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Supporting Information

High-order Framework Nucleic Acid for Targeted-delivery of Antisense Peptide Nucleic Acid to Overcome Drug Resistance

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Experimental section

Materials and reagents. Regular Fmoc-PNA monomers were purchased from PANAGENE Inc. (Daejeon, Korea). Fmoc-AEEA-OH, Fmoc-Cys(Trt)-OH, and Rink Amid MBHA resin were supplied by CSBio Ltd. (USA). 5(6)-Carboxyfluorescein (FAM) was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd (China). ZORBAX Eclipse XDB-C18 column and InfinityLab Porashell 120 EC-C18 column were provided by Agilent Technologies Inc. (USA). Yarra 3μm SEC-4000 column and SecurityGuard guard cartridge kit were bought from Phenomenex (USA). Regular DNA oligonucleotides were synthesized and purified by Shanghai Jie Li Biotechnology Co., Ltd. (China). Sulfhydryl DNA oligonucleotides were synthesized and purified by Huzhou Hippo Biotechnology Co., Ltd. (China). All DNA sequences used in this work were listed in Table S1. The Amicon ultra 3K Da, 30K Da and 100K Da centrifugal filters were purchased from Sigma-Aldrich Inc. (USA). 30% Acrylamide/Bisacrylamide (29:1) solution was supplied by Sangon Biotech (Shanghai) Co., Ltd. (China). Agarose was purchased from Bio-Rad Laboratories, Inc. (USA). GelRed gel stain solution was obtained from Biotium (USA). Doxorubicin (DOX), RPMI 1640 cell culture medium, and RIPA cell lysis solution were supplied from Beijing Solarbio Science & Technology Co., Ltd. (China). The Dox-resistant human MCF-7 breast cancer cell line (MCF-7ADR) was obtained from Shanghai Jinyuan Biotechnology Co., Ltd. (China). Fetal bovine serum was purchased from PAN Biotech (Germany). Hoechst 33342 was provided by Dojindo Laboratories (Japan). Anti-P glycoprotein primary antibody was supplied by Abcam (USA). Anti-Bcl-2 primary antibody was obtained from Cell Signaling Technology, Inc (USA). Anti-GAPDH primary antibody and HRP-labeled secondary antibody were purchased from Affinity Biosciences (USA). Ultra-sensitive chemiluminescent substrate kit and thiazolyl blue tetrazolium bromide power were purchased from Beyotime Biotechnology, Inc. (China). Other common chemical reagents were purchased from Sinopharm Chemical Reagent Co. Ltd. (China). All Reagents used for HPLC were of chromatographic grade and others were of analytic grade. All solutions were prepared using ultrapure water which was purified by a Millipore Milli-Q water system.

Synthesis and characterization of asPNA oligomers. The sequences of asPNA oligomers used in this study are shown in Table S2. asPNA oligomers were synthesized using Fmoc-solid phase synthesis protocols according to the previous report.[1] FAM was modified at the N-terminus as a fluorescent labeling. Cysteine was attatched to the C-terminus via a Fmoc-AEEA-OH linker, which was used to form a disulfide bond with mercapto-DNA. asPNA oligomers were cleaved from the resin using a cocktail solution containing a 94: 1: 2.5: 2.5 volume ratio of trifluoroacetic acid: triisopropylsilane: 1,2-Ethanedithiol: H_2O. The resulting mixture was precipitated with diethyl ether, purified by reverse-phase (RP)-HPLC, characterized by an AB Sciex LC-Q-TOF 4600 Mass Spectrometer, and freeze-dried. As shown in Fig. S1, the observed (Obs.) molecular weights of all four asPNA oligomers were in good agreement with the calculated (Cal.) molecular weights and all four asPNA oligomers showed a high purity after HPLC purification. All asPNA stock solutions were prepared using sterile deoxygenated

ultrapure water. The concentrations of asPNA oligomers were calculated using the following extinction coefficients: ε_{260}[A]=13.7 mL/(μmol×cm), ε_{260}[T]=8.6 mL/(μmol×cm), ε_{260}[C]=6.6 mL/(μmol×cm), ε_{260}[G]=11.7 mL/(μmol×cm) on a Cary 5000 UV-Vis-NIR spectrophotometer and normalized to 200 μM for future use.

Synthesis of DNA Linker-asPNAs. To synthesize DNA Linker-2-FAM-P-gp-asPNA conjugates, DNA linker-2 and FAM-P-gp-asPNA were mixed in the 30% DMSO aqueous solution at a 1:1 molar ratio and incubated overnight in the dark at 37 ℃. Then the reaction product was purified by RP-HPLC. DNA Linker-3-FAM-Bcl-2-asPNA was also synthesized and purified by the same method. The concentrations of purified DNA Linker-asPNAs were determined using the ssDNA mode on a DS-11 Spectrophotometer.

Preparation of tetrahedral framework nucleic acid monomers. Three tetrahedral framework nucleic acid monomers T_0, T_1, and T_2 were self-assembled from four component DNA oligonucleotides (sequences were listed in Table S1) through a one-step annealing process. Specifically, four equimolar (usually 1 μM) component DNA oligonucleotides were mixed in TM buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.0). The mixture was heated to 95 ℃ for 5 min in the IKA dry blocker heater I, then rapidly cooled to 4 ℃ in the refrigerator, and kept for 10 min. As-prepared framework nucleic acid monomers were purified by size exclusion chromatography (SEC)-HPLC[2] and quantified using the dsDNA mode in a DS-11 Spectrophotometer.

Preparation of G_1, G_2 and Apt-G_2-asPNAs. G_1, G_2, and apt-G_2-PNAs were prepared through one-pot hybridization method according to stoichiometric ratios of constituent units. Specifically, Apt-G_2-asPNAs were prepared by mixing T_0, T_1, T_2, Linker-1-MUC1 aptamer, Linker-2-P-gp-asPNA, and Linker-3-Bcl-2-asPNA with a molar ratio of 1:4:12:12:12:12 and incubated at 37 ℃ for 2 h. G_1 (T_0:T_1=1:4) and G_2 (T_0:T_1:T_2=1:4:12) were also prepared by a similar process. The concentrations of as-prepared high-order framework nucleic acid were calculated via the dsDNA mode on a DS-11 Spectrophotometer and increased by ultrafiltration concentration (100K Da Amicon centrifugal filters; 2500 g × 5 min). G_2 and G_2-asPNAs were also prepared by a similar one-pot method.

HPLC purification. All HPLC purifications were operated on an Agilent Technologies 1260 Infinity Ⅱ LC system. asPNA oligomers were purified by RP-HPLC. The column was Agilent ZORBAX Eclipse XDB-C18. The mobile phases A and B were 0.1% TFA in water and 90% acetonitrile in water, respectively. asPNA oligomers would be eluted at 20～25 min with the following settings (gradient elution): flow rate, 4 mL/min; 0~2 min at 0% B, 2~5 min linearly varying from 0% B to 10% B, 5~25 min linearly varying from 10% B to 30% B, after 25 min washing and balancing for the next elution. DNA Linker-asPNAs were purified by RP-HPLC. The column was Agilent InfinityLab Porashell 120 EC-C18. The mobile phases A and B were 50 mM TEAA in water (pH 7.0) and acetonitrile, respectively. DNA Linker-asPNAs would elute at 15～20 min with the following settings (gradient elution): flow rate, 1 mL/min; 0min at 5% B followed by a

linear gradient from 5% B to 25% B over 25 min, after 25 min washing and balancing for the next elution. Collected HPLC fractions were centrifuged at 8000~10000 g for 15 min using 3K Da Amicon ultrafilters until all liquids were concentrated to about 100 μL and washed at least three times with aseptic ultrapure water to remove residual mobile phase. Framework nucleic acid monomers were purified by SEC-HPLC. The column was Phenomenex Yarra 3μm SEC-4000. The mobile phase was 25 mM Tris-HCl, 450 mM NaCl (pH 7.4). Framework nucleic acid monomers would elute at ~8 min with 1 mL/min flow rate (isocratic elution). Collected HPLC fractions were concentrated and exchanged by sterile TM buffer, using 30K Da Amicon centrifugal filters with a centrifugation condition of 3500 g × 15 min each time.

Gel electrophoresis characterization. All DNAs in the following samples were stained with GelRed for imaging on a Bio-Rad ChemiDoc MP imaging system. For DNA Linker-asPNAs analysis, 10% denatured polyacrylamide gel electrophoresis (PAGE) was performed in 1 × TAE-Mg electrophoresis buffer at 150 V for 90 min. For framework nucleic acid monomers analysis, 6% native PAGE was carried out in 1 × TAE-Mg electrophoresis buffer at 100 V for 90 min on ice. For dendritic frame nucleic acids analysis, 2% agarose gel electrophoresis was employed in 1 × TAE-Mg electrophoresis buffer at 100 V for 90 min on ice.

AFM characterization. The freshly cleaved mica surface was modified with 10~20 μL of 0.5% (v/v) APTES for 2 min, washed for 1min with ultra-pure water, and blow-dried with an ear syringe. 10~20 μL sample was dripped on mica surface and adsorbed for 5 min. After that, mica surface was washed for about 30s with ultra-pure water and blow-dried with compressed air. The samples were scanned using ScanAsyst-Air tips and ScanAsyst in air mode in a Bruker Dimension FastScan atomic force microscopy.

asPNA release. To investigate whether the linkage between the DNA linker and FAM-asPNA comprised a disulfide bond in DNA Linker-FAM-asPNAs (DNA Linker-2-FAM-P-gp-asPNA and DNA Linker-3-FAM-Bcl-2-asPNA), Either DNA Linker-FAM-asPNA and TCEP were mixed at a molar ratio of 1 to 1000 and incubated at 37 ℃ for 1 h. Then reaction mixtures were analyzed through the fluorescence of GelRed and FAM on the 10% denatured PAGE. To study asPNA release efficiency in Apt-G_2-asPNAs, FAM-P-gp-asPNA instead of P-gp-asPNA was hybridized with Apt-G_2-asPNA to prepare Apt-G_2-asPNAs. 40 uL of 62.5 nM Apt-G_2-FAM-asPNA (corresponding to 600 nM FAM-P-gp-asPNA) and 10 uL of 25 mM GSH in 1 × PBS (pH 7.0) incubated at 37℃ for 0, 0.25, 0.5, 1, 1.5, 2, 2.5, and 3 h, respectively. Then the reaction products at each time point were washed three times with 1 × PBS by ultrafiltration using 100K Da Amicon centrifugal filters. The volume of each intercepted solution was normalized to 100 uL and the FAM fluorescence intensity at 520 nm was recorded by a Tecan Spark multimode microplate reader. The efficiency of asPNA release was calculated by the following equation: release efficiency = [(fluorescence intensity at 0 h - fluorescence intensity at other time point)/ fluorescence intensity at 0 h] × 100%. Meanwhile, DNA amounts before and after GSH reduction were compared via the dsDNA mode on a DS-11

Spectrophotometer.

Cell culture. MCF-7ADR cells were cultured in RPIM 1640 complete medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin in an atmosphere of 5% CO2 at 37 ℃.

Confocal imaging analysis. To observe the uptake of Apt-G_2-asPNA, MCF-7ADR cells were seeded in glass-bottom confocal dishes and cultured overnight. Then the cells were incubated with free asPNA, G_2-asPNA, and Apt-G_2-asPNA with a final FAM-P-gp-asPNA concentration of 300 nM, respectively. After incubation for 6 h, the cells were washed three times with 1×PBS, and cell nucleus was stained with 500 uL of 10 ug/ mL Hoechst 33342 for 15min. After three washes, images were recorded by Leica TCS SP8 laser confocal fluorescent microscopy with 488 nm excitation for FAM.

Flow cytometry analysis. To quantify the uptake of Apt-G_2-asPNA, MCF-7ADR cells were plated in 6-well plates and cultured overnight. Then the cells were incubated with free asPNA, G_2-asPNA, and Apt-G_2-asPNA with a final FAM-P-gp-asPNA concentration of 300 nM, respectively. After incubation for 6 h, the cells were washed three times with 1×PBS, digested by trypsin-EDTA, and collected by centrifugation at 3000 g for 5 min. Finally, the cells were suspended in 1×PBS and detected in the FITC channel using a BD LSRFortessa flow cytometer and an offline FlowJo 10.0 software. To quantify the uptake of Dox, MCF-7ADR cells were plated in 6-well plates and cultured overnight. Then the cells were incubated with free asPNAs, G_2-asPNAs, and Apt-G_2-asPNAs with a final asPNA concentration of 300 nM for 48 h, respectively. After that, the cells were exposed to 10 μM Dox for 6 h. Then the cells were washed three times with 1×PBS, trypsinized, and collected by centrifugation at 3000 g for 5 min. Finally, the cells were suspended in 1×PBS and detected in the PE-Texas Red channel using a BD LSRFortessa flow cytometer and an offline FlowJo 10.0 software.

Western blot assay. After seeding in 6-well plates and culturing overnight, MCF-7ADR cells were incubated with free asPNAs, 25 nM G_2-asPNAs, 25 nM Apt-G_2-asPNAs (each "asPNAs" corresponding to 300 nM P-gp-asPNA and 300 nM Bcl-2-asPNA), 125 nM Apt-G_2-asPNA containing 1.5 μM P-gp-asPNA, and 125 nM Apt-G_2-asPNA containing 1.5 μM Bcl-2-asPNA for 96 h, respectively. Then the cells were washed twice with cold 1×PBS and lysed in RIPA buffer to extract soluble cell proteins. 50 μg of cell proteins was separated using SDS-PAGE and transferred to a PVDF membrane. Then the PVDF membrane was blocked with 5% skim milk, incubated with primary antibodies (anti-P-gp, anti-Bcl-2, anti-GAPDH) overnight at 4 ℃, and incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 2 h at ambient temperature. Finally, the blots were developed using an ultra-sensitive chemiluminescent substrate kit and imaged on a Bio-Rad ChemiDoc MP imaging system.

Cell viability assay. After seeding in 96-well plates and culturing overnight, MCF-7ADR cells were co-incubated with 1 × PBS, G_2, G_2-asPNAs, or Apt-G_2-asPNAs (corresponding

to G_2: 25 nM, P-gp-asPNA: 300 nM, Bcl-2-asPNA: 300 nM) in conjunction with various concentrations of Dox (0, 1, 5, 10, 15, 20 μM). After co-incubation for 96 h, the cells were incubated with a final MTT reagent concentration of 0.5 mg/mL for another 4 h. Then the cell supernatant was removed and 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize dark purple formazan crystals. Colorimetric detection was done at a wavelength of 490 nm using a Tecan Spark multimode microplate reader.

Supporting Tables

Table S1. Sequences of DNA oligonucleotides used in this work. Underlined sequences are the linker sequences between tetrahedral FNA monomers or between T_2 and DNA Linker-1-MUC1 aptamer or DNA Linker-2 or DNA Linker-3. Specifically, the sequences denoted by A, B, C, D, and E are complementary to those denoted by A', B', Linker-1, Linker-2, and Linker-3, respectively.

Names of tetrahedral FNA monomers	Names of DNA oligonucleotides	Sequence (5'-3')
T_0	**A-S1**	GTGCTTGGTAACATAGGTGGACAGCCAGTTGAGACGAACATTCCTAAGTCTGAAATTTATCACCCGCCATAGTAGACGTATCACCAGG
	A-S2	GTGCTTGGTAACATAGGTGGACAGCGCTACACGATTCAGACTTAGGAATGTTCGACATGCGAGGGTCCAATACCGACGATTACAGCTT
	A-S3	GTGCTTGGTAACATAGGTGGACAGCGTGATAAAACGTGTAGCAAGCTGTAATCGACGGGAAGAGCATGCCCATCCACTACTATGGCGG
	A-S4	GTGCTTGGTAACATAGGTGGACAGCCTCGCATGACTCAACTGCCTGGTGATACGAGGATGGGCATGCTCTTCCCGACGGTATTGGACC
T_1	**A'-S1**	GCTGTCCACCTATGTTACCAAGCACCAGTTGAGACGAACATTCCTAAGTCTGAAATTTATCACCCGCCATAGTAGACGTATCACCAGG
	B-S2	TCTACCATCAACGAAGCCTAACGCCGCTACACGATTCAGACTTAGGAATGTTCGACATGCGAGGGTCCAATACCGACGATTACAGCTT
	B-S3	TCTACCATCAACGAAGCCTAACGCCGTGATAAAACGTGTAGCAAGCTGTAATCGACGGGAAGAGCATGCCCATCCACTACTATGGCGG
	B-S4	TCTACCATCAACGAAGCCTAACGCCCTCGCATGACTCAACTGCCTGGTGATACGAGGATGGGCATGCTCTTCCCGACGGTATTGGACC
T_2	**B'-S1**	GGCGTTAGGCTTCGTTGATGGTAGACAGTTGAGACGAACATTCCTAAGTCTGAAATTTATCACCCGCCATAGTAGACGTATCACCAGG
	C-S2	AAAAAAAAAAAAAAAAAAAAAAAAGCTACACGATTCAGACTTAGGAATGTTCGACATGCGAGGGTCCAATACCGACGATTACAGCTT
	D-S3	GAGTGTGTAACGGCCAACAGACATAGTGATAAAACGTGTAGCAAGCTGTAATCGACGGGAAGAGCATGCCCATCCACTACTATGGCGG
	E-S4	AAAGCCGGTAAGGTGAGGAATCCGACTCGCATGACTCAACTGCCTGGTGATACGAGGATGGGCATGCTCTTCCCGACG

		GTATTGGACC
	DNA Linker-1-MUC1 aptamer	TTTTTTTTTTTTTTTTTTTTTTTTTTTGCAGTTGATCCTTTG GATACCCTGG
	DNA Linker-2	TATGTCTGTTGGCCGTTACACACTCCCC-SH
	DNA Linker-3	TCGGATTCCTCACCTTACCGGCTTTCCC-SH

Table S2. Sequences of asPNA oligomers used in this work.

Name	**Sequence (N' to C')**
P-gp-asPNA	TTCAAGATCCATCCCGACCTCGCG-mPEG-Cys
FAM-P-gp-asPNA	FAM-TTCAAGATCCATCCCGACCTCGCG-mPEG-Cys
Bcl-2-asPNA	TCTCCCAGCGTGCGCCAT-mPEG-Cys
FAM-Bcl-2-asPNA	FAM-TCTCCCAGCGTGCGCCAT-mPEG-Cys

Supporting Figures

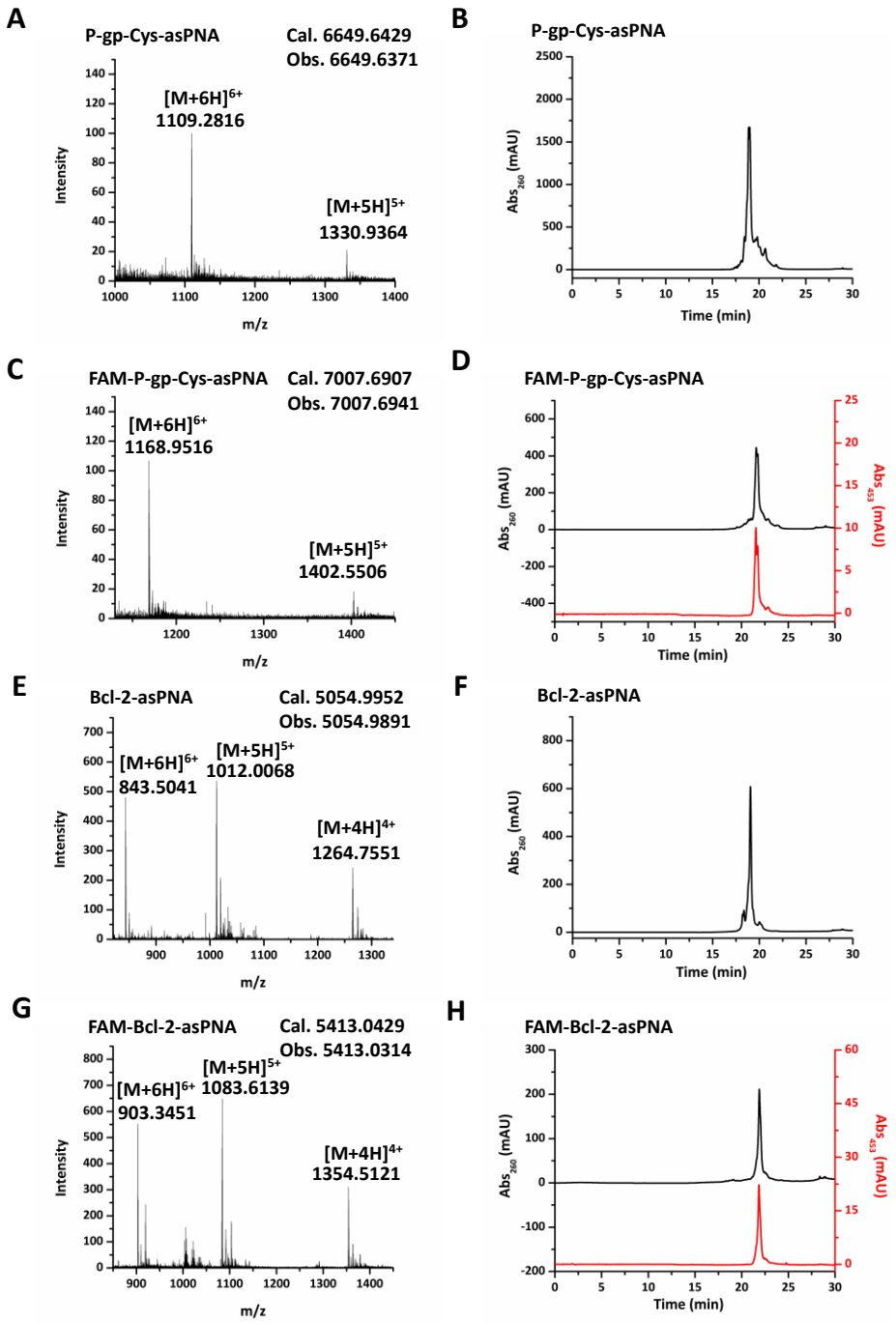

Fig. S1 ESI-MS and RP-HPLC analysis of (A, B) P-gp-asPNA, (C, D) FAM-P-gp-asPNA, (E, F) Bcl-2-asPNA, and (D, E) FAM-Bcl-2-asPNA after purification using a semi-preparative column (Agilent ZORBAX Eclipse XDB-C18). RP-HPLC analysis was performed using an analytical column (Agilent InfinityLab Porashell 120 EC-C18) with the following settings: the mobile phases A and B were 0.1% TFA in water and 90% acetonitrile in water, respectively; flow rate, 1 mL/min; 0~10 min at 5% B, 10~25 min linearly varying from 5% B to 35% B, 25~30 min linearly varying from 35% B to 100% B; samples were detected by PNA absorption at 260 nm and FAM absorption at 453 nm.

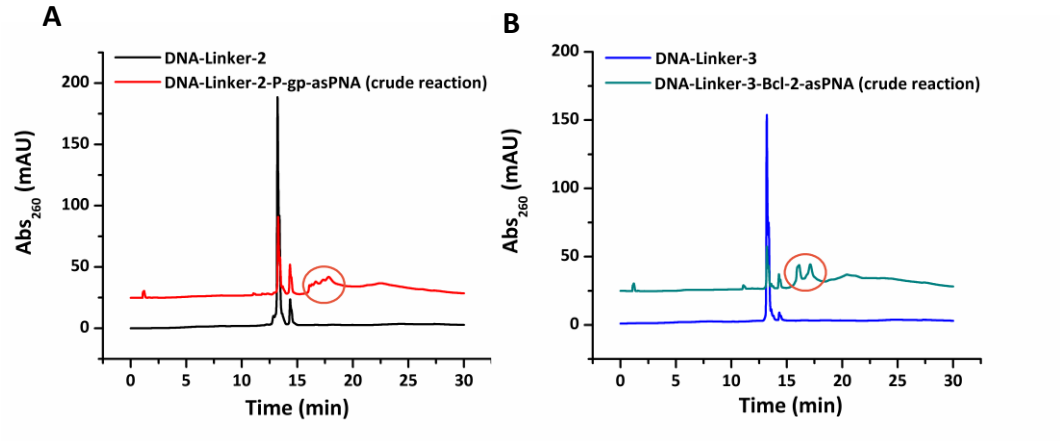

Fig. S2 RP-HPLC elution profiles of (A) free DNA Linker-2 and the conjugation reaction mixture of DNA Linker-2 and FAM-P-gp-PNA, and (B) free DNA Linker-3 and the conjugation reaction mixture of DNA Linker-3 and FAM-Bcl-2-asPNA. The peaks at ~13 and ~14 minutes represent unreacted DNA Linker and DNA Linker self-oxide, respectively. The split peaks at 16~18 minutes (orange circle) stand for DNA Linker-asPNA conjugates. Samples were detected by DNA absorption at 260 nm and FAM absorption at 460 nm (data not shown).

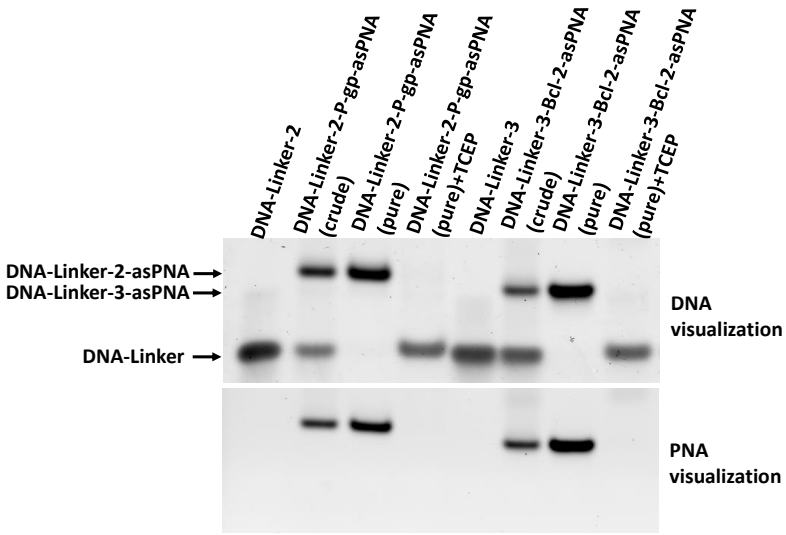

Fig. S3 10% denatured PAGE evaluation of DNA Linker-asPNAs conjugation, purity, and disulphide reduction. Gel was visualized by GelRed fluorescence to detect DNAs (top) and FAM fluorescence to detect labelled asPNAs (bottom).

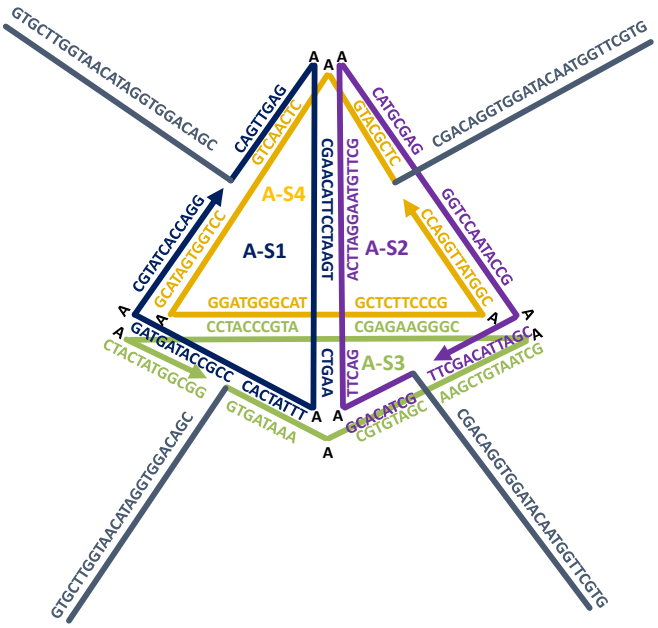

Fig. S4 Sequence and design of tetrahedral FNA monomer T_0. T_1 and T_2 were similar except that overhang sequences were different.

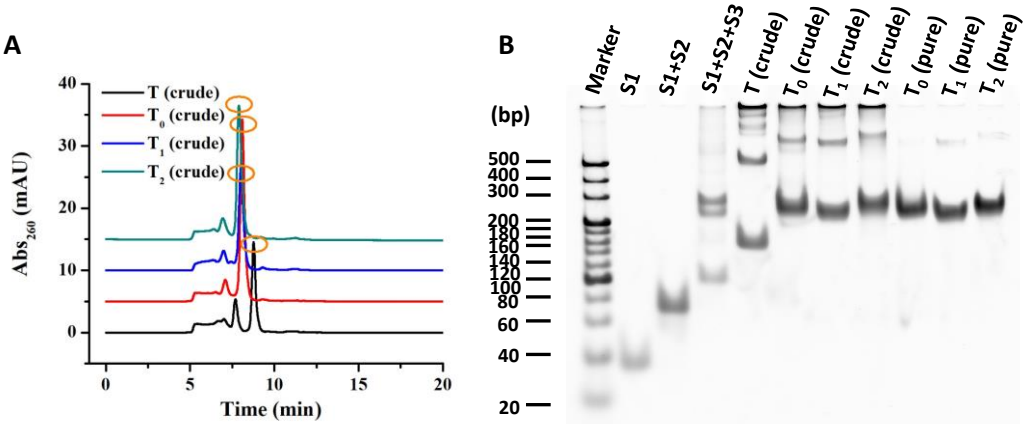

Fig. S5 (A) SEC-HPLC elution profiles of tetrahedral frame nucleic acids (T, T_0, T_1, and T_2) with the desired peaks marked by orange circles. (B) 6% native PAGE analysis of tetrahedral frame nucleic acids before and after HPLC purification.

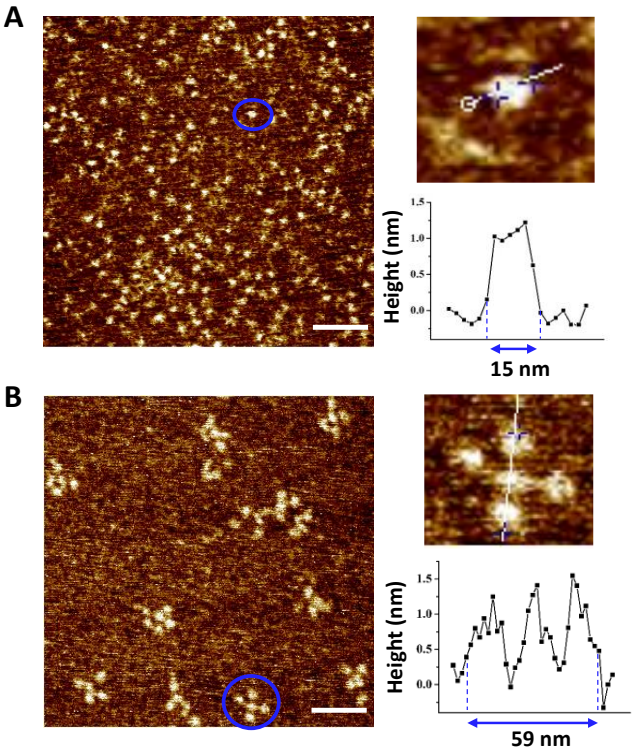

Fig. S6 AFM images and diameter profiles of a representative (A) T_0 and (B) G_1. Scale bars: 100 nm.

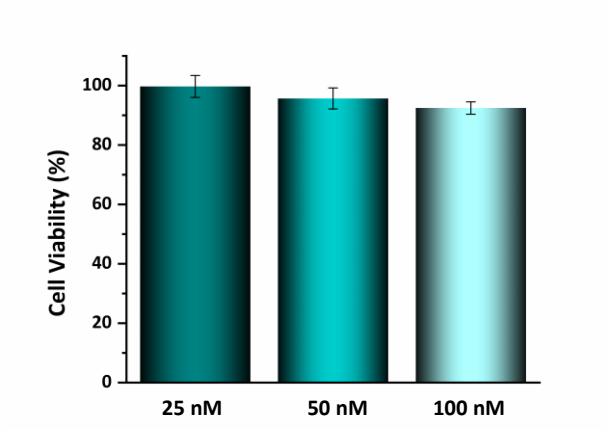

Fig. S7 Cell viability of MCF-7ADR cells after incubation with different concentrations of G_2 for 96 h.

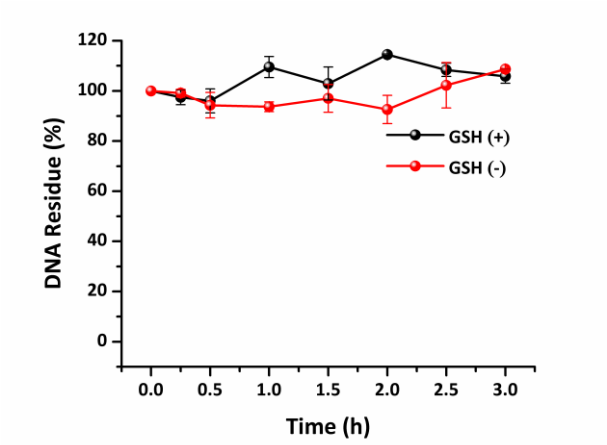

Fig. S8 The percentage of residual DNA amounts of Apt-G_2-asPNA incubated with or without 5 mM GSH in 1×PBS (pH 7.0).

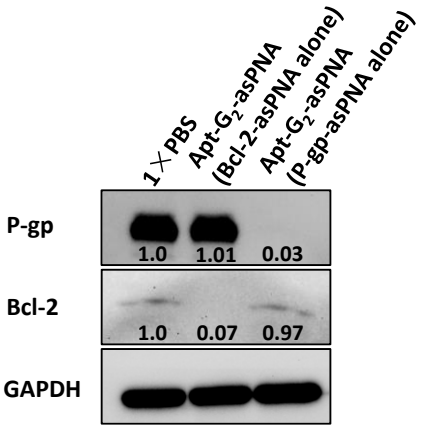

Fig. S9 Western blot analysis of P-gp and Bcl-2 protein levels in MCF-7ADR cells after 96 h incubation with Apt-G_2-asPNA containing 1.5 μM Bcl-2-asPNA alone and Apt-G_2-asPNA containing 1.5 μM P-gp-asPNA alone. GAPDH was used as internal reference protein.

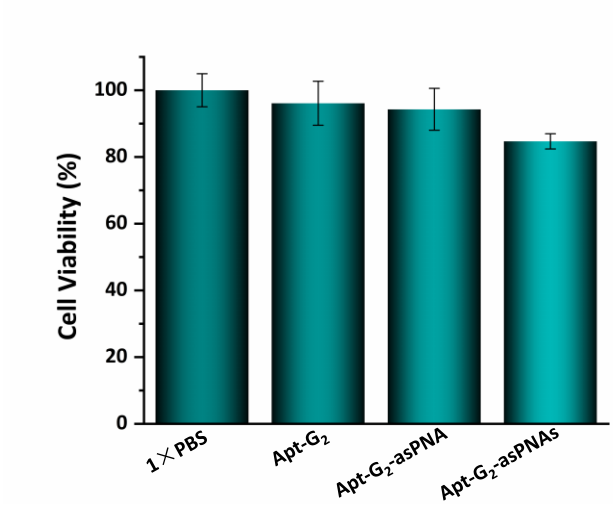

Fig. S10 Cell viability of MCF-7ADR cells after treatment with Apt-G_2, Apt-G_2-asPNA containing P-gp-asPNA alone, and Apt-G_2-asPNAs for 96 h. The concentrations of G_2 in all materials were 25 nM.

Underlying Raw Images

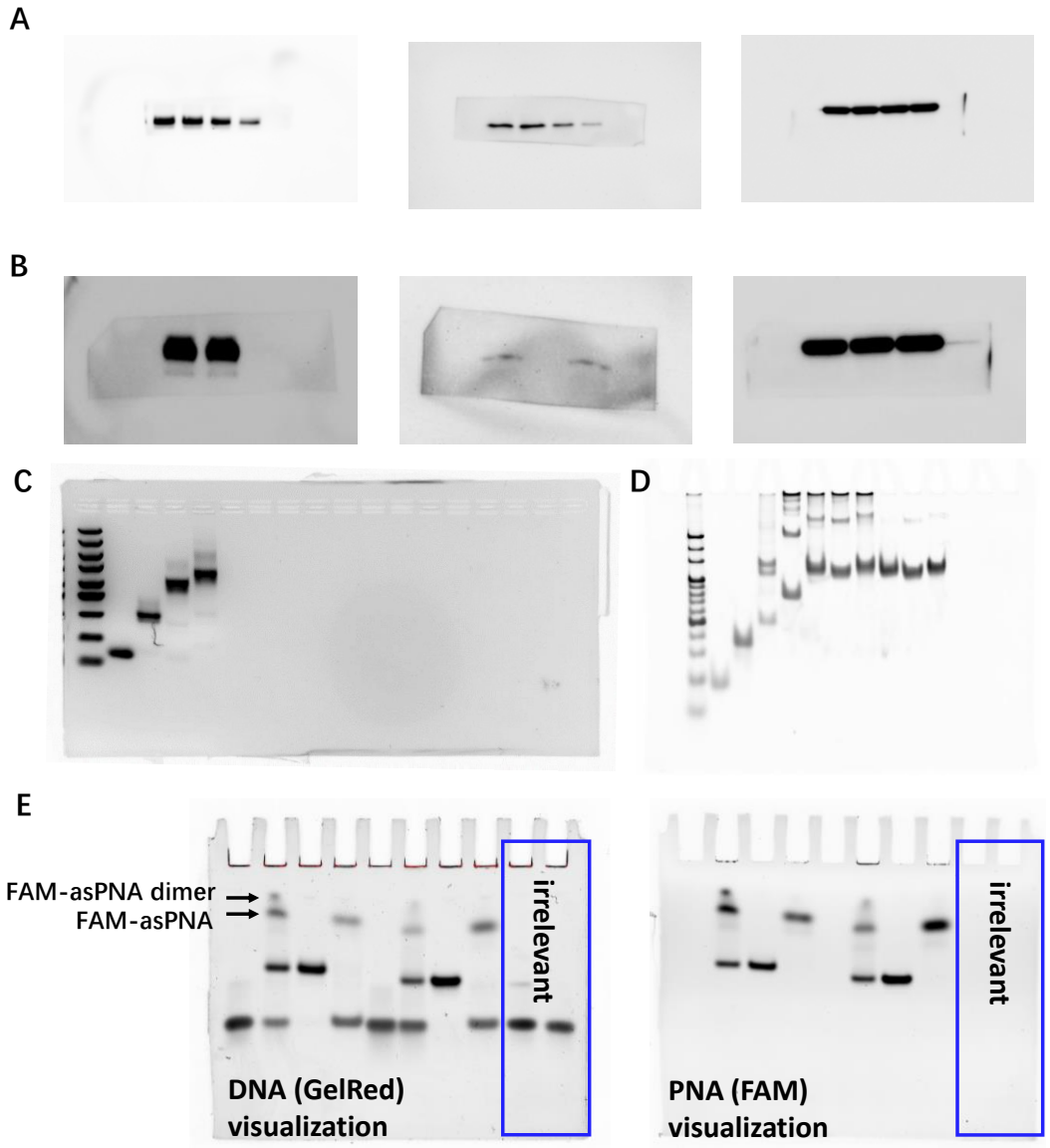

Fig. S11 The underlying raw images of western blots of Fig 3B (A) and Fig. S9 (B), and electrophoresis photographs of Fig. 2C (C), Fig. S5B (D) and Fig. S4 (E). Notes: To explain more clearly about the coupling, purification and reductive release of asPNA and DNA Linker from the angle of DNA, only the bands in Fig. S11E (left picture) containing DNA are shown in Fig. S4. The signals of pure FAM-asPNA are from FAM dye instead of DNA stain and thus not shown in Fig S4.

Notes and references

1. S. Xing, X. Xu, P. Fu, M. Xu, T. Gao, X. Zhang and C. Zhao, *Colloid. Surface B*, 2019, **181**, 333-340.
2. S. Xing, D. Jiang, F. Li, J. Li, Q. Li, Q. Huang, L. Guo, J. Xia, J. Shi, C. Fan, L. Zhang and L. Wang, *ACS Appl. Mater. Interfaces*, 2015, **7**, 13174-13179.