Electronical supporting information

for

Bioinspired molecular engineering of bivalent aptamers by ligand-induced self-dimerization

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Experimental Section

Reagents and Materials

Oligonucleotide sequences used in this study presented in Table S1 were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). Cyanine dye (CyT) was supported by Professor Tang's group at the Institute of Chemistry, Chinese Academic of Science. All other chemicals were of analytical reagent grade and used without further purification. Ultrapure water, prepared by the Milli-Q Gradient ultrapure water system (Millipore), was used in all experiments.

Sample preparation

Binding buffer was prepared from Dulbecco's Phosphate-buffered Saline (PBS) by adding 5 mM $MgCl_2$, 2 g/L BSA (Fisher Scientific), and 100 mg/L yeast tRNA (Sigma-Aldrich, St. Louis, MO, USA). Washing buffer was prepared in the same way, but without the addition of BSA. The DNA stock solutions were prepared by dissolving oligonucleotides directly in 20 mM Tris-HCl buffer (pH 7.4) containing 300 mM K^+ and 0.625 mM Mg^{2+}, followed by annealing in a thermocycler (heating at 90 °C for 5 min first and cooling down slowly to room temperature over a few hours).

For stability analysis, the aptamer samples were incubated with 20 mM Tris-HCl buffer (pH 7.4, with 300 mM K^+ and 0.625 mM Mg^{2+}) containing 0.25% exonuclease I (Exo I) or RPMI 1640 with 10% fetal bovine serum (FBS) at 37 °C. At designated time points, samples were heated at 95 °C for 5 min to denature the enzyme and subsequently stored at -20 °C until all samples were collected. Samples were then thawed on ice for electrophoresis or flow cytometry.

Construction of bivalent aptamers

HPLC-purified oligonucleotide sequences (Table S1) used in this study were purchased from Sangon Biotech Co. Ltd. (Shanghai, China). To construct bivalent aptamers, a monomeric aptamer was first dissolved in 20 mM Tris-HCl, pH 7.4, 300 mM KCl and 0.625 mM Mg^{2+} and then annealed in a thermocycler (first heated at 90 ºC for 5 min and then cooled down slowly to room temperature). The concentration of DNA was determined with UV-vis spectrophotometry before usage. Ultrapure water, prepared by the Milli-Q Gradient ultrapure water system (Millipore), was used in all experiments.

Cell Experiments

Cell cultures: All cell lines used in this work were purchased from American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium (Gibco®, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gibco®, Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (PS, Life Technologies, Carlsbad, CA) at 37 °C in 5% CO_2 atmosphere before use. Dulbecco's Phosphate-buffered Saline (D-PBS, Gibco) was used to wash cells.

To study the cell binding capability of bivalent aptamers, CCRF-CEM cells were collected and dispersed in the binding buffer (1×10^6 cells/mL), followed by treatment with FAM-labeled library DNA (100 nM), FAM-labeled monomer Sgc8 (100 nM), and FAM-labeled Sgc8-5G (50 nM) at 4 ºC in 200 μL of binding buffer (pH 7.4) for 30 min. Thereafter, cells were isolated and washed with washing buffer three times. Finally, the cells were resuspended in 200 μL of DPBS for flow cytometry analysis. For cellular internalization studies, the cells were washed with washing buffer via centrifugation at 1000 rpm and then incubated with FAM-labeled aptamers or ssDNA library in 200 μL of 1640 RPMI medium for 4 h at 37 °C. Cells were washed with washing buffer twice, and then the samples were suspended in 200 μL of DPBS for flow cytometry analysis.

To study the physiological stability of bivalent aptamers by flow cytometry, FAM-labeled library DNA, FAM-labeled monomer Sgc8, and FAM-labeled Sgc8-5G were treated with 0.25 U/μL Exo I or 10% fetal bovine serum for different time periods, respectively, followed by incubation with CCRF-CEM cells at 4 ºC in 200 μL of binding buffer (pH 7.4) for 30 min. The cell binding capability of these samples were studied by flow cytometry analysis.

For confocal imaging analysis of the cell binding capability, CCRF-CEM cells and Ramos cells were seeded in cell culture dishes and treated with Cy5-labeled library DNA, Cy5-labeled monomer Sgc8, and Cy5-labeled Sgc8-5G at 4 °C in 200 μL of binding buffer (pH 7.4) for 2 h, respectively. Afterwards, the cells were washed with washing buffer and imaged by a confocal laser scanning microscope (CLSM, Zeiss). The cell binding capability of the XQ-2d-based bivalent aptamer was studied under the same procedures as above, instead of using PL45 cells as the model cells.

The dissociation constants (Kd) of aptamer-cell interaction were obtained by fitting the curve of fluorescence intensity of specific binding to the concentration of aptamers using the equation Y = BmaxX/ (Kd + X) and GraphPad Prism 7 software. Each data point in the binding curves represents the average of three identical scans. Flow cytometry was performed with a BD FACSVerse™ system. Confocal microscopy was carried out on a FV1000-X81 confocal microscope (Olympus).

Characterization

Fluorescence spectra were acquired at 25 ± 1 ºC, using a Hitachi F-4500 spectrophotometer equipped with a temperature-controlled circulator. Samples were prepared by diluting to a concentration of 2 μM in 20 mM Tris-HCl, pH 7.4, 300 mM KCl and 0.625 mM Mg^{2+}, heating to 95 ºC, and cooling slowly (overnight) to room temperature. Fluorescence measurements were performed on a cuvette after adding 2 μM CyT, stirring gently for 10 min, and keeping in darkness for 2 h.

CD spectra were recorded on a JASCO J-815 spectrophotometer equipped with a temperature-controlled circulator. CD measurements were carried out at 25 ºC in the wavelength range of 220-350 nm, using a response time of 1 s, a step size of 1 nm, and a 2 nm bandwidth. Samples were also prepared by slow cooling from 95 ºC to room temperature. Each data point represents the average of three identical scans after correction for the corresponding buffer blank. The G4 structures showed a positive peak at 264 nm and a negative peak at 242 nm in the presence of K^+.

Polyacrylamide gel electrophoresis (PAGE) was used to evaluate the sequence integrity after treatment with 10% FBS and Exo I or the dimerization of monomeric aptamers. PAGE was performed in 1× TBE buffer solution (0.09 M Tris-boric acid and 0.002 M EDTA) with 12% native gels. 5 μL DNA samples mixed with 1 μL of 6 × loading buffer were loaded on the gel, and electrophoresis was run at 64 V for 2 h at room temperature. After electrophoresis, the gels were analyzed with a molecular imager (BIO-RAD).

Table S1 Sequences used in this work.

Sequence Name	Sequence (from 5'-3')
cy5-XQ-2d-5G	GGGGGATTTTTTTTTTTTGCTCATAGGGTTAGGGGCTGCTGGCCAGATACTC AGATGGTAGGGTTACTAT /iCy5/ GAGCTTTTTTTTTTAATGGGGG
cy5-XQ-2d-5T	TTTTTATTTTTTTTTTTTGCTCATAGGGTTAGGGGCTGCTGGCCAGATACTC AGATGGTAGGGTTACTAT /iCy5/ GAGCTTTTTTTTTTAATTTTTT
cy5-XQ-2d	GCTCATAGGGTTAGGGGCTGCTGGCCAGATACTCAGATGGTAGGGTTACTA T /iCy5/ GAGC
cy5-lib-5G	GGGGGATTTTTTTTTTTTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN /iCy5/ NNNNTTTTTTTTTTAATGGGGG
cy5-lib	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNN /iCy5/ NNNN
FAM-XQ-2d-5G	GGGGGATTTTTTTTTTTTGCTCATAGGGTTAGGGGCTGCTGGCCAGATACTC AGATGGTAGGGTTACTA /i6FAMdT/ GAGCTTTTTTTTTTAATGGGGG
FAM-XQ-2d	GCTCATAGGGTTAGGGGCTGCTGGCCAGATACTCAGATGGTAGGGTTACTA /i6FAMdT/ GAGC
FAM-lib	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNN /i6FAMdT/ NNNN
FAM-sgc8-5G	GGGGGATTTTTTTTTTTTTCTAACTGCTGCGCCGCCGGGAAAATACTGTACG GTT /i6FAMdT/ AGATTTTTTTTTAATGGGGG
FAM-sgc8-5T	TTTTTATTTTTTTTTTTTTCTACTGCTGCGCCGCCGGGAAAATACTGTACGG TT /i6FAMdT/ AGATTTTTTTTTTAATTTTTT
FAM-sgc8	TCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTT /i6FAMdT/ AGA
FAM-Lib-5G	GGGGGATTTTTTTTTTTTTTAATTCATACTGTAATCGATATATCCAGCCTTGA ATA /i6FAMdT/ GCT TTTTTTTTTTAATGGGGG
FAM-TH-S1	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCAT /i6FAMdT/ AGTA
TH-S2	TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCC AATAC
TH-S3	TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCT CTTC
TH-S4	TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCG CAT
FAM-sgc8-haripin	TCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTT/i6FAMdT/AGATTTC TCTCGCACCATCTCTCTCCTTCTGGGTGCGAGAG
FAM-sgc8-VEGF	TCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTT/i6FAMdT/AGATTTG GGCGGGCGGGGGCGGG
FAM-sgc8-ckit2	TCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTT/i6FAMdT/AGATTTC GGGCGGGCGCGAGGGAGGG
FAM-sgc8-inverted dT	TCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTT/i6FAMdT/AGAT(inver ted)
FAM-sgc8-ds1	GCAGTCTACTATGGCTTTTTTTTTTTCTAACTGCTGCGCCGCCGGGAAAAT ACTGTACGGTT /i6FAMdT/ AGATTTTTTTTTTCGGTATCATCTGACG
FAM-sgc8-ds2	CGTCAGATGATACCGTTTTTTTTTTTCTAACTGCTGCGCCGCCGGGAAAAT ACTGTACGGTT /i6FAMdT/ AGATTTTTTTTTTGCCATAGTAGACTGC

Fig. S1 PAGE analysis of the effect of K^+ concentration on the formation of Sgc8-5G-dimer. A nondenaturing 12% acrylamide gel was used. The gel was stained with SYBR Gold and visualized on GE Typhoon Trio. The bands from left to right were the monomeric aptamer Sgc8-5G incubated in Tris-HCl buffer containing different concentrations of K^+. The K^+ concentrations from left to right were: 0, 10, 20, 50, 100, 200, 300, 400, 500, 750, and 1000 mM, respectively.

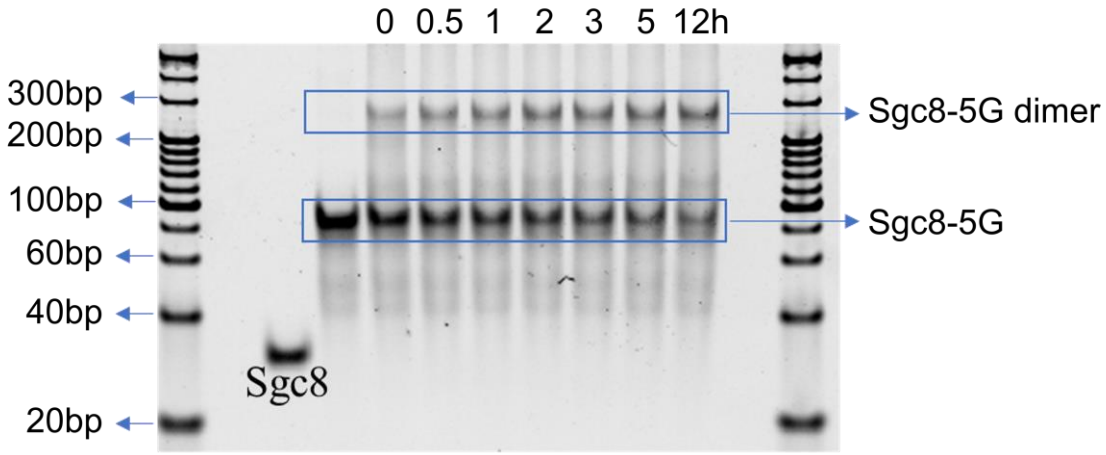

Fig. S2 PAGE analysis of the effect of incubation time on the K^+-mediated dimerization of Sgc8-5G (12% acrylamide gel). The gel was stained with SYBR Gold and visualized on GE Typhoon Trio. The bands from left to right were Sgc8, Sgc8-5G and Sgc8-5G after incubation in Tris-HCl buffer containing 300 mM K^+ for indicated time intervals.

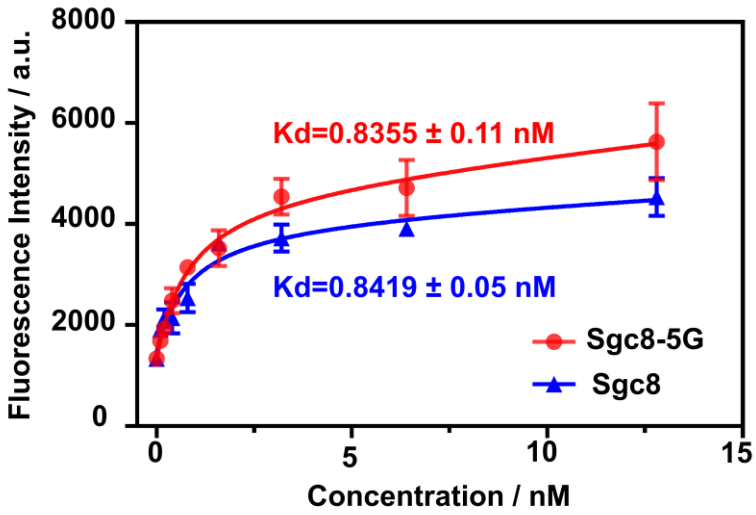

Fig. S3 Binding affinity analysis of Sgc8-5G and Sgc8 at 4 °C.

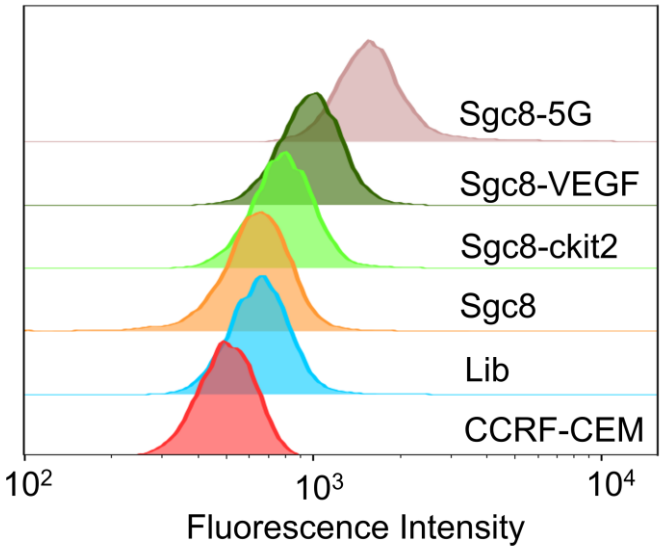

Fig. S4 Flow cytometry assay of the internalization ability of library DNA (Lib), Sgc8, G4-containing monomer Sgc8 (Sgc8-VEGF and Sgc8-ckit2), and G4-containing bivalent Sgc8 (Sgc8-5G) in CCRF-CEM cells at 37 °C. The concentration was 100 nM.

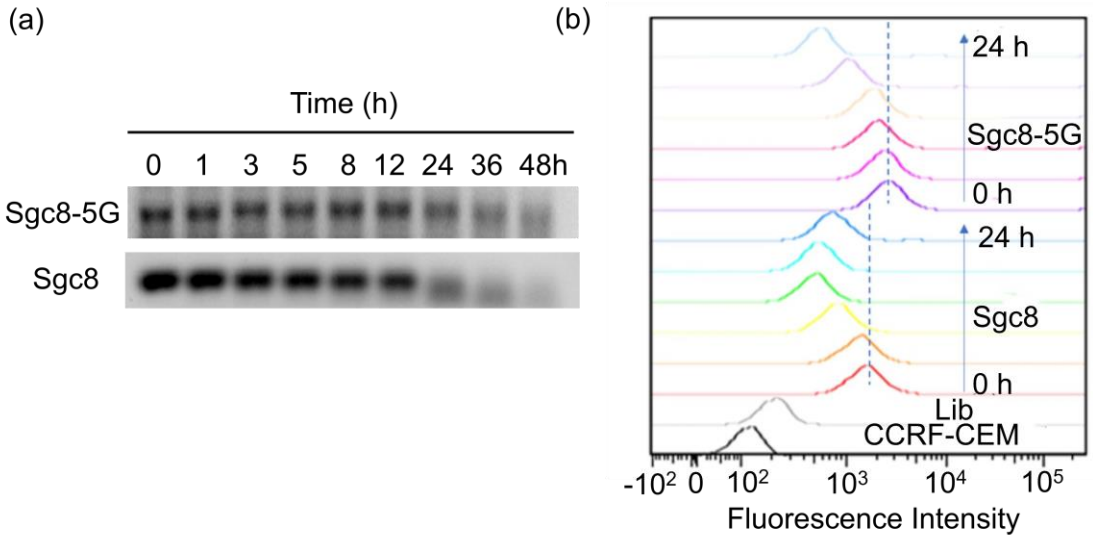

Fig. S5 Analysis of the cell binding of Sgc8-5G and Sgc8 towards CCRF-CEM cells after treatment with 10% FBS. (a) Stability analysis of Sgc8-5G and Sgc8 after treatment with 10% FBS for indicated time intervals, as determined by Polyacrylamide gel electrophoresis. (b) Binding ability analysis of Sgc8-5G and Sgc8 after treatment with 10% FBS, as determined by flow cytometry. The concentrations of Lib, Sgc8, and Sgc8-5G in the flow cytometric assay were 100, 100, and 50 nM, respectively. The peaks from bottom to top were CCRF-CEM only, Lib, Sgc8 and Sgc8-5G treated with 10% FBS for 0, 3, 5, 8, 12, and 24 h, respectively.

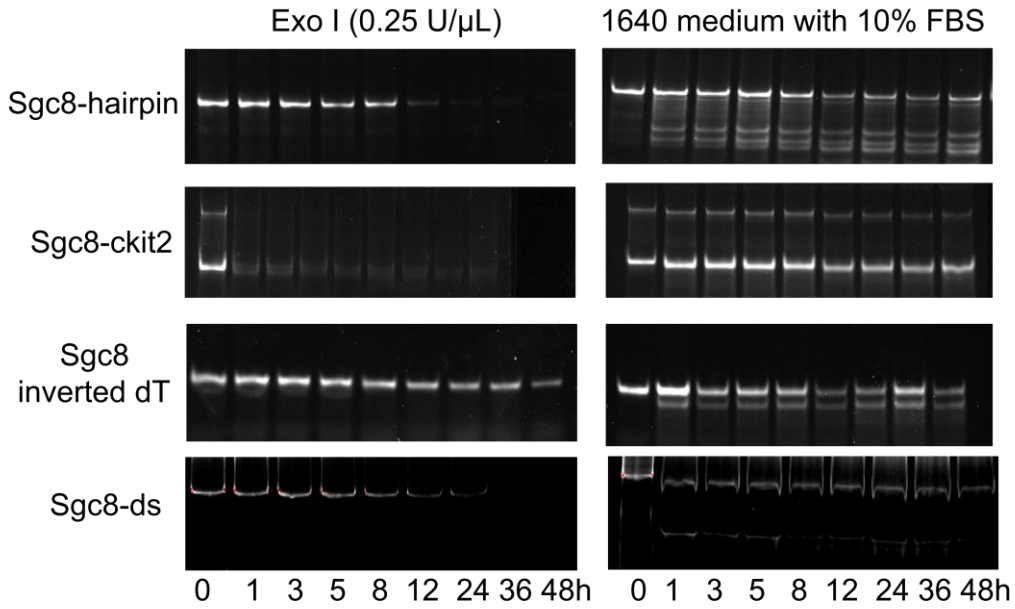

Fig. S6 PAGE analysis of Sgc8 aptamers with varied structures including Sgc8 attaching a hairpin structure (Sgc8-hairpin), Sgc8 with a G4 structure (Sgc8-ckit2), Sgc8 with inverted dT modification at 3′-terminus (Sgc8-inverted dT), and a sgc8 dimer formed via hybridization (Sgc8-ds), after incubation with 0.25 U/μL Exo I (left) or 10% FBS (right) for indicated time intervals. The concentration for all aptamers was 1 μM.

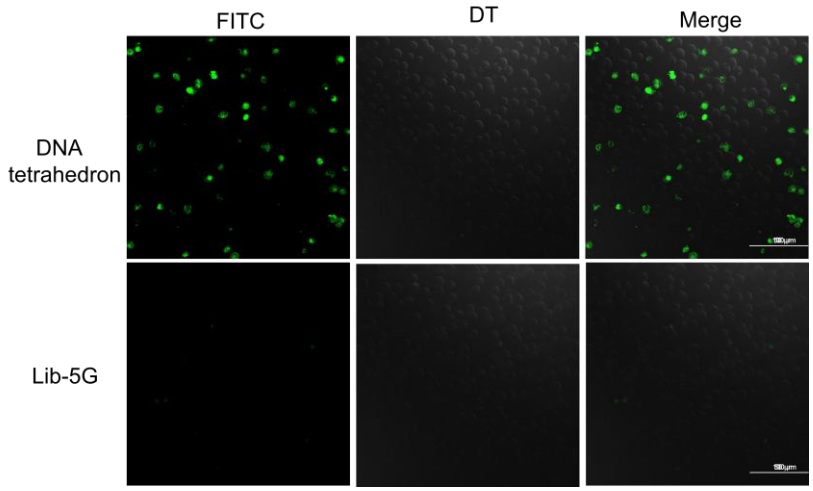

Fig. S7 Confocal imaging of CCRF-CEM cells after treatment with FAM-labeled DNA tetrahedron or Lib-5G at a concentration of 50 nM and at 37 °C for 1 h, respectively.

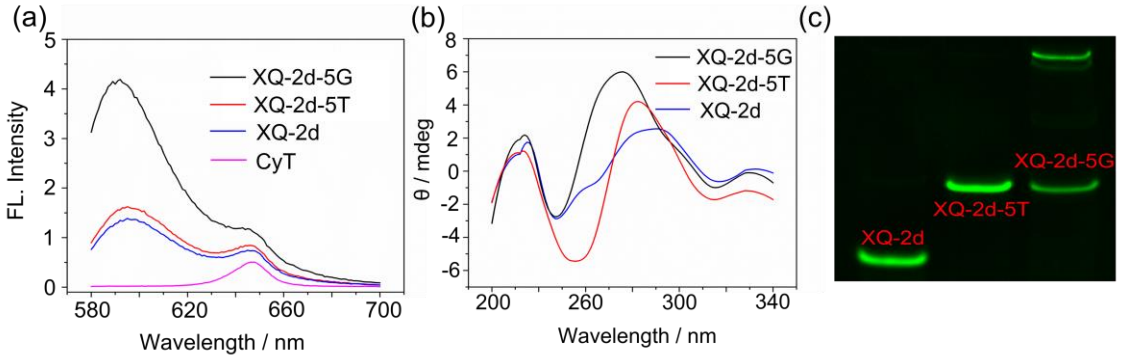

Fig. S8 (a) Fluorescence spectra of 2 μM CyT and CyT mixed with XQ-2d-5G, XQ-2d-5T, or XQ-2d in 20 mM Tris-HCl (300 mM KCl, 0.625 mM Mg^{2+}, pH 7.4) buffer at 25°C. (b) Circular dichroism spectra of XQ-2d-5G, XQ-2d-5T and XQ-2d in 20 mM Tris-HCl (300 mM KCl, 0.625 mM, pH 7.4) buffer at 25°C. (c) Gel electrophoresis image of XQ-2d (Lane 1), XQ-2d-5T (Lane 2), and XQ-2d-5G with K^+ (Lane 3) by 12% PAGE.

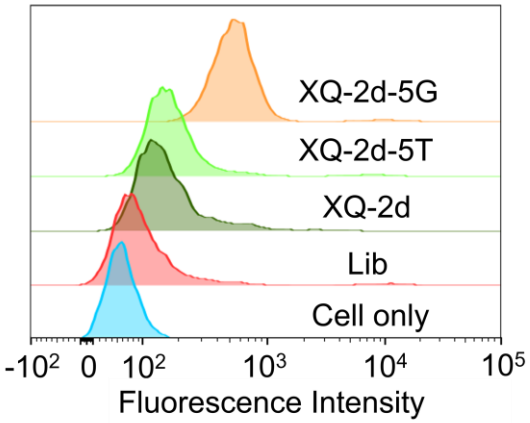

Fig. S9 Flow cytometry of the binding ability of XQ-2d-5G, XQ-2d and XQ-2d-5T in PL45 cells. The concentrations of Lib, XQ-2d, XQ-2d-5T, Lib-5G and XQ-2d-5G were 100 nM, 100 nM, 100 nM, 100 nM and 50 nM, respectively. The incubation temperature was 4 ºC.

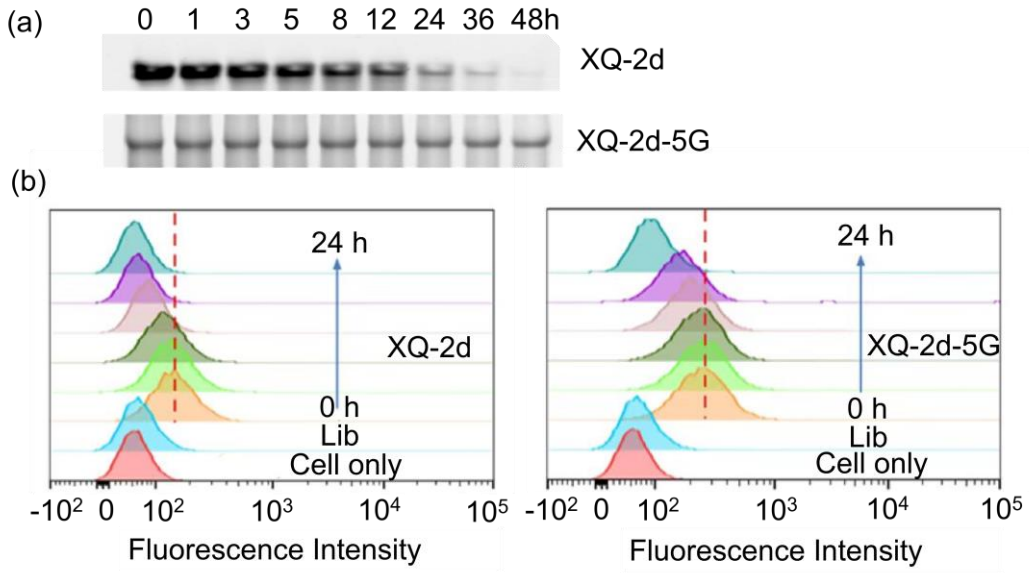

Fig. S10 Stability analysis of XQ-2d and XQ-2d-5G after treatment with 10% FBS. (a) Stability analysis of XQ-2d and XQ-2d-5G after treatment with 10% FBS for indicated time intervals, as determined by polyacrylamide gel electrophoresis. (b) Binding ability analysis of XQ-2d (left) and XQ-2d-5G (right) after treatment with 10% FBS, as determined by flow cytometry. The concentrations of Lib, XQ-2d, and XQ-2d-5G in the flow cytometric assay were 100, 100, and 50 nM, respectively. The peaks from bottom to top were PL45 cells only, Lib, XQ-2d, and XQ-2d-5G treated with 10% FBS for 0, 3, 5, 8, 12, and 24 h, respectively.

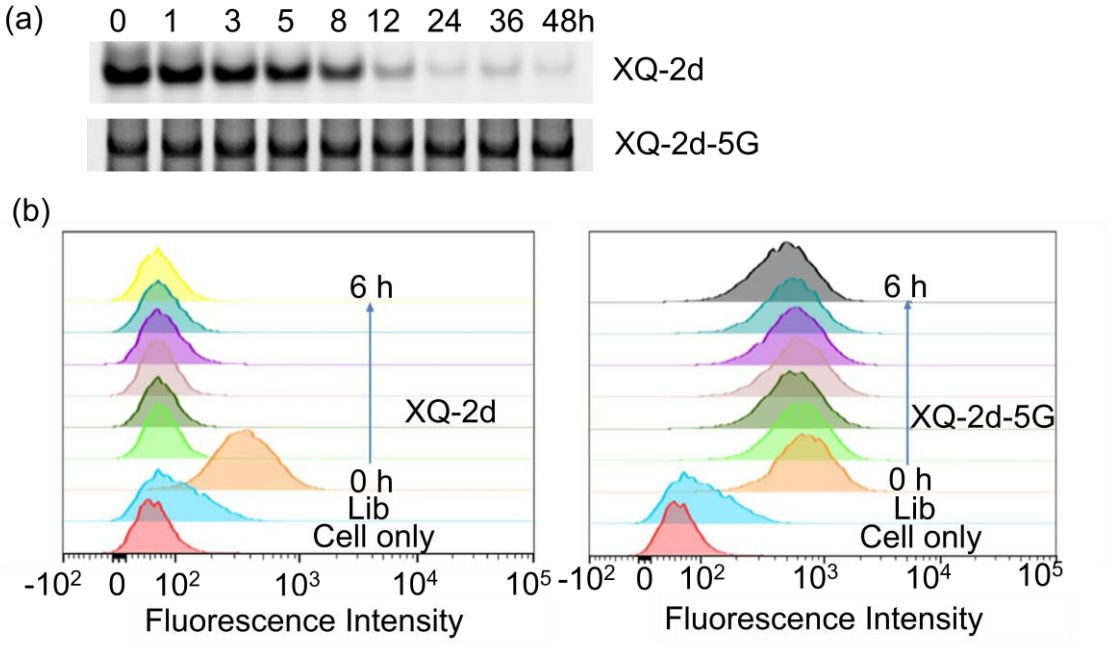

Fig. S11 Stability analysis of XQ-2d and XQ-2d-5G after treatment with Exo I. (a) Stability analysis of XQ-2d and XQ-2d-5G after incubation in 0.25 U/μL Exo I for indicated time intervals, as determined by polyacrylamide gel electrophoresis. (b) Binding ability analysis of XQ-2d (left) and XQ-2d-5G (right) after treatment with Exo I, as determined by flow cytometry. The concentrations of Lib, XQ-2d and XQ-2d-5G in the flow cytometric assay were 100, 100, and 50 nM, respectively. The peaks from bottom to top were cells only, Lib, XQ-2d, and XQ-2d-5G incubated in 0.25 U/μL Exo I for 0, 1, 2, 3, 5, and 6 h, respectively.

Author Contributions

S. X., L. H., Y. L., and W. T. conceptualized the idea of this project; S. X. and L. H. performed most of the experiments and wrote the manuscript; F. C. and W. D. did part of the cellular characterizations; F. C., W. D., S. X., L. L., C. C., and Y. Y. analyzed the data; Y. L. and W. T. supervised this project and edited the paper. All authors discussed and provided comments on this work.