Electronic Supplementary Information

A DNA tetrahedron dimer for dual membrane protein logic recognition and interaction inhibition

Yuanmeng Zhang,[a] Muyang Lin,[a,b] Jingkang Yao[a] and Xiaowen Xu*[a]

[a.] School of Chemistry and Chemical Engineering, [b.] Taishan College, Shandong University, 250100 Jinan, P. R. China. E-mail: xuxw@sdu.edu.cn; Fax: +86 531 88363916.

Experimental Section

Materials and reagents. DNA oligonucleotides (Table S1) were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). Ammonium persulfate (APS), diethyl pyrocarbonate (DEPC) treated water, ethylenediaminetetraacetic acid tetrasodium (EDTA), 40% acrylamide/bisacrylamide (19: 1) solution, Tris base and N, N, N', N'-tetramethylethylenediamine (TEMED) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Alexa Fluor 488-labeled wheat germ agglutinin for cell membrane staining and SYBR Gold were purchased from Thermo Fisher Scientific. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Missouri, USA). Annexin V-FITC apoptosis detection kit was purchased from Beyotime Biotechnology (Shanghai, China). HER protein was purchased from T&L Biological Technology (Beijing, China). Primary antibodies of β-actin, p-HER2, Bax and HRP-linked antibody were purchased from Cell Signaling Technology (Shanghai, CST-US subsidiary in China). Fetal bovine serum (FBS), DMEM cell medium and phosphate-buffered saline (PBS) were purchased from Biological Industries (Israel). Ultrapure water (18.25 MΩ•cm) obtained from the UP water purification system was used throughout the experiment.

Table S1. Sequences of oligonucleotides used in this study.[a]

Name	Sequence (5' to 3')
S1	TATCACCCAGTTGACAGTGTAGCATGTAATAGATGCGAGCCAATAC
S2	TCAACTGGGTGATAAAACGACACTTGGGAATCTACTATGGCTCTTC
S3	CGGTATCAACTATGCTTTTTTTTTTTTTTTTTCAGACGGAATGTGCTTCCCAAGTGTCGTAAGTATTGGCTCGCAT
S4	ACATTCCGTCTGAAACATTACATGCTACACGAGAAGAGCCATAGTATTTTTTTTGGTGGTGGTGGTTGTGGTGGTGGTGG
S3'	TTCAGACGGAATGTGCTTCCCAAGTGTCGTAAGTATTGGCTCGCATTTTTTTTTTTTTTTTGCAATGCCGCAATCC
S4'	ACATTCCGTCTGAAACATTACATGCTACACGAGAAGAGCCATAGTATTTTTTTTTCTAAAAGGATTCTTCCCAAGGGGATCCAATTCAAACAGC
S5	BHQ-2-TGTGGCATATCCCAAGGGGATCCATGACACAAC
S6	GCATAGTTGATACCGGGATTGCGGCATTGCTTGGGATATGCCACA-Cy3
S7	AGTGGTGGTTGTGTCATGGATCCCC
BN	GGATCCATGACACAACCACCACT
BH	TGGATCCCCTTGGGATATG
HER2 aptamer	TCTAAAAGGATTCTTCCCAAGGGGATCCAATTCAAACAGC
S4-Cy3	ACATTCCGTCTGAAACATTACATGCTACACGAGAAGAGCCATAGTATT/iCy3/TTTTTTGGTGGTGGTGGTTGTGGTGGTGGTGG
S4-OA	ACATTCCGTCTGAAACATTACATGCTACACGAGAAGAGCCATAGTA
S5-OA	BHQ2-GGATCCATGACACAA
S6-OA	GCATAGTTGATACCGGGATTGCGGCATTGCAGTGGTGGTTGTGTCATGGATCC-Cy3
BN-OA	GGATCCATGACACAACCACCACT
cDNA-HER2-apt	GCTGTTTGAATTGGATCCCCTTGGGAAGAATCCTTTTAGA
cDNA-NCL-apt	CCACCACCACCAACACCACCACCACC

[a] The protruding sequences that hybridize the linking complex in DNA tetrahedron are marked in blue. The HER2-binding aptamer sequence is marked in green, while the nucleolin-binding

aptamer sequence is marked in orange.

Instrumentation. UV-vis absorption spectra were recorded on a TU-1901 spectrometer (Persee, China). Polyacrylamide gels were imaged on a GelDocTM XR^+ imaging system (Bio-RAD Laboratories Inc., USA). Fluorescence emission spectra were recorded with F-320 fluorescence spectrophotometer (Gangdong, Tianjin). Western blot analysis was obtained on Amersham Imager 600 (GE Healthcare, USA). Dynamic light scattering measurements were performed using a Zetasizer Nano ZS (Malvern, UK). The dimer sample was characterized with atomic force microscopy (Bruker, USA). Confocal laser scanning microscopic (CLSM) imaging were performed on a Leica TCS SP8 confocal microscope (Leica, Germany). Flow cytometric analysis was performed on a NovoCyte 3130 flow cytometer (Agilent Technologies Inc., USA). ITC experiment was performed with Microcal VP-ITC instrument (Malvern, England). Cryo-electron microscopy imaging was performed on on the Tundra cryo-electron microscope (Thermo Scientific, USA). Absorbance measurement in MTT assay was measured using a multifunctional microplate reader (Tecan, Switzerland).

Preparation of DNA tetrahedron dimer. The two types of DNA tetrahedron were annealed respectively. To assemble the tetrahedron, S1, S2, S3, S4, BN or S1, S2, S3', S4', BH at 1:1:1:1:1 molar ratio were mixed in TAE-Mg^{2+} (40 mM Tris, 1.5 mM $Mg(CH_3COO)_2$, 0.01 mM EDTA, pH 8.0) buffer solution. The mixed solution was heated at 95 °C for 5 min and then slowly cooled to room temperature. In the same way, the same molar amount of S5, S6, S7 are mixed and annealed to obtain the linking DNA complex. The DNA tetrahedron dimer was obtained by mixing the two types of DNA tetrahedron and the linking DNA complex with equal proportion and incubating at 37 °C for 4 h. The synthesized DNA tetrahedron dimer was stored at 4 °C. For preparation of control DNA tetrahedron containing only one aptamer, S1, S2, S3, S4 and BN-OA ("OA" is the abbreviation of "One Aptamer") are annealed to form tetrahedron N, and S1, S2, S3' and S4-OA are annealed to form tetrahedron. The two types of tetrahedron were incubated with partially-hybridized duplex of S5-OA/S6-OA that acts as a linker at 37 °C for 4 h, obtaining the control DNA

tetrahedron.

Polyacrylamide gel electrophoresis. The DNA samples were mixed with loading buffer and loaded into 6% polyacrylamide gel. The 6% polyacrylamide gel was prepared by mixing 16.25 mL of ultrapure water, 3.75 mL of 40% acrylamide/bisacrylamide solution (19:1), 5 mL of 5×TAE-Mg^{2+} buffer (200 mM Tris-Ac, 7.5 mM $Mg(CH_3COO)_2$, 0.05 mM EDTA, pH 8.0), 180 μL of 0.1 g/mL APS and 18 μL TEMED. Gel was run in 1×TAE buffer at 4 °C and 100 V for 3.5 h, stained with 1×SYBR Gold for 30 min, and photographed by imaging system.

Agarose gel electrophoresis. 2% agarose gel was formulated by dissolving 0.6 g agarose powder in 30 mL of 1×TAE-Mg^{2+} buffer. The solution was heated in the microwave until it got clear and transparent, which was then cooled to about 60 °C and poured into the gel tray. The gel was left at room temperature for 30 min for solidification. The loading sample concentration was 300 nM. The gel was run at 100 V for 2 h, stained with 1×SYBR Gold for 30 min, and photographed by imaging system.

Dynamic light scattering characterization. The DNA nanostructure was characterized by dynamic light scattering on Zetasizer Nano ZS. 50 μL of 100 nM DNA tetrahedron monomer or DNA tetrahedron dimer was transferred to 50-μL disposable cuvettes (Sarstedt, Germany) for measurements.

Atomic force microscopy characterization. A fresh mica was placed into a petri dish containing 20 μL APTES. After incubating for 5 min, it was rinsed three times with ultra-pure water. 20 μL of 100 nM DNA tetrahedron dimer or DNA tetrahedron monomer was dropped on the mica and deposited for 10 min, then the mica was rinsed with ultrapure water, followed by addition of 20 μL ultrapure water. The sample was characterized by an atomic force microscope with a peakforce tapping mode.

Fluorescence measurement. The fluorescence emission spectra were recorded from 550 nm to 700 nm under the excitation wavelength of 538 nm, and concentration of the DNA tetrahedron dimer used was 50 nM.

Cell culture. HL-7702, HeLa and SK-BR-3 cells were grown in DMEM medium supplemented with 10 % FBS and 100 U/mL penicillium/streptomycin. All the cells were incubated at 37 °C in a humid environment containing 5 % CO_2.

Stability test. To test the stability of DNA tetrahedron dimer for cellular application, the DNA tetrahedron dimer was incubated with cell culture media containing 10% fetal bovine serum for periods of time, and the products were then characterized by 2% agarose gel.

Confocal fluorescence imaging. In order to test the response of the DNA tetrahedron dimer to different cell types, SK-BR-3 cells (or HeLa, HL-7702 cells) were seeded on confocal dishes overnight. The medium was removed, the cells were washed with PBS buffer, and the fresh medium containing 200 nM DNA tetrahedron dimer was incubated with the cells at 37 °C for 2 h for confocal imaging.

To study the interaction of DNA tetrahedron monomer (with blocked nucleolin aptamer) or DNA tetrahedron dimer with cells, 200 nM DNA nanostructures were incubated with cells for a series of times for fluorescence imaging.

For the cell membrane and DNA nanostructure co-localization investigation, WGA-AF488 cell membrane stain was added 30 minutes prior to the imaging, then the cells were washed with PBS and replaced with fresh medium containing 20 mM Ca^{2+} and 50 mM K^{+} for imaging.

The control DNA tetrahedron with only nucleolin-binding aptamer was incubated with HeLa cells for imaging as mentioned above.

Flow cytometry. Three different types of cells were seeded with a density of 2×10^5 cells/well into a 24-well plate overnight. The medium was removed and 500 μL medium containing 200 nM DNA tetrahedron dimer was added and incubated at 37 °C for 1 h. After discarding the medium, the cells were washed with PBS, digested and collected into a centrifuge tube. The cells were collected by centrifuging at 1000 rpm for 3 min and then suspended in 100 μL PBS containing 2% FBS for the flow cytometric analysis.

Isothermal titration calorimetry (ITC) experiment. ITC experiment was performed

with Microcal VP-ITC instrument (Malvern). The sample cell contained about 1.44 mL of 40 nM HER2 aptamer or HER2 aptamer-BH duplex, and the titrant of 200 nM HER2 protein was loaded in the syringe. We introduced 25 total injections of 10 μL each and a purge injection of 5 μL. The purge injection was not included in the calculations. The measurements were performed in PBS buffer at 25 °C.

Cryo-electron microscopy imaging. Briefly, the carbon-coated copper grid was hydrophilized by easiGlow glow discharger, which was then clamped by the cryosampler clamp and secured to the robotic arm of the sampler. When the copper grid and sampling hole were aligned, 3 μL of 150 nM DNA tetrahedron dimer was added onto the copper grid, and then the copper grid is quickly inserted into liquid ethane. The prepared copper grid was transferred to the box that was submerged under liquid nitrogen for freezing, and then transferred to the working area, followed by attachment of a C-ring. The sample was imaged on the Tundra cryo-electron microscope (Thermo Scientific) with 100 kV accelerated voltage. Hundreds of images were obtained, and the three-dimensional model was re-constructed by cryosparc software.

Western Blotting Analysis. SK-BR-3 cells were seeded on the 6-well plate, and the experiment was ready to perform when the cell density reached 90%. The cells were incubated with 400 nM probes for 12 h or 24 h, respectively. The cells were then lysed with RIPA lysis buffer containing phosphatase inhibitors and protease inhibitors. Bax and p-HER2 were separated using 12% or 6% SDS-PAGE, and the proteins were transferred with 0.22 μm and 0.45 μm PVDF membranes activated by methanol, respectively. After the membrane was cut according to the molecular weight, it was incubated with the corresponding primary antibody in small containers at 4 °C overnight. The excess un-conjugated primary antibody was removed by washing. The cut membrane was then incubated with the second antibody with excess unconjugated antibody removed by washing. Finally, the membrane was visualized through enhanced chemiluminescence imaging.

Cell apoptosis analysis. SK-BR-3 cells were seeded in 24-well plates at a density of 2×10^5 cells/well and incubated overnight. The cells were incubated with 200 nM

DNA tetrahedron dimer for 12 h and then washed with PBS. After the cells were transferred to the round-bottom tubes, 195 μL Annexin V binding solution, 5 μL Annexin V-TITC binding solution and 5 μL PI binding solution were added successively. After incubation at room temperature for 20 min, the samples were characterized by flow cytometry.

Cell viability assay. The cells were seeded into 96-well cell culture plates with a density of 4000 cells per well and cultured at 37 °C with 5% CO_2. The original medium was removed after 24 h. The cells were treated with the fresh medium containing 200 nM DNA tetrahedron dimer for 24 h, and then 20 μL of 5 mg/mL MTT was added. After incubation at 37 °C for 4 h, the MTT solution was carefully discarded and 100 μL DMSO was added to dissolve the purple formazan. The absorbance at 490 nm per well was measured and the cell viability was calculated. The same method was used to analyze the effect of different concentrations of DNA tetrahedron dimer on cell viability.

Additional Figures.

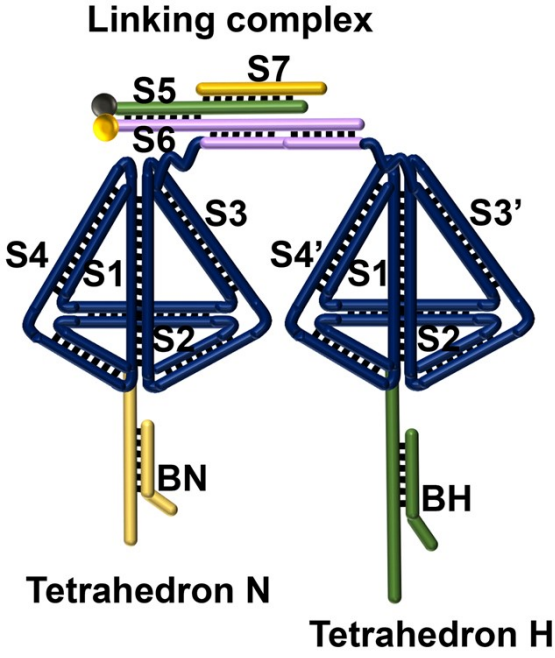

Fig. S1 Enlarged structure with detailed constituent strands of the DNA tetrahedron dimer.

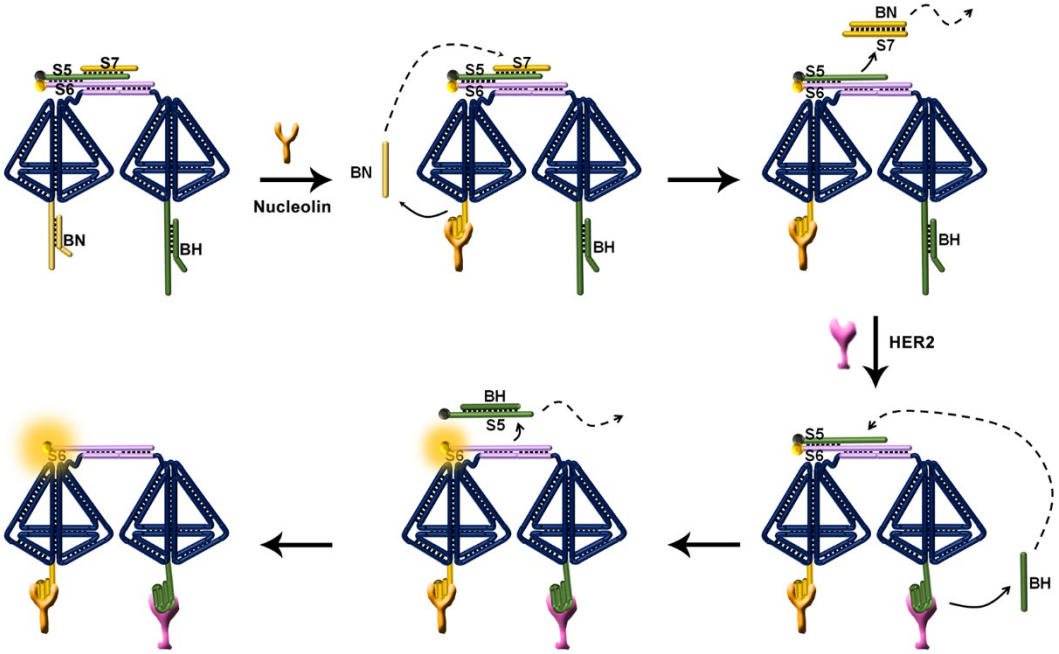

Fig. S2 Schematic illustration showing the details of recognition-driven strand exchange process.

As shown in Fig. S2, in the presence of nucleolin, BN is dehybridized from the nucleolin aptamer as the result of nucleolin aptamer binding to nucleolin. The dissociated BN then hybridizes with S7 via the exposed toehold on S7, leading to the formation of BN-S7 duplex and the dissociation of S7 from the DNA tetrahedron dimer, meanwhile exposing part sequence of S5. When HER2 is present, the HER2 aptamer will bind HER2 to form the aptamer-HER2 complex, repelling BH. The repelled BH then hybridizes with S5, forming BH-S5 duplex and inducing the detachment of S5. Since BHQ-2-labeled S5 is dissociated far from Cy3-labeled S6 on the DNA tetrahedron dimer, the fluorescence of Cy3 is restored to cause the lighting-up of the DNA tetrahedron dimer.

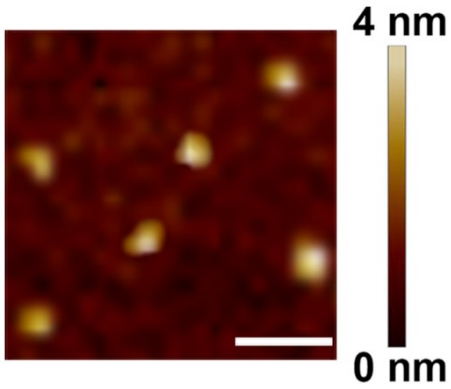

Fig. S3 Atomic force microscopy characterization of the DNA tetrahedron monomer. Scale bar: 20 nm.

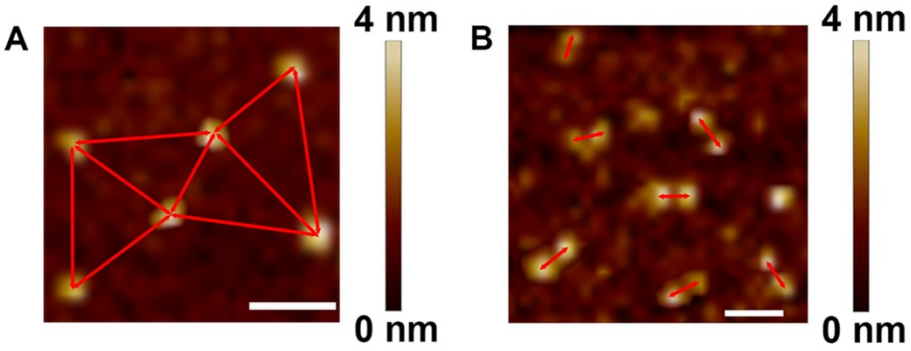

Fig. S4 Statistical analysis of the average distance of each DNA tetrahedron for DNA tetrahedron monomer (A) and dimer (B) in atomic force microscopy characterization. Scale bar: 20 nm.

The control data of AFM image of DNA tetrahedron monomer is characterized and shown in Fig. S3. It can be observed that DNA monomer nanostructures are individually dispersed on the mica, and no DNA dimer or multimer is formed. By statistical analysis, the average distance between individual DNA nanostructure center in monomer is about 28 nm, while the average distance between individual DNA nanostructure center in dimer is about 10 nm in dimer (Fig. S4). It shows that the two DNA tetrahedrons can be brought together by the linking DNA complex in dimer and has huger steric hindrance.

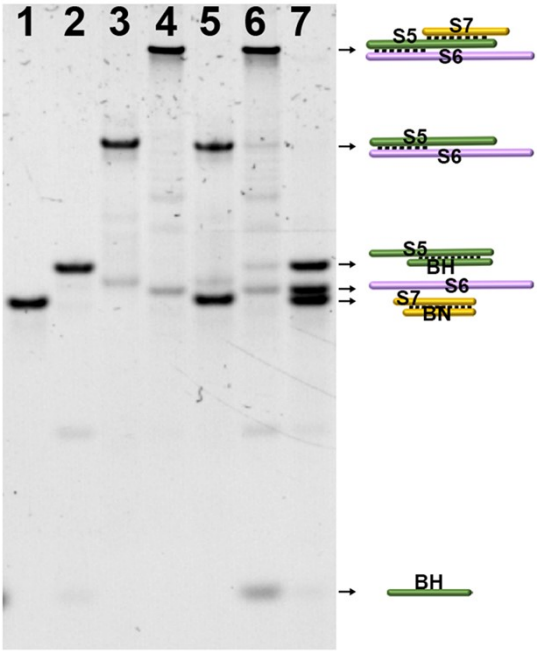

Fig. S5 Native polyacrylamide gel electrophoresis characterization of the strand displacement reaction between the blocking strands and the linking DNA complex. Lane 1: S7 + BN; lane 2: S5 + BH; lane 3: S5 + S6; lane 4: S5 + S6 + S7; lane 5: linking DNA complex + BN; lane 6: linking DNA complex + BH; lane 7: linking DNA complex + BN + BH.

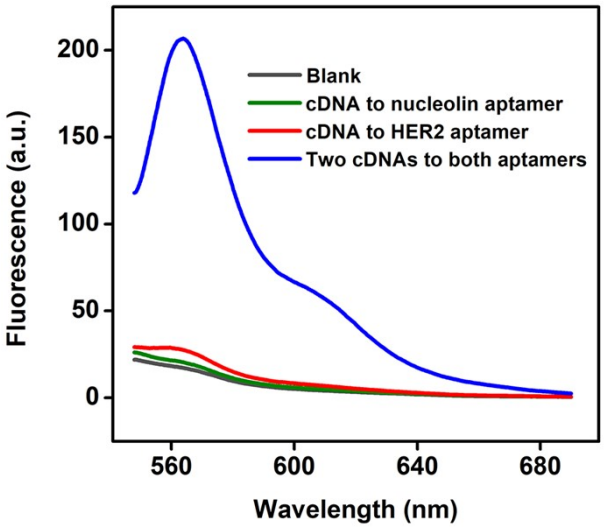

Fig. S6 Fluorescence emission spectra of the DNA tetrahedron dimer in the absence of complementary DNA to aptamers, in the presence of complementary DNA to nucleolin aptamer, in the presence of complementary DNA to HER2 aptamer, and in the presence of two complementary DNA to both aptamers.

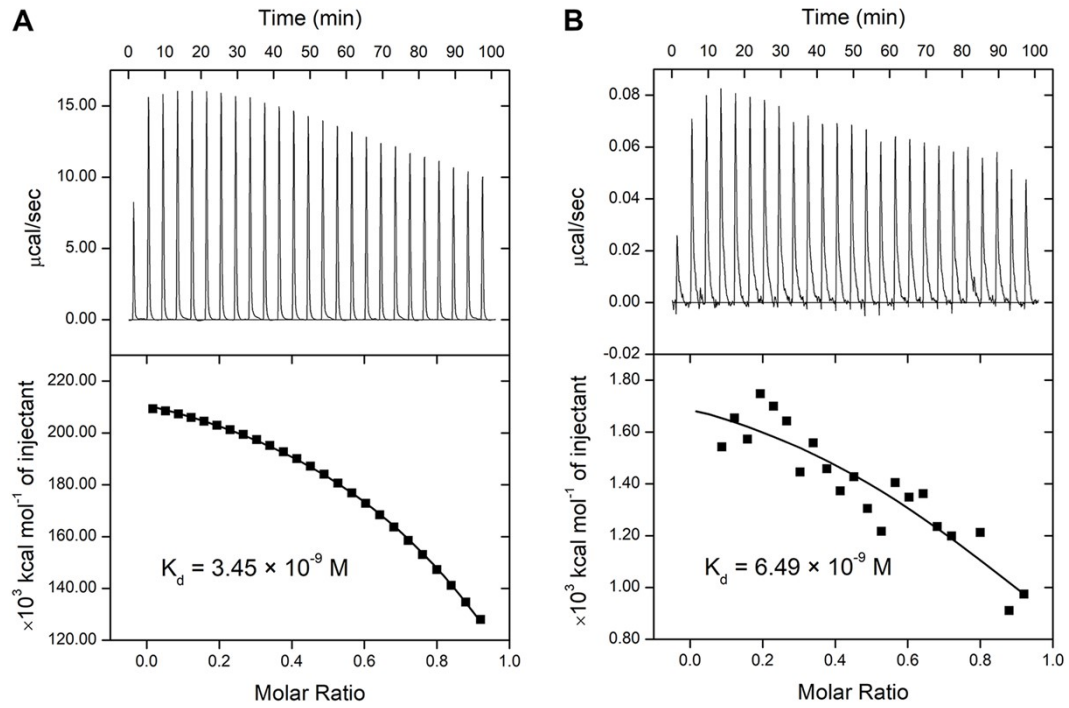

Fig. S7 Isothermal titration calorimetry (ITC) data for injection of HER2 protein to (A) HER aptamer and (B) HER2 aptamer/BH duplex solution.

As shown in Figure S7, it can be observed that the gradual binding of HER2 aptamer or HER2 aptamer/BH duplex to HER2 with each injection. By fitting to single-site binding model, it is obtained that the equilibrium dissociation constants (K_d) for HER2 aptamer binding to HER2 is 3.45×10^{-9} M, while K_d for HER2 aptamer/BH duplex binding to HER2 is 6.49×10^{-9} M. It is shown that the binding affinity is reduced to half when the aptamer is blocked with a partially complementary sequence. The blocking of BH to aptamer inhibits the direct binding to HER2, causing some thermodynamical dots fluctuation. Also, in comparison with HER2 aptamer, the time returning to baseline is retarded for HER2 aptamer/BH duplex, indicating a slower binding kinetics of HER2 aptamer/BH duplex to HER2. Although the binding affinity is reduced and the binding kinetics is slowed down, the aptamer can still bind to target protein, releasing the blocking complementary strand.

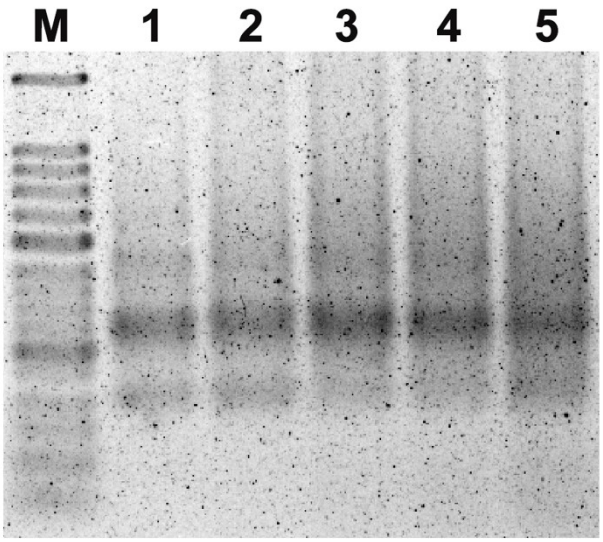

Fig. S8 Stability test of 200 nM DNA tetrahedron dimer in DMEM medium with 10% fetal bovine serum. Lane M: DNA marker; lane 1: incubation of 0 h; lane 2: incubation of 2 h; lane 3: incubation of 4 h; lane 4: incubation of 6 h; lane 5: incubation of 8 h.

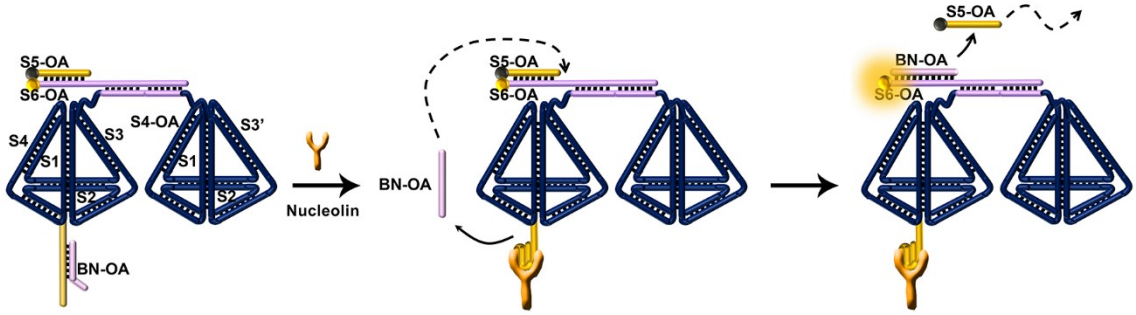

Fig. S9 Schematic illustration of the DNA tetrahedron containing only one aptamer for nucleolin recognition.

For the control DNA tetrahedron dimer containing only nucleolin-binding aptamer, no matter whether HER2 is expressed or not, in the presence of nucleolin, the nucleolin aptamer extended on tetrahedron N will bind nucleolin and repel BN-OA to induce dehybridization. The dissociated BN-OA then invades S6-OA via the exposed toehold of unpaired bases, hybridizing with S6-OA to displace S5-OA. The fluorophore of Cy3 is far away from the quencher of BHQ2 with fluorescence restoration.

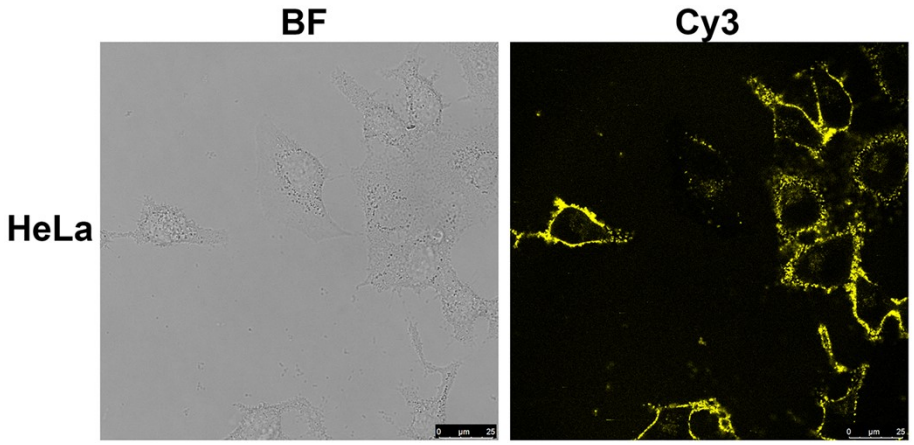

Fig. S10 Confocal fluorescence image of incubation the DNA tetrahedron dimer containing only nucleolin-binding aptamer with HeLa cells.

#	Sample	Gate	Count	%E1	Mean X	CV X
1	HL-7702	E1	9,617	100.00%	6,401	37.11%
2	HeLa	E1*	9,662	100.00%	8,061	44.04%
3	SK-BR-3	E1*	9,144	100.00%	51,635	61.86%

Fig. S11 Quantitative data of flow cytometry analysis provided by NovoCyte 3130 flow cytometer software.

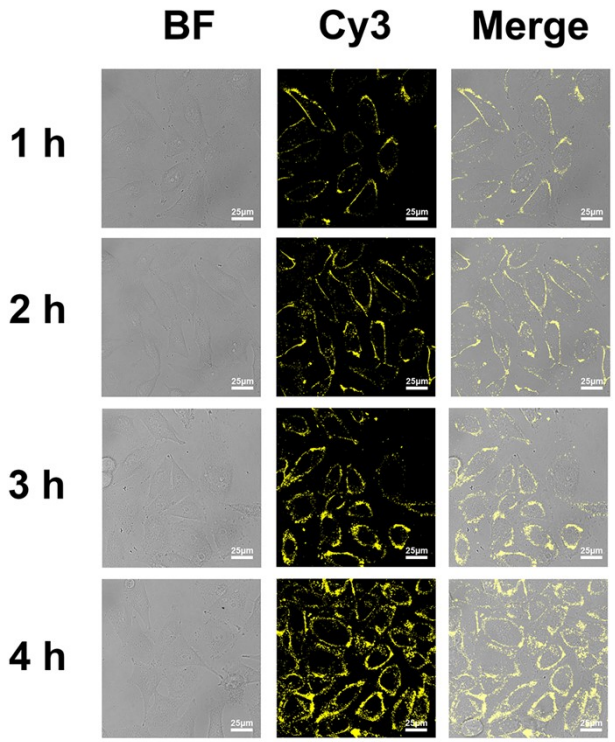

Fig. S12 Confocal fluorescence images collected at different time intervals after incubation of 200 nM DNA tetrahedron dimer (200 nM) with SK-BR-3 cells.

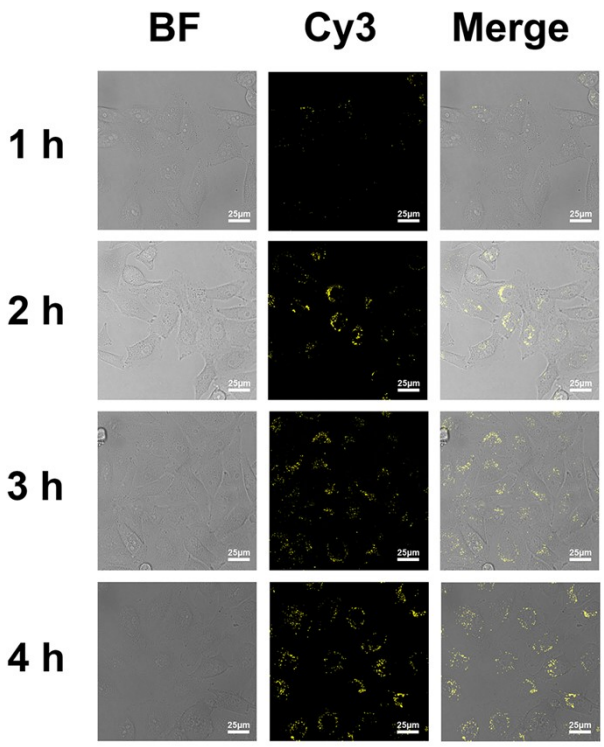

Fig. S13 Confocal fluorescence images collected at different time intervals after incubation of 200 nM DNA tetrahedron monomer that extends a blocked nucleolin-binding aptamer (fluorescently labeled tetrahedron-N) with SK-BR-3 cells.

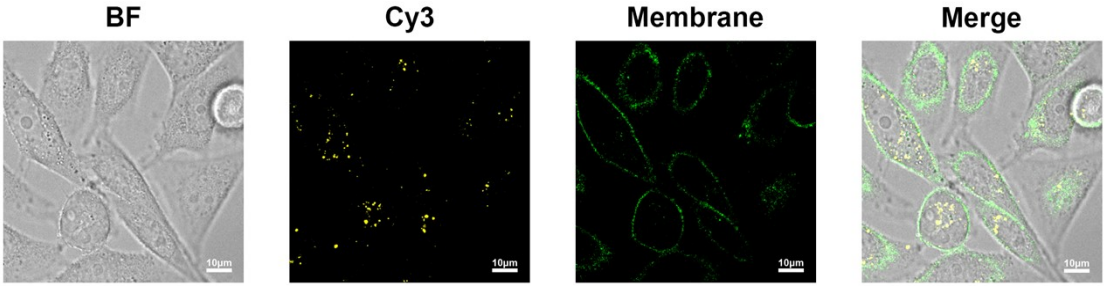

Fig. S14 Confocal fluorescence images of the cell membrane-stained SK-BR-3 cells treated with 200 nM DNA tetrahedron monomer.

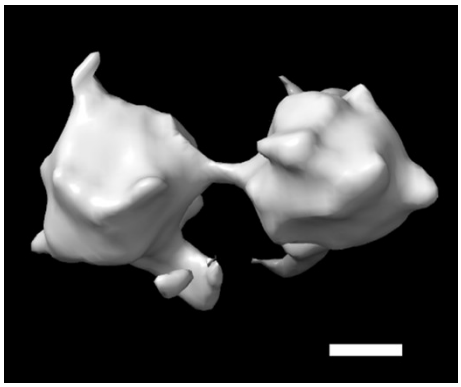

Fig. S15 Cryo-electron microscopy of the DNA tetrahedron dimer. Scale bar: 4 nm.

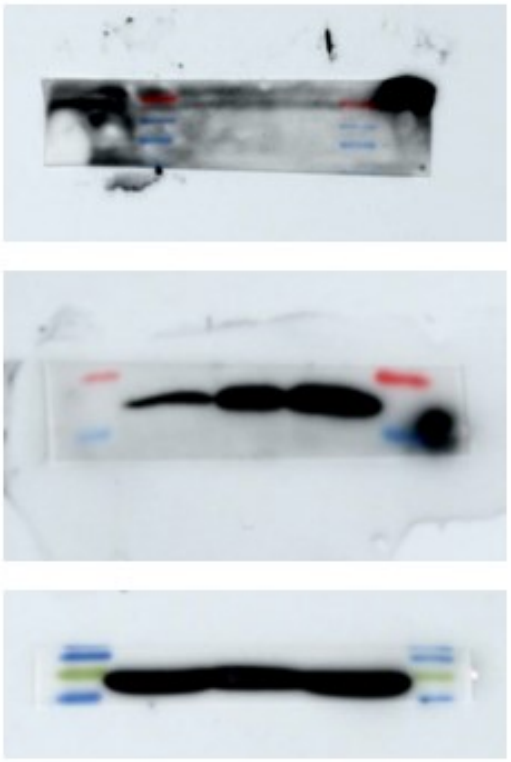

Fig. S16 Raw data images for the western blot experiments. To save the high cost of primary antibodies and secondary antibodies, the whole membrane was first cut according to the molecular weight of colored protein markers that indicate specific proteins (Biji T. Kurien, Western Blotting for the Non-Expert, Springer Nature Switzerland AG 2021). Then three cut membranes were respectively incubated with corresponding primary antibodies and secondary antibody in small containers containing small volume of antibodies, which were then visualized through enhanced chemiluminescence imaging.

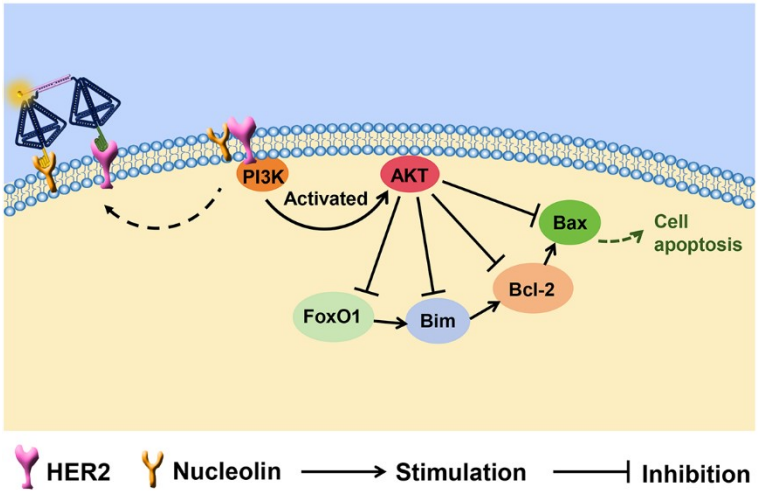

Fig. S17 Schematic illustration of the relationship of HER2 and Bax in signal of apotosis process.

As shown in Fig. S17, when dimerized with nucleolin, HER2 acts as a receptor tyrosine kinase and activates downstream PI3K (intracellular phosphatidylinositol kinase), which phosphorylates PIP3 (inositol 3,4,5 trisphosphate) and recruits Akt to the cytoplasmic membrane, thereby activating the Akt proteins.[1] Akt proteins are inhibitory to the apoptosis-related proteins FoxO1, Bim, Bcl-2 and Bax.[2] Therefore, when HER2 is isolated from nucleolin by the DNA tetrahedron dimer, the above-mentioned downstream pathway activity is inhibited, the amount of apoptosis-related proteins, especially Bax, show an increased expression level.

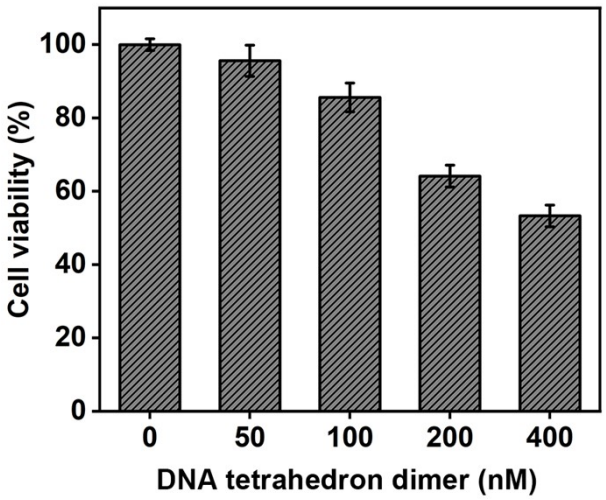

Fig. S18 Cell viability assay for SK-BR-3 cells treated with different concentrations of DNA tetrahedron dimer.

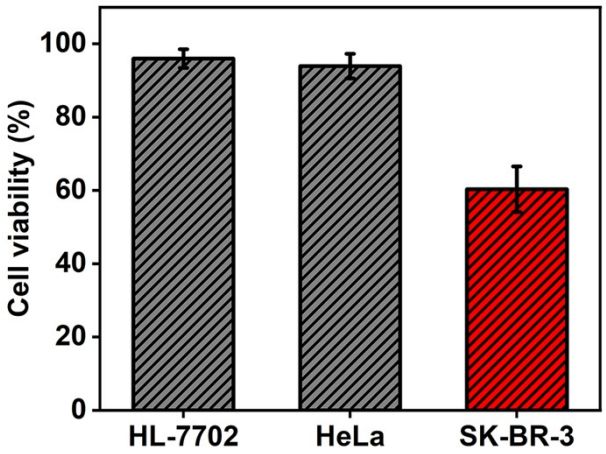

Fig. S19 Cell viability assay for different types of cells treated with 200 nM DNA tetrahedron dimer.

References:

1. A. R. Rasti, A. Guimaraes-Young, F. Datko, V. F. Borges, D. L. Aisner, E. Shagisultanova, *JCO Precis. Oncol.*, 2022, **6**, e2100370.
2. M. Sadidi, S. I. Lentz, E. L. Feldman, *Biochimie*, 2009, **91**, 577-585.