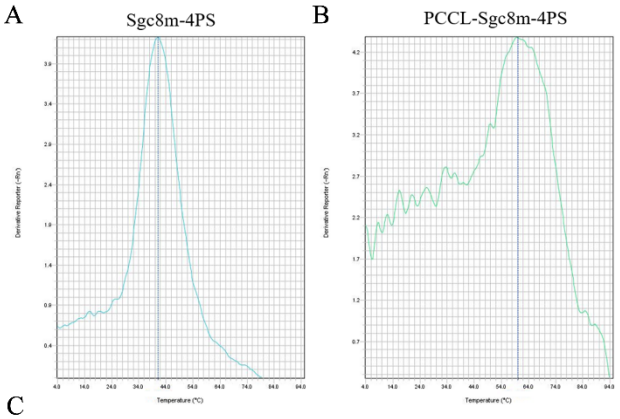
Figure S12. Melting temperature analysis of aptamer SL1 (A), SL1m (B), PCCL-SL1m (C), and the melting temperature values of aptamers (D).



C

	Sgc8m-4mA	PCCL-Sgc8m-4mA
Tm (℃)	39.59±0.37	64.43±0.65

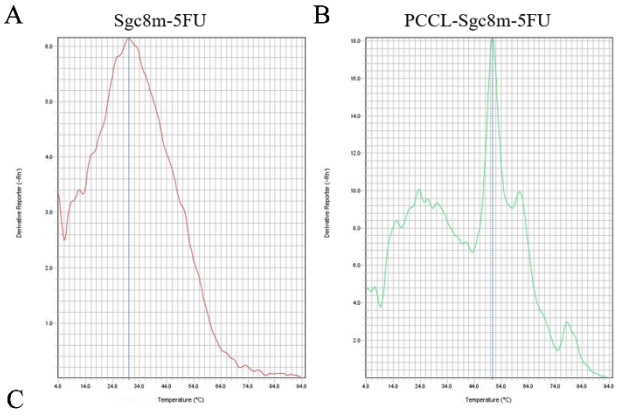
Figure S13. Melting temperature analysis of aptamer Sgc8m-4mA (A), PCCL- Sgc8m-4mA (B), and the melting temperature values of aptamers (C).



C

	Sgc8m-4ps	PCCL-Sgc8m-4ps
Tm (°C)	39.59±0.23	64.43±0.66

Figure S14. Melting temperature analysis of aptamer Sgc8m-4PS (A), PCCL- Sgc8m-4PS (B), and the melting temperature values of aptamers (C).



C

	Sgc8m-5FU	PCCL-Sgc8m-5FU
Tm (°C)	39.59±0.78	64.43±0.12

Figure S15. Melting temperature analysis of aptamer Sgc8m-5FU (A), PCCL- Sgc8m-5FU (B), and the melting temperature values of aptamers (C).

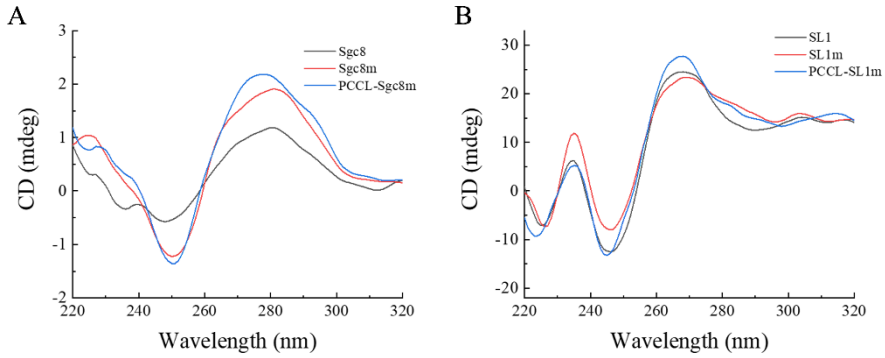


Figure S16. CD spectra of PCCL-Sgc8m in DPBS supplemented with 5 mM $MgCl_2$ (A) and PCCL-SL1m in 20 mM Tris-HCl (pH 7.6) buffer supplemented with in 5 mM KCl (B) at room temperature.

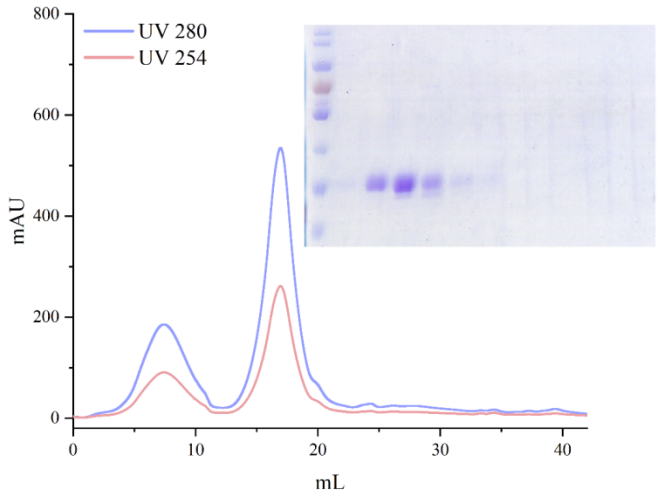


Figure S17. 10% SDS-PAGE analysis of PTK7 Ig3-4 after purification with anion-exchange chromatography.

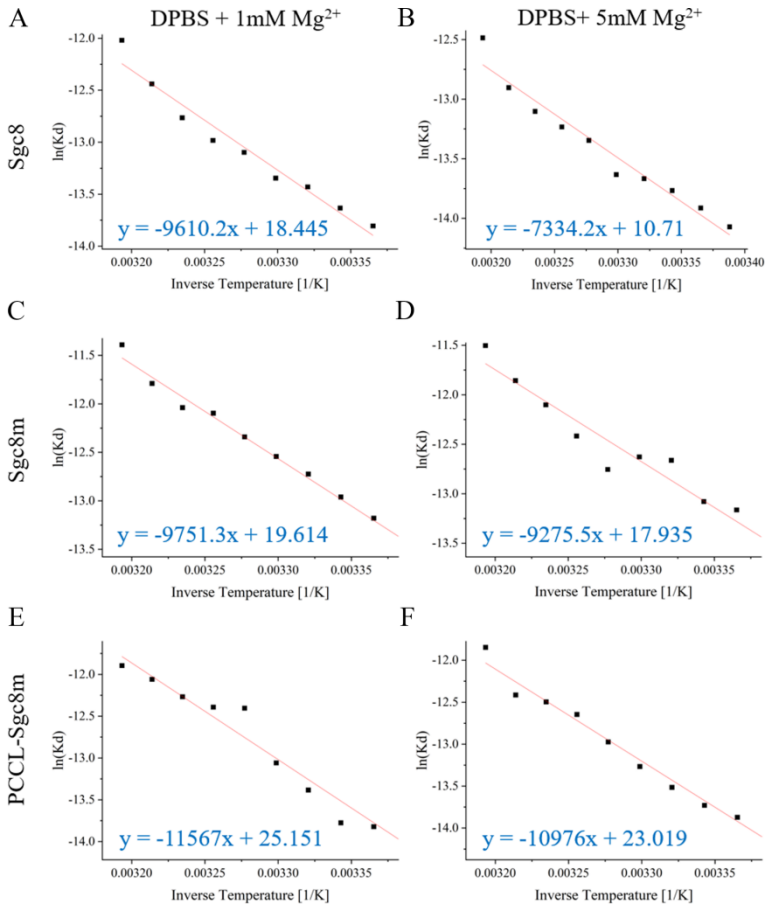


Figure S18. Van't Hoff plot of the interaction of Sgc8, Sgc8m and PCCL-Sgc8m to PTK7 Ig3-4 in DPBS supplemented with 1 mM $MgCl_2$ or 5 mM $MgCl_2$.

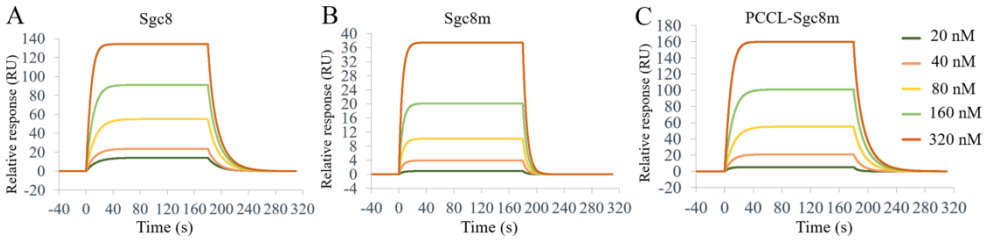


Figure S19. The SPR sensorgrams of the interaction of Sgc8 (A), Sgc8m (B) and PCCL-Sgc8m (C) with PTK7 Ig3-4 at concentrations of 20, 40, 80, 160, and 320 nM in DPBS.

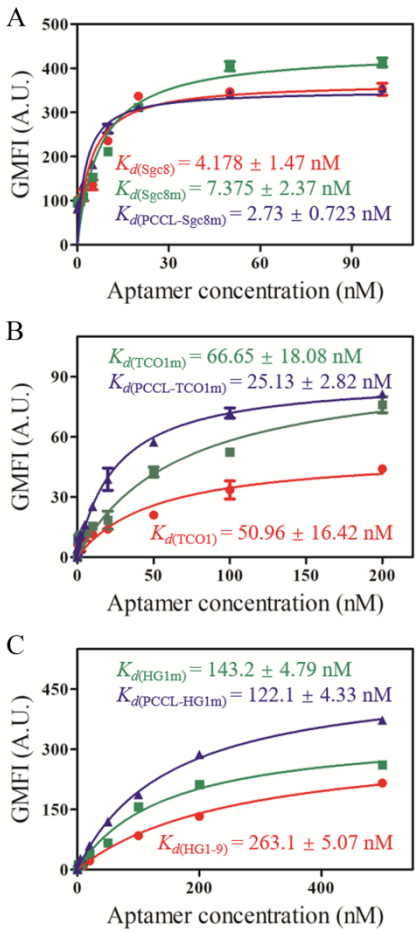


Figure S20. Flow cytometric analysis of the equilibrium dissociation constants of PCCL-Sgc8m (A), PCCL-TCO1m (B) and PCCL-HG1m (C), The incubation temperature was 37 °C. GMFI: geometric mean fluorescence intensity.

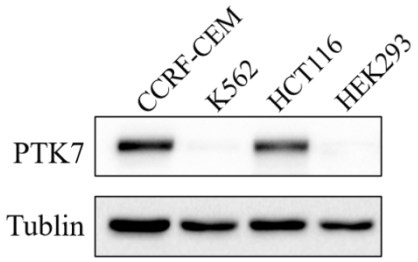


Figure S21. Western blot assay of PTK7 expression in CCRF-CEM cells, K562 cells, HCT116 cells and HEK293 cells.

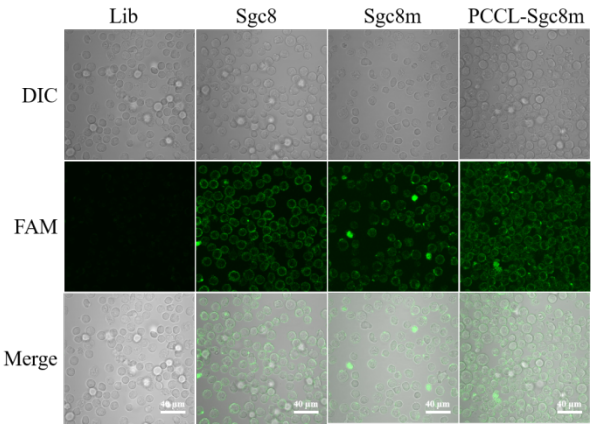


Figure S22. CLSM imaging analysis of the binding of FAM-labeled control DNA lib, unlocked aptamer Sgc8 and Sgc8m, and PCCL-Sgc8m on CCRF-CEM cells. The DNA concentration was 200 nM. The incubation temperature was 37 °C.

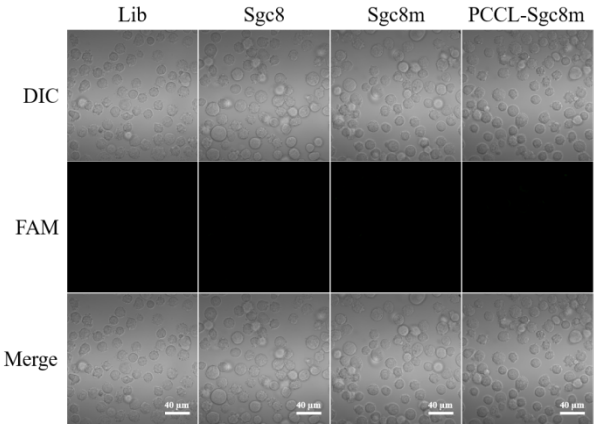


Figure S23. CLSM imaging analysis of the binding of FAM-labeled control DNA lib, unlocked aptamer Sgc8 and Sgc8m, and PCCL-Sgc8m on K562 cells. The DNA concentration was 200 nM. The incubation temperature was 37 °C.

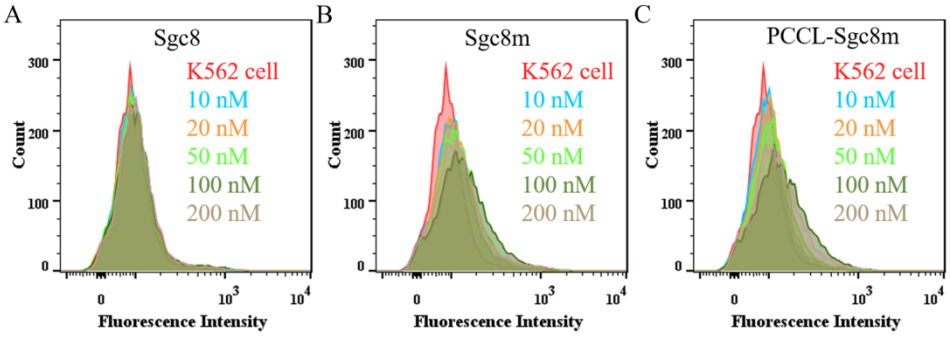


Figure S24. Flow cytometry assay of binding ability of FAM-labeled Sgc8 (A), Sgc8m (B) and PCCL-Sgc8m (C) on K562 cells. The incubation temperature was 4 °C.

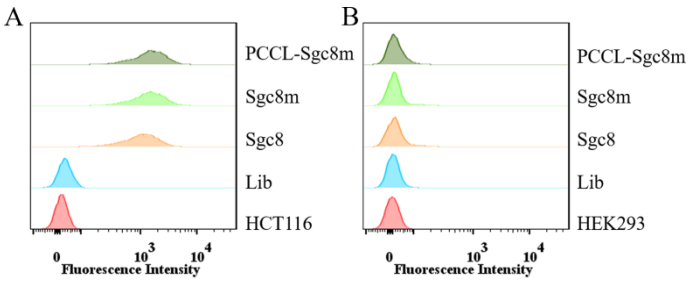


Figure S25. Flow cytometry assay of binding ability of FAM-labeled Sgc8, Sgc8m and PCCL-Sgc8m on HCT116 cells (A) and HEK293 cells (B). The aptamer concentration was 20 nM. The incubation temperature was 4 °C.

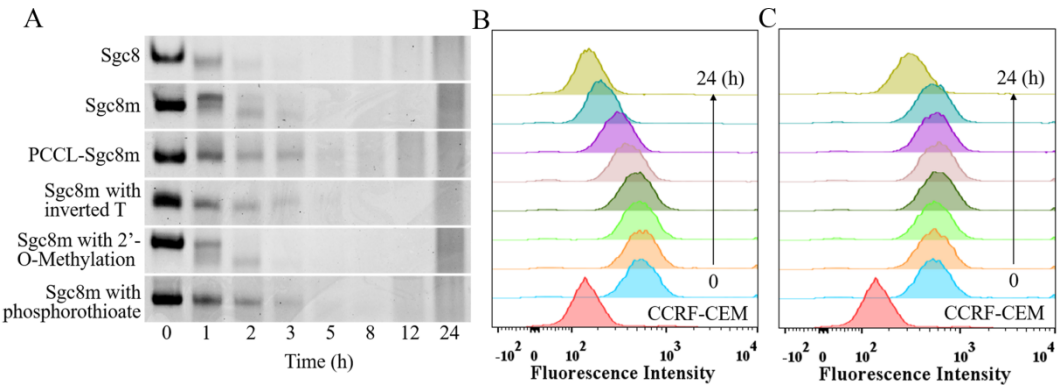


Figure S26. Stability analysis of aptamers after treatment with 50% FBS. (A) PAGE analysis of the stability of PCCL-Sgc8m in 50% FBS compared with the unlocked aptamers with or without chemical modifications. Flow cytometric analysis of the binding of FAM-labeled Sgc8m (B) and PCCL-Sgc8m (C) to CCRF-CEM cells after treatment with 50% FBS for 0, 1, 2, 3, 5, 8, 12, 24 h. The concentrations of DNA for the flow cytometric assay were 10 nM. The incubation temperature in the flow cytometric assay was 4 °C.

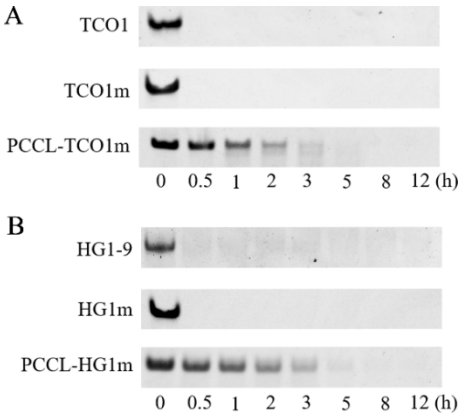


Figure S27. PAGE analysis of the stability of PCCL-TCO1m (A) and PCCL-HG1m (B) in 0.5 U/μL Exo I compared with the unlocked aptamers.

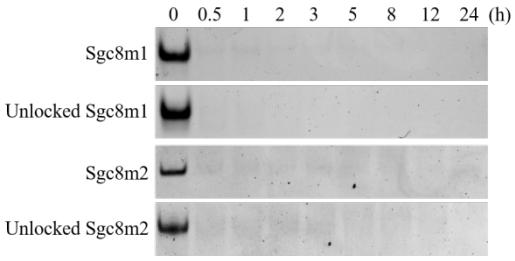


Figure S28. Stability analysis of Sgc8m1, Sgc8m2, unlocked Sgc8m1 and unlocked Sgc8m2 after treatment with 0.5 U/μL Exo I for different time, as determined by polyacrylamide gel electrophoresis. Unlocked Sgc8m1 and unlocked Sgc8m2 were prepared by 365-nm-UV-illuminating Sgc8m1 and Sgc8m2 in the presence of 8-methoxypsoralen, respectively. The concentrations of aptamers in PAGE were 1 μM.

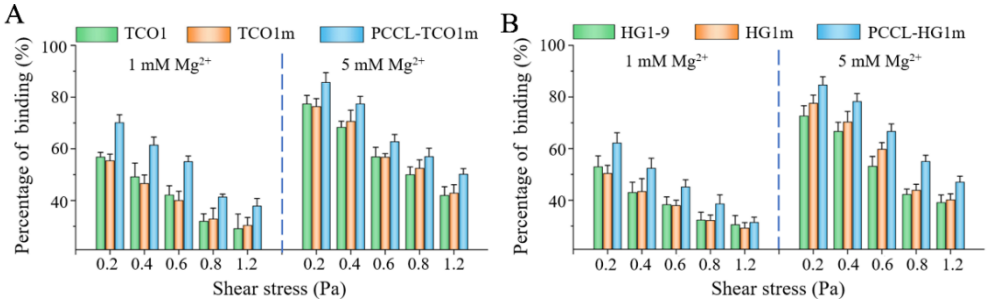


Figure S29. Evaluation of the interaction of FAM-labeled PCCL-TCO1m, TCO1m and TCO1 with CCRF-CEM cells (A) and FAM-labeled PCCL-HG1m, HG1m and HG1-9 with K562 cells (B) under well-defined shear conditions.

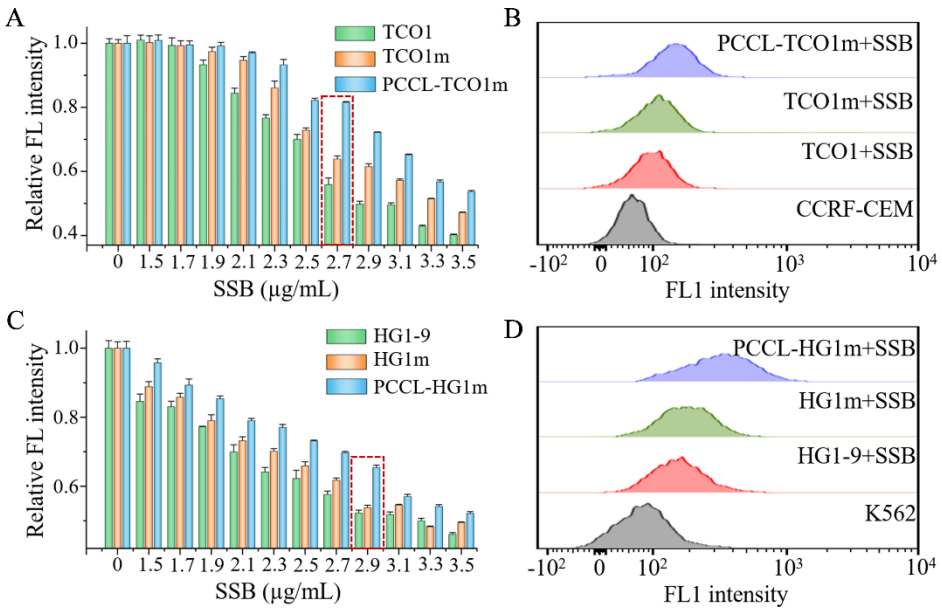


Figure S30. Flow cytometric analysis of the binding ability of FAM-labeled PCCL-TCO1m, TCO1m and TCO1 with CCRF-CEM cells (A) and FAM-labeled PCCL-HG1m, HG1m and HG1-9 with K562 cells (C) in the presence of SSB at different concentrations., The DNA concentration was 50 nM, and the data are presented as mean standard deviation (n=3). Flow cytometric analysis of the binding of FAM-labeled PCCL-TCO1m, TCO1m and TCO1 with CCRF-CEM cells (B) in the presence of 2.7 μg/mL SSB and FAM-labeled PCCL-HG1m, HG1m and HG1-9 with K562 cells (D) in the presence of 2.9 μg/mL SSB.

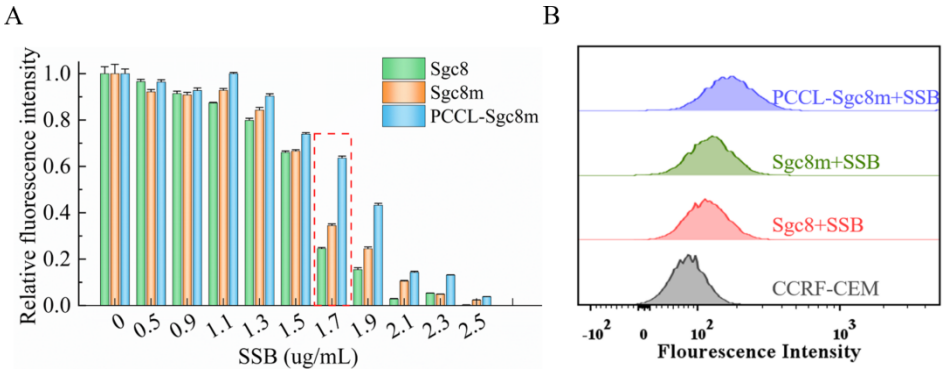


Figure S31. Flow cytometric analysis of the stability of aptamer-CCRF-CEM cells complexes in the presence of SSB at different concentrations. The aptamer concentration was 10 nM. The incubation temperature was 4 °C.

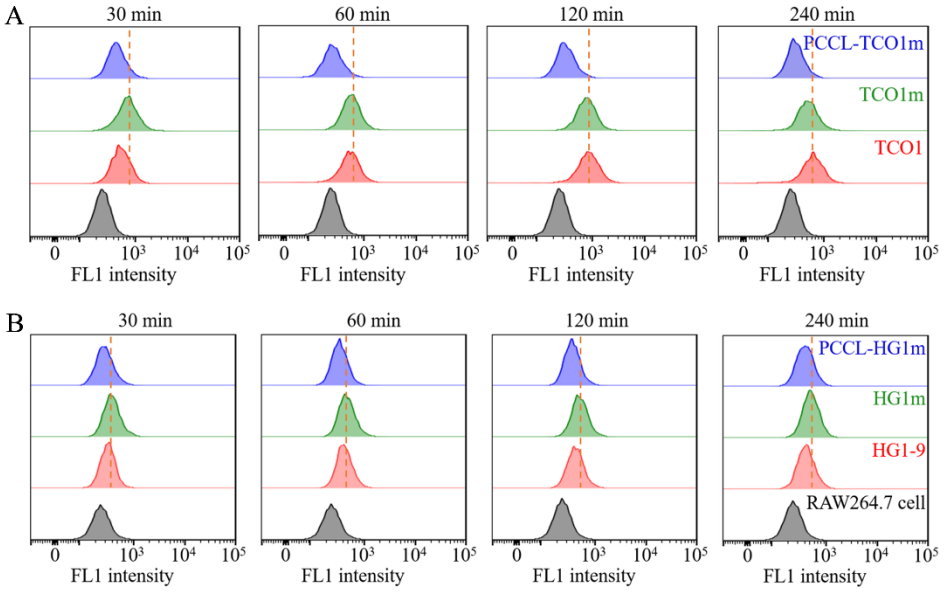


Figure S32. Macrophage uptake analysis of FAM-labeled PCCL-TCO1m, TCO1m and TCO1 (A) and FAM-labeled PCCL-HG1m, HG1m and HG1-9 (B) during a 4-h incubation period of with RAW264.7 cells pre-stimulated by LPS for 8 h.

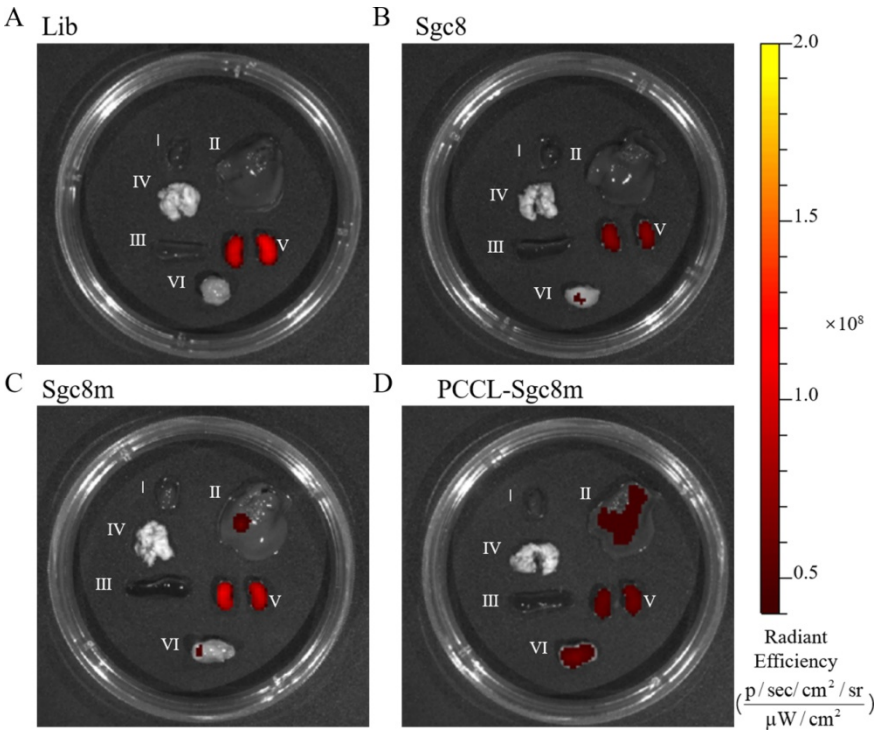


Figure S33. The *ex vivo* fluorescence analysis of main organs and tumors from mice injected with Cy5-labeled Lib (A), Cy5-labeled Sgc8 (B), Cy5-labeled Sgc8m (C) and Cy5-labeled PCCL-Sgc8m (D) at tail vein. (I) heart, (II) liver, (III) spleen, (IV) lung, (V) kidney and (VI) tumor.

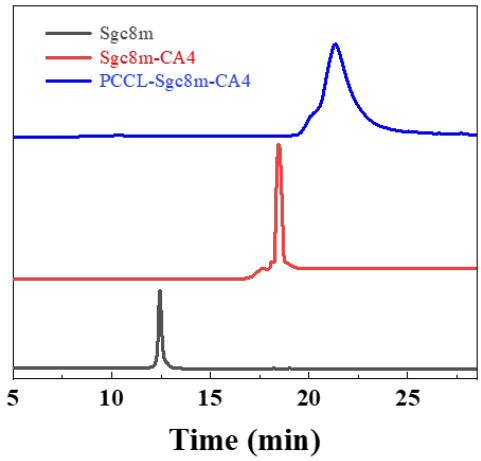


Figure S34. HPLC purification profile of PCCL-Sgc8-CA4 conjugations, Sgc8m-CA4 and Sgc8.

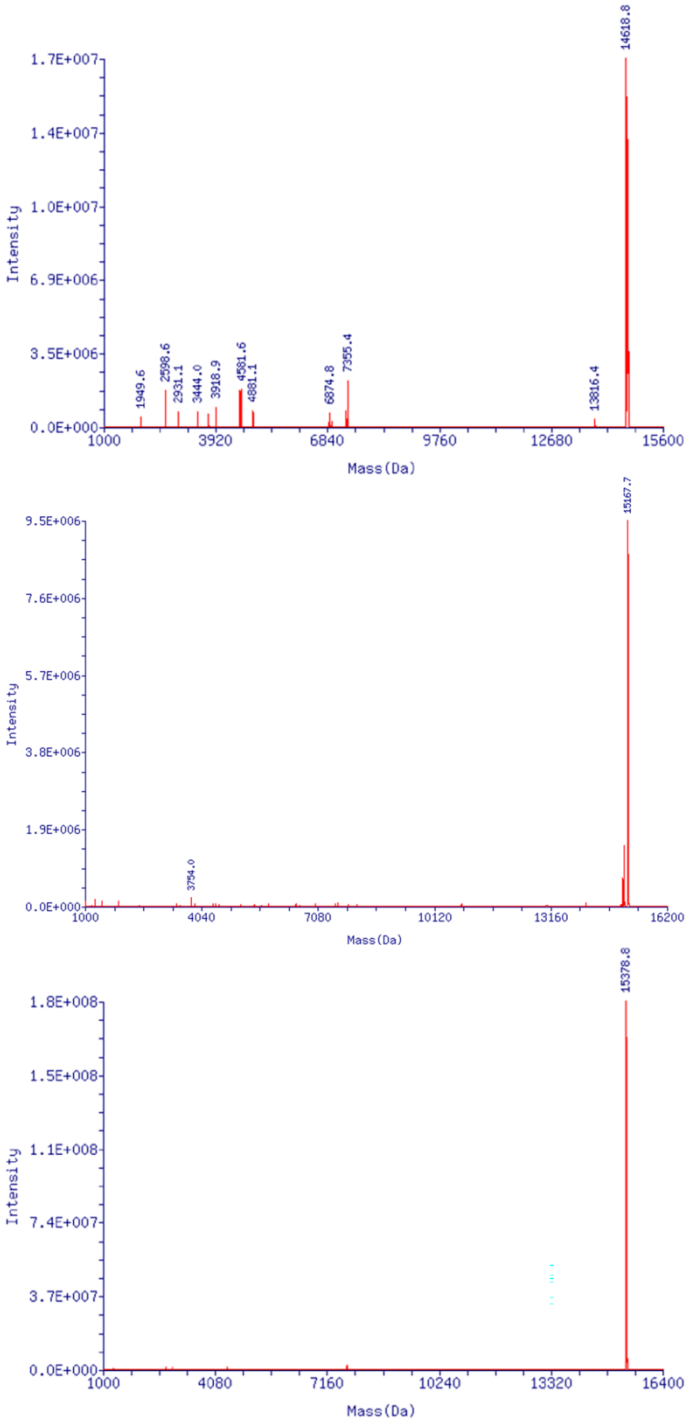


Figure S35. ESI-MS analysis of Sgc8m-DBCO (top), Sgc8m-CA4 conjugation (middle) and PCCL-Sgc8m-CA4 conjugation (bottom). Calculated molecular weight of Sgc8m-DBCO:14623.5, Found: 14618.8; Calculated molecular weight of Sgc8m-CA4:15169.1, Found: 15167.7; Calculated molecular weight of PCCL-Sgc8m-CA4: 15385.1, Found: 15378.8.

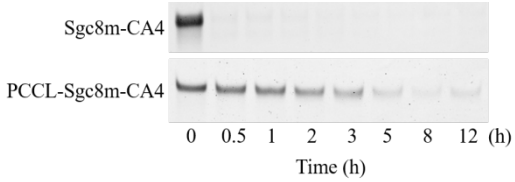


Figure S36. Stability analysis of Sgc8m-CA4 and PCCL-Sgc8m-CA4 after treatment with 0.5 U/μL Exo I for different time, as determined by PAGE. The concentration of various aptamers in PAGE analysis was 1 μM.

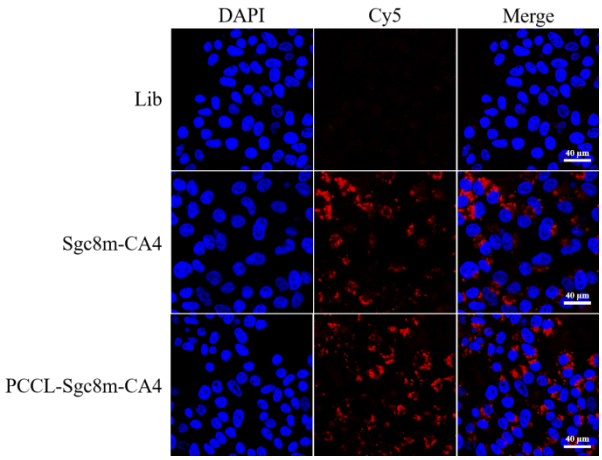


Figure S37. Cellular uptake analysis of Cy5-modified Sgc8m-CA4 and PCCL-Sgc8m-CA4 in HCT116 cells after 1 h incubation via CLSM imaging. Cell nuclei were stained with DAPI emitting blue fluorescence. Scale bar: 40 μm.

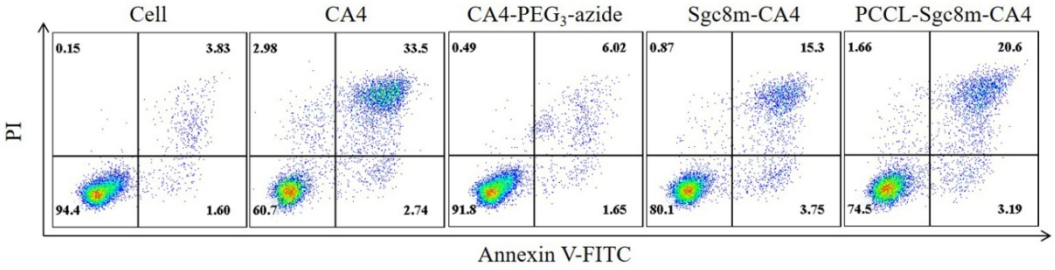


Figure S38. Apoptosis analysis of HCT116 cells after treatment with free CA4, CA4-PEG$_3$-azide, Sgc8m-CA4 and PCCL-Sgc8m-CA4.

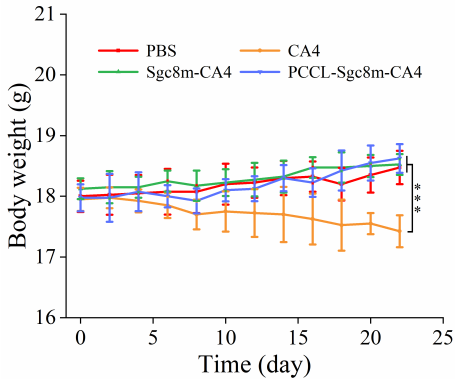


Figure S39. Relative body weight change curves of HCT116 tumor-bearing xenografted mice during the treatment period of 22 days.

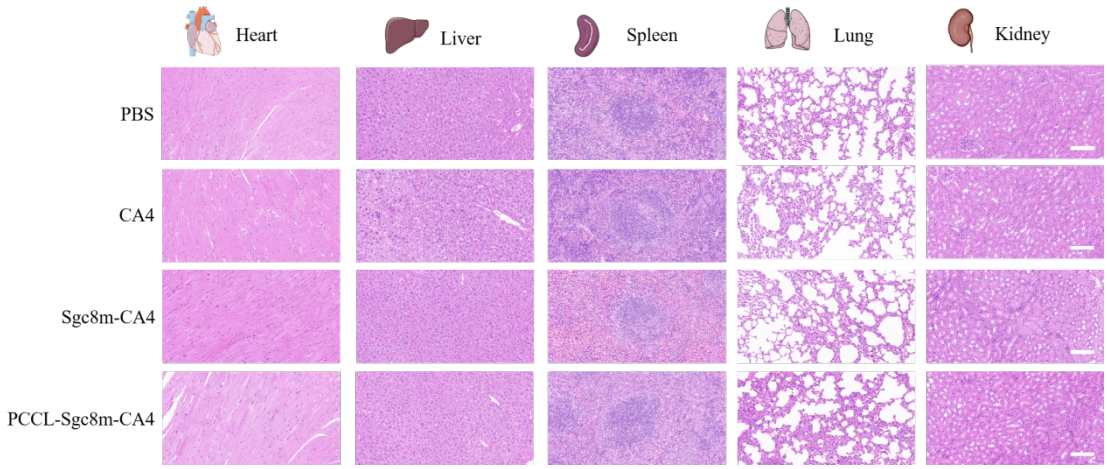


Figure S40. H&E pathological staining analysis of major organs from mice received different treatment. Scale bars: 100 μm.

References

1. Hemminki, K., Zhang, L. F., Krüger, J., Autrup, H., Törnqvist, M., Norbeck, H. E. Exposure of bus and taxi drivers to urban air pollutants as measured by DNA and protein adducts. *Toxicol. Lett.*, **1994**, 72, 171-174.

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3. Zhou, F., Wang, P., Peng, Y., Zhang, P., Huang, Q., Sun, W., He, N., Fu, T., Zhao, Z., Fang, X., Tan, W. Molecular Engineering-Based Aptamer-Drug Conjugates with Accurate Tunability of Drug Ratios for Drug Combination Targeted Cancer Therapy. *Angew. Chem. Int. Ed.*, **2019**, 58,, 11661-11665.