SUPPORTING INFORMATION

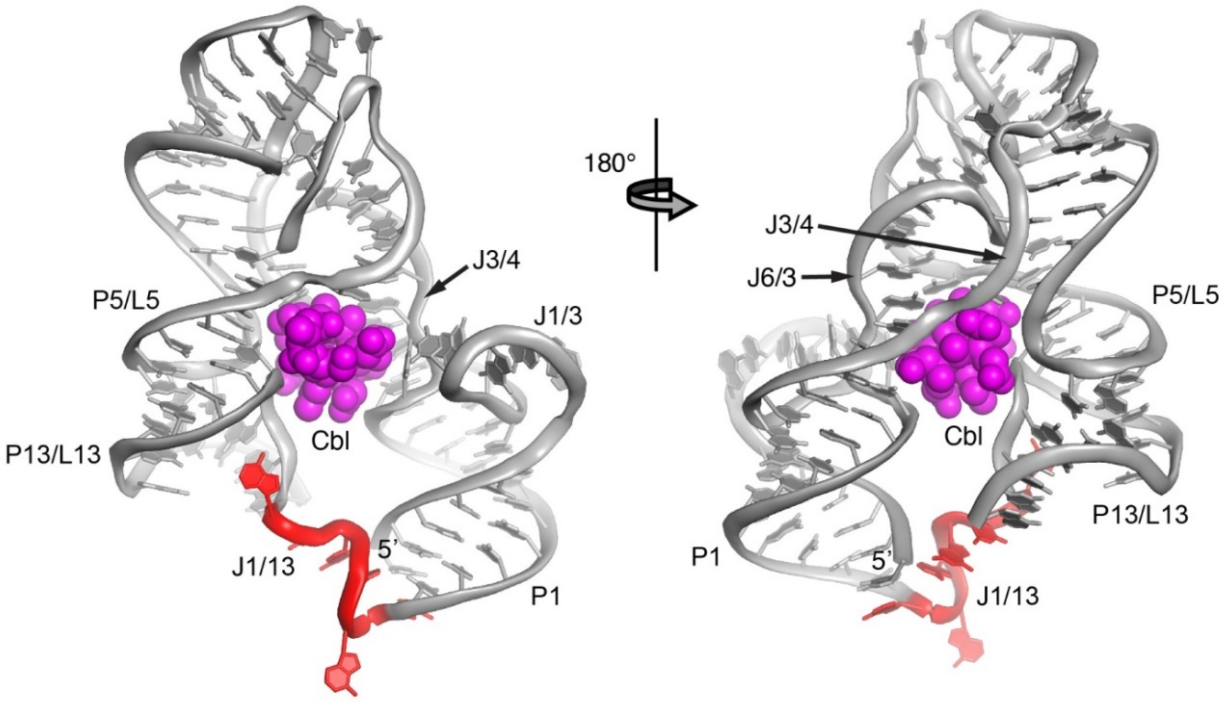
Unveiling the Promise of Peptide Nucleic Acids as Functional Linkers for an RNA Imaging Platform

Authors: Aleksandra J. Wierzba,[1,2] Erin M. Richards,[1,2] Shelby R. Lennon,[1] Robert T. Batey,[1,*] Amy E. Palmer,[1,2,*]

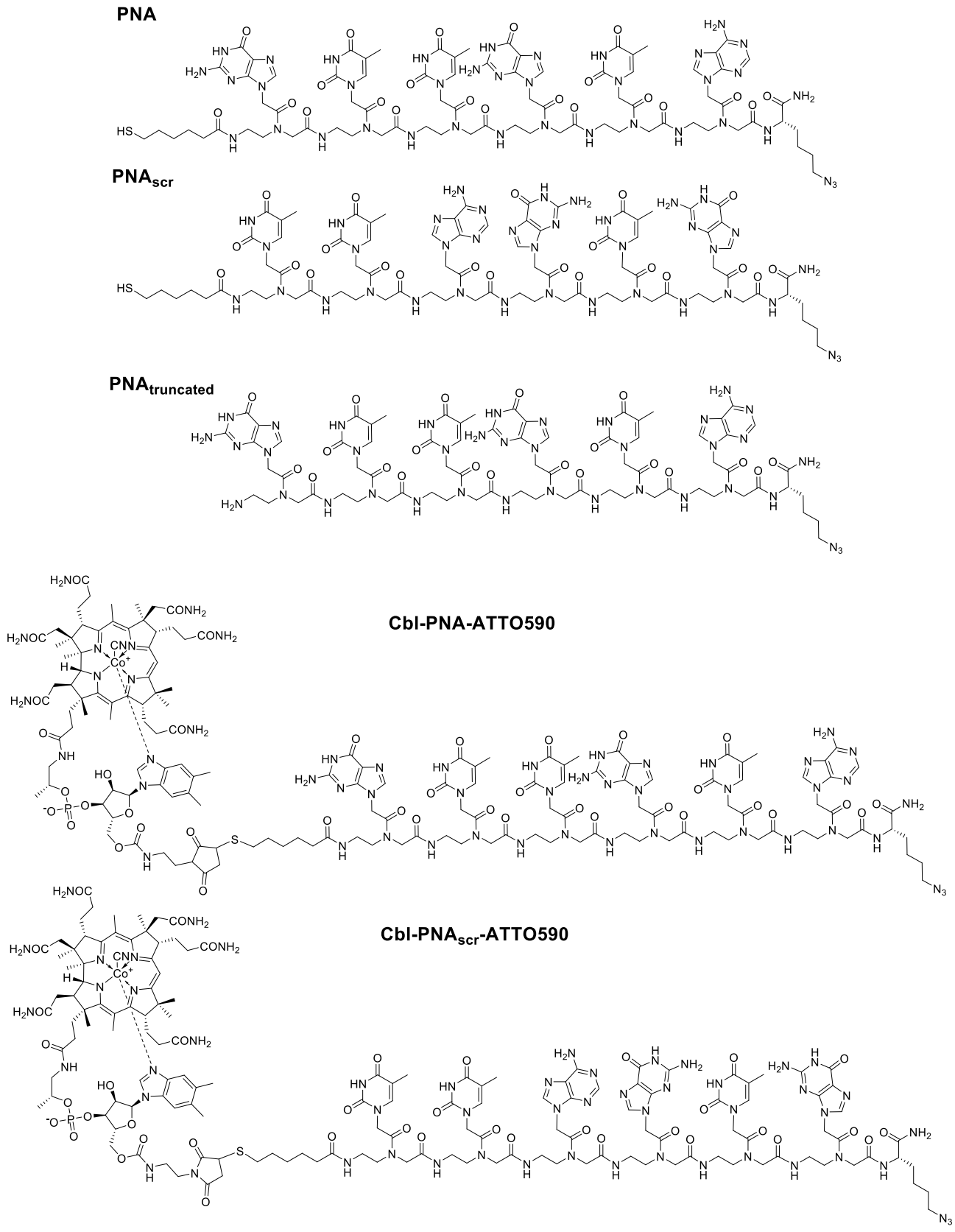
Affiliations:
[1]Department of Biochemistry, University of Colorado, Boulder, CO 80309-0596, USA
[2]BioFrontiers Institute, University of Colorado, Boulder, CO 80303-0596, USA
*To whom correspondence should be addressed. R.T.B.: Tel +1 303 735 2159; Fax +1 303 492 5894; Email: Robert.Batey@colorado.edu; A.E.P.: Tel +1 303 492 1945; Fax +1 303 492 5894; Email: Amy.Palmer@colorado.edu

Table of contents

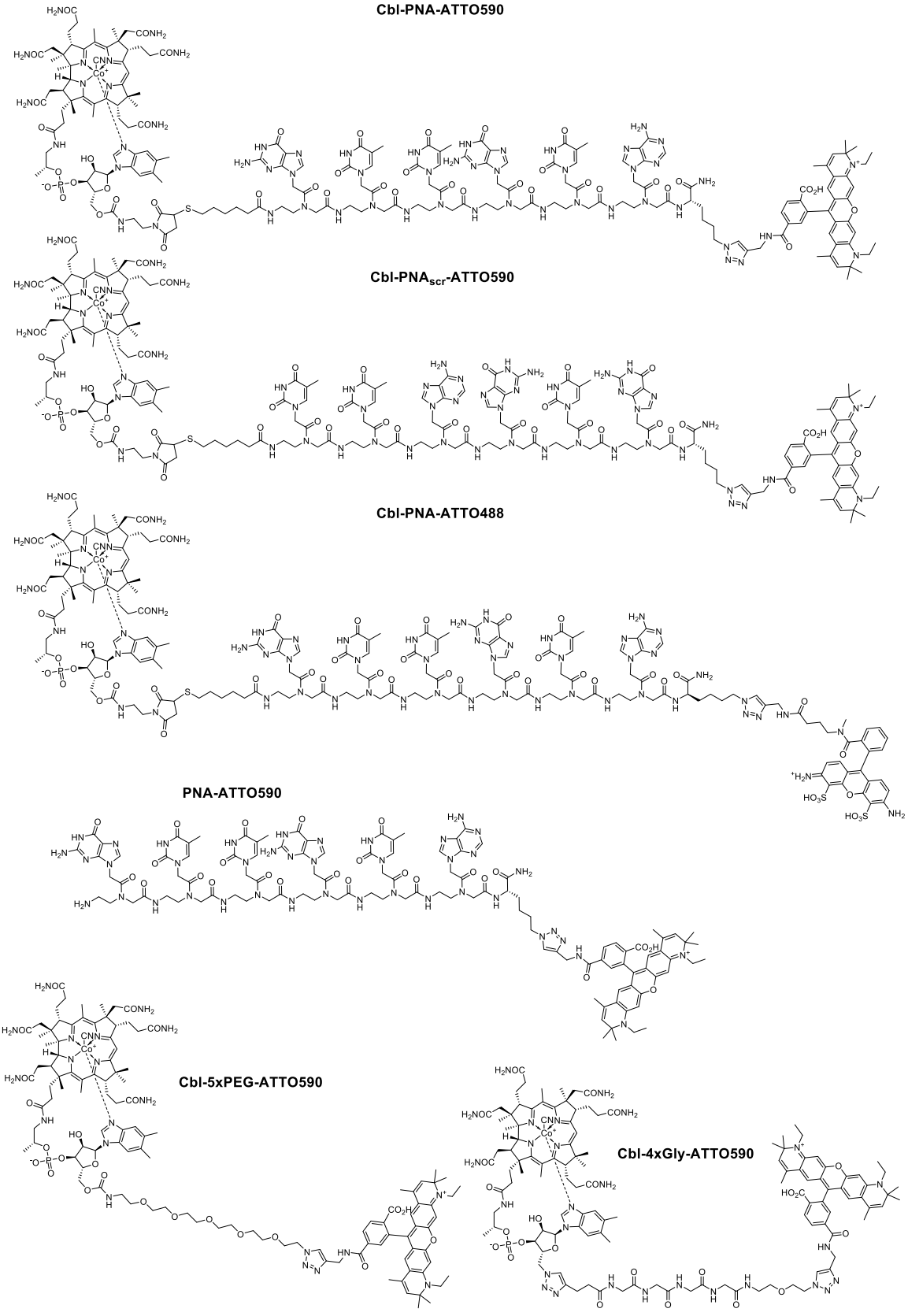
1. Supporting Figures



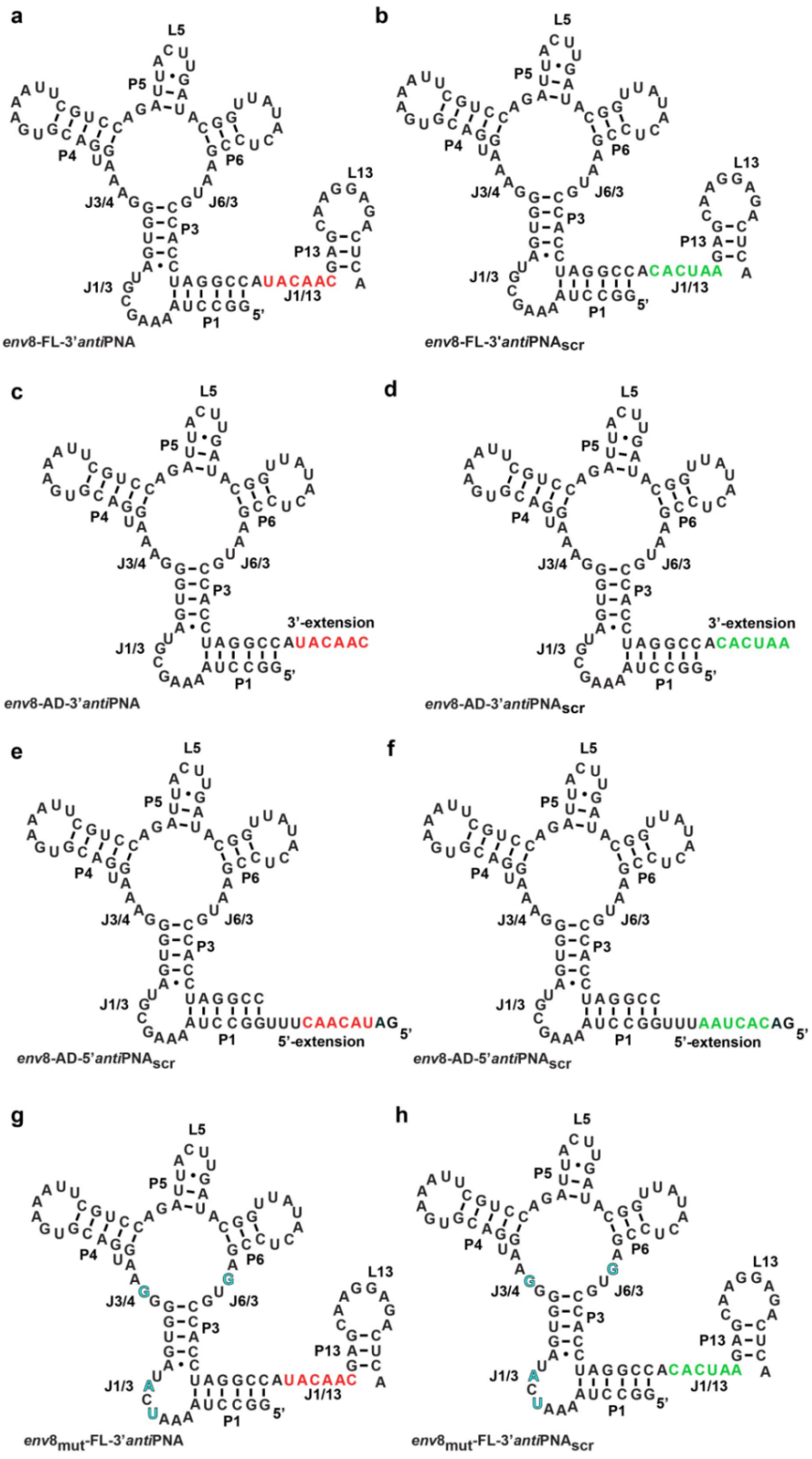
Supporting Figure S1. Crystal structure of wild type *env*8 (*env*8-FL-3'*anti*PNA) bound to cobalamin (PDB: 4FRG).(1) Key structural regions denoted as P (paired), J (junction) and L (loop). J1/13 fragment is marked in red and cobalamin in magenta.



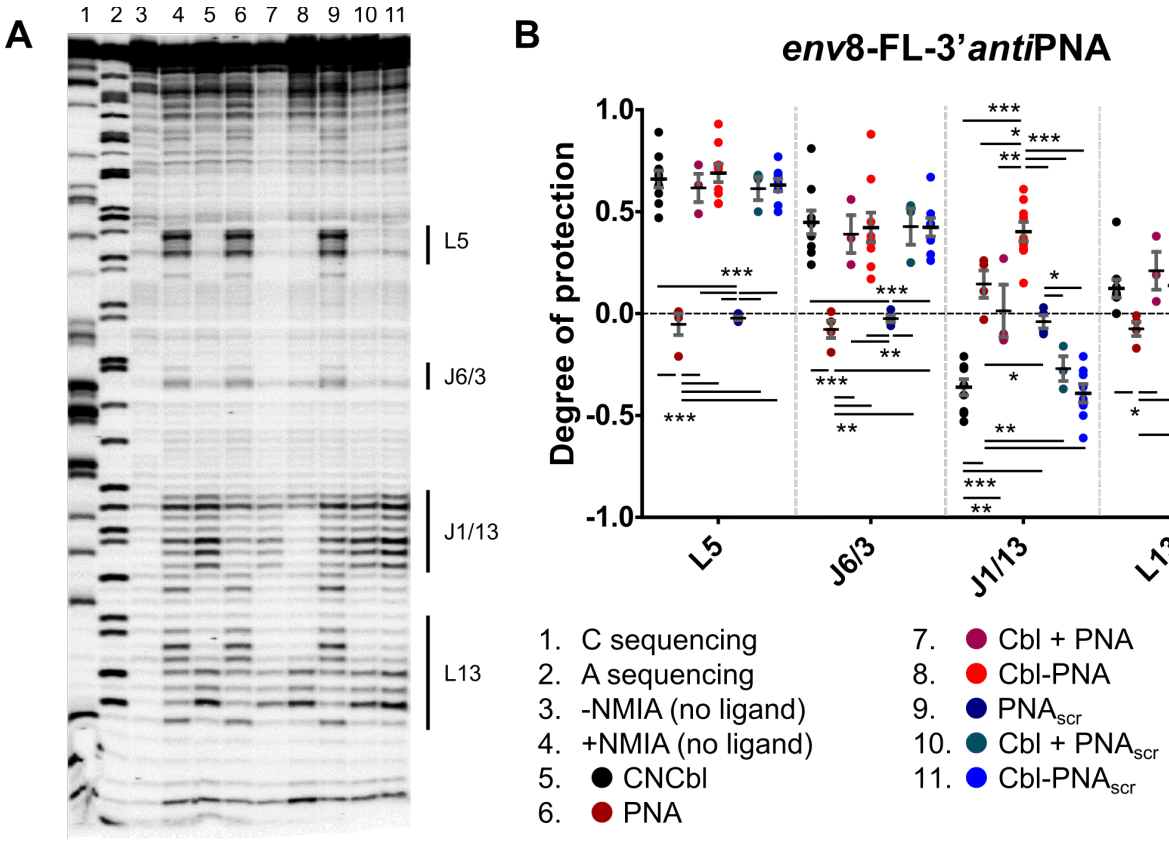
Supporting Figure S2. Chemical structures of PNA linkers and Cbl-PNA conjugates.



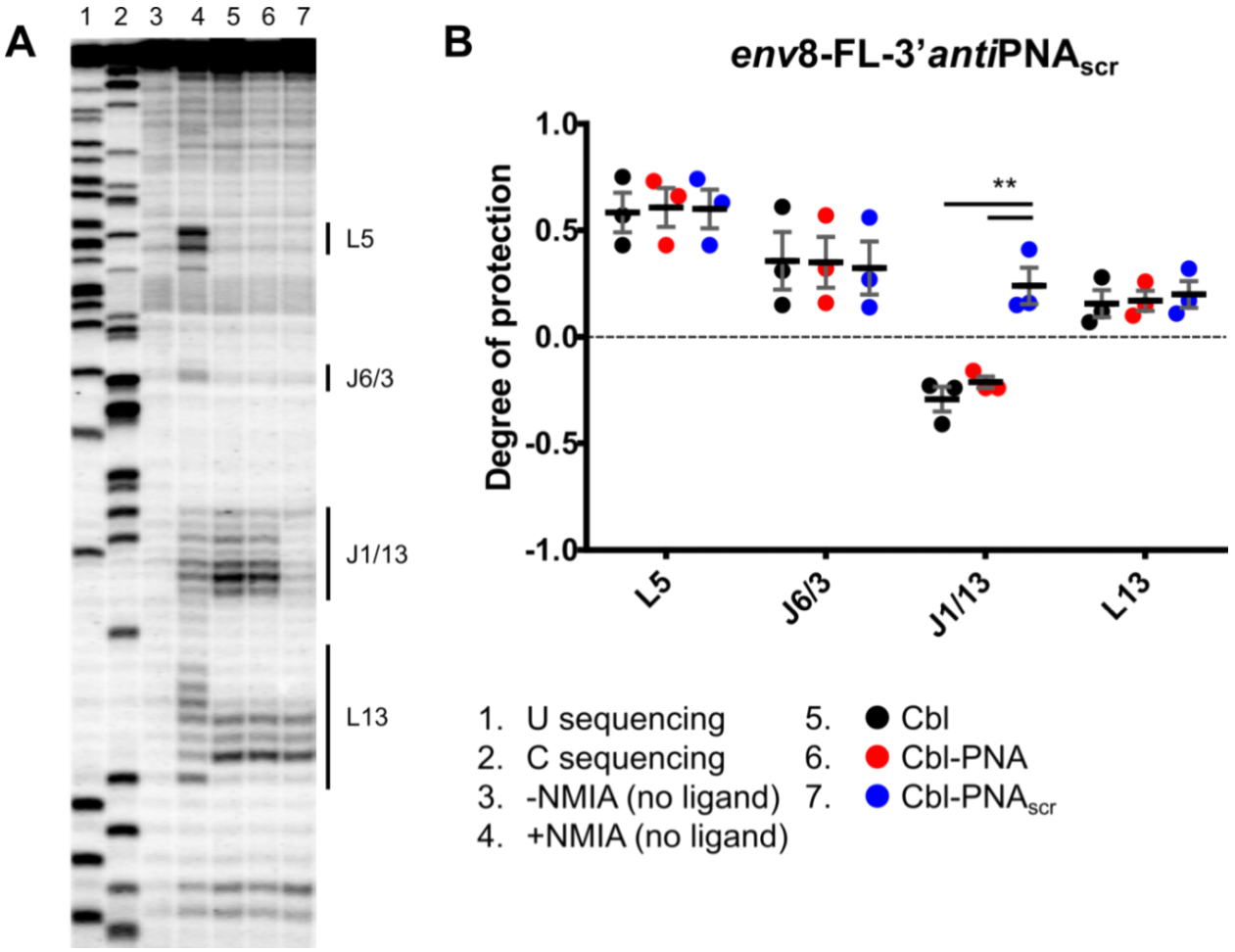
Supporting Figure S3. Chemical structures of Cbl-based probes and PNA-ATTO590 probe.



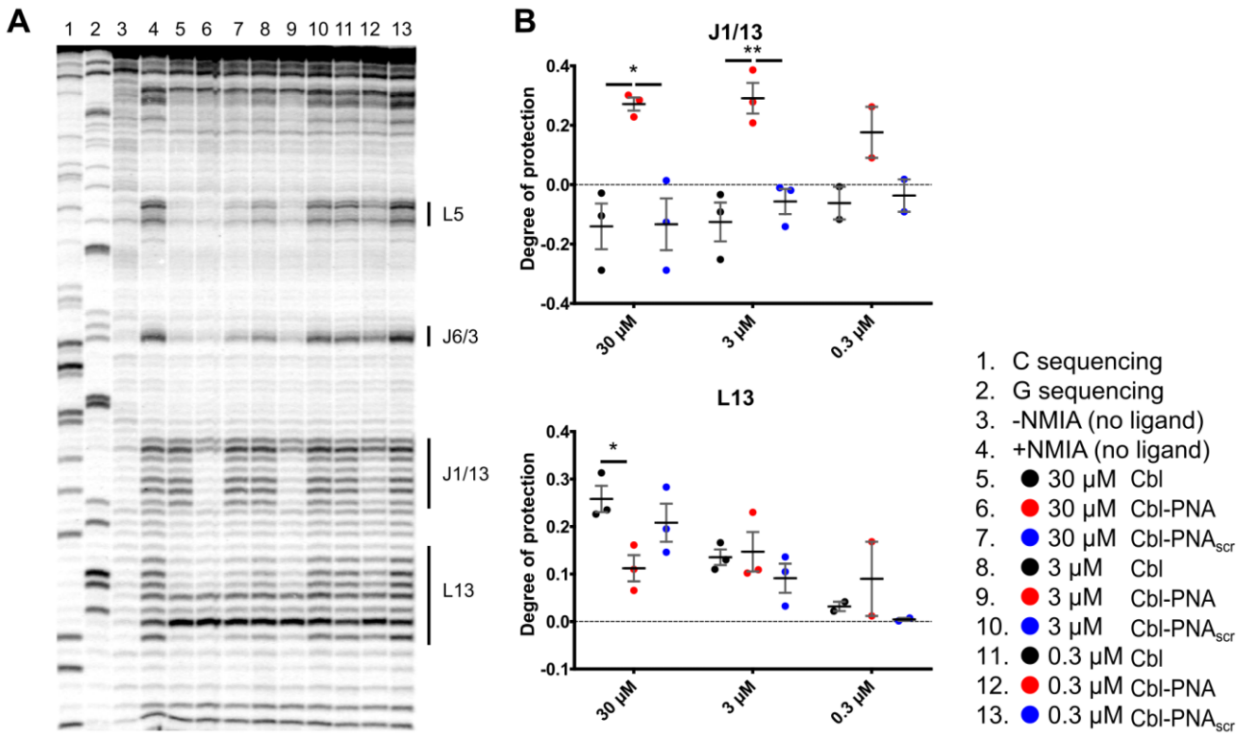
Supporting Figure S4. Secondary RNA structures used in the study. The *anti*PNA and *anti*PNA$_{scr}$ fragments are color-coded with red and green, respectively. Mutated nucleotides in **g** and **h** are colored blue.



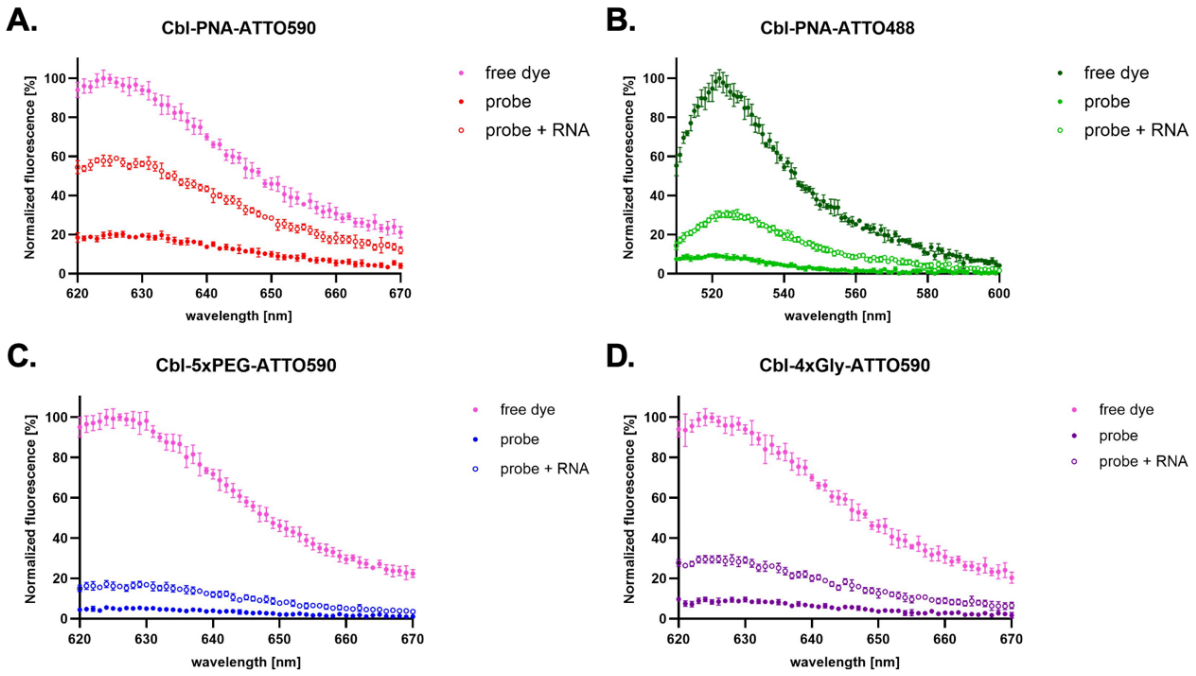
Supporting Figure S5. Extension of Figure 2. **A**. Full representative *env*8-FL-3'*anti*PNA SHAPE gel in the presence of several ligands. Lanes are loaded according to the key. Cbl-dependent SHAPE pattern changes in L5, J6/3, and L13 occur when Cbl is present in the ligand (lanes 5, 7, 8, 10, & 11 compared to lane 4). Sequence specific annealing of the PNA in the context of the Cbl-PNA ligand is seen by ligand dependent protections in J1/13 (lane 8 compared to lane 4). **B**. Quantifications of all marked SHAPE gel regions with the same ligands as A. Ligands are represented by colored dots according to the key. Degree of protection is in comparison to the +NMIA condition. Unpaired t-tests, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Not significant p-values (>0.5) are not marked. n=3-9. Error bars represent SEM.



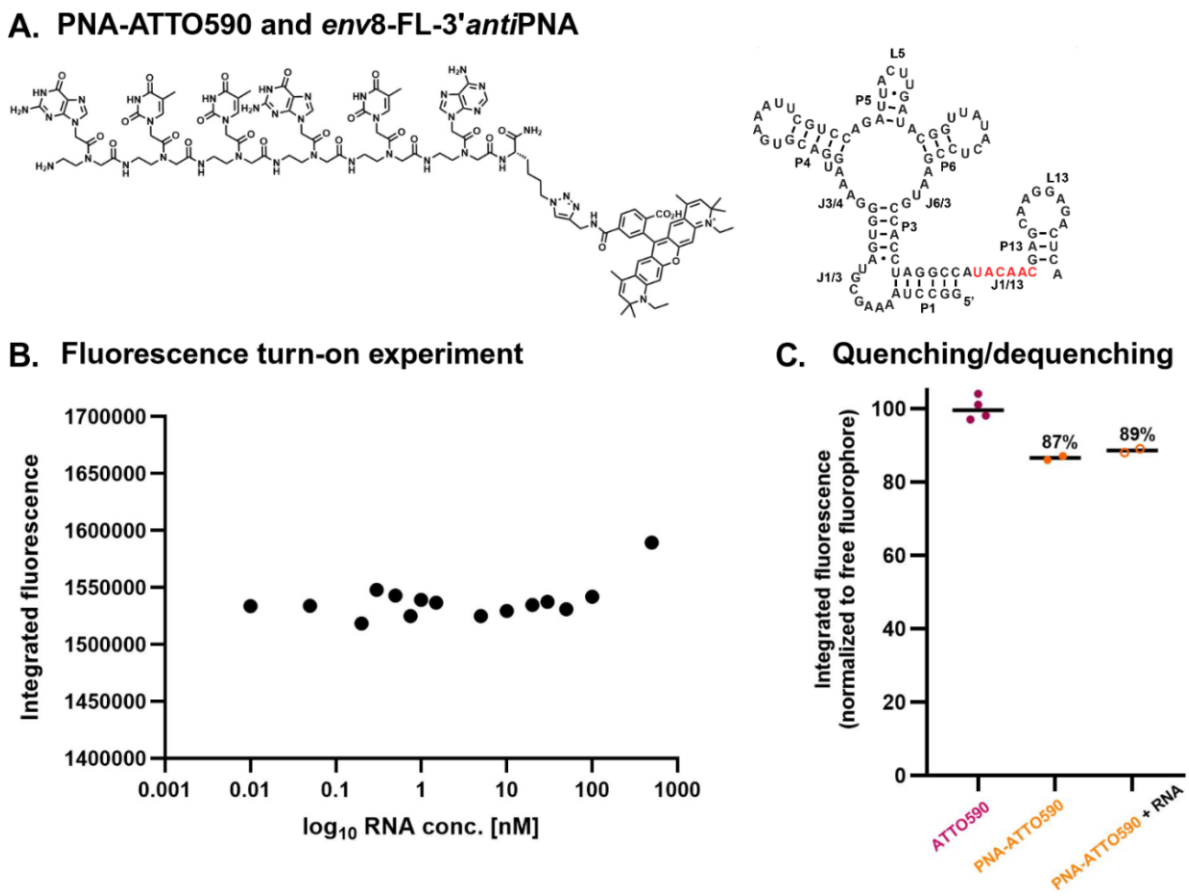
Supporting Figure S6. Extension of Figure 2. **A**. Full representative *env*8-FL-3'*anti*PNA$_{scr}$ SHAPE gel in the presence of several ligands. Lanes are loaded according to the key. Cbl-dependent SHAPE pattern changes in L5, J6/3, and L13 indicate Cbl binding (lanes 5, 6, & 7 compared to lane 4). Sequence specific annealing of the PNA$_{scr}$ is seen by ligand dependent protections in J1/13 (lane 7 compared to lane 4). **B**. Quantifications of all marked SHAPE gel regions with the same ligands as A. Ligands are represented by colored dots according to the key. Degree of protection is in comparison to the +NMIA condition. Unpaired t-tests, ** $p < 0.01$. Not significant p-values (>0.5) are not marked. n=3. Error bars represent SEM.



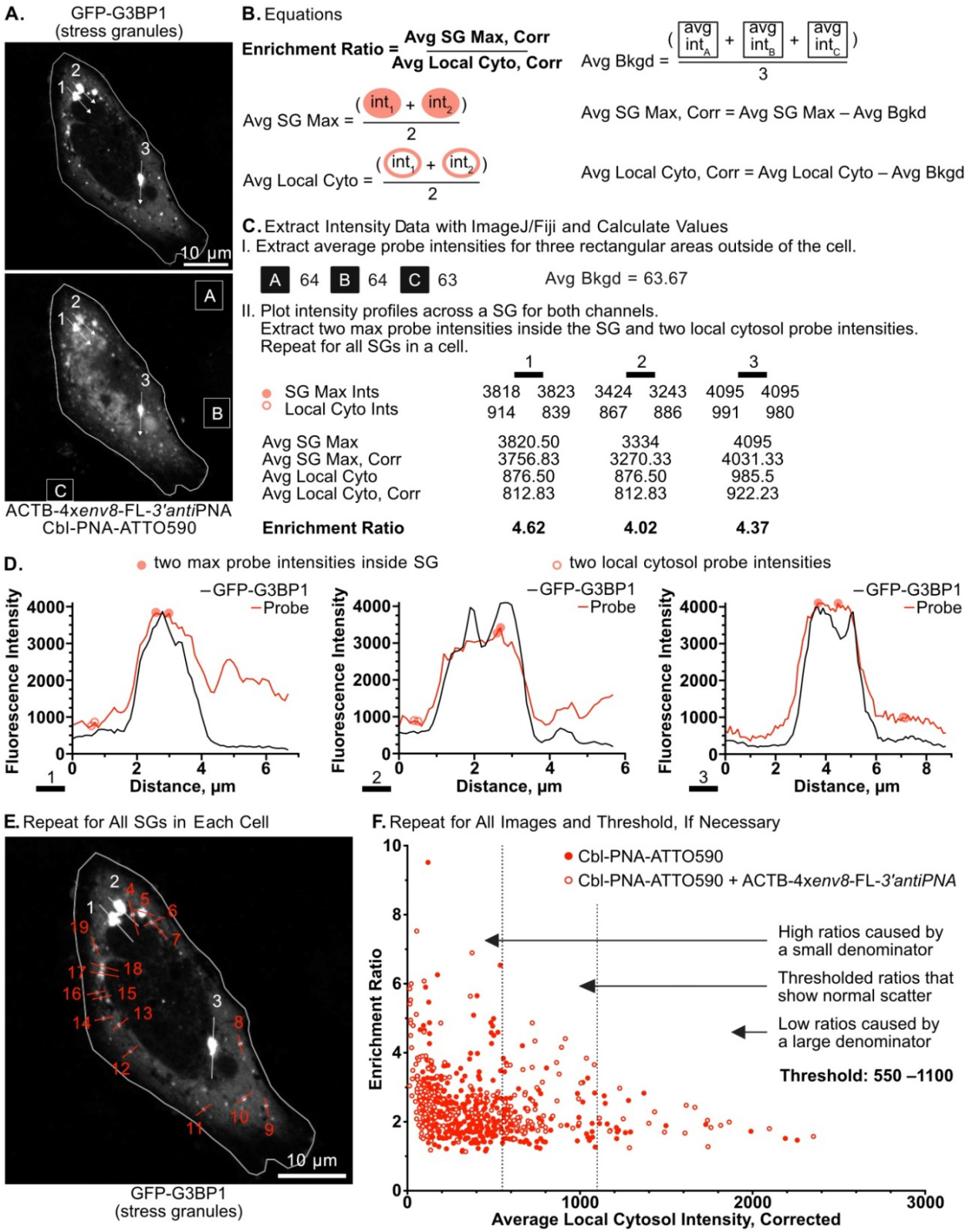
Supporting Figure S7. Extension of Figure 4. **A**. Full representative *env*8$_{mut}$-FL-3'*anti*PNA SHAPE gel in the presence of several ligands at different concentrations. Lanes are loaded according to the key. Cbl-dependent SHAPE pattern changes in L5, J6/3, and L13 indicate Cbl binding. Sequence specific annealing of the PNA is seen by ligand dependent protections in J1/13 (lanes 6, 9, & 12 compared to lane 4). **B**. Quantifications of marked SHAPE gel regions not quantified in Figure 6 with the same ligands and concentrations as A. Ligands are represented by colored dots according to the key. Degree of protection is in comparison to the +NMIA condition. Unpaired t-tests, ** $p < 0.01$, * $p < 0.05$. Not significant p-values (>0.5) are not marked. n=2-3. Error bars represent SEM.



Supporting Figure S8. Fluorescence emission spectra of cobalamin probes in the presence and absence of *env*8-FL-3'*anti*PNA, compared to the emission spectra of the free dye. **A.** Probe: Cbl-PNA-ATTO590 (c_{probe} =1 nM), RNA: *env*8-FL-3'*anti*PNA (c_{RNA} = 50 nM), free dye: ATTO590 (c_{dye} = 1 nM). **B.** Probe: Cbl-PNA-ATTO488 (c_{probe} = 1 nM), RNA: *env*8-FL-3'*anti*PNA (c_{RNA} = 50 nM), free dye: ATTO488 (c_{dye} = 1 nM). **C.** Probe: Cbl-5xPEG-ATTO590 (cprobe = 1 nM), RNA: *env*8-FL-3'*anti*PNA (c_{RNA} = 500 nM), free dye: ATTO590 (c_{dye} = 1 nM). **D.** Probe: Cbl-4xGly-ATTO590 (c_{probe} = 1 nM), RNA: *env*8-FL-3'*anti*PNA (c_{RNA} = 500 nM), free dye: ATTO590 (c_{dye} = 1 nM). n= 6. Error bars represent SEM.

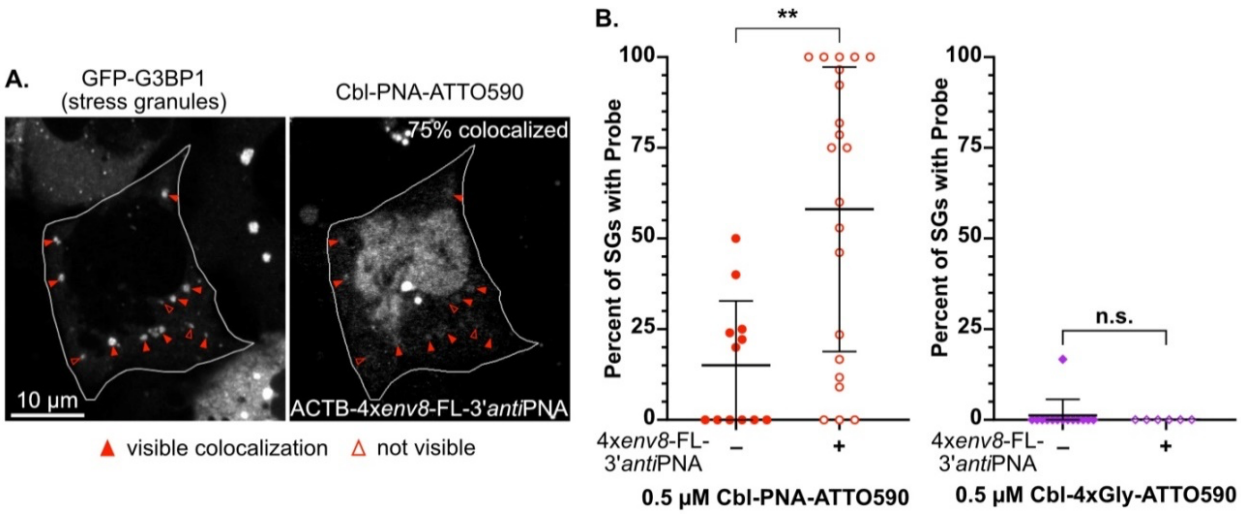


Supporting Figure S9. Control experiments indicating the relevance of cobalamin as a quencher in the Cbl-PNA-ATTO590 probe and PNA linker contribution towards quenching. **A.** Structures of PNA-ATTO590 probe and wild type *env*8. **B.** Titration of PNA-ATTO590 probe with *env*8-FL-3'*anti*PNA ($c_{PNA\text{-}ATTO590}$ = 1 nM). **C**. The levels of probe quenching and dequenching in the presence of RNA: *env*8-FL-3'*anti*PNA (c_{probe} = 1 nM, c_{RNA} = 500 nM).

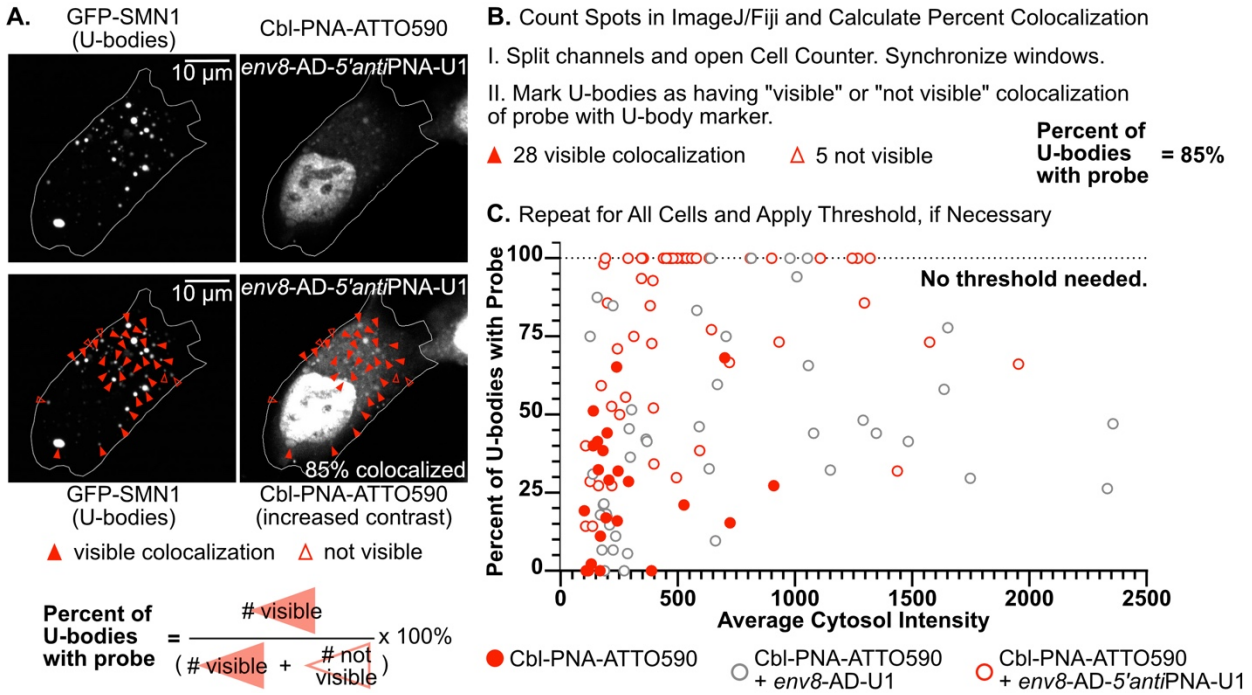


Supporting Figure S10. A. Representative images of a cell stably expressing GFP-G3BP1, co-transfected with NLS-TagBFP and ACTB-4x*env*8-FL-3'*anti*PNA, and beadloaded with 5 µM Cbl-PNA-ATTO590. Cell outline in white. Labeled white arrows 1, 2, and 3 showing example line scans across SGs. Labeled white

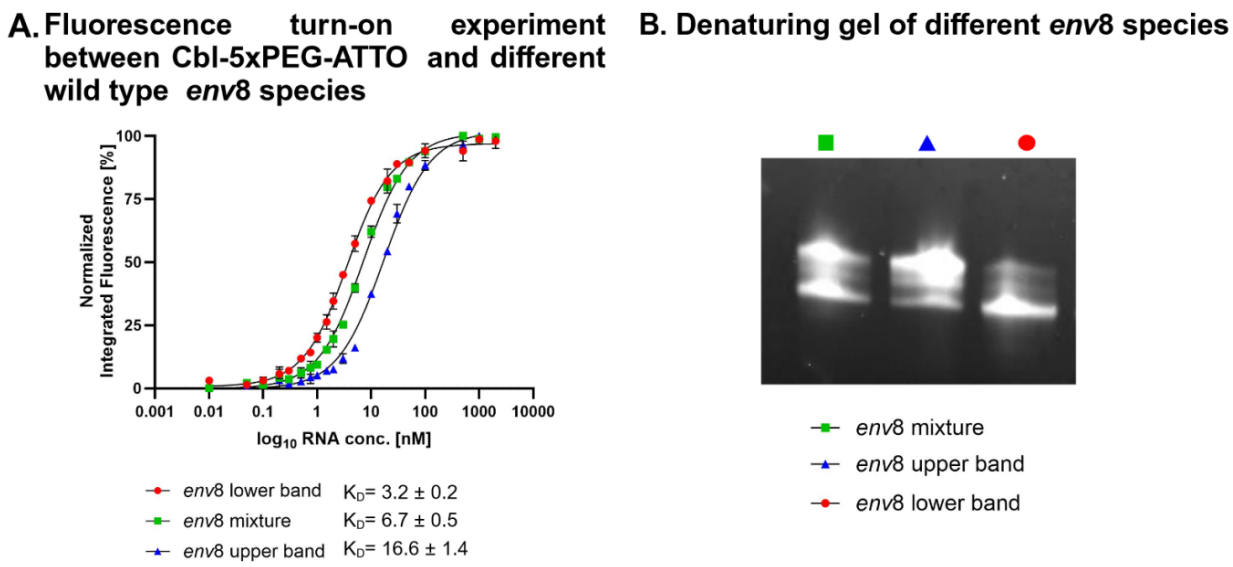
squares showing example boxes outside of the cell. Scale bar = 10 μm. **B.** All equations necessary for calculation of enrichment ratio overlayed with shapes and colors to orient the reader to example intensity values in Panels C and D. **C.** (I) Values for the average probe intensities of the boxes outside the cell corresponding to the labeled boxes in Panel A and the calculation of average background probe intensity. (II) Values taken from the line scans in Panel D corresponding to the labeled arrows in Panel A and the calculation of average SG max probe intensities, background-corrected average SG max probe intensities, average local cytosol probe intensities, background-corrected average local cytosol probe intensities, and enrichment ratios. **D.** Line plots corresponding to the line scans of the labeled arrows in Panel A showing the fluorescence intensity in the GFP-G3BP1 (SG marker) channel and the probe channel versus distance in microns. Each plot shows two filled-in red circles for the two chosen max probe intensities inside the SG and two red outlined circles for the two chosen local cytosol probe intensities. **E.** GFP-G3BP1 channel for the same representative image as in Panel A with three white labeled arrows showing the three line scans with example values in Panels C and D and with more red labeled lines showing the remaining line scans that would be drawn to finish analysis of this cell. Scale bar = 10 μm. **F.** Dot plot of enrichment ratios versus background-corrected average local cytosol probe intensity. Each dot represents one SG. Filled in red circles are from cells without ACTB-4x*env*8-FL-3'*anti*PNA and red outlined circles are from cells co-transfected with ACTB-4x*env*8-FL-3'*anti*PNA. Dashed vertical lines at 550 and 1100 show the chosen thresholds for background-corrected average local cytosol probe intensity. These thresholds were chosen to avoid irregularities caused by particularly large or small denominators in the enrichment ratio equation.



Supporting Figure S11. A. Representative image of a cell stably expressing GFP-G3BP1, co-transfected with NLS-TagBFP and ACTB-4x*env8*-FL-3'*anti*PNA, and beadloaded with 0.5 µM Cbl-PNA-ATTO590. Cell outline in white. Filled-in red triangles show SGs with visible colocalization while outlined red triangles show SGs without visible colocalization. Scale bar = 10 µm. **B.** Dot plots of percent of SGs with visible colocalization in cells co-transfected with NLS-TagBFP without or with ACTB-4x*env8*-FL-3'*anti*PNA and beadloaded with 0.5 µM Cbl-PNA-ATTO590. Each dot represents one cell. n= 6-21 cells across 2-4 imaging dishes for each condition as those quantified in Figure 6 Panel D. Bars show mean and standard deviation. p-values from Kolmogorov-Smirnov test (nonparametric cumulative distribution t-test). $p < 0.0001$ = ****, $p < 0.001$ = ***, $p < 0.01$ = **, $p < 0.05$ = *, $p \geq 0.05$ = n.s.



Supporting Figure S12. A. Representative images of a cell co-transfected with GFP-SMN1 and *env*8-AD-5'*anti*PNA-U1 and beadloaded with 50 µM Cbl-PNA-ATTO590. Cell outline in white. Filled-in red triangles show U-bodies with visible colocalization while outlined red triangles show U-bodies without visible colocalization. Scale bar = 10 µm. **B.** Equation to calculate percent of U-bodies with probe, counts for the representative image in Panel A, and calculation of percent of U-bodies visible in the probe channel. **C.** Dot plot of percent of U-bodies with probe *versus* average local cytosol probe intensity. Average local cytosol probe intensity was calculated by averaging the average probe intensities in three boxes inside the cytosol. Each dot represents one cell. Filled in red circles are from cells without Riboglow aptamer, grey outlined circles are from cells co-transfected with NLS-TagBFP and *env*8-AD-U1, and red outlined circles are from cells co-transfected with NLS-TagBFP and *env*8-AD-5'*anti*PNA-U1. There is no obvious structure to the data caused by average local cytosol intensity, so no thresholds were taken into account.



Supporting Figure S13. A. Fluorescence turn-on assay showing K_D dependence on the purity of the wild type *env*8 ($c_{Cbl\text{-}5xPEG\text{-}ATTO590}$= 1 nM, n=4-8. Error bars represent SEM)**. B**. Denaturing gel showing successful separation on the products (8% acrylamide gel, see Methods). *Note: Upper env8 transcription product is presumably a result of dsRNA self-templated addition by T7.*(2)

2. Supporting Tables

Table S1. RNA sequences. The *anti*PNA and *anti*PNA$_{scr}$ fragments are color-coded with red and green, respectively.

Name	Sequence
*env*8-FL-3'*anti*PNA	GGC CUA AAA GCG UAG UGG GAA AGU GAC GUG AAA UUC GUC CAG AUU ACU UGA UAC GGU UAU ACU CCG AAU GCC ACC UAG GCC AUA CAA CGA GCA AGG AGA CUC A
*env*8-FL-3'*anti*PNA_SHAPE	GGG CCU UCG GCC AAG GCC UAA AAG CGU AGU GGG AAA GUG ACG UGA AAU UCG UCC AGA UUA CUU GAU ACG GUU AUA CUC CGA AUG CCA CCU AGG CCA UAC AAC GAG CAA GGA GAC UCU CGA UCC GGU UCG CCG GAU CCA AAU CGG GCU UCG GUC CGG UUC
*env*8-FL-3'*anti*PNA$_{scr}$	GGC CUA AAA GCG UAG UGG GAA AGU GAC GUG AAA UUC GUC CAG AUU ACU UGA UAC GGU UAU ACU CCG AAU GCC ACC UAG GCC ACA CUA AGA GCA AGG AGA CUC A
*env*8-FL-3'*anti*PNA$_{scr}$_SHAPE	GGG CCU UCG GGCC AAG GCC UAA AAG CGU AGU GGG AAA GUG ACG UGA AAU UCG UCC AGA UUA CUU GAU ACG GUU AUA CUC CGA AUG CCA CCU AGG CCA CAC UAA GAG CAA GGA GAC UCU CGA UCC GGU UCG CCG GAU CCA AAU CGG GCU UCG GUC CGG UUC
*env*8-AD-3'*anti*PNA	GGC CUA AAA GCG UAG UGG GAA AGU GAC GUG AAA UUC GUC CAG AUU ACU UGA UAC GGU UAU ACU CCG AAU GCC ACC UAG GCC AUA CAA C
*env*8-AD-3'*anti*PNA$_{scr}$	GGC CUA AAA GCG UAG UGG GAA AGU GAC GUG AAA UUC GUC CAG AUU ACU UGA UAC GGU UAU ACU CCG AAU GCC ACC UAG GCC ACA CUA A
*env*8-AD-5'*anti*PNA	GAUA CAA C UUU GGC CUA AAA GCG UAG UGG GAA AGU GAC GUG AAA UUC GUC CAG AUU ACU UGA UAC GGU UAU ACU CCG AAU GCC ACC UAG GCC
*env*8-AD-5'*anti*PNA$_{scr}$	GACA CUA A UUU GGC CUA AAA GCG UAG UGG GAA AGU GAC GUG AAA UUC GUC CAG AUU ACU UGA UAC GGU UAU ACU CCG AAU GCC ACC UAG GCC
*env*8$_{mut}$-FL-3'*anti*PNA	G GAG GC CUA AA UCA UAG UG GGG AAG UGA CGU GAA AUU CGU CCA GAU UAC UUG AUA CGG UUA UAC UCC GAG UGC CAC CUA GGC CAU ACA ACG AGC AAG GAG ACU CA
*env*8$_{mut}$-FL-3'*anti*PNA_SHAPE	GGC CUU CGG GCC AAG GCC UAA AUC AUA GUG GGG AAG UGA CGU GAA AUU CGU CCA GAU UAC UUG AUA CGG UUA UAC UCC GAG UGC CAC CUA GGC CAU ACA ACG AGC AAG GAG ACU CAU CGA UCC GGU UCG CCG GAU CCA AAU CGG GCU UCG GUC CGG UUC
*env*8$_{mut}$-FL-3'*anti*PNA$_{scr}$	GGC CUA AA UCA UAG UG GGG AAG UGA CGU GAA AUU CGU CCA GAU UAC UUG AUA CGG UUA UAC UCC GAG UGC CAC CUA GGC CAC ACU AAG AGC AAG GAG ACU CA
*env*8$_{mut}$-FL-3'*anti*PNA$_{scr}$_SHAPE	GGC CUU CGG GCC AAG GCC UAA AUC AUA GUG GGG AAG UGA CGU GAA AUU CGU CCA GAU UAC UUG AUA CGG UUA UAC UCC GAG UGC CAC CUA GGC CAC ACU AAG AGC AAG GAG ACU CAU CGA UCC GGU UCG CCG GAU CCA AAU CGG GCU UCG GUC CGG UUC

Table S2. Primer, ultramer and g-block sequences.

Note: See next page for continuation.

RNA	primer/ultramer/gblock	Sequence
*env*8-FL-3'*anti*PNA	PCR'd plasmid	TAA TAC GAC TCA CTA TAG GGC CTA AAA GCG TAG TGG GAA AGT GAC GTG AAA TTC GTC CAG ATT ACT TGA TAC GGT TAT ACT CCG AAT GCC ACC TAG GCC ATA CAA CGA GCA AGG AGA CTC A
	Forward primer	GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG GCC TAA AAG CGT AG
	Reverse primer	TGA GTC TCC TTG CTC GTT GTA TGG CCT AGG TGG CAT TCG GAG TAT A
*env*8-FL-3'*anti*PNA$_{scr}$	Ultramer	TAA TAC GAC TCA CTA TAG GGC CTT CGG GCC AAG GCC TAA AAG CGT AGT GGG AAA GTG ACG TGA AAT TCG TCC AGA TTA CTT GAT ACG GTT ATA CTC CGA ATG CCA CCT AGG CCA CAC TAA GAG CAA GGA GAC TCT CGA TCC GGT TCG CCG GAT CCA AAT CGG GCT TCG GTC CGG TTC
	Forward primer	GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG GCC TAA AAG CGT AG
	Reverse primer	TGA GTC TCC TTG CTC TTA GTG
*env*8-AD-3'*anti*PNA	Ultramer	TAA TAC GAC TCA CTA TAG GGC CTT CGG GCC AAG GCC TAA AAG CGT AGT GGG AAA GTG ACG TGA AAT TGG TCC AGA TTA CTT GAT ACG GTT ATA CTC CGA ATG CCA CCT AGG CCA TAC AAC TCG ATC CGG TTC GCC GGA TCC AAA TCG GGC TTC GGT CCG GTT C
	Forward primer	GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG GCC TAA AAG CGT AG
	Reverse primer	GTT GTA TGG CCT AGG TGG C
*env*8-AD-3'*anti*PNA$_{scr}$	Ultramer	TAA TAC GAC TCA CTA TAG GGC CTT CGG GCC AAG GCC TAA AAG CGT AGT GGG AAA GTG ACG TGA AAT TCG TCC AGA TAA CTT GAT ACG GTT ATA CTC CGA ATG CC ACCT AGG CCA CAC TAA TCG ATC CGG TTC GCC GGA TCC AAA TCG GGC TTC GGT CCG GTT C
	Forward primer	GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG GCC TAA AAG CGT AG
	Reverse primer	TTA GTGT GGC CTA GGT GGC
*env*8-AD-5'*anti*PNA	Ultramer	TAA TAC GAC TCA CTA TAG GGC CTT CGG GCC AAT ACA ACT TTG GCC TAA AAG CGT AGT GGG AAA GTG ACG TGA AAT TCG TCC AGA TTA CTT GAT ACG GTT ATA CTC CGA ATG CCA CCT AGG CCT CGA TCC GGT TCG CCG GAT CCA AAT CGG GCT TCG GTC CGG TTC
	Forward primer	TAA TAC GAC TCA CTA TAG TAC AAC TTT GGC CTA AAA GCG TAG
	Reverse primer	GGC CTA GGT GGC ATT CGG
*env*8-AD-5'*anti*PNA$_{scr}$	Ultramer	TAA TAC GAC TCA CTA TAG GGC CTT CGG GCC AAC ACT AAT TTG GCC TAA AAG CGT AGT GGG AAA GTG ACG TGA AAT TCG TCC AGA TTA CTT GAT ACG GTT ATA CTC CGA ATG CCA CCT AGG CCT CGA TCC GGT TCG CCG GAT CCA AAT CGG GCT TCG GTC CGG TTC
	Forward primer	TAA TAC GAC TCA CTA TAG CAC TAA TTT GGC CTA AAA GCG TAG TGG
	Reverse primer	GGC CTA GGT GGC ATT CGG

Table S2 continued. Primer, ultramer and g-block sequences.

RNA	primer/ultramer/gblock	Sequence
*env*8$_{mut}$-FL-3'*anti*PNA	Ultramer	GGA GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG GCC TAA ATC ATA GTG GGG AAG TGA CGT GAA ATT CGT CCA GAT TAC TTG ATA CGG TTA TAC TCC GAG TGC CAC CTA GGC CAT ACA ACG AGC AAG GAG ACT C A
	Forward primer	GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG GAG GCC TAA ATC ATA G
	Reverse primer	TGA GTC TCC TTG CTC GTT GTA TGG CCT AGG TGG CAC TCG GAG TAT A
*env*8$_{mut}$-FL-3'*anti*PNA$_{scr}$	Ultramer	GGA GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG GGC TAA ATC ATA GTG GGG AAG TGA CGT GAA ATT CGT CCA GAT TAC TTG ATA CGG TTA TAC TAA GAG TGC CAC CTA GGC CAC ACT AAG AGC AAG GAG ACT CA
	Forward primer	GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG GAG GCC TAA ATC ATA G
	Reverse primer	TGA GTC TCC TTG CTC TTA GTG
*env*8-FL-3'*anti*PNA_SHAPE	gBlock	GGG CCT TCG GCC AAG GCC TAA AAG CGT AGT GGG AAA GTG ACG TGA AAT TCG TCC AGA TTA CTT GAT ACG GTT ATA CTC CGA ATG CCA CCT AGG CCA TAC AAC GAG CAA GGA GAC TCT CGA TCC GGT TCG CCG GAT CCA AAT CGG GCT TCG GTC CGG TTC
	Forward primer	GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG
	Reverse primer	GAA CCG GAC CGA AGC CCG
*env*8-FL-3'*anti*PNA$_{scr}$_SHAPE	Ultramer	GGG CCT TCG GGC CAA GGC CTA AAA GCG TAG TGG GAA AGT GAC GTG AAA TTC GTC CAG ATT ACT TGA TAC GGT TAT ACT CCG AAT GCC ACC TAG GCC ACA CTA ATC GAT CCG GTT CGC CGG ATC CAA ATC GGG CTT CGG TCC GGT TC
	Forward primer	GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG
	Reverse primer	GAA CCG GAC CGA AGC CCG
*env*8$_{mut}$-FL-3'*anti*PNA_SHAPE	Ultramer	GGA GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG GCC TAA ATC ATA GTG GGG AAG TGA CGT GAA ATT CGT CCA GAT TAC TTG ATA CGG TTA TAC TCC GAG TGC CAC CTA GGC CAT ACA ACG AGC AAG GAG ACT C A
	Forward primer	GCG CGC GAA TTC TAA TAC GAC TCA CTA TAG GCC TTC GGG CCA AGG CCT AAA TCA TAG
	Reverse primer	GAA CCG GAC CGA AGC CCG ATT TGG ATC CGG CGA ACC GGA TCG ATG AGT CTC CTT GCT CGT
*env*8-AD-5'*anti*PNA-U1	gBlock	AAG ATC TCA TAC TTA CCT GAT ACA ACT TTG GCC TAA AAG CGT AGT GGG AAA GTG ACG TGA AAT TCG TCC AGA TTA CTT GAT ACG GTT ATA CTC CGA ATG CCA CCT AGG CCG CAG GGG AGA TAC CAT GAT CA
	Forward primer	ATG CGA AGA TCT CAT ACT TAC CTG ATA C
	Reverse primer	ACT GTG ATC ATG GTA TCT CCC C

Table S3. Reaction conditions for PCR and *in vitro* transcription.

PCR[a] (Reaction volume = 100 µL)	
Reagent (stock concentration)	**Volume [µL]**
MilliQ H_2O	84
Pfu Buffer[b] (10x)	10
dNTPs (10 mM)	2
Forward primer (100 µM)	1
Reverse primer (100 µM)	1
Template DNA (1 µM)	1
Pfu polymerase[e]	1
Transcription[c] (Reaction volume = 1 mL)	
Reagent (stock concentration)	**Volume [µL]**
MilliQ H_2O	590
Transcription buffer[d] (10x)	100
$MgCl_2$ (2 M)	12
DTT (1 M)	8
rATP (100 mM)	40
rGTP (100 mM)	40
rCTP (100 mM)	40
rUTP(100 mM)	40
PCR template	100
Inorganic pyrophosphatase (IPP)[e]	10
T7 RNA polymerase[e]	20

[a]The reaction is amplified in a PCR machine with the program: initial melt for 2 min at 95°C; 30 cycles of: 95°C for 30 s, 50°C for 30 s, and 72°C for 45 s; 10 min final extension at 72°C; hold at 4°C. [b]Pfu buffer: 200 mM Tris-HCl, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, 1 mg/mL BSA. [c]The reaction is carried out at 37°C for 2.5 h. [d]Transcription buffer: 400 mM Tris pH 8.0, 100 mM DTT, 20 mM spermidine, 0.1% Triton X-100.[e]reagents made in house.

Table S4. Roadmap for fluorescence turn-on assay between Cbl-PNA-ATTO590 and *env*8-FL-3'*anti*PNA.

Each titration point contains: probe (1 nM), RNA (dissolved in water; concentration depends on the titration point), 1xRNA buffer, 0.01% nonidet P40 (surfactant; eliminates the issue of fluorophore sticking to eppendorf tubes and 384-well plates), 10% DMSO (ensures solubility of the probe)

The reactions are prepared in 1.5 mL eppendorfs. Two technical replicates are prepared in one eppendorf (total volume 2 x 60 µL = 120 µL). Every reaction contains equal volume of the master mix (see below) and different concentrations of the RNA. 55 µL of the final reaction mixture is pipetted twice into a Corning 384-well plate.

Master mix contains: probe, 10 x RNA buffer, DMSO, nonidet P40, H_2O (enough to bring all the components to the expected concentrations, see below)

Notes:

- 10xRNA buffer contains: 1M KCl, 100 mM NaCl, 10 mM $MgCl_2$, 500 mM HEPES pH=8
- $c_{RNA\ stock}$= 40 µM (MilliQ water)
- $c_{probe\ stock}$= 335 µM (DMSO)

The table contains the volumes of reagents needed to perform the titration. For calculations, see below (color-coding is used for each reagent for easier text follow-up):

Titration point	c_{RNA} [nM]	V_{RNA} [uL] (stock dilution)	V_{H2O} [uL]	$V_{master\ mix}$ [uL]	V_{total} [uL]= V_{RNA} + V_{H2O} + $V_{master\ mix}$
1	0	0	15	105	120
2	0.01	3 (1: 100000)	12		
3	0.05	15 (1: 100000)	0		
4	0.1	3 (1: 10000)	12		
5	0.2	6 (1: 10000)	9		
6	0.3	9 (1: 10000)	6		
7	0.5	15 (1: 10000)	0		
8	0.75	2.25 (1:1000)	12.75		
9	1	3 (1:1000)	12		
10	1.5	4.5 (1:1000)	10.5		
11	2	6 (1:1000)	9		
12	3	9 (1:1000)	6		
13	5	15 (1:1000)	0		
14	10	3(1:100)	12		
15	20	6 (1:100)	9		
16	50	15 (1:100)	0		

How to calculate the volume of the RNA stock solution in each reaction (V_{RNA} [uL])?

Example for titration point 16:

$c_{RNA\ stock}$= 40 µM

$V_{reaction\ total}$ = 120 µL

$c_{RNA\ for\ point\ 16}$ = 50 nM

V_{RNA} = ($c_{RNA}/c_{RNA\ stock}$)* $V_{reaction\ total}$ = [(50/1000)/40]*120 = 0.15 µL of RNA stock solution or 15 µL of RNA stock solution diluted at 1:100 ratio

Use the same approach to calculate the remaining titration points. See the table above for calculation results.

How much of each RNA dilution is required?

Start with diluting RNA stock to 1:10 and then proceed with serial dilutions. Prepare 30% more of each solution to have sufficient amount for the serial dilutions.

Below are summarized RNA volumes required for the titration:

$V_{RNA\ stock}$ (1:10) = 10 µL

$V_{RNA\ stock}$ (1:100, titration points 14-16) = 3+6+15+0.3*(3+6+15) = 32 µL

$V_{RNA\ stock}$ (1:1000, titration points 8-13) = (2.25+3+4.5+6+9+15)+0.3*(2.25+3+4.5+6+9+15)= 52 µL

$V_{RNA\ stock}$ (1:10000, titration points 4-7) = 3+6+9+15+0.3*(3+6+9+15)= 43 µL

$V_{RNA\ stock}$ (1:100000, titration points 2-3) = 3+15+0.3*(3+15)= 23 µL

How to calculate the amount of water?

The volume of the master mix remains constant for each titration point. However, since different volumes of RNA dilutions are used, additional water volume must be added to each eppendorf tube to bring the total volume in each well to the same level. Use the highest volume of the RNA dilution as a reference (in this case, 15 µL), and add this amount of water to the first eppendorf, which contains no RNA (the first titration point). For the remaining wells, subtract the volume of RNA from 15 µL (see the blue column in the table above for the results).

How to prepare the master mix?

Master mix contains: probe, 10x RNA buffer, DMSO, nonidet P40, H_2O

Begin by estimating the number of reactions. There are 16 titration points, but it's advisable to add extra volume in case of any repeats. Adding an extra 25% results in 20 reactions of volume 120 µL:

Probe:
$c_{probe\ stock}$= 335 µM
$C_{probe\ in\ reaction}$= 1 nM

$V_{probe\ stock\ for\ 20\ reactions}$= [($c_{probe\ in\ reaction}$/$c_{probe\ stock}$)* $V_{reaction\ total}$]*20 = [(1/(335*1000))*120]*20 = 0.0072 µL or 7.2 µL of probe stock solution diluted at 1:100 ratio

10x RNA buffer:

The reaction contains 1x RNA buffer, and the total reaction volume is 120 µL, so in each reaction there is 12 µL of 10x RNA buffer. Multiply 12 µL by the amount of the reactions:

$V_{10xRNAbuffer}$ = 12 µL * 20 = 240 µL

DMSO:

The final DMSO concentration in the reaction is 10%, so there is 12 µL of DMSO in each 120 µL reaction, analogous to the 10x RNA buffer.

V_{DMSO}= 12 µL * 20 = 240 µL

Nonidet P40:

Use 1% solution of nonidet. The final concentration of nonidet P40 in the reaction is 0.01%, so there is 1.2 µL of 1% nonidet P40 in each 120 µL reaction. For 20 reactions:

$V_{1\%nonidetP40}$= 1.2 µL * 20 = 24 µL

Water

This is the remaining amount of water that allows to obtain the expected volume and concentrations for all the ingredients.

$V_{H2O\ in\ the\ master\ mix\ for\ 20\ reactions}$= [$V_{reaction\ total}$ - (V_{RNA}+V_{H2O}) - $V_{10xRNAbuffer}$ - V_{DMSO} - $V_{1\%nonidetP40}$ – ($V_{probe\ stock\ for\ 20\ reactions}$/20)*20= [120 - 15 – 12 – 12 – 1.2 – (7.2/20)]*20 = 1588.8 µL

Master mix = $V_{probe\ stock\ for\ 20\ reactions}$ + $V_{10xRNAbuffer}$ + V_{DMSO} + $V_{1\%nonidetP40}$ + $V_{H2O\ in\ the\ master\ mix}$= 7.2 + 240 + 240 + 24 + 1588.8 = 2100 µL

The volume of master mix required for reach reaction:

$V_{master\ mix/reaction}$ = 2100/20 = 105 µL.

Other way of calculating $V_{master\ mix/reaction}$:

$V_{master\ mix/reaction} = V_{reaction\ total} - (V_{RNA}+V_{H2O}) = 120 – 15 = 105$ µL

Preparing the reactions and plating

- Prepare 16 numbered eppendorfs of 1.5 mL volume.
- Thaw RNA stock on ice; prepare RNA dilutions in H_2O; heat at 90°C for 3 minutes before incubating on ice for at least 10 min
- Prepare master mix during RNA incubation
- Pipet water volumes according to the amounts from the table above (V_{H2O})
- Pipet master mix into each eppendorf (105 µL per reaction); for consistency vortex master mix every 3 eppendorfs
- Spin down all eppendorfs
- Spin down RNA dilutions and pipet into eppendorfs according to the amounts from the table above (V_{RNA})
- Vortex and spin down all eppendorfs
- Plate 55 µL of each reaction on 384 well plate (each reaction is pipetted twice; vortex each eppendorf before plating)
- Pipet one buffer well (55 µL) for background subtracting purpose (buffer for background subtraction (1 mL): 100 µL of 10x RNA buffer, 100 µL DMSO, 10 µL of 1% nonidet P40, 790 µL H_2O)
- Incubate at room temperature in the dark for 1 hour before reading
- Read the plate using suitable program (see Methods)

Note: The above roadmap was appropriately adjusted depending on the titration range required for each probe. Every RNA stock solution concentration used in the study was 40 µM and every probe stock concentration was 335 µM.

Table S5. Imaging settings for live cell experiments. Laser wattages were collected with a PM100A Optical Power Meter (Thorlabs) and a S130C 400 nm – 1100 nm sensor (Thorlabs) on the 5 mW setting. Reported wattages were collected just before the objective in the light path (at an open slot on the objective wheel) and are reported as the max wattage in the manually scanned area.

Experiment	Imaging Settings
SG assay RNA: ACTB-4x*env8*-FL-3'*anti*PNA Probe: 5 μM Cbl-PNA-ATTO590 or 5 μM Cbl-5xPEG-ATTO590	405 nm (NLS-TagBFP): 0.7 (0.065 mW) – 5% (0.090 mW), 90 gain 488 nm (GFP-G3BP1): 0.5 (0.029 mW) – 1.5% (0.035 mW), 40 gain 561 nm (Cbl-PNA-ATTO590, Cbl-5xPEG-ATTO590): 20% (0.344 mW), 40 gain 2X averaging, 12.1 μs dwell time Nyquist sampling (1.2 AU) defined by 561-nm laser Single plane image
SG assay RNA: ACTB-4x*env8*-FL-3'*anti*PNA Probe: 5 μM Cbl-4xGly-ATTO590	405 nm (NucBlue): 2% (0.072 mW), 90 gain 488 nm (GFP-G3BP1): 0.5 (0.029 mW) – 2% (0.041 mW), 40 gain 561 nm (Cbl-4xGly-ATTO590): 20% (0.344 mW), 40 gain 640 nm (PB-HaloTag-ACTB-0x + HaloTag-JF669 ligand): 0.5 (0.017 mW) – 5% (0.125 mW), 90 gain 2X averaging, 12.1 μs dwell time Nyquist sampling (1.2 AU) defined by 561-nm laser Single plane image
SG assay RNA: (1/8)NORAD-3x*env8*-FL-3'*anti*PNA, ACTB-4x*env8*-FL-3'*anti*PNA, or (1/2)NORAD-4x*env8*-FL-3'*anti*PNA Probe: 5 μM Cbl-PNA-ATTO590	405 nm (NLS-TagBFP): 1 (0.067 mW) – 10% (0.119 mW), 90 gain 488 nm (GFP-G3BP1): 0.3 (0.027 mW) – 1.2% (0.034 mW), 40 gain 561 nm (Cbl-PNA-ATTO590): 8% (0.144 mW), 40 gain 2X averaging, 12.1 μs dwell time Nyquist sampling (1.2 AU) defined by 561-nm laser Single plane image
SG assay RNA: (1/2)NORAD-4x*env8*-FL-3'*anti*PNA Probe: 5 μM Cbl-PNA-ATTO488	405 nm (NLS-TagBFP): 2 (0.072 mW) – 10% (0.119 mW), 90 gain 488 nm (Cbl-PNA-ATTO488): 20% (0.198 mW), 30 gain 640 nm (HaloTag-G3BP1 + HaloTag-JF669 ligand): 2 (0.055 mW) – 6% (0.148 mW), 90 gain 2X averaging, 12.1 μs dwell time Nyquist sampling (1.2 AU) defined by 488-nm laser Single plane image
SG assay RNA: ACTB-4x*env8*-FL-3'*anti*PNA Probe: 0.5 μM Cbl-PNA-ATTO590 or 0.5 μM Cbl-4xGly-ATTO590	405 nm (NLS-TagBFP): 0.7 (0.065 mW) – 10% (0.119 mW), 90 gain 488 nm (GFP-G3BP1): 0.3 (0.027 mW) – 6% (0.075 mW), 40 gain 561 nm (Cbl-PNA-ATTO590, Cbl-4xGly-ATTO590): 30% (0.512 mW), 40 gain 2X averaging, 12.1 μs dwell time Nyquist sampling (1.2 AU) defined by 561-nm laser Single plane image
U-body assay RNA: *env8*-AD-U1 or *env8*-AD-5'*anti*PNA-U1 Probe: 50 μM Cbl-PNA-ATTO590	405 nm (NucBlue): 2 (0.072 mW) – 5%, 90 gain 488 nm (EGFP-SMN1): 0.2 (0.023 mW) – 1% (0.031 mW), 20 – 40 gain 561 nm (Cbl-PNA-ATTO590): 5% (0.093 mW), 40 gain 2X averaging, 12.1 μs dwell time Nyquist sampling (1.2 AU) defined by 561-nm laser Single plane image

3. Supporting Information: Methods

3.1. RNA synthesis and purification

For all *in vitro* experiments, RNAs were synthesized using template DNA amplified through PCR. DNA templates were amplified using a series of overlapping oligonucleotides ordered from IDT (Supporting Table S2). Transcriptions used T7 RNA polymerase (for details see Supporting Table S3).(3) The RNAs were purified using denaturing PAGE (8% acrylamide:bisacrylamide 29:1, 8 M urea, 1x Tris-Borate-EDTA (TBE) buffer). This was polymerized with 10% (w/v) ammonium persulfate (500 µL for 100 mL of gel) and tetramethylethlyenediamine (TEMED, 50 µL for 100 mL of gel). The gel was preheated at 14 W for about 30 minutes prior to loading. 1x TBE buffer was used as running buffer. RNAs were mixed with an equal volume of denaturing dye (85% formamide, 0.5x TBE, 50 mM EDTA, pH 8.0, with xylene cyanol and bromophenol blue tracking dyes) and heated at 90° C for 3 minutes prior to loading without cooling. RNA samples were loaded and the gel ran for about 2.5 hours at 14 W at room temperature. *In vitro* transcription of wild type *env*8 RNA led to two major products. Analysis of Cbl binding to the upper band, the lower band, or a mixture of two bands revealed the lower band to be the correct species (Supporting Figure S12). All subsequent binding experiments were conducted with the purified lower band. Full length transcripts were visualized by UV shadowing, excised from the gel, and eluted from the gel by soaking at 4 °C in MilliQ water. RNA was concentrated into MilliQ water using centrifugal concentrators (Amicon) with 10 KDa molecular weight cutoff. Final RNA concentrations were calculated using A260 and molar extinction coefficients determined from the summation of the individual bases. For selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) assays, RNA was gel purified twice and stored at -80 °C until use. Other gel purified RNA was purified once and stored at -20 °C until use. Secondary structures and sequences of all riboswitch RNAs used in this study are shown in Supporting Figure S4 and Supporting Table S1, respectively.

3.2. Fluorescence turn-on experiments

All *in vitro* experiments were conducted in RNA buffer (100 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 50 mM HEPES, pH 8.0) with the addition of 0.01% nonidet P40 and 10% DMSO (v/v). For the Cbl-fluorophore probes, the extinction coefficient of the fluorophores was used to determine the concentration (120000 $M^{-1}cm^{-1}$ for ATTO590 and 90000 $M^{-1}cm^{-1}$ for ATTO488, source: ATTO-TEC). To determine the binding affinity of each probe to the RNA of interest, a series of titration experiments were performed. Relevant amounts of RNA were titrated into 120 µL reactions into Eppendorf tubes, ensuring a final concentration of 1 nM cobalamin probe (for details, see Supporting Table S4). All reactions were performed in technical duplicate and in at least biological triplicate. The reactions were pipetted into a Corning 384-well plate (2 x 55 µL). The reactions were allowed to equilibrate for 60 minutes at room temperature in the dark before reading. Extended equilibration times (up to 24 hrs) did not significantly alter the K_D for the titration of *env*8-FL-3'*anti*PNA and Cbl-PNA-ATTO590, therefore we settled on the 60 min time point. ATTO590 fluorescence

was excited at 590 ± 8 nm and fluorescence emission was from collected from 615-675 nm using a BMG Labtech CLARIOstarPLUS microplate reader. ATTO488 was excited at 485 ± 5 nm and fluorescence emission was from collected from 505-565 nm using the same instrument. Fluorescence values were background corrected by subtracting the fluorescence values of a buffer control at each wavelength and integrated over all wavelengths. The corrected and integrated fluorescence values were plotted versus log(nM RNA) in GraphPad Prism. Technical replicates were fit to the quadratic binding equation with one transition $Y = m + (n - m) * (((c + x + K) - sqrt(sqr(c + x + K) - (4 * c * x)))/(2 * c))$ where Y is the corrected integrated fluorescence value, m is the lower baseline, n is the upper baseline, c is the probe concentration, x is the RNA concentration, and K is the K_D.(4) Calculated K_Ds are the average of multiple biological replicates, and errors were calculated from those average K_Ds. Biological replicates were combined for the final graphs shown in the figures. To combine replicates and calculate the fraction of probe bound to RNA, each was normalized to values between 0 and 100, where 0 was the value of the lower baseline and 100 was the value of the upper baseline as calculated by the quadratic binding equation. The fold turn-on of 1 nM probes was calculated by dividing the fluorescence value obtained from titration point at c_{RNA} = 50 nM (for Cbl-PNA-ATTO590 and Cbl-PNA-ATTO488) and c_{RNA} = 500 nM (for Cbl-5xPEG-ATTO590 and Cbl-4xGly-ATTO590) by the fluorescence value obtained for the free probe. Same fluorescence values were used to determine quenching/dequenching levels for each probe. Quenching levels were derived from titration point at c_{RNA} = 50 nM (for Cbl-PNA-ATTO590 and Cbl-PNA-ATTO488) and c_{RNA} = 500 nM (for Cbl-5xPEG-ATTO590 and Cbl-4xGly-ATTO590) while dequenching levels from fluorescence values obtained for free probe at 1 nM. Each value was divided by the fluorescence level obtained for the free fluorophore ($c_{free\ dye}$ = 1 nM) to obtain quenching/dequenching levels. Data were normalized to values between 0 and 100, where 0 was the value of the lower baseline and 100 was the value of the upper baseline.

3.3. Stress granule (SG) assay setup and imaging

175,000 U-2 OS cells stably expressing GFP-G3BP1 or HaloTag-G3BP1 were seeded into home-made 3.5-cm cell culture-treated imaging dishes with a ~10 mm center hole covered by cover glass (No. 1.5, VWR) 2 days before imaging. About 24 hours before imaging, cells were transfected using TransIT (Mirus Bio) and ~292 fmol ACTB-4xenv8-FL-*3'antiPNA* (as described previously,(5) Addgene plasmid #112058), (1/2)NORAD-4x*env*8-FL-3'*anti*PNA (Addgene plasmid #199208), or (1/8)NORAD-4x*env*8-FL-3'*anti*PNA (Addgene plasmid #199209). For all experiments, unless noted otherwise, NLS-TagBFP (Addgene plasmid #55265) was used in an equimolar amount as a co-transfection marker which was shown to co-express from the same cell as the other transfected plasmid 94% of the time.(5) In the 5 μM Cbl-4xGly-ATTO590 experiments only, an equimolar amount of PB-HaloTag-ACTB-0x was used instead as a co-transfection marker. On the day of imaging, cells were beadloaded as previously described(5) with 3 μL of 5 μM or 0.5 μM probe (2% DMSO, 98% D-PBS by volume). 2 mL of cell culture media with 0.5 mM sodium arsenite (Sigma-Aldrich) was added immediately after beadloading to induce stress granules. For the 5 μM Cbl-4xGly-ATTO590 experiments only, 10 nM HaloTag-JF669 (Lavis Labs) and 2 drops of NucBlue live cell stain

(Life Technologies) were also added at the time of beadloading to stain the HaloTag-ACTB co-transfection protein, and illuminate the nucleus, respectively. For the 5 μM Cbl-PNA-ATTO488 experiments, cells were beadloaded with 6 μL of 5 μM probe and 100 nM HaloTag-JF669 ligand and 2 drops of NucBlue live cell stain (Life Technologies) were added at the time of beadloading to stain the HaloTag-G3BP1 protein and illuminate the nucleus. Cells were allowed to recover at 37 °C and 5% CO_2 for 30 minutes to 1 hour. Then cells were prepped for imaging by removing the media, rinsing once with 1 mL FluoroBrite DMEM (Fisher) supplemented with 10% FBS and 0.5 mM sodium arsenite, and imaged in 1 mL FluoroBrite DMEM supplemented with 10% FBS and 0.5 mM sodium arsenite. Cells were then imaged in a LiveCell stage top environmental chamber (Pathology Devices, Inc.) at 37 °C, 5% CO_2, and 95% humidity for up to 2 hours. Images were collected on a Nikon Ti-E A1R laser scanning confocal microscope with a 100X (1.45 NA) Plan Apo Lambda oil objective (Nikon) and 405-nm (Coherent OBIS), 488-nm (Coherent OBIS), 561-nm (Coherent Sapphire), and 640-nm (Coherent OBIS) lasers. Signal from excitation with 405-nm and 488-nm lasers were collected with Nikon PMT detectors and signal from excitation with 561-nm and 640-nm lasers were collected with GaAsp PMT detectors. Imaging settings are defined in Supporting Table S5. Expression of transfected plasmids for longer than 30 hours or collection of a z-stack that was then maximum-intensity projected for analysis resulted in significantly decreased dynamic range between the probe alone and plus aptamer RNA conditions.

3.4. Stress granule (SG) assay analysis

Confocal images as .nd2 files were imported into Fiji/ImageJ using the Bio-Formats Importer. Channels were separated and the expression of the co-transfection marker and presence of beadloaded probe were confirmed. The line selection tool was used to draw a line across a SG in the SG marker channel, save the selection in the ROI Manager, and make a line plot of the fluorescence intensities with Plot Profile. The ROI manager was used to bring up the same line selection in the probe channel and make a line plot of the fluorescence intensities with Plot Profile. The two line profiles were aligned and two max probe intensities inside the SG (as defined by high SG marker intensity) were recorded in an Excel spreadsheet with the image date, construct, probe identity, probe concentration, cell number, and SG number. The same was done for two average probe intensities outside of the SG (as defined by low SG marker intensity), avoiding the nucleus because some of the Riboglow probe localizes to the nucleus. Line profiles of each SG in the cytosol with well-defined edges and larger than 2 px-by-2 px were collected, and probe intensities inside and outside the SG were recorded. To correct for imaging media fluorescence and detector background noise, three boxes larger than 15 px-by-15 px were drawn outside of cells in the image using the rectangle selection tool. The mean probe intensity of each background box was determined with Measure and recorded in the Excel spreadsheet for that image. The average background probe intensity, the average SG max probe intensity, the average local cytosol probe intensity, the background-corrected average SG max probe intensity, the background-corrected average local cytosol probe intensity, and the enrichment ratio were calculated in Excel with the equations in Supporting Figure S10B. For each imaging set, the enrichment

ratio was plotted as a function of the background-corrected average local cytosol probe intensity. Thresholds for the background-corrected average local cytosol probe intensity were determined by the shape of the data, setting the lower threshold to remove high enrichment values caused by a small denominator and setting the higher threshold to remove low enrichment values caused by a large denominator. Statistical tests were only performed on data sets that used the same imaging settings and threshold values. A Q-test was performed on each data set to determine if the highest or lowest enrichment ratio should be removed from the thresholded data set. No more than one data point was removed from each thresholded data set. A Kolmogorov-Smirnov test (nonparametric cumulative distribution t-test) was performed on graphs with two data sets, and a Kruskal-Wallis test (nonparametric one-way ANOVA) was performed with each condition compared against each other condition on graphs with more than two data sets. Graphing of data and statistical tests were done in GraphPad Prism.

3.5. U-body assay setup and imaging

175,000 U-2 OS cells were seeded into home-made 3.5-cm cell culture-treated imaging dishes with a ~10 mm center hole covered by cover glass (No. 1.5, VWR) 2 days before imaging. About 24 hours before imaging, cells were transfected using TransIT (Mirus Bio) and ~448 fmol *env*8-AD-U1 as described previously,(5) Addgene plasmid #112059) or *env*8-AD-5'*anti*PNA-U1 (described above). ~65 fmol EGFP-SMN1 (Addgene plasmid #37057) was used as a co-transfection marker and a U-body marker protein. On the day of imaging, 1 mL of cell culture media with 10 μM thapsigargin (Calbiochem/VWR) was added 3 hours before imaging to induce U-bodies. One hour before imaging, cells were beadloaded as previously described(5) with 3 μL of 50 μM probe (25% DMSO, 75% D-PBS by volume). 1 mL of cell culture media with 10 μM thapsigargin and 2 drops of NucBlue live cell stain (Life Technologies) were added immediately after beadloading to keep inducing U-bodies and to stain the nucleus. Cells were allowed to recover at 37 °C and 5% CO_2 for 30 minutes to 1 hour. Cells were prepped for imaging by removing the media, rinsing once with 1 mL FluoroBrite DMEM (Fisher) supplemented with 10% FBS and 10 μM thapsigargin. Cells were imaged in 1 mL FluoroBrite DMEM supplemented with 10% FBS and 10 μM thapsigargin in a LiveCell stage top environmental chamber (Pathology Devices, Inc.) at 37 °C, 5% CO_2, and 95% humidity for up to 2 hours. Images were collected on a Nikon Ti-E A1R laser scanning confocal microscope with a 100X (1.45 NA) Plan Apo Lambda oil objective (Nikon) and 405-nm (Coherent OBIS), 488-nm (Coherent OBIS), 561-nm (Coherent Sapphire), and 640-nm (Coherent OBIS) lasers. Signal from excitation with 405-nm and 488-nm lasers were collected with Nikon PMT detectors and signal from excitation with 561-nm and 640-nm lasers were collected with GaAsp PMT detectors. Imaging settings are defined in Supporting Table S5.

3.6. U-body assay analysis

Confocal images as .nd2 files were imported into Fiji/ImageJ using the Bio-Formats Importer. Channels were separated and the expression of the co-transfection marker/U-body marker protein and presence of beadloaded probe were confirmed. The U-body marker protein channel was initialized in Cell Counter and

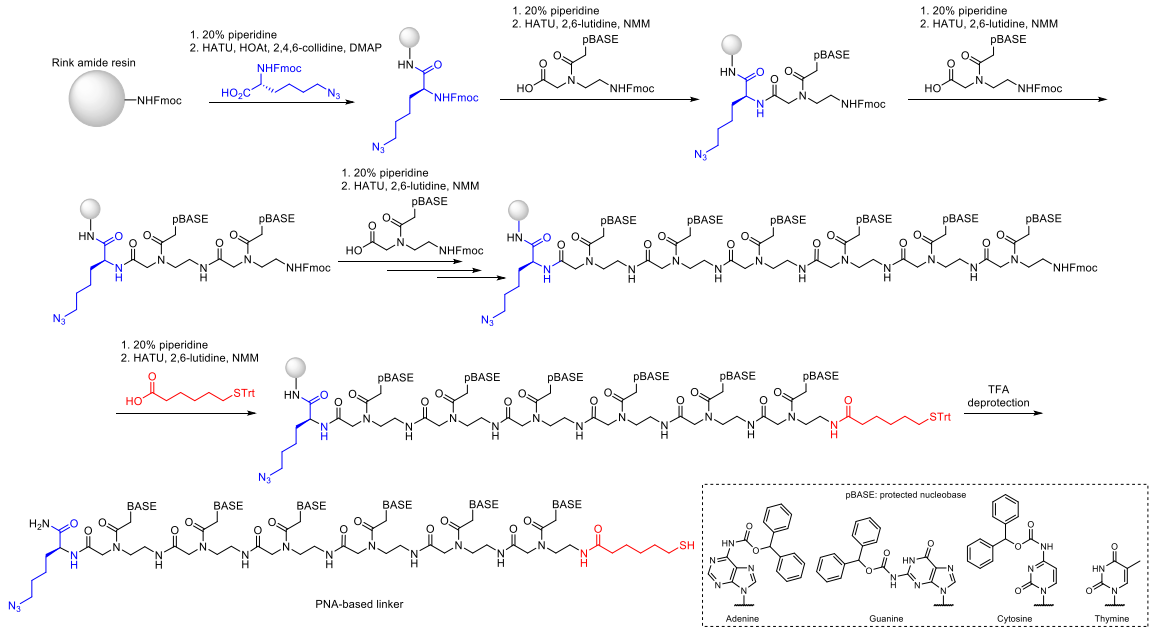
channels were synced with Synchronize Windows. One counter type in Cell Counter was used to mark U-bodies that visibly had probe and another counter type was used to mark U-bodies that did not visibly have probe. This was repeated for all U-bodies in the cytosol with well-defined edges and larger than 2 px-by-2 px. The number of U-bodies with visible colocalization and the number of U-bodies with no visible colocalization were recorded in an Excel spreadsheet with the image date, construct, probe identity, probe concentration, and cell number. For each cell, three boxes larger than 4 px-by-4 px were drawn inside the cytosol using the rectangle selection tool. The mean probe intensity of each cytosol box was determined with Measure and recorded in Excel. The percent of U-bodies with probe was calculated with the equation in Supporting Figure S12 in Excel. For each imaging set, the percent of U-bodies with probe was plotted as a function of average cytosol intensity, but it was determined that no threshold for the average cytosol intensity was required. Statistical tests were only performed on data sets that used the same imaging settings. A Q-test was performed on each data set to determine if the highest or lowest percent of U-bodies with probe should be removed from the data set. No more than one data point was removed from each data set. A Kruskal-Wallis test (nonparametric one-way ANOVA) was performed with each condition compared against each other condition. Graphing of data and statistical tests were done in GraphPad Prism.

4. Supporting Information: Probe synthesis and characterization

4.1. General Information

Commercially available reagents and solvents were used as received. PNA monomers were obtained from PNA Bio and Rink amide resin from Chem-Impex. ATTO590 propargylamide and ATTO488 propargylamide were obtained from Millipore Sigma. As supplied ATTO590 consists of a mixture of two isomers with similar spectral properties (para and meta isomer). For simplicity only one isomer is presented on the schemes. The scale of the reaction with ATTO dyes did not provide sufficient amount of the products for NMR analyses, thus the HPLC and HR MS analyses were performed to characterize and confirm the purity of the probes. The synthesis of Cbl-PNA conjugates does not require purification of the respective PNA linker; therefore, crude material was used to synthesize these conjugates and only small amount of each PNA linker was purified via semipreparative HPLC and characterized via HPLC and HR MS. ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Bruker 400 MHz spectrometer with the residual solvent peak used as an internal standard. Data are reported as follows: chemical shift, peak multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants (Hz), and number of protons. High-resolution ESI mass spectra were recorded on Waters Synapt G2 HDMS qTOF. All reactions and product purities were monitored using RP-HPLC techniques. Semipreparative chromatography was performed using LiChroprep RP-18 (40–63 mm) with HPLC grade water and MeCN as eluents. HPLC analytical measurement conditions: column, Kromasil 100-5-C18, 250 mm, 4.6 mm; detection, UV/Vis; pressure, 10 MPa; temperature, 22ºC, flow 1 mL/min or phenomenex Jupiter 5u C18 300A, 250 mm, 4.6 mm; detection, UV/Vis; pressure, 10 MPa; temperature, 22ºC, flow 1 mL/min. HPLC semipreparative measurement conditions: Kromasil 100-5-C18, 250 mm, 4.6 mm; detection, UV/Vis; pressure, 20 MPa; temperature, 22ºC, flow 3 mL/min. Abbreviations: AcOEt – ethyl acetate; Bhoc – benzhydryloxycarbonyl protecting group; CDT – 1,1′-Carbonyldi-(1,2,4-triazole); DIPEA – N,N-Diisopropylethylamine; DMAP – 4-Dimethylaminopyridine; Et_2O – diethyl ether; Fmoc – fluorenylmethoxycarbonyl protecting group; HATU – 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; HOAt – 1-Hydroxy-7-azabenzotriazole; MeCN – acetonitrile; MeOH – methanol; NMM – 4-Methylmorpholine; NMP – N-Methyl-2-pyrrolidone; RP HPLC – Reverse-phase high-performance liquid chromatography; TBTA –Tris[(1-benzyl-1H-1,2,3-triazol-4 yl)methyl]amine; TFA – trifluoroacetic acid.

4.2. Synthesis of PNA linkers



Scheme S1. Schematic representation of PNA linker synthesis using Fmoc chemistry.

PNA linkers were synthesized according to previously published protocol(6) with modifications. PNA linkers were synthesized manually by Fmoc chemistry on 0.05 mmol scale using 2.5 molar excess of the Fmoc/Bhoc protected PNA monomers, 3.0 molar excess of Fmoc-Lys(N_3)-OH, 3.0 molar excess of 6-(tritylthio)hexanoic acid and Rink amide resin (loading 0.4 mmol/g). Fmoc deprotection of the resin was carried with 20% piperidine in DMF (1x for 5 min and 1x for 15 min). Fmoc-Lys(N_3)-OH was activated with the mixture of HATU (3.0 equiv), HOAt (3.0 equiv.), 2,4,6-collidine (6.0 equiv.) and catalytic amount of DMAP in DMF/NMP (1:1; v/v). Coupling of Fmoc-Lys(N_3)-OH was carried for 2 h. Deprotection of the Fmoc form the lysine was carried with 20% piperidine in DMF (1x for 5 and 1x for 15 min). PNA momomers were activated with the mixture of HATU (2.3 equiv), NMM (2.5 equiv) and 2,6-lutidine (3.75 equiv) in DMF/NMP (1:1; v/v). Each coupling of the PNA monomer was performed twice for 40 min. Fmoc deprotection of each PNA monomer was performed with 20% of piperidine in DMF (2 × 2 min). 6-(tritylthio)hexanoic acid was activated with HATU (2.8 equiv), NMM (3.0 equiv) and 2,6-lutidine (4.5 equiv) in DMF/NMP (1:1; v/v). Coupling of 6-(tritylthio)hexanoic acid was performed twice for 30 min. The resin was washed consecutively with DCM (2x), DMF (5x) and DCM (3x) after each Fmoc deprotection and with DMF (3x) and DCM (3x) consecutively after each coupling step. To deprotect and cleave the product the resin was treated with TFA/ triisopropylsilane/m-cresol mixture (95:2.5:2.5 v/v/v) for 1 h. The reaction mixture was then precipitated with ice-cold Et_2O, centrifuged and dried.

Note: The crude PNA linkers had sufficient purity to be successfully used in the next step (Cbl-PNA conjugate synthesis). Purification of a small sample (approx. 5 mg) for characterization purposes was performed using semipreparative RP-HPLC (see HPLC method below).

HPLC purification method for PNA linkers (λ= 254 nm):

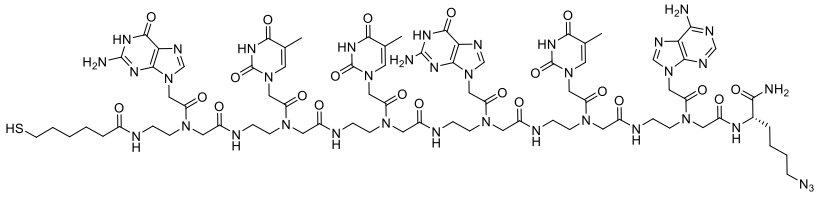
Time [min]	Water + 0.02%TFA [%]	Acetonitrile [%]
Initial	90	10
5	90	10
24	50	50
25	90	10
27	90	10

HPLC analytical method for PNA linkers (λ= 254 nm):

Time [min]	Water + 0.02%TFA [%]	Acetonitrile [%]
Initial	90	10
10	30	70
13	30	70
14	90	10
16	90	10

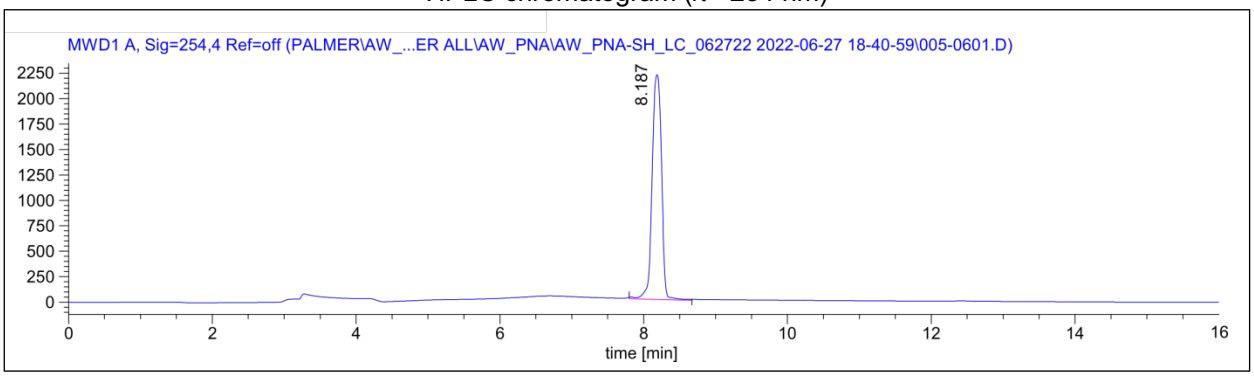
4.3. Characterization of PNA linkers

PNA (sequence G-T-T-G-T-A)

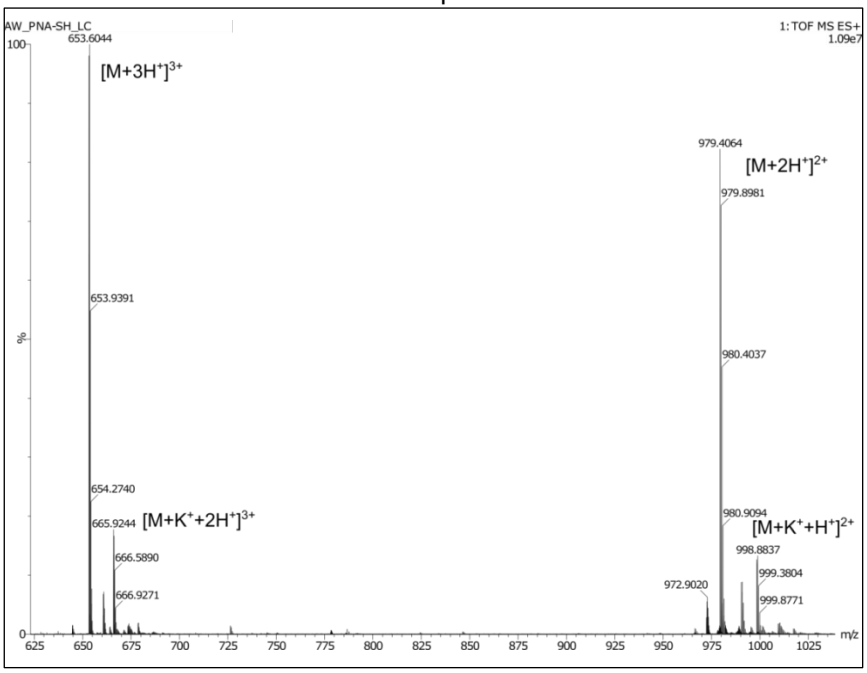


The compound was obtained as a white solid; crude mass = 60 mg. HRMS (ESI) m/z $[M + 2H]^{2+}$ calculated for $C_{78}H_{106}N_{38}O_{22}S$, 979.4027; found, 979.4064. t_R (RP-HPLC): 8.19 min.

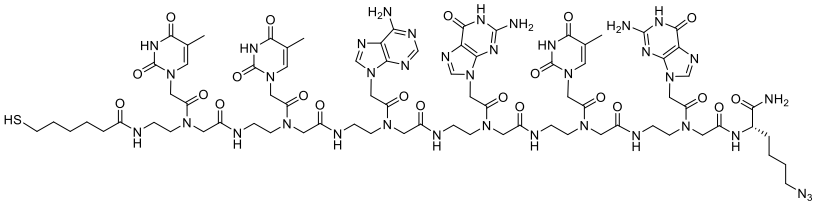
HPLC chromatogram (λ= 254 nm)



ESI MS spectrum

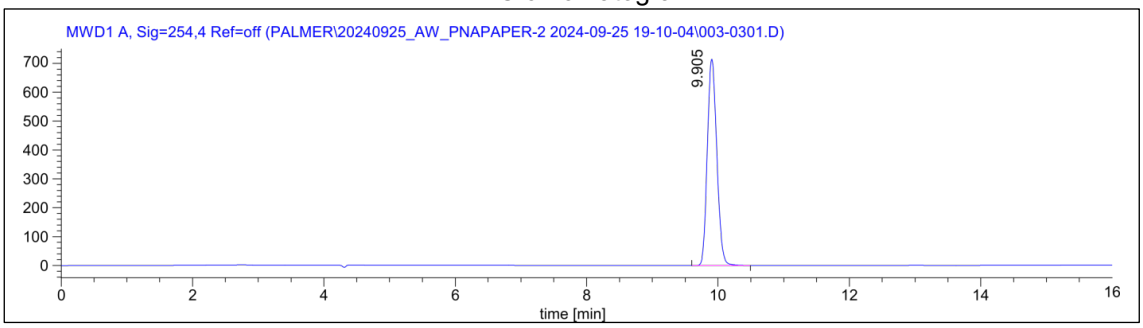


PNA$_{scr}$ (sequence T-T-A-G-T-G)

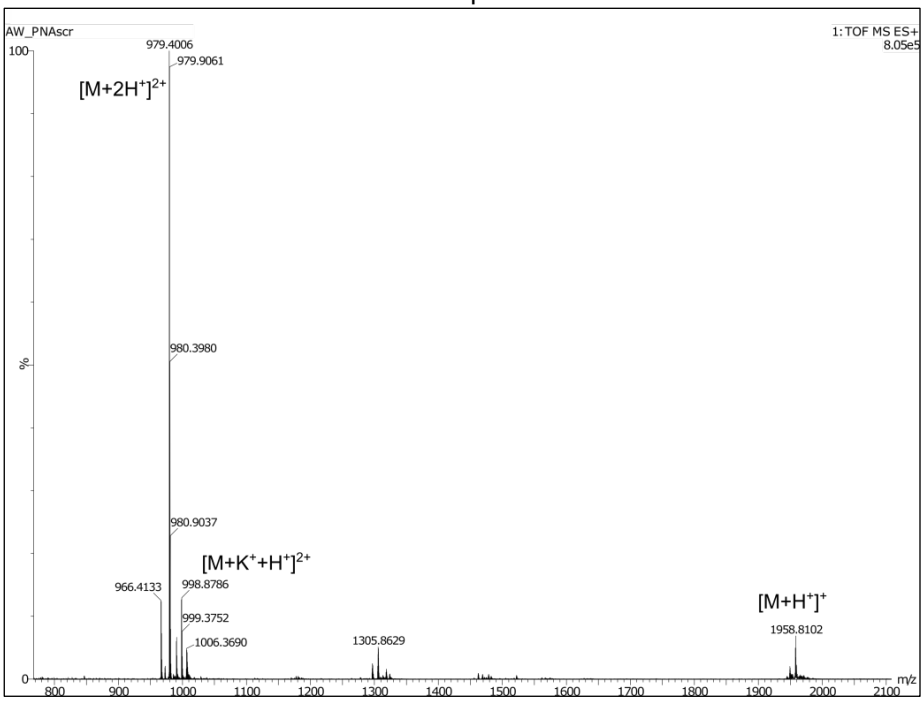


The compound was obtained as a white solid; crude mass = 73 mg. HRMS (ESI) m/z $[M + 2H]^{2+}$ calculated for $C_{78}H_{106}N_{38}O_{22}S$, 979.4027; found, 979.4006. t_R (RP-HPLC): 9.91 min.

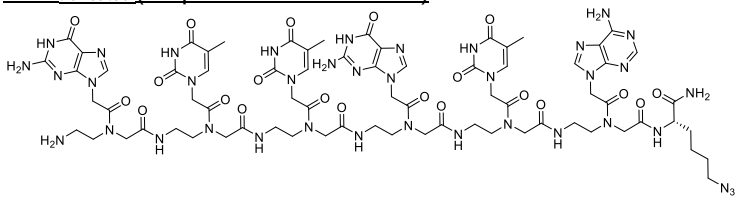
HPLC chromatogram



ESI MS spectrum

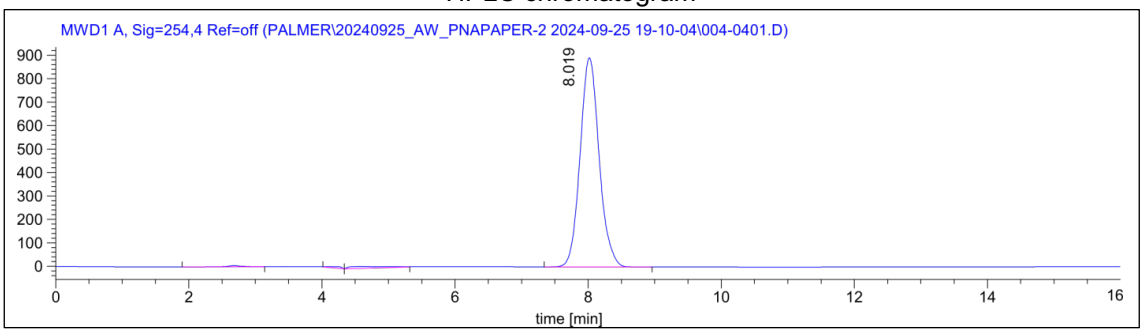


$PNA_{truncated}$ (sequence G-T-T-G-T-A)

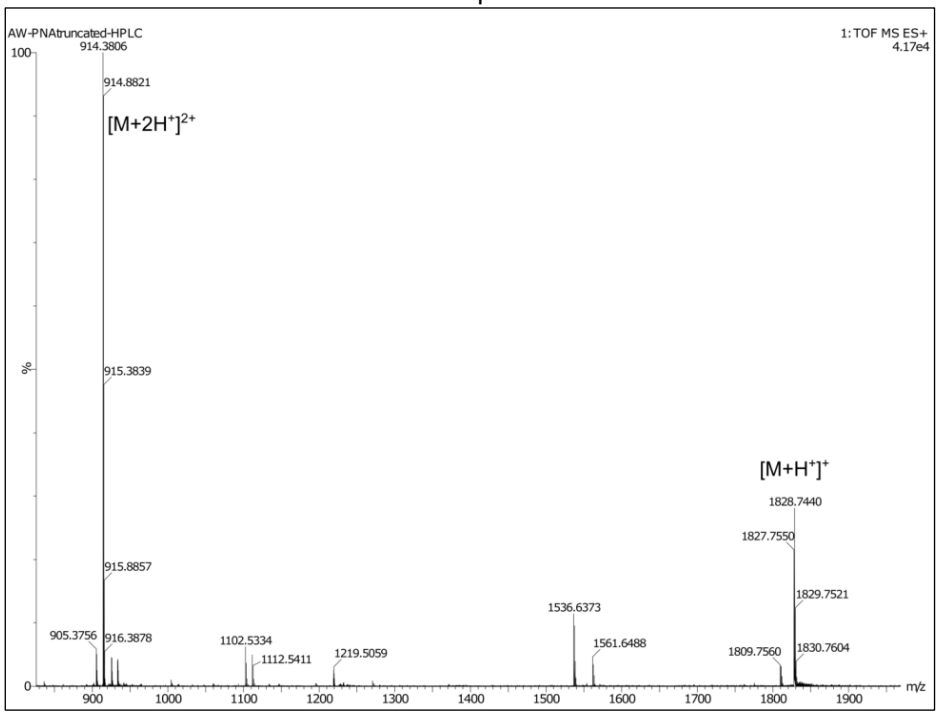


The compound was obtained as a white solid; crude mass = 65 mg. HRMS (ESI) m/z $[M + H]^+$ calculated for $C_{72}H_{95}N_{38}O_{21}$, 914.3801; found, 914.3806. t_R (RP-HPLC): 8.02 min.

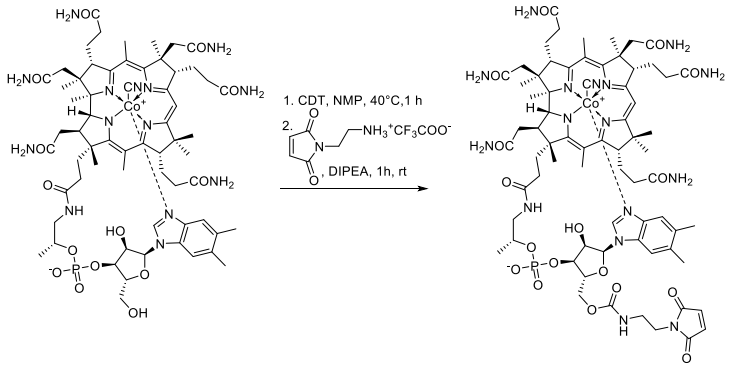
HPLC chromatogram



ESI MS spectrum



4.4. Synthesis of cobalamin derivative with maleimide functionality



Scheme S2. Synthesis of maleimide-functionalized cobalamin derivative.

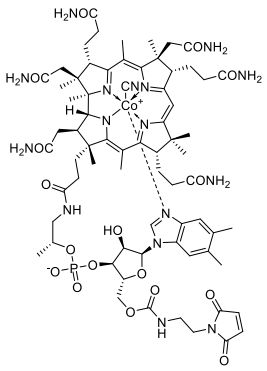
Cobalamin (70 mg, 0.05 mmol, 1 equiv.) was dissolved in dry N-Methyl-2-pyrrolidone (NMP, 3.0 mL) at 40 °C under an argon atmosphere. To a stirring solution under argon solid CDT (21 mg, 0.125 mmol, 2.5 equiv.) was added. When full consumption of the substrate (monitored by the RP HPLC) was observed (approx. 1.0 h), heating bath was removed and 1-(2-aminoethyl)-1H-pyrrole-2,5-dione as TFA salt (12.7 mg, 0.05 mmol, 1 equiv.) was added in one portion along with DIPEA (13 µL, 0.075 mmol, 1.5 equiv.). The resulting solution was stirred for 1h at room temperature. Subsequently, the reaction mixture was poured into AcOEt (10 mL), and centrifuged. The solid residue was redissolved in MeOH and precipitated with Et2O (10 mL), and centrifuged. After drying, the remaining solid was dissolved in water and purified by RP column chromatography with a mixture of MeCN and H2O as eluents (gradually from 10% to 25% v/v).

HPLC analytical method for Cbl-maleimide (λ= 254 and 361 nm):

Time [min]	Water + 0.02%TFA [%]	Acetonitrile [%]
Initial	90	10
10	30	70
13	30	70
14	90	10
16	90	10

4.5. Characterization of cobalamin derivative with maleimide functionality

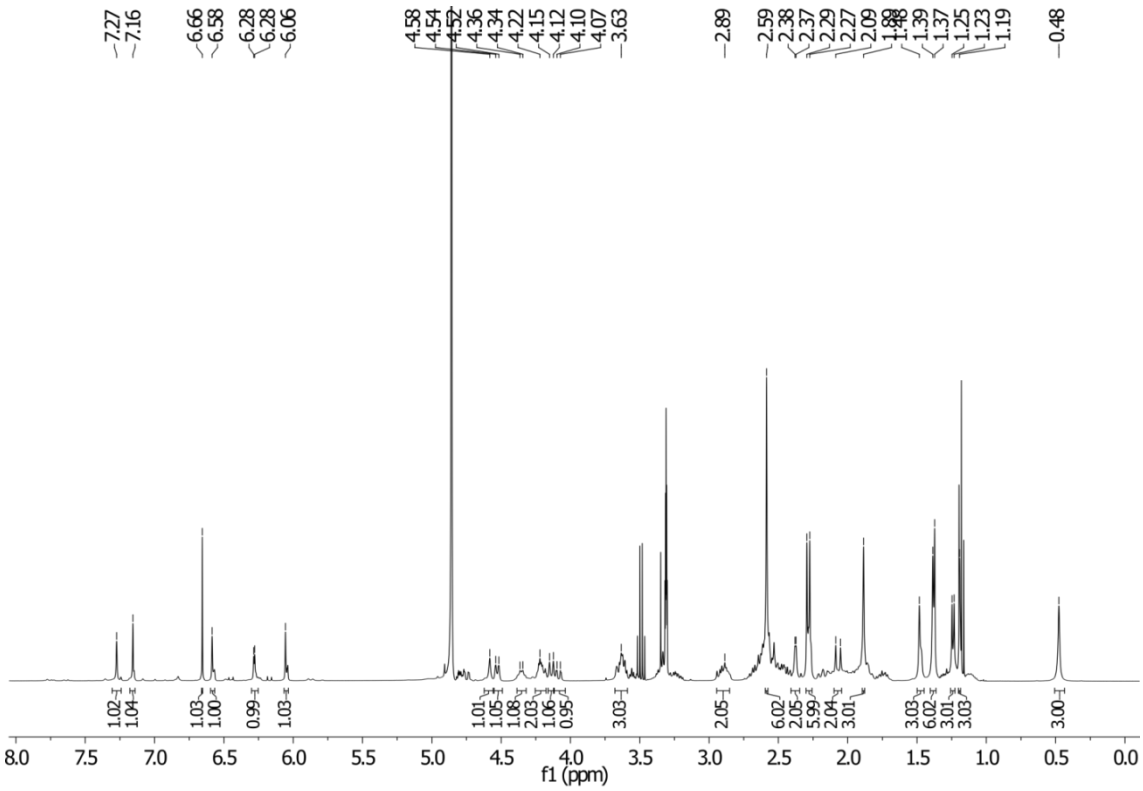
Cbl-maleimide

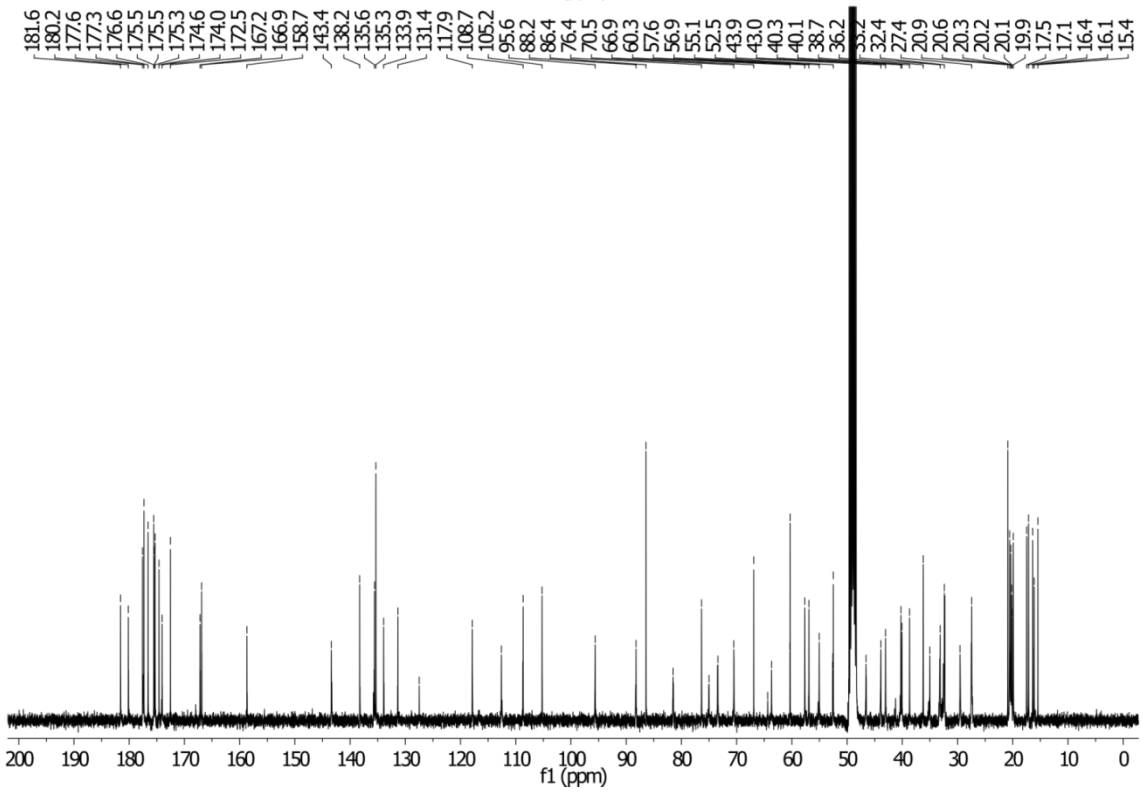


The compound was obtained as a red solid; yield: 40%. HRMS (ESI) m/z $[M + 2H]^{2+}$ calculated for $C_{70}H_{96}CoN_{16}O_{17}P$, 761.3099; found, 761.3108. t_R (RP-HPLC): 7.44 min.

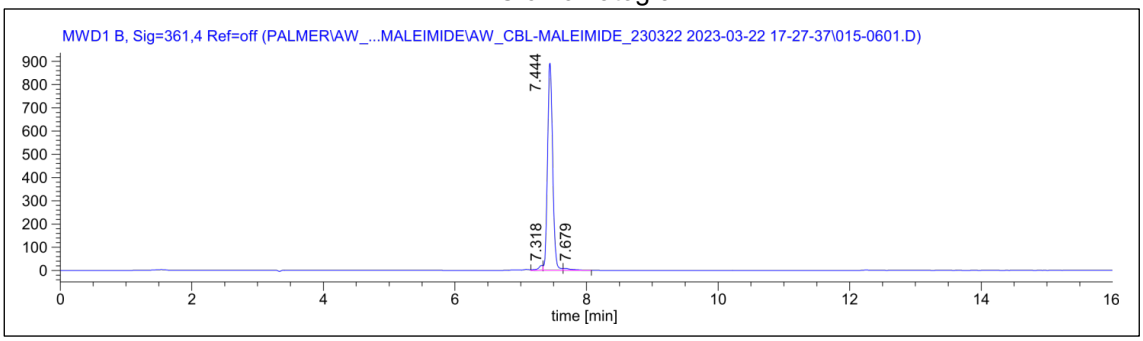
^{1}H NMR (400 MHz, CD_3OD) δ 7.27 (s, 1H), 7.16 (s, 1H), 6.66 (s, 1H), 6.58 (s, 1H), 6.28 (d, J = 2.9 Hz, 1H), 6.06 (s, 1H), 4.83 – 4.71 (m, 2H), 4.58 (s, 1H), 4.53 (d, J = 9.0 Hz, 1H), 4.39 – 4.32 (m, 1H), 4.25 – 4.18 (m, 2H), 4.14 (d, J = 11.4 Hz, 1H), 4.09 (dd, J = 12.3, 2.3 Hz, 1H), 3.70 – 3.59 (m, 3H), 3.57 – 3.50 (m, 1H), 3.41 – 3.32 (m, 1H), 3.28 – 3.18 (m, 1H), 2.96 – 2.83 (m, 2H), 2.72 – 1.82 (m, 17H) 2.59 (s, 6H), 2.38 (d, J = 2.7 Hz, 2H), 2.28 (d, J = 8.2 Hz, 6H), 2.07 (d, J = 13.8 Hz, 2H), 1.89 (s, 3H), 1.48 (s, 3H), 1.37 (d, 3H), 1.39 (d, 3H), 1.32 – 1.23 (m, 1H) 1.24 (d, J = 6.3 Hz, 3H), 1.19 (s, 3H), 1.14 – 1.07 (m, 1H), 0.48 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 181.56, 180.15, 177.59, 177.33, 176.59, 175.55, 175.50, 175.31, 174.62, 174.04, 172.53, 167.19, 166.90, 158.68, 143.40, 138.25, 135.58, 135.34, 133.93, 131.35, 127.47, 117.87, 112.60, 108.66, 105.25, 95.60, 88.21, 86.40, 81.53, 76.36, 75.01, 73.40, 70.50, 66.90, 64.37, 63.67, 60.34, 60.31, 57.65, 56.91, 55.05, 52.63, 52.58, 52.50, 46.58, 43.89, 43.03, 40.26, 40.07, 38.68, 36.21, 35.01, 33.15, 32.40, 29.54, 27.43, 20.90, 20.57, 20.33, 20.18, 20.14, 19.90, 17.49, 17.14, 16.39, 16.13, 15. 44.

^{1}H and ^{13}C NMR spectra recrded in CD_3OD (for clarity only selected signals were integrated in ^{1}H NMR spectra)

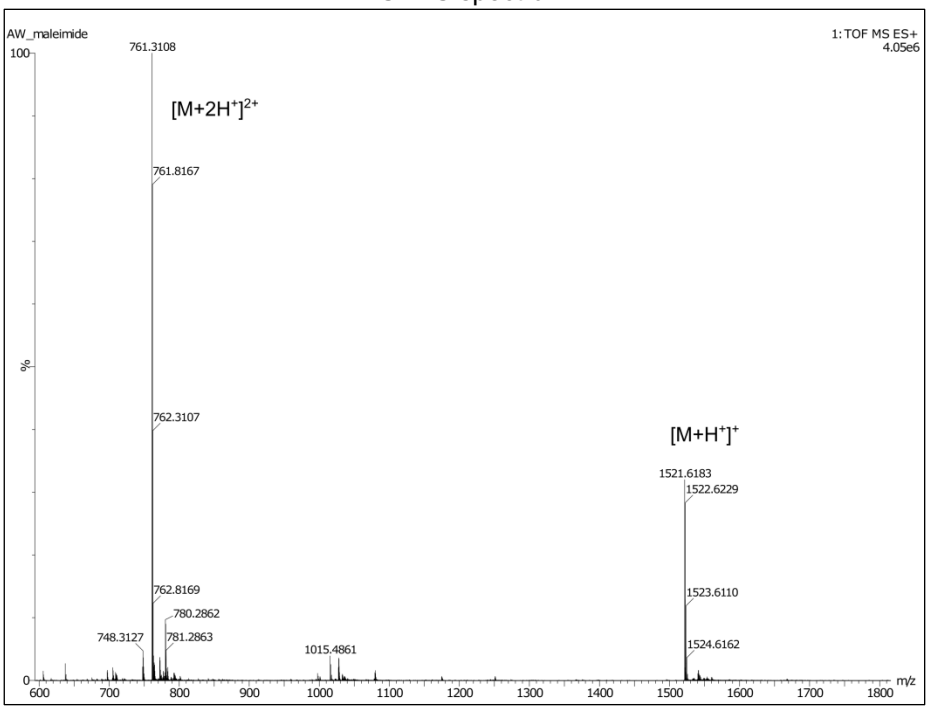




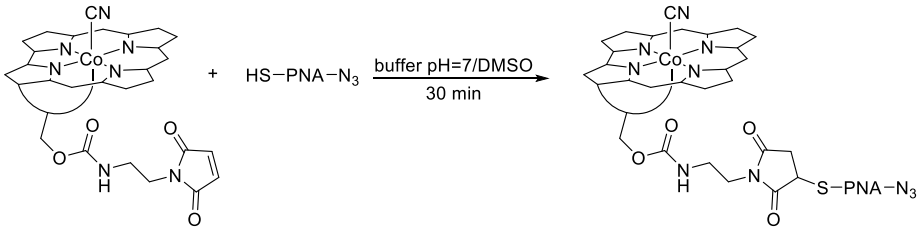
HPLC chromatogram



ESI MS spectrum



4.6. Synthesis of Cbl-PNA conjugates



Scheme S3. Schematic representation of Cbl-PNA conjugate synthesis.

Cbl-maleimide derivative (3 μmol, 2 equiv.) was dissolved in a potassium phosphate monobasic buffer (pH=7, 200 μL), while the PNA linker (1.5 μmol, 1 equiv.) was separately dissolved in DMSO (200 μL). The Cbl solution in the buffer was then added to the PNA solution in DMSO (*Note: the order is crucial, as PNA linker is insoluble in the buffer and tends to precipitate if added in reverse*), and the mixture was stirred for 30 min. Subsequently, the reaction mixture was diluted with MeOH (up to 2 mL), precipitated with Et2O (10 mL), and centrifuged. After drying, the remaining solid was dissolved in water and purified by RP column chromatography with a mixture of MeCN and H2O as eluents (gradually from 10% to 25% v/v) or via semipreparative HPLC (see method below).

HPLC purification method for Cbl-PNA conjugates (λ= 254 nm and 361 nm):

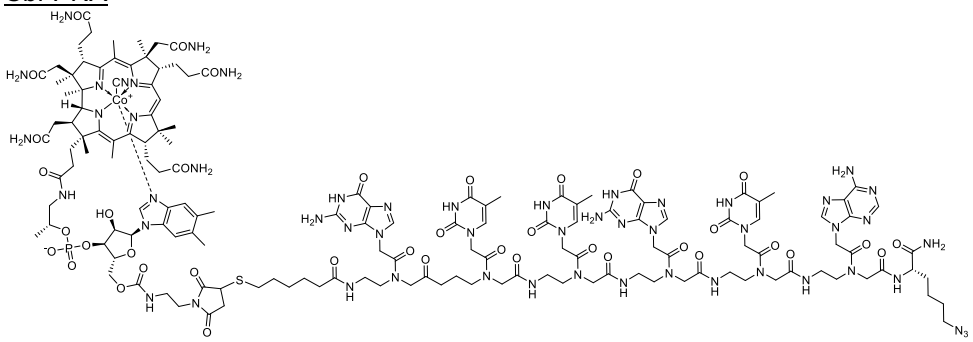
Time [min]	Water + 0.02%TFA [%]	Acetonitrile [%]
Initial	90	10
5	90	10
24	50	50
25	90	10
27	90	10

HPLC analytical method for Cbl-PNA conjugates (λ= 254 and 361 nm):

Time [min]	Water + 0.02%TFA [%]	Acetonitrile [%]
Initial	90	10
10	30	70
13	30	70
14	90	10
16	90	10

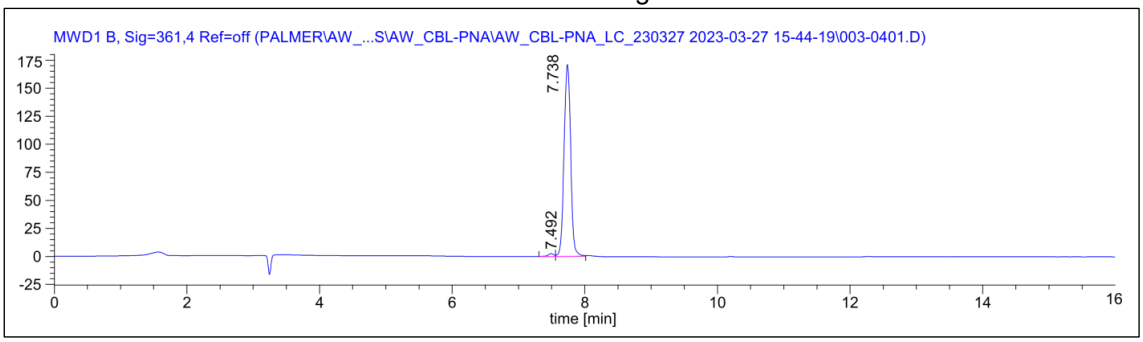
4.7. Characterization of Cbl-PNA conjugates

Cbl-PNA

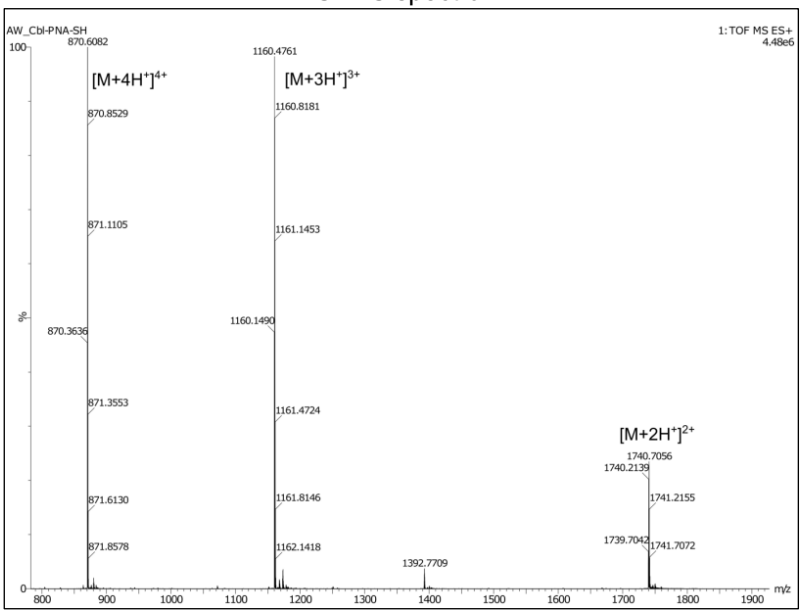


The compound was obtained as a red solid; yield: 30% (*Note: The yield is affected by the use of crude PNA linker as the limiting reagent and can be improved by using purified PNA linker*). HRMS (ESI) m/z $[M + 2H]^{2+}$ calculated for $C_{148}H_{200}CoN_{54}O_{39}PS$, 1739.7053; found, 1739.7042. t_R (RP-HPLC): 7.74 min.

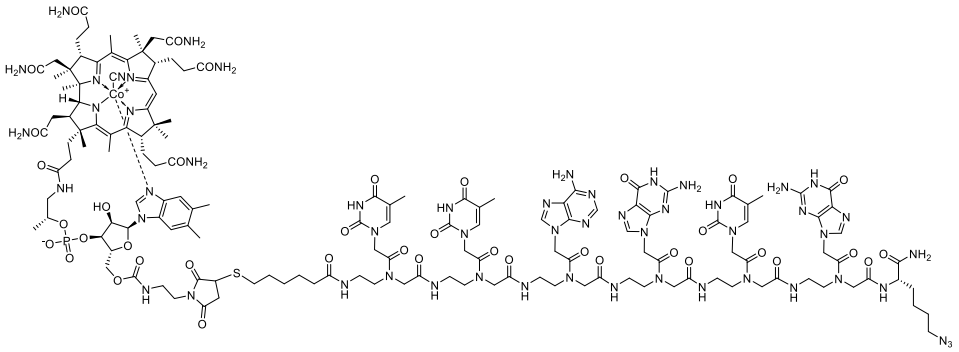
HPLC chromatogram



ESI MS spectrum

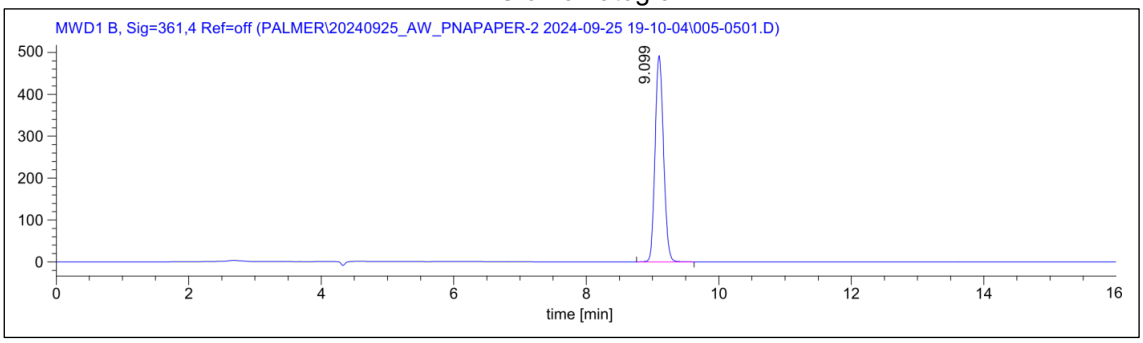


Cbl-PNA$_{scr}$

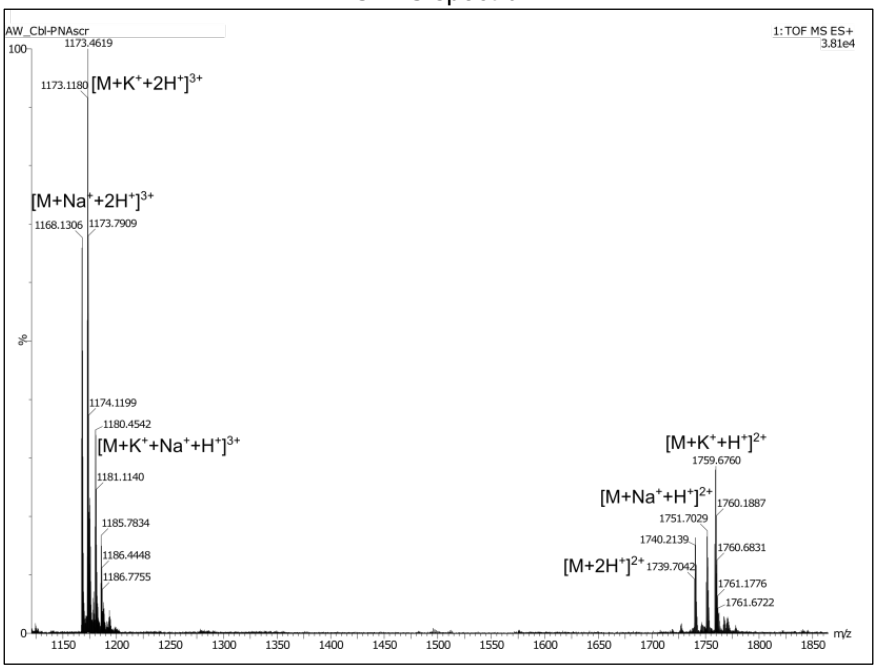


The compound was obtained as a red solid; yield: 42%. (*Note: The yield is affected by the use of crude PNA$_{scr}$ linker as the limiting reagent and can be improved by using purified PNA$_{scr}$ linker*). HRMS (ESI) m/z $[M + 2H]^{2+}$ calculated for $C_{148}H_{200}CoN_{54}O_{39}PS$, 1739.7053; found, 1739.7042. t_R (RP-HPLC): 7.73 min.

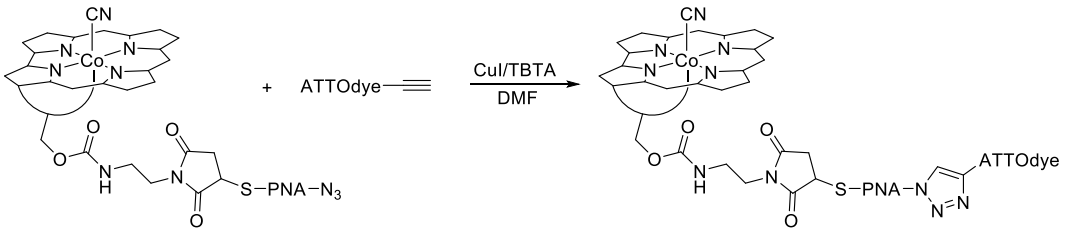
HPLC chromatogram



ESI MS spectrum



4.8. Synthesis of Cbl-PNA-dye probes



Scheme S4. Schematic representation of Cbl-PNA-dye probe synthesis.

Synthesis of Cbl-fluorophore probes was performed as previously described(5) with modifications. Catalyst solution: CuI (1 mg, 5 µmol) and TBTA (5 mg, 10 µmol) were dissolved in DMF (250 µL) and stirred for 20 min. Cbl-PNA conjugate (2 µmol, 2 equiv.) was dissolved in DMSO (20 µL) and subsequently diluted with DMF (200 µL) (*Note: DMSO addition is required as Cbl-PNA conjugates exhibit challenging solubility in DMF*). Dye propargylamide (1 µmol, 1 equiv.) was dissolved in DMF (30 µL) and added to the solution of Cbl-PNA conjugate. Subsequently the catalyst solution was added (see above) and the mixture was stirred at 35°C overnight. When full conversion of the dye was achieved (determined via HPLC, see *Notes* below) the reaction mixture was diluted with MeOH (5 mL), poured into Et_2O (15 mL) and the precipitate was centrifuged and dried. The dried solid was then dissolved in minimal amount of DMSO, and purified via semipreparative HPLC (see method below).

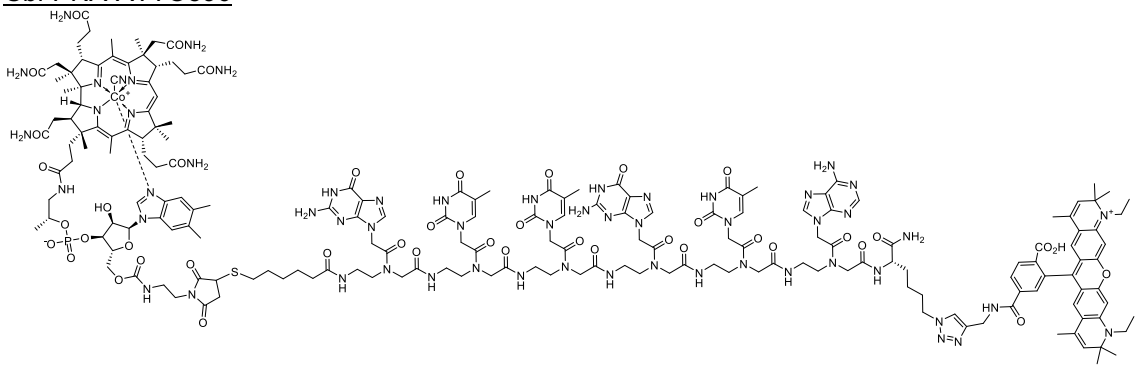
Notes: All reactions were run until full conversion of the dye was achieved (determined via HPLC). Full conversion of ATTO590 propargylamide was achieved by running the reaction overnight at 35°C. In contrast, the reaction with ATTO488-propargylamine required heating to 40°C and an extended reaction time of 48 h to reach full conversion. Reactions involving Cbl-PNA conjugates generally demand a high catalyst load, which results in the formation of a byproduct with an iodine atom incorporated into the triazole ring, forming alongside the desired product in approximately a 1:1 ratio (confirmed by HPLC and HR MS; data not shown). Further reaction optimization was not pursued due to the high cost of the ATTO fluorophores. Although the byproduct exhibits performance comparable to the desired probe (data not shown), all experiments in this study were conducted using the purified probe, which does not contain iodine.

HPLC analytical method for probes with ATTO590 dye (λ= 254, 361, 590 nm):					
Purification method			Analytical method		
Time [min]	Water + 0.02%TFA [%]	Acetonitrile [%]	Time [min]	Water + 0.02%TFA [%]	Acetonitrile [%]
Initial	90	10	Initial	90	10
5	90	10	10	30	70
23	35	65	13	30	70
24	20	80	14	20	80
27	20	80	15	20	80
28	90	10	16	50	50
30	90	10	17	90	10
			20	90	10

HPLC analytical method for probes with ATTO488 dye (λ= 254, 361, 488 nm):					
Purification method			Analytical method		
Time [min]	Water + 0.02%TFA [%]	Acetonitrile [%]	Time [min]	Water + 0.02%TFA [%]	Acetonitrile [%]
Initial	90	10	Initial	90	10
3	90	10	2	80	20
4	80	20	15	80	20
18	80	20	16	70	30
19	50	50	17	70	30
20	50	50	18	90	10
21	90	10	20	90	10
25	90	10			

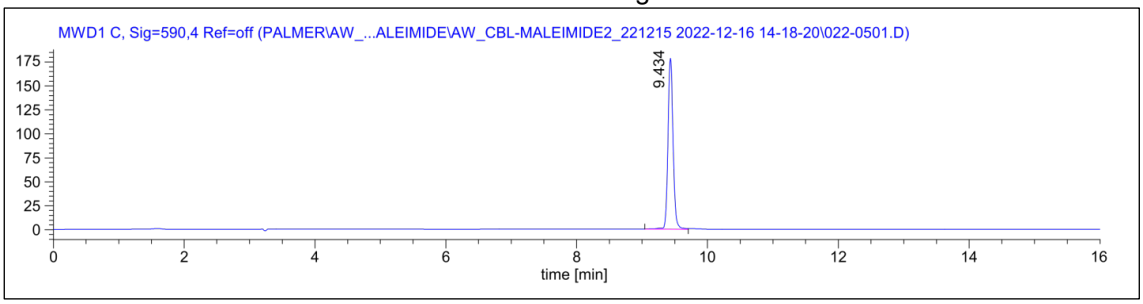
4.9. Characterization of Cbl-PNA-dye probes

Cbl-PNA-ATTO590

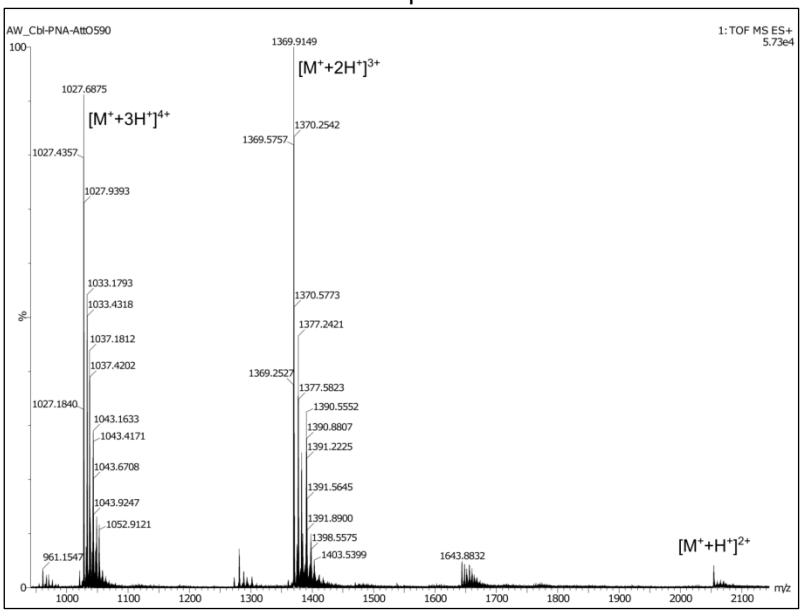


The compound was obtained as a violet solid. HRMS (ESI) m/z $[M^+ + 3H^+]^{4+}$ calculated for $C_{188}H_{243}CoN_{57}O_{43}PS^+$, 1027.1837; found, 1027.1840. t_R (RP-HPLC): 9.34 min.

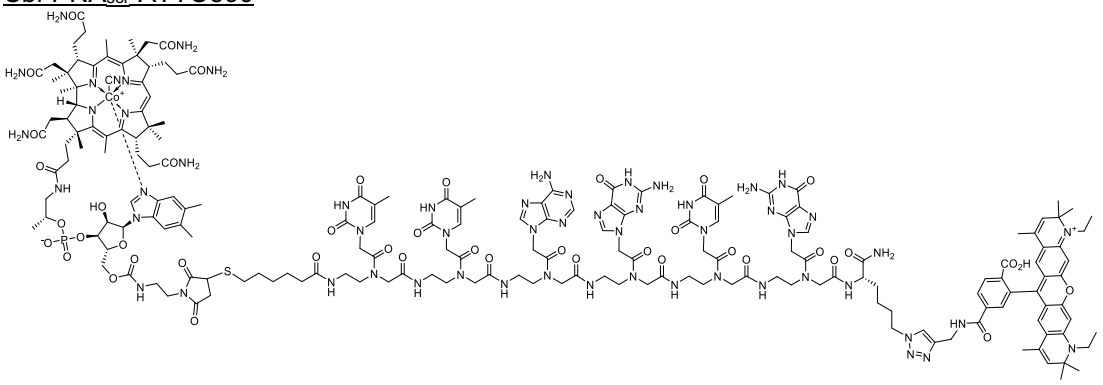
HPLC chromatogram



ESI MS spectrum

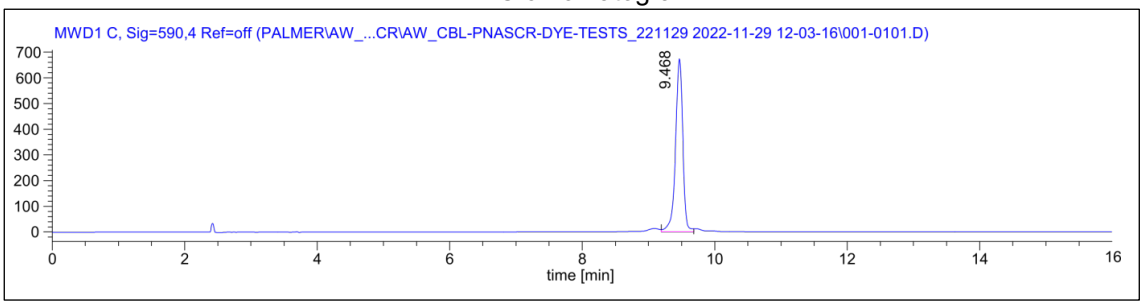


Cbl-PNA$_{scr}$-ATTO590

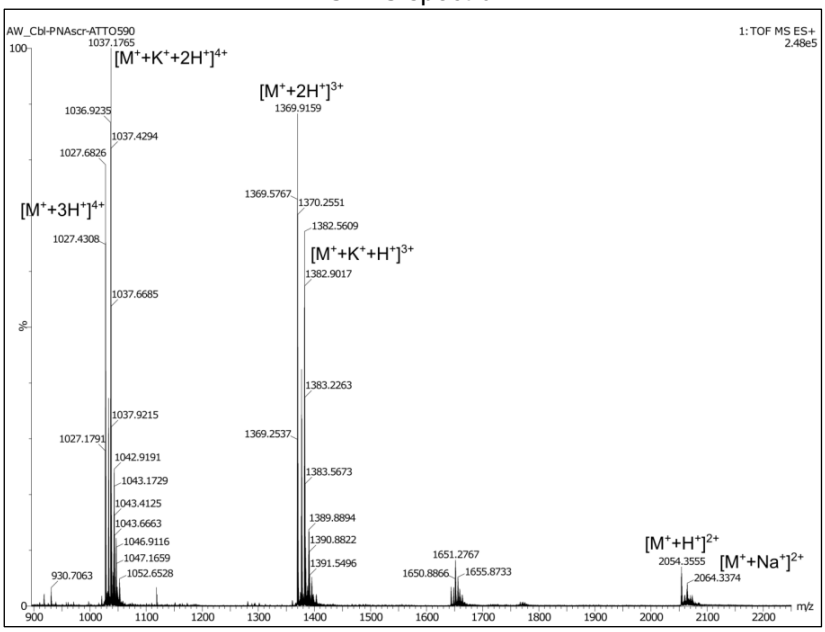


The compound was obtained as a violet solid. HRMS (ESI) m/z $[M^+ + K^+ + H^+]^{3+}$ calculated for $C_{188}H_{241}CoN_{57}O_{43}PSK^+$, 1381.8945; found, 1381.8956. t_R (RP-HPLC): 9.47 min.

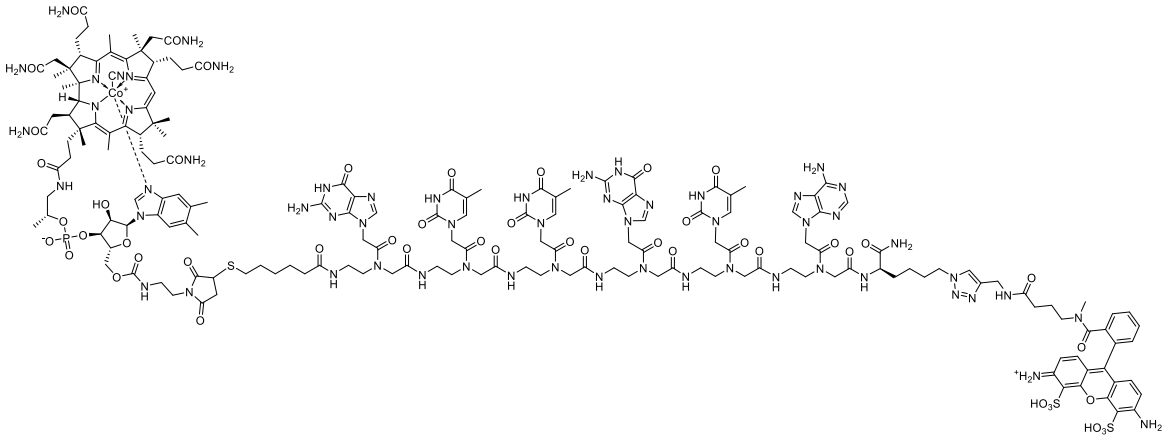
HPLC chromatogram



ESI MS spectrum

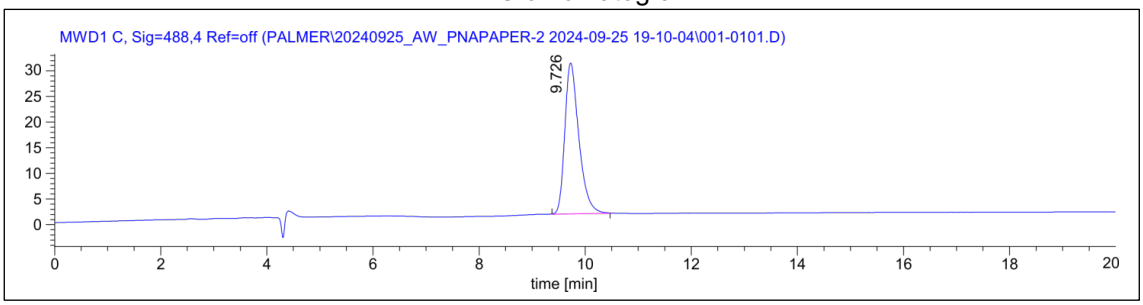


Cbl-PNA-ATTO488

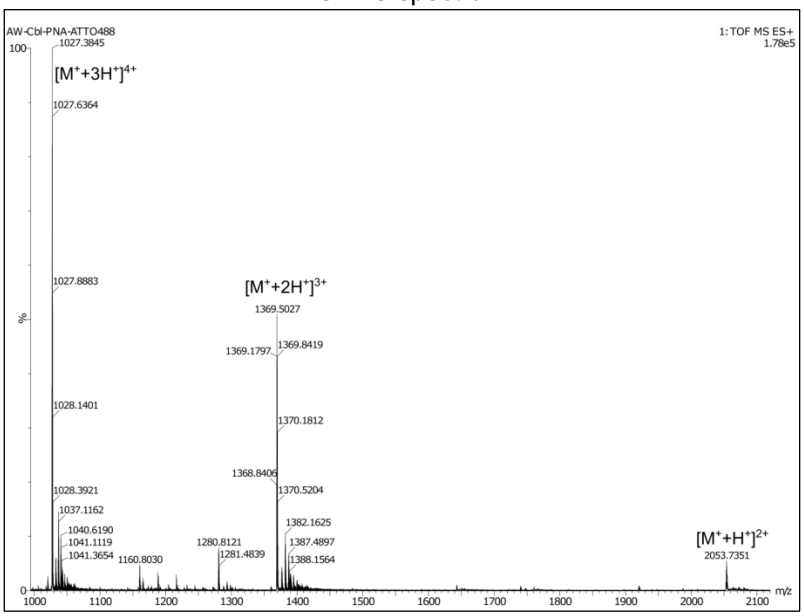


The compound was obtained as a red solid. HRMS (ESI) m/z $[M^+ + 3H^+]^{4+}$ calculated for $C_{176}H_{228}CoN_{58}O_{48}PS_3^+$, 1026.8848; found, 1026.8810. t_R (RP-HPLC): 9.73 min.

HPLC chromatogram

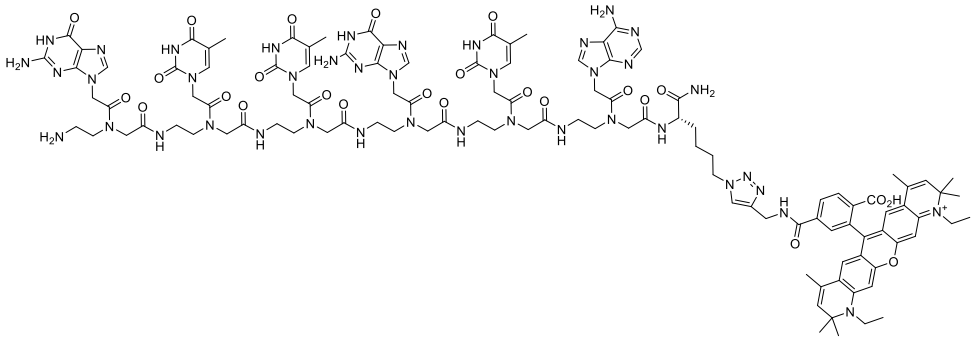


ESI MS spectrum



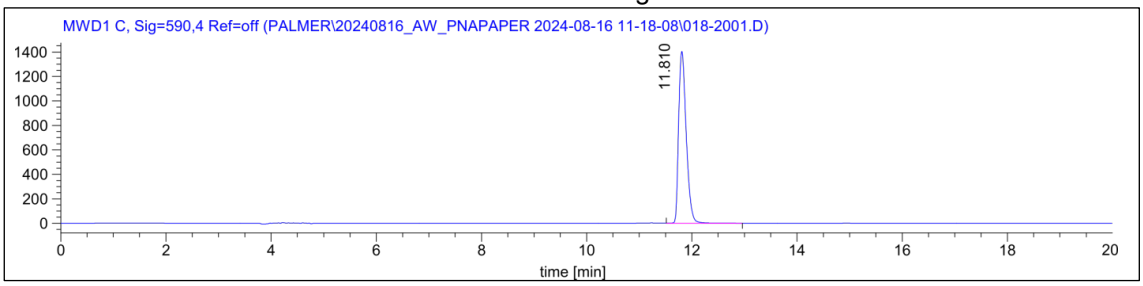
4.10. Synthesis and characterization of PNA-ATTO590 probe

PNA-ATTO590 conjugate

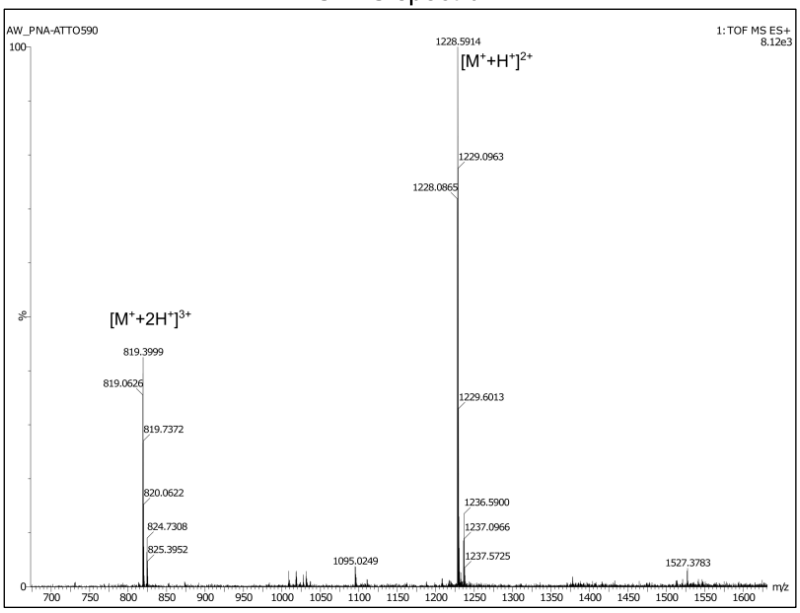


The compound was synthesized according to the protocol 4.8 with the following modifications: 1) 6 equiv. of PNA_{trunc} were used. 2) 125 µL of the catalyst solution was used. 3) The reaction was performed at room temperature overnight. The compound was obtained as a violet solid. HRMS (ESI) m/z $[M^+ + H^+]^+$ calculated for $C_{112}H_{137}N_{41}O_{25}^+$, 1228.0349; found, 1228.0865. t_R (RP-HPLC): 11.81 min.

HPLC chromatogram



ESI MS spectrum



5. References

1. J. E. Johnson Jr, F. E. Reyes, J. T. Polaski, R. T. Batey, B12 cofactors directly stabilize an mRNA regulatory switch. *Nature* **492**, 133–137 (2012).
2. Y. Gholamalipour, A. Karunanayake Mudiyanselage, C. T. Martin, NAR breakthrough article 3 end additions by T7 RNA polymerase are RNA self-templated, distributive and diverse in character—RNA-Seq analyses. *Nucleic Acids Res.* **46**, 9253–9263 (2018).
3. A. L. Edwards, A. D. Garst, R. T. Batey, "Determining Structures of RNA Aptamers and Riboswitches by X-Ray Crystallography BT - Nucleic Acid and Peptide Aptamers: Methods and Protocols" in G. Mayer, Ed. (Humana Press, 2009), pp. 135–163.
4. I. Jarmoskaite, I. Alsadhan, P. P. Vaidyanathan, D. Herschlag, How to measure and evaluate binding affinities. *Elife* **9**, 1–34 (2020).
5. E. Braselmann, *et al.*, A multicolor riboswitch-based platform for imaging of RNA in live mammalian cells. *Nat. Chem. Biol.* **14**, 964–971 (2018).
6. A. J. Wierzba, M. Wojciechowska, J. Trylska, D. Gryko, "Vitamin B12 – Peptide Nucleic Acid Conjugates BT - Peptide Conjugation: Methods and Protocols" in W. M. Hussein, R. J. Stephenson, I. Toth, Eds. (Springer US, 2021), pp. 65–82.