Electronic Supplementary Information (ESI)

DNA–affibody nanoparticles for inhibiting breast cancer cells overexpressing HER2

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Experimental Section

Chemicals and Apparatus

All chemicals were purchased and used without further purification. The DNAs were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). N^{ε}-malemidocaproyloxysuccinimide ester was obtained from AstaTech Inc. (Bristol, PA). Doxorubicin hydrochloride was purchased from Oakwood Products Inc. (Columbia, SC). Trastuzmab was obtained from BioVision Inc. (Milpitas, CA). Ni-NTA agarose was obtained from QIAGEN Inc. (Valencia, CA). Sephadex G-25, imidazole, sodium chloride, sodium acetate, polyacrylamide, trizma base, acetic acid, ethylenediaminetetraacetic acid (EDTA), magnesium chloride and ethanol were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Amicon® ultra centrifugal filters were purchased from Merck Millipore Ltd. (Darmstadt, Germany). Gibco® RPMI 1640 medium, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). antibotic−antimycotic (100×), fetal bovine serum (FBS), and DAPI were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

UV spectral measurements were made using an Agilent Technologies Cary 60 UV/Vis spectrometer. DNA gels were imaged using a VWR UV-transilluminator-20 gel imager. The nanoparticles were scanned using a Bruker Dimension FastScan atomic force microscopy. The surface zeta potential was measured using Malvern Zetasizer 3600 instrument. The fluorescence spectra were measured using a Varian Cary Eclipse Fluorescence Spetrophotometer.

Preparation of DNA–affibody

The sequences of the four single-strand DNA were as follows:

DNA1: 5′-NH_2-AGG CAG TTG AGA CGA ACA TTC CTA AGT CTG AAA TTT ATC ACC CGC CAT AGT AGA CGT ATC ACC-3′;

DNA2: 5′-NH_2-CCT CGC ATG ACT CAA CTG CCT GGT GAT ACG AGG ATG GGC ATG CTC TTC CCG ACG GTA TTG GAC-3′;

DNA3: 5′-CTT GCT ACA CGA TTC AGA CTT AGG AAT GTT CGA CAT GCG AGG GTC CAA TAC CGA CGA TTA CAG-3′;
DNA4: 5′-GGT GAT AAAACG TGT AGC AAG CTG TAA TCG ACG GGA AGA GCA TGC CCA TCC ACT ACT ATG GCG-3′.

The sequence of the affibody used in this study was MIHHHHHHLQVDNKFNKEMRNAYWEIALLPNLNNQQKRAFIRSLYDDPSQSANLLAEA KKLNDAQAPKVDC. The affibody was expressed in *E. coli* cells and purified using a Ni-NTA column.

DNA_1 or DNA_2 (200 µg, 10.3 nmol) was dissolved in 160 µL of phosphate-buffered saline (PBS, 10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl) and treated with 40 µL of 10 mM N^{ε}-malemidocaproyloxysuccinimide ester (EMCS) in dimethyl sulfoxide. The reaction mixture was incubated at room temperature for 3 h and stopped by the addition of 20 µL of 3M NaOAc. After the addition of 600 µL of ethanol and incubation at 4 °C for 30 min, the reaction mixture was centrifuged at 15000g for 30 min. After washing with 70% ethanol, the DNA was dissolved in 50 µL of PBS buffer and treated with 300 µg (38.1 nmol) of affibody in 300 µL of PBS buffer. After incubation at room temperature for 1 – 5 h, the reaction mixture was purified on a DEAE-Sepharose column (1 × 0.7 cm). The column was eluted with PBS buffer containing 0.2 – 0.9 M NaCl. The purified DNA–affibody chimera was analyzed by 8% denaturing polyacrylamide gel electrophoresis (PAGE). The gel was run at 110 V for 1 h, and stained with ethidium bromide.
The elution from the previous step was continued by purification on a Ni-NTA chromatography column. The elution solution (900 µL) was loaded on a column containing 100 µL of Ni-NTA resin. Then the column was washed five times with 100 µL of 50 mM Tris·HCl, pH 8.0, containing 300 mM NaCl and 10 mM imidazole. Finally, the Ni-NTA column was eluted three times with 100 µL of 50 mM Tris·HCl, pH 8.0, containing 300 mM NaCl and 150 mM imidazole. Aliquots of each fraction were analyzed by 15% SDS-PAGE. The affibody**–**DNAs so obtained were concentrated using Amicon® ultracentrifugal filters (MW cutoff 10 kDa).

Preparation of DNA tetrahedron–affibody nanoparticle

DNA_1–affibody (10.0 nmol), DNA_2–affibody (10.0 nmol), DNA_3 (10.0 nmol), and DNA_4 (10.0 nmol) were added 8 mL of 10 mM Tris·HCl, pH 8.0, containing 12 mM $MgCl_2$. The reaction mixture was incubated at 70 °C for 10 min then cooled to room temperature over a period of 30 min. The obtained DNA tetrahedron–affibody nanoparticle (95.4 kDa) was analyzed by 5% native polyacrylamide gel electrophoresis (PAGE). The gel was run at 110 V for 1 h, and stained with ethidium bromide.

Preparation of DNA tetrahedron–affibody–doxorubicin nanoparticle

The DNA tetrahedron–affibody nanoparticle prepared in the previous step was concentrated using Amicon® ultra centrifugal filters (MW cutoff 50 kDa). The concentrated DNA tetrahedron–affibody nanoparticle (5 μM) in 100 μL of 10 mM Tris·HCl, pH 8.0, containing 12 mM $MgCl_2$ was treated with 5 μL of 10 mM doxorubicin and incubated at room temperature for 10 min. Excess DOX was removed on a Sephadex G-25 column.

Atomic force microscopy (AFM) characterization

For DNA tetrahedron–affibody nanoparticle imaging, 10-μL samples (10 nM) were deposited onto a freshly peeled mica surface for 2 min. Next, 10 μL of 100 mM $NiCl_2$ solution was added to assist adsorption. Finally, 55 μL of TAE/Mg^{2+} buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, 12 mM $MgCl_2$, pH 8.0) was added onto the mica and another 55 μL of TAE/Mg^{2+} buffer was added on the atomic force microscope (AFM) tip. The samples were imaged in ScanAsyst in Fluid mode (with a ScanAsyst-liquid+ tip) with Dimension FastScan AFM (Bruker).

Quantification of the DOX/DNA ratio in the DNA tetrahedron–DOX nanoparticle.

The UV absorption of the DNA tetrahedron (0.5 μM) was measured in the range 220 – 600 nm in a solution of 10 mM Tris-HCl, pH 8.0, containing 12 mM $MgCl_2$. Then different amounts of DOX (5 – 30 μM) were added to the solution, and the UV absorption was measured. Finally, excess DOX was removed using a Sephadex G-25 column, and the purified DNA tetrahedron–DOX nanoparticle was measured. A standard curve between the A_{505}/A_{260} (Y axis) and the ratio of DOX/tetrahedron (X axis)

was prepared. The amount of DOX binding to the DNA tetrahedron in the detection sample was calculated using the equation y = 0.0026x + 0.0082.

Measurement of surface zeta potential

The surface zeta potential of the DNA tetrahedron–affibody nanoparticle and DNA tetrahedron–affibody–DOX nanoparticle was measured in the range -140 – 140 mV in a solution of 10 mM Tris-HCl, pH 8.0, containing 12 mM $MgCl_2$. Each sample (3 μM) was scaned for 100 times.

Release assay of doxorubicin

Three samples in 1 mL volumn were parepared as following: sample 1, 300 μM DOX in 10 mM Tris-HCl, pH 8.0, containing 12 mM $MgCl_2$; sample 2, 6 μM DNA tetrahedron–affibody–DOX (containing 300 μM DOX) in 10 mM Tris-HCl, pH 8.0, containing 12 mM $MgCl_2$; sample 3, 6 μM DNA tetrahedron–affibody–DOX (containing 300 μM DOX) and 600 units of DNase I in 10 mM Tris-HCl, pH 8.0, containing 12 mM $MgCl_2$ and 1 mM $CaCl_2$. Each sample was transferred into a dialysis tube (1 mL, MW cutoff 10 kDa), which was floated in 100 mL 1 x phosphate-buffered saline (PBS, pH 7.4) and continuously stirred at room temperature. At the determined times, 100 μL of PBS buffer was taken out and the fluorescent intensity was measured. The fluorescence spectra of DOX were measured using a Varian Cary Eclipse Fluorescence Spetrophotometer with the excitation slit as 10 nm and emission slit as 10 nm. The samples were excited at 490 nm, and the emission spectra were recorded at the range of 510 – 700 nm.

DNA stability assay in fetal bovine serum

Three samples in 100 μL of 10 mM Tris-HCl, pH 8.0, containing 12 mM $MgCl_2$ were parepared as following: sample 1, 24 μM single strand DNA_1; sample 2, 6 μM DNA tetrahedron–affibody; sample 3, 6 μM DNA tetrahedron–affibody–DOX (containing 300 μM DOX). Each sample was added into 100 μL of fetal bovine serum and incubated at 37 ^oC. At the determined times, 5 μL of reaction mixture was taken out and added into 5 μL of loading buffer (formamide containing 100 mM EDTA, 80 ^oC). The reaction mixture

was analyzed by 15% denaturing polyacrylamide gel (7 M urea). After electrophoresis in 89 mM Tris buffer, pH 8.0, containing 89 mM boric acid and 2 mM EDTA at 100 V for 1 h, the gel was stained with ethidium bromide for 30 min and visualized using UV light. The extent of reaction (expressed as the percentage of DNA cleavage) was quantified by utilizing ImageQuant version 5.2 software. The cleavage rate constants (k_{cl}) were determined by fitting the data to the equation [100 – % cleavage] = $100e^{-kt}$.

Biological activity of nanoparticles

BT474 breast cancer cells (ATCC® HTB-20, overexpression of HER2) and MDA-MB-231 breast cancer cells (ATCC® HTB-26, low expression of HER2 receptor) were cultured at 37 °C in a 5% CO_2 atmosphere and grown in Gibco® RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibotic-antimycotic mix antibiotic supplement before use.

Exponentially growing BT474 cells and MDA-MB-231 cells were harvested and plated in 96-well plates at a concentration of 2×10^4 cells/well for BT474 cells and 5×10^4 cells/well for MDA-MB231 cells. After incubation at 37 °C for 24 h, the cells were treated with trastuzumab, DNA tetrahedron–affibody **III** and DNA tetrahedron–affibody–doxorubicin nanoparticle **IV** (1:50 **III–**doxorubicin) at different concentrations for an additional 48, 72 or 96 h. Then 20 µL of MTT (5 mg/mL) was added to each well and the plates were incubated at 37 °C for 4 h. The supernatant was discarded, and 100 µL of DMSO was added to each well. The absorbance was recorded at 490 nm after 15 min. Inhibition of cell growth was obtained by the following formula: Inhibition of cell growth (%) = $(OD_{negative\ control} - OD_{treatment}) \times 100\% / (OD_{negative\ control} - OD_{background})$. Data are reported as the mean of three independent experiments, each run in quintuplicate.

HER2 binding assay of BT474 and MDA-MB-231 Cells

BT474 cells and MDA-MB-231 cells were grown on glass bottom microwell disks at a cell density of 10,000 cells/well at 37 °C for 48 h. When the cell confluency reached about 70%, the cells were treated with doxorubicin and DNA tetrahedron–affibody–doxorubicin nanoparticle **IV** (1:50 **III**–doxorubicin) at 1 µM concentration for 1 h. Then the cells were stained using 2.5 µg/mL DAPI (Invitrogen) for 30 min after the cells were

rinsed with phosphate buffered saline (PBS) for two times. Finally, the cells were fixed with 4% paraformaldehyde for 10 min. The fluorescent images were obtained using a fluorescence microscope (Nikon Ti-U, Japan) with red and green filters. Thereafter, all images were recorded and the target cells counted using a 40× oil objective. To ensure accurate intensity measurements, the exposure time (3000 ms) and laser time were kept the same. The mean pixel intensity within the region of interest was calculated. Data are reported as the mean of three independent experiments, each run in quintuplicate. The data was expressed as mean ± SD.

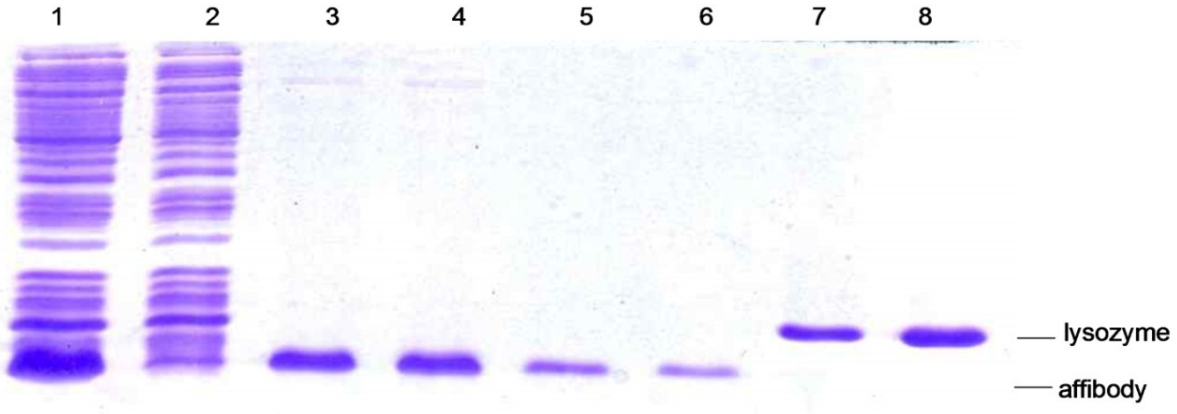

Fig. S1. Purification of affibody using a Ni-NTA column. The results were analyzed on a 15% SDS-PAGE gel followed by Coomassie Brilliant Blue R-250 staining. Lane 1, crude lysate of *E. coli* expression; lane 2, flow through from Ni-NTA column; lane 3, elution with 150 mM imidazole; lane 4, second elution with 150 mM imidazole; lane 5, third elution with 150 mM imidazole; lane 6, fourth elution with 150 mM imidazole; lane 7, 1 μg of lysozyme; lane 8, 2 μg of lysozyme.

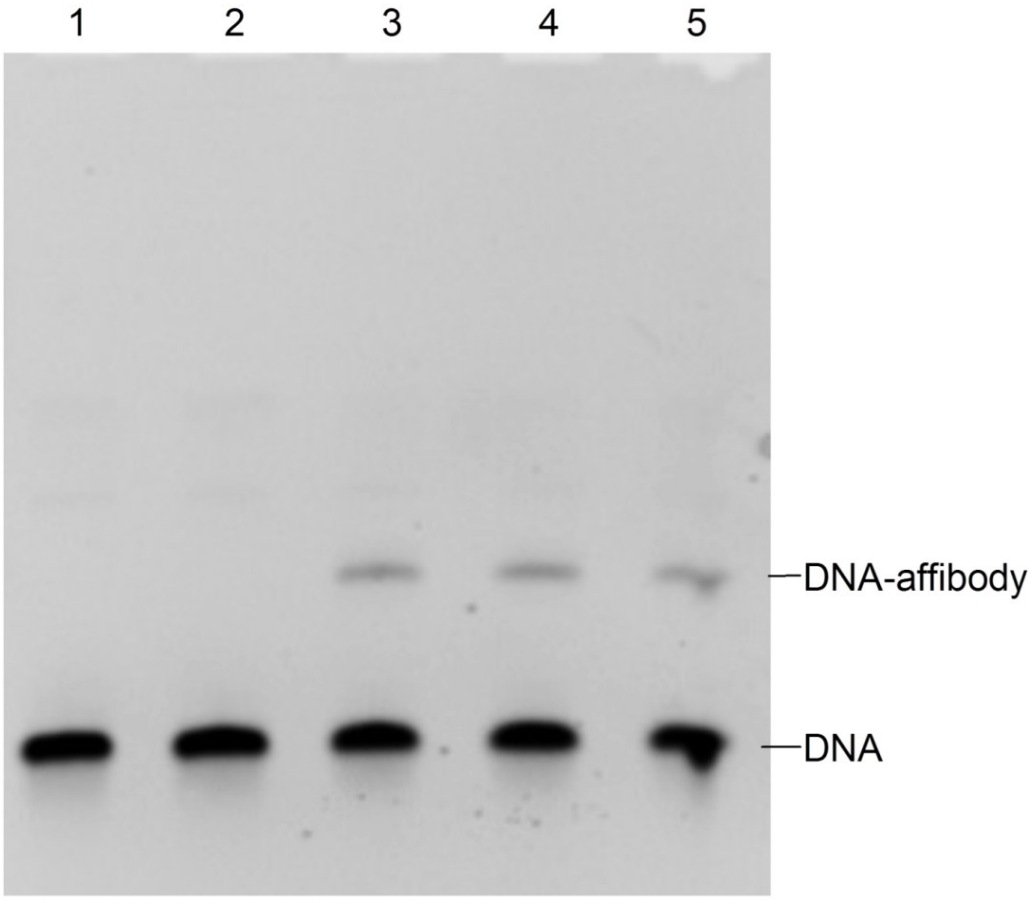

Fig. S2. Coupling reaction of the affibody with EMCS-linked DNA. The results were analyzed on a 15% denaturing polyacrylamide gel (7 M urea) followed by ethidium bromide staining. Lane 1, DNA_1; lane 2, EMCS-DNA_1; lane 3, EMCS-DNA_1 treated with affibody for 1 h; lane 4, EMCS-DNA_1 treated with affibody for 3 h; lane 5, EMCS-DNA_1 treated with affibody for 5 h.

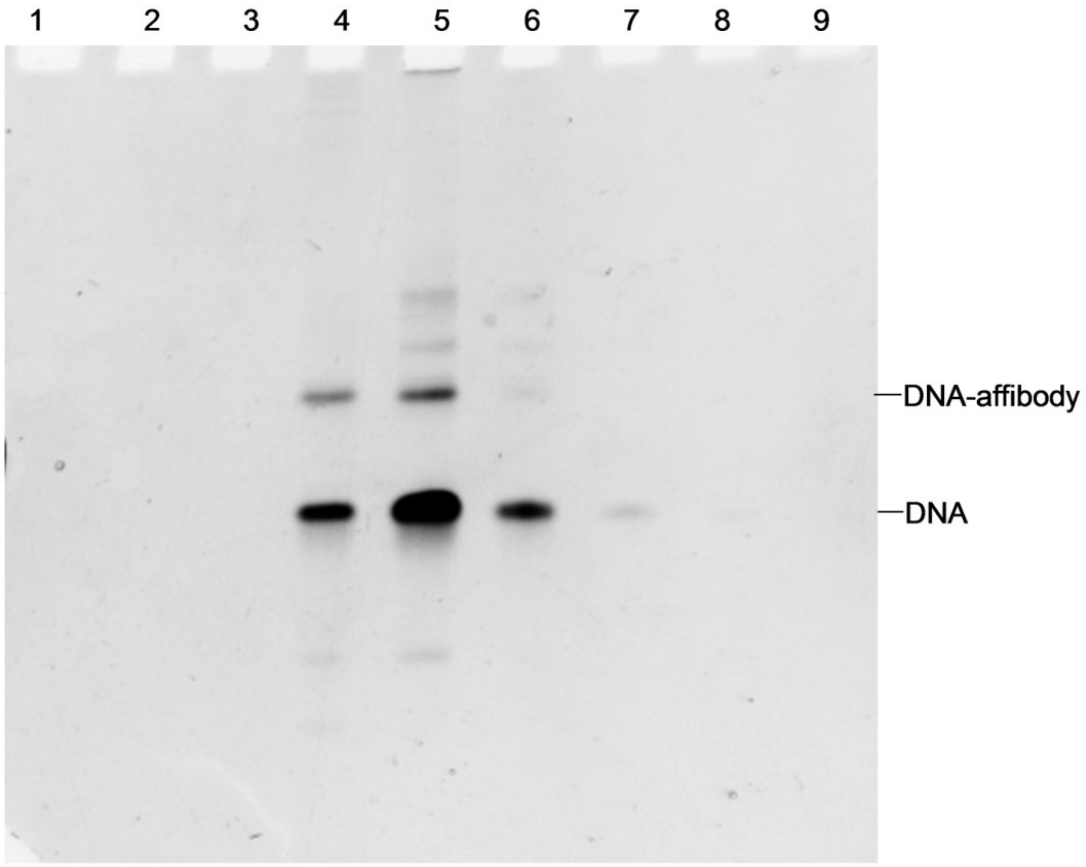

Fig. S3. Purification of DNA–affibody using a DEAE-Sepharose CL-6B column. The results were analyzed on a 15% denaturing polyacrylamide gel (7 M urea) followed by ethidium bromide staining. Lane 1, flow through from the DEAE-Sepharose CL-6B column; lane 2, elution with 200 mM NaCl; lane 3, elution with 300 mM NaCl; lane 4, elution with 400 mM NaCl; lane 5, elution with 500 mM NaCl; lane 6, elution with 600 mM NaCl; lane 7, elution with 700 mM NaCl; lane 8, elution with 800 mM NaCl; lane 9, elution with 900 mM NaCl.

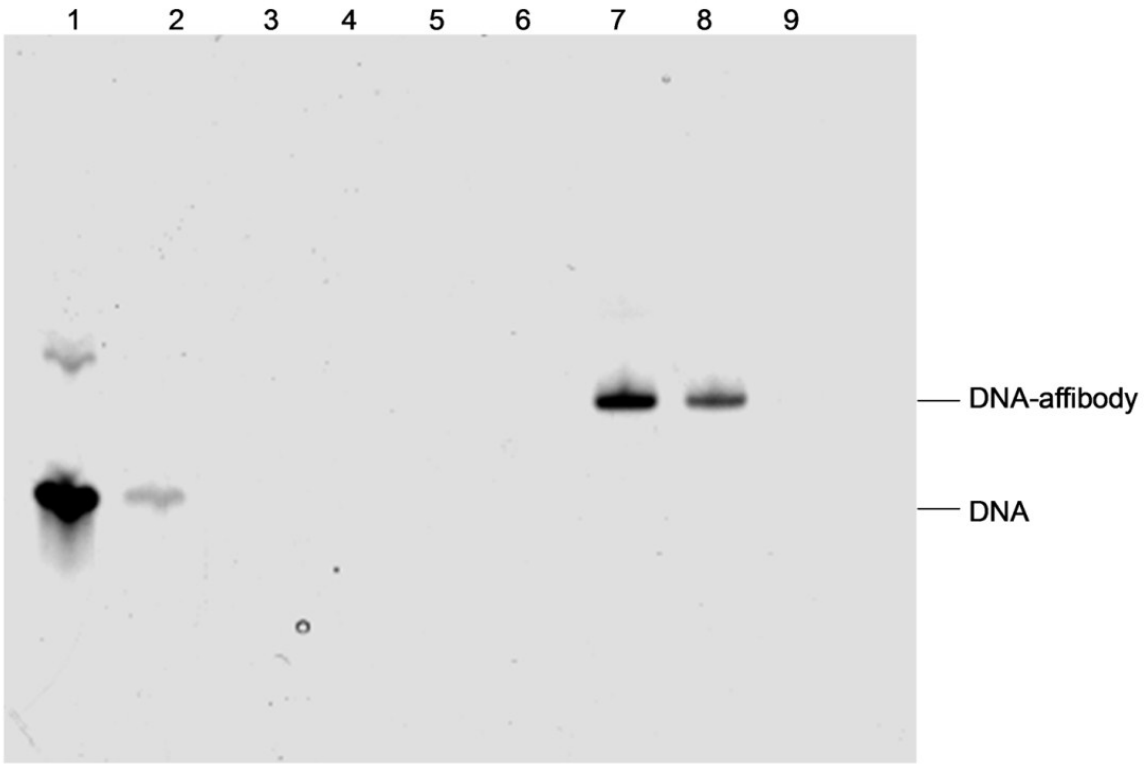

Fig. S4. Purification of DNA–affibody using a Ni-NTA coloumn. The results were analyzed on a 15% denaturing polyacrylamide gel (7 M urea) followed by ethidium bromide staining. Lane 1, flow through from Ni-NTA column; lane 2, first washing with 10 mM imidazole; lane 3, second washing with 10 mM imidazole; lane 4, third washing with 10 mM imidazole; lane 5, fourth washing with 10 mM imidazole; lane 6, fifth washing with 10 mM imidazole; lane 7, first elution with 150 mM imidazole; lane 8, second elution with 150 mM imidazole; lane 9, third elution with 150 mM imidazole.

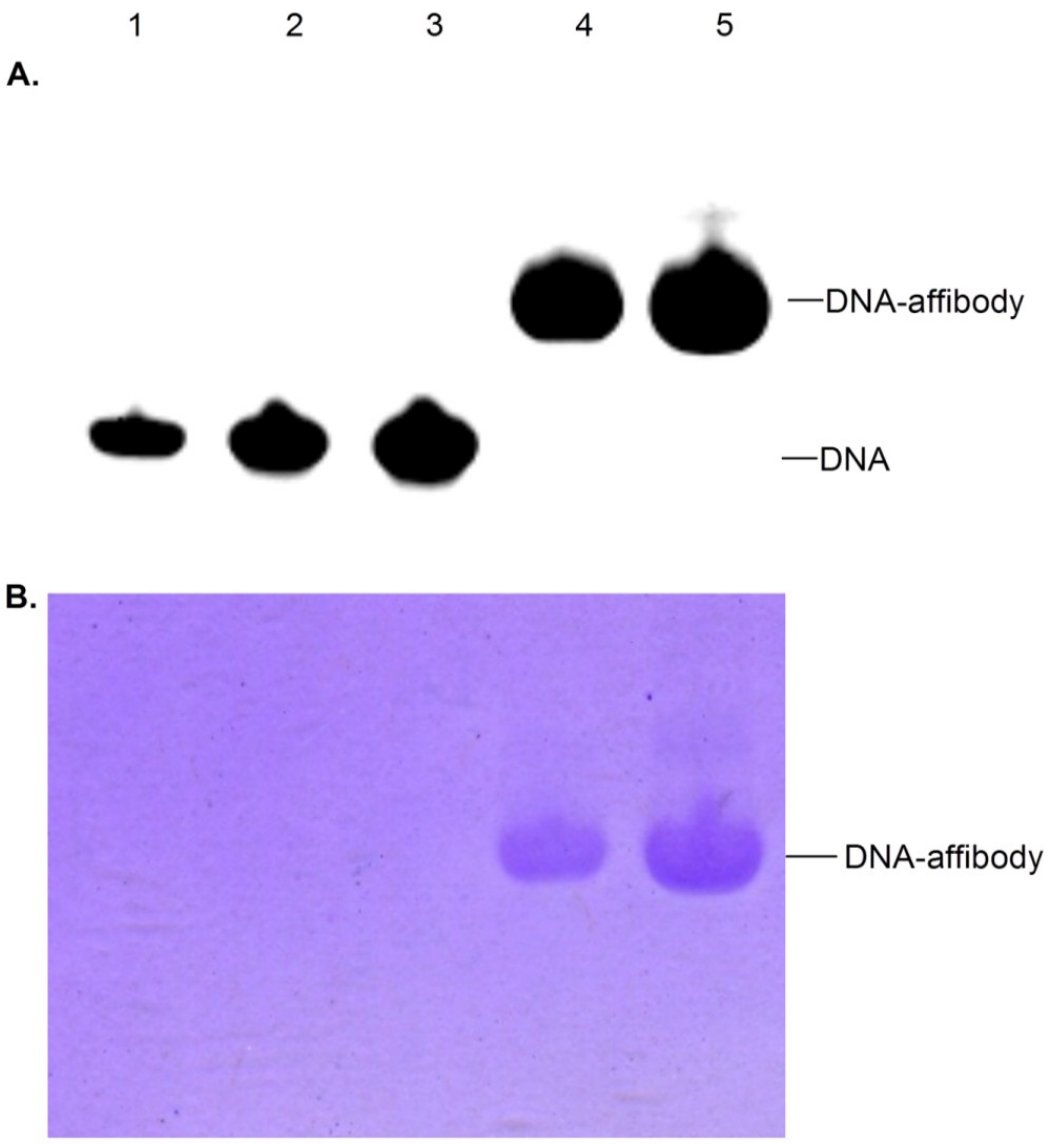

Fig. S5. Quantification of DNA–affibody. The results were analyzed on a 15% denaturing polyacrylamide gel (7 M urea). The gel was stained with ethidium bromide (**A**) followed by Coomassie Brilliant Blue R-250 (**B**). Lane 1, DNA_1 (1 μg); lane 2, DNA_1 (2 μg); lane 3, DNA_1 (3 μg); lane 4, DNA_1–affibody (4 μg); lane 5, DNA_2–affibody (6 μg).

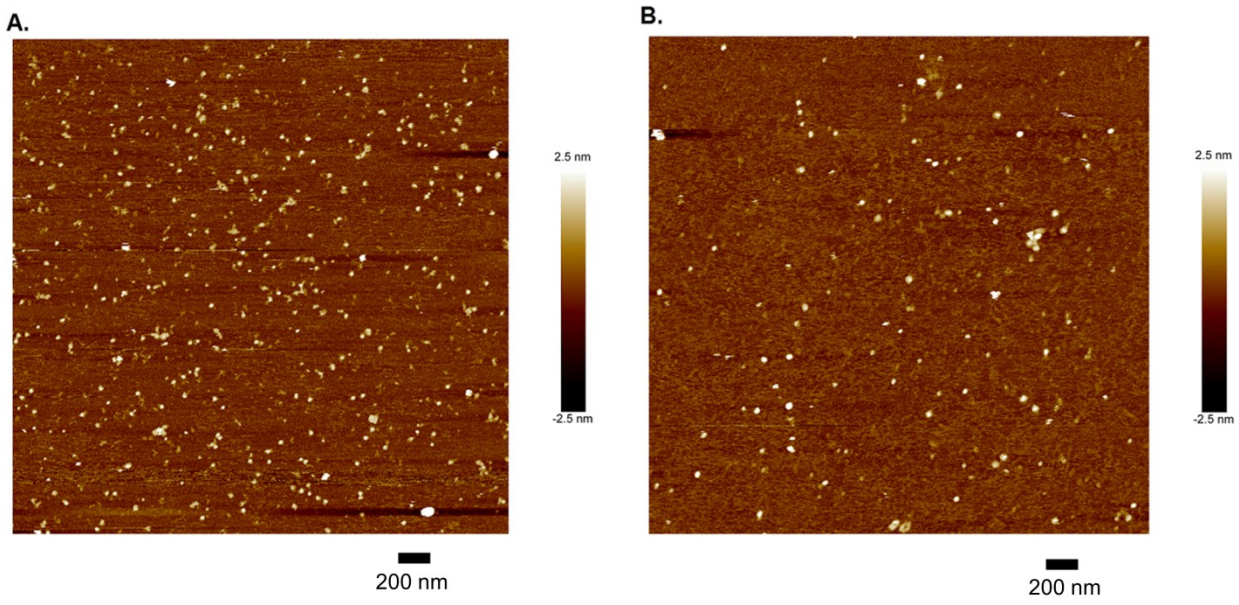

Fig. S6. AFM micrographs of nanoparticles. (**A**) Structure of DNA tetrahedron–affibody nanoparticle (**III**). (**B**) Structure of DNA tetrahedron–affibody–DOX nanoparticle (**IV**). Scale bars are 200 nm.

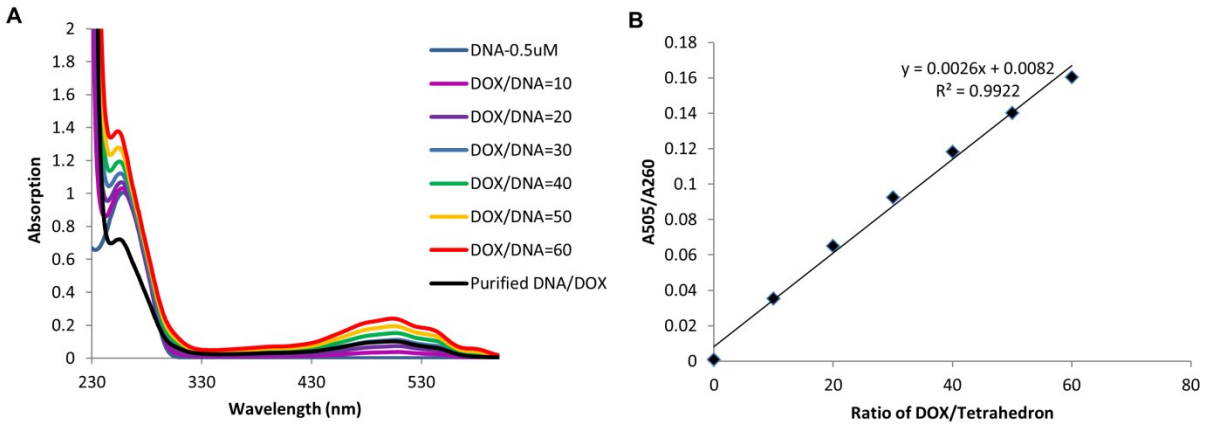

Fig. S7. Quantification of DOX/DNA ratio in the DNA tetrahedron–DOX nanoparticle. (**A**) UV absorption of DNA tetrahedron (0.5 μM) after adding different amounts of DOX (5 – 30 μM). The excess DOX was removed through a Sephadex G-25 column for the detection samples (black line). (**B**) The standard curve between the A_{505}/A_{260} (Y axis) and the ratio of DOX/DNA tetrahedron (X axis). The amount of DOX binding to tetrahedron in the detection sample was calculated using the equation in the panel **B**. The maximum ratio of DOX/DNA was 52.9 ± 2.1 based on triplicate assays.

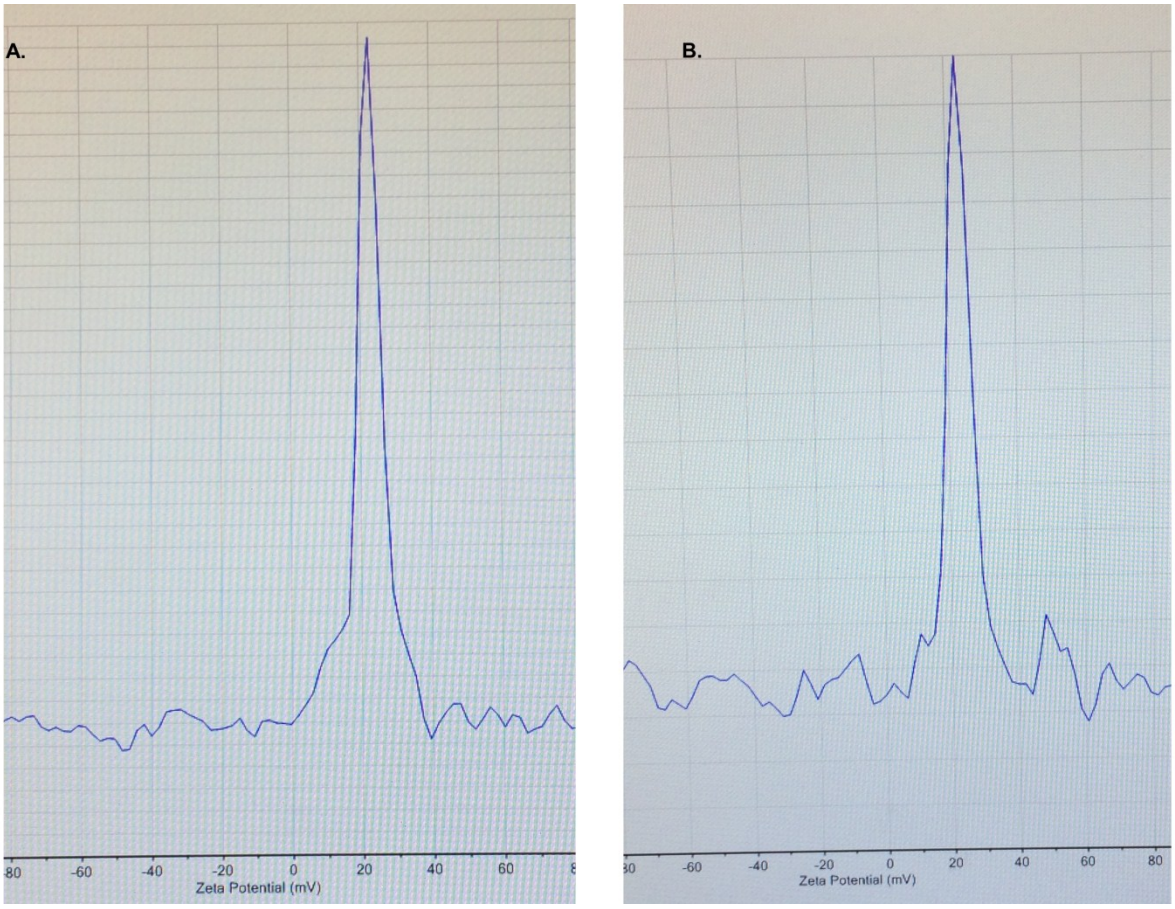

Fig. S8. The surface zeta potential of nanoparticles. (**A**) The surface zeta potential of DNA tetrahedron–affibody nanoparticle (**III**). (**B**) The surface zeta potential of DNA tetrahedron–affibody–DOX nanoparticle (**IV**).

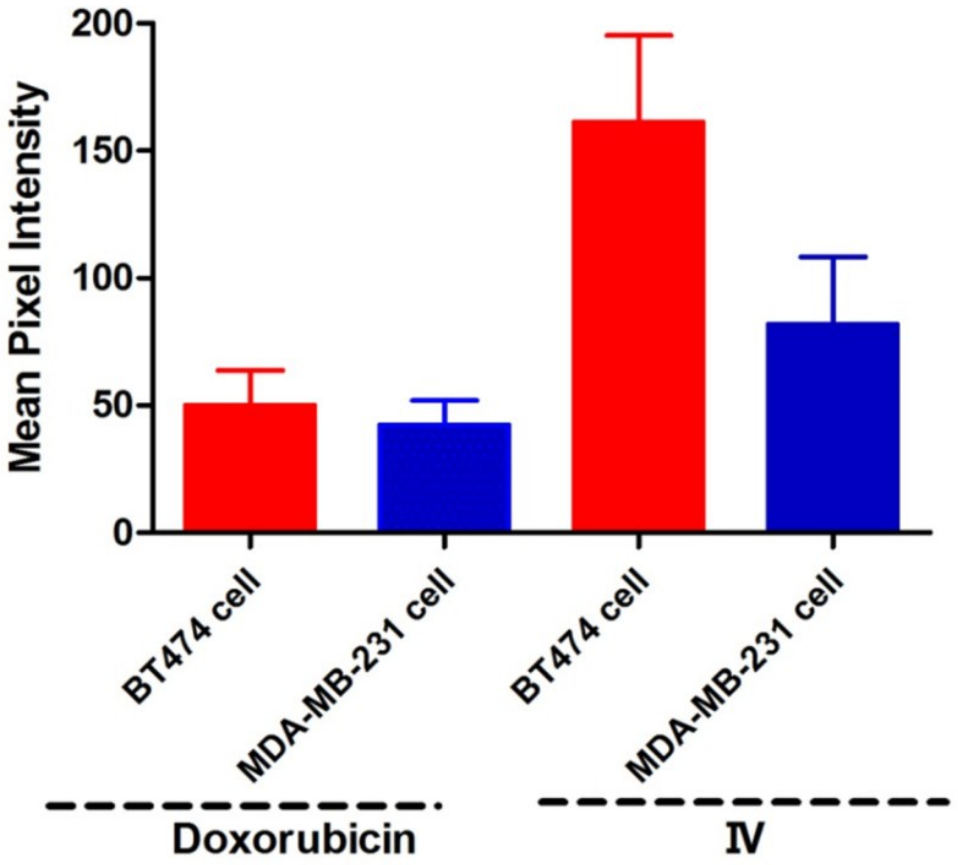

Fig. S9. Quantification of the fluorescence density of doxorubicin and DNA tetrahedron–affibody–doxorubicin nanoparticle on the surface of BT474 and MDA-MB-231 cells. The fluorescent images were obtained using a fluorescence microscope (Nikon Ti-U, Japan). The mean pixel intensity within the region of interest was calculated. The data was expressed as mean ± SD.

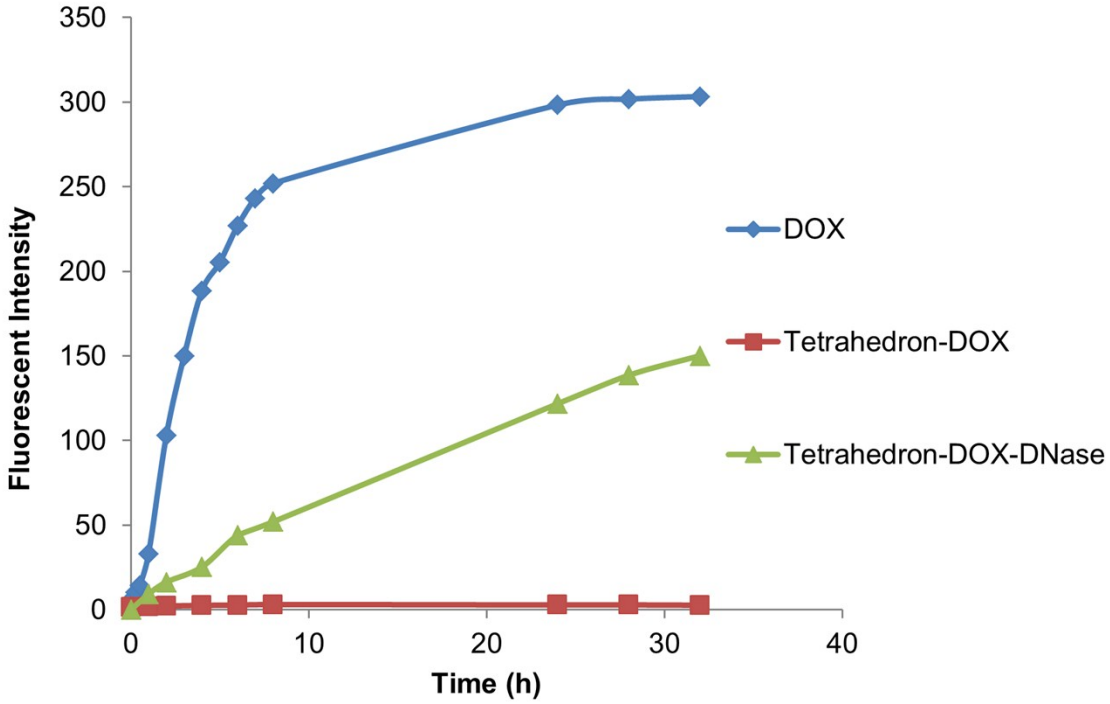

Fig. S10. Doxorubicin release assay from the DNA tetrahedron–affibody–DOX nanoparticle. The fluorescence spectra of DOX were measured using a Varian Cary Eclipse Fluorescence Spetrophotometer with the excitation slit as 10 nm and emission slit as 10 nm. The samples were excited at 490 nm, and the emission spectra were recorded at the range of 510 – 700 nm.

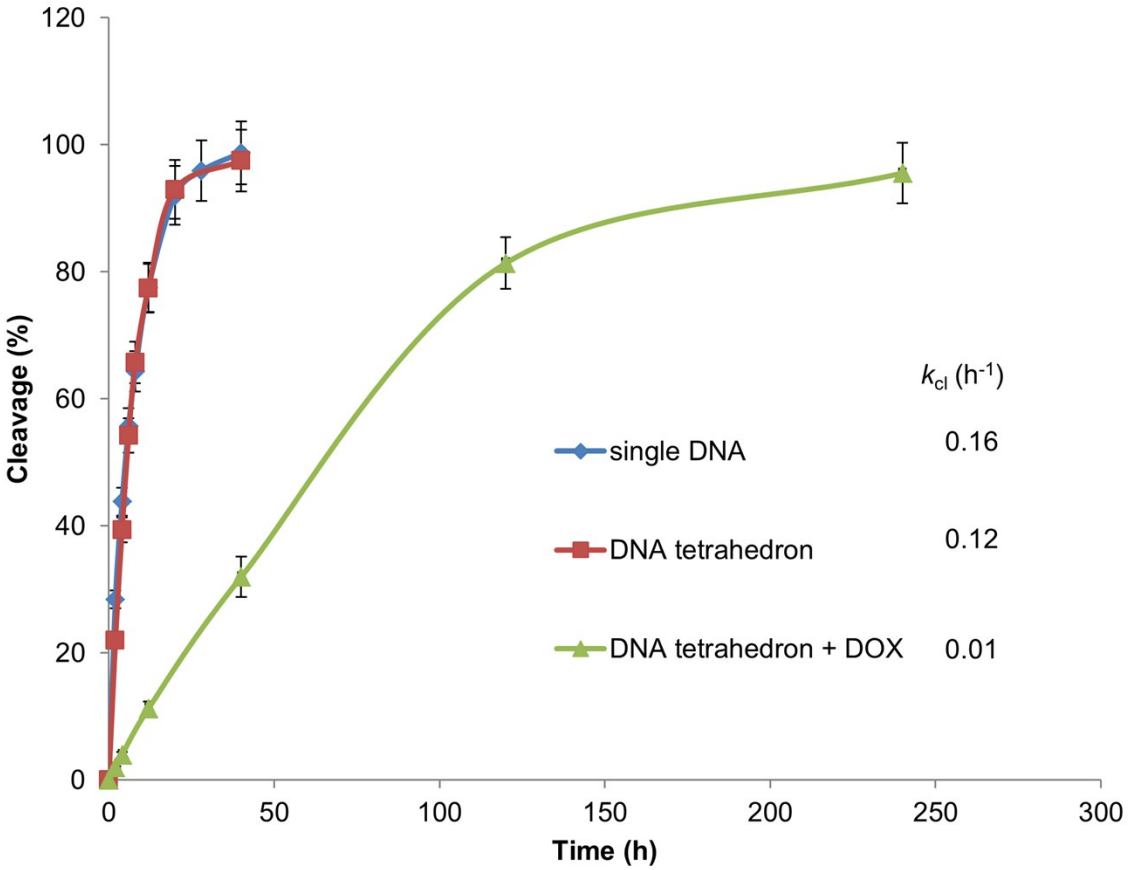

Fig. S11. The stability of single strand DNA and DNA tetrahedron nanoparticles in 50% fetal bovine serum. The reaction mixture was analyzed by 15% denaturing polyacrylamide gel (7 M urea). After electrophoresis in 89 mM Tris buffer, pH 8.0, containing 89 mM boric acid and 2 mM EDTA at 100 V for 1 h, the gel was stained with ethidium bromide for 30 min and visualized using UV light. The extent of reaction (expressed as the percentage of DNA cleavage) was quantified by utilizing ImageQuant version 5.2 software. The cleavage rate constants (k_{cl}) were determined by fitting the data to the equation [100 – % cleavage] = $100e^{-kt}$.

Table S1. Ratio of inhibition of breast cancer cell lines between DNA tetrahedron–affibody–doxorubicin (**IV**) and DOX. The concentrations of DOX in the nanoparticle **IV** were same to the free DOX.

Time (h)	DOX concentration (μM)	Ratio of inhibition (**IV**/DOX)	
		BT474 cells	MDA-MB-231 cells
48	0.16	2.6	0.42
	0.32	1.8	0.64
	0.64	1.1	0.50
72	0.16	2.1	0.97
	0.32	1.4	0.75
	0.64	1.1	0.49
96	0.16	1.9	0.75
	0.32	1.6	0.51
	0.64	1.1	0.61