Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Title: Handcuffed antisense oligonucleotides for light-controlled cell-free expression

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Python function for hairpin search

```
def hairpin(input_DNA, filename):

    ''' Reverse Complement'''
    tab = str.maketrans("ACTG", "TGAC") #Makes Complement
    def reverse_complement_table(seq): #Reverses Sequence
      return seq.translate(tab)[::-1]

    arr = np.empty((0,7))

    ''' ASO parameters'''
    len_overlap = (a, b)
    len_aso = (c, d)

    ''' Iteration'''
    for j in range(len_overlap[0], len_overlap[-1]+1):
      for k in range(len_aso[0], len_aso[-1]+1):
        for i in range(0, len(input_DNA)-j):
          q=(input_DNA[i:i+j])
          RC = reverse_complement_table(q)
          if input_DNA[i+k+j:i+k+2*j] == RC:
            x = np.array([j, i+1, q, k, input_DNA[i+j:i+k+j], i+1+k, RC])
            arr = np.vstack([arr, x])

    ''' Save File'''
    df = pd.DataFrame(data=arr, columns=["Overlap Length", "Start Site", "Start Sequence", "Spacer Length", "Spacer Sequence", "Complementary Site", "Reverse Complement"])
    df.to_csv(filename + '.csv')
```

Oligonucleotide Sequences

General

Unmodified oligonucleotides (ONs) were purchased from Merck in desalted and lyophilised form and resuspended in 10 mM Tris pH 8. Amine-containing oligonucleotides were purchased from IDT, HPLC-purified in lyophilised form and dissolved in 10 mM potassium phosphate buffer, pH 7.4.

Oligonucleotides used in PCR

Index	Primer Name	Sequence (5'-3')
1	T7 Forward Primer	GAAATTAATACGACTCACTATAGGGTCTAG
2	Reverse Primer	GATATAGTTCCTCCTTTCAG

Supplementary Table 1: Primers used for generation of linear template DNA by PCR

Oligonucleotides used in screening

Index	Name	Sequence (5'-3')
1	252[4]	CTTCGGGCATGGCGGACTTGAAG
2	330[4]	CGCCCTCGAACTTCACCTCGGCG
3	146[4]	GCACGGGCAGCTTGCCGGTGGTGC
4	249[4]	CTTCGGGCATGGCGGACTTGAAGAAG
5	3[4]	GGTGAACAGCTCCTCGCCCTTGCTCACC
7	221[2]	GCTTCATGTGGTCGGGGTAGC
8	134[2]	TGGTGCAGATGAGCTTCA
9	125[3]	AGCTTCAGGGTCAGCT
10	154[3]	GGCACGGGCAGCTTGCC
11	202[3]	GGCGAAGCACTGCAGGCC
12	169[4]	GAAGAAGTCGTGCTGCTTC
13	229[1]	GTGCTGCTTCATGTGGTC

Supplementary Table 2: Sequences tested for antisense activity against *mVenus*. Numbers in brackets indicate stem-length of the hairpin.

Amine-modified oligonucleotides

Index	Name	Sequence (5'-3')
1	252[4]	/5AmMC6/CTTCGGGCATGGCGGACTTGAAG/3AmMO/
2	221[2]	/5AmMC6/GCTTCATGTGGTCGGGGTAGC/3AmMO/
3	169[4]	/5AmMC6/GAAGAAGTCGTGCTGCTTC/3AmMO/
4	229[1]	/5AmMC6/GTGCTGCTTCATGTGGTC/3AmMO/
5	229[1]	/5AmMC6/GTGCTGCTTCATGTGGTC

Supplementary Table 3: DNA sequences with terminal amine modifiers.

DNA sequences of genes

Entry	Gene	Sequence 5'-3'
1	*mVenus*	GAAATTAATACGACTCACTATAGGGTCTAGAAATAATTTTGTTTAACTTTAAGAAGG AGGTATACAT**ATG**GTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATC CTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGG GCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGCTCATCTGCACCACCGG CAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTCGGCTACGGCCTGCAGT GCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGC CCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAG ACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGA AGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAA CTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAACGGCATCAAGG CCAACTTCAAGATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCAC TACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTA CCTGAGCTACCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATG GTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTA CAAG**TAA**TGAGGATCCCGGGAATTCTCGAGTAAGGTTAACCTGCAGGAGGCCTTTA ATTAAGGTGGTGCGGCCGCGCTAGCGGTCCCGGGGGATCGATCCGGCTGCTAACA AAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATA ACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTA TATC

Supplementary Table 4: Sequences of linear DNA templates used for cell-free expression, produced by PCR. T7-Promoter and -Terminator Regions are underlined, protein start- and stop-codons are in bold. mRNA position counting starts at the bolded start codon ATG.

Chemical Biology Methods

General

All experiments were performed under reduced ambient lighting (overhead lights turned off). Data was plotted using python's matplotlib and seaborn libraries. Error bars and confidence intervals were computed using seaborn and show a 95% confidence interval. T-tests were performed using python's scipy library, using the stats.ttest_ind() method and a one-tailed t-test was applied. Gel electrophoresis data was analysed using ImageLab software.

Polyacrylamide gel electrophoresis

Gels were cast by hand with the BioRad Mini-PROTEAN® handcasting equipment at a thickness of 0.75 mm. Gels with bound Streptavidin were run denaturing at 8% PAA/BisAA (19:1) (unless otherwise indicated), 7 M Urea, 1x TBE Buffer and polymerised using 0.8% APS and 0.05% TMEDA at 250 V in 1X TBE buffer. Samples were loaded with 95% formamide, 0.015% SDS, 5 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol. Gels were stained with Gel-Red® nucleic acid stain (Biotium) and with QuickBlue protein stain (LubioScience).

Agarose gel electrophoresis

RNA Gels were prepared at 1.5% agarose in 1x TBE buffer, prestained with Sybr® Green II and run at 110 V in 1x TBE buffer. Samples were prepared using RNA loading dye (NEB B0363S) and heated to 70 °C for 10 minutes then cooled on ice before loading to denature the RNA. Samples were run against a low-range ssRNA ladder (NEB, N0364S) or ssRNA ladder (NEB, N0362S).

Linear DNA template preparation

Linear DNA encoding for the fluorescent protein mVenus was prepared by Polymerase Chain Reaction (PCR) as reported previously.[1] PCR reactions were carried out using DreamTaq DNA polymerase MasterMix (2x, ThermoFisher), Forward and Reverse Primers (**Supplementary Table 1,** Entry 1 and 2) at 0.25 µM concentration and 0.04 ng/µL of HindIII-digested *mVenus* plasmid as template in a total reaction volume of 50 µL. The PCR was carried out according to the manufacturer's protocol for 35 cycles with an annealing temperature of 47 °C for 30 seconds, an extension time of 72 °C for 1 minute (15s/kbp) and a final extension at 72 °C for 10 minutes. The resulting DNA was then purified using the GeneJet PCR purification columns (ThermoFisher) following the manufacturer's protocol and eluted in 50 µL H_2O.

In vitro transcription of mRNA

In vitro transcription was performed with 10 ng/µL of linear *mVenus* template DNA (as prepared above) using the HiScribe RNA Synthesis kit (NEB) in a total reaction volume of 10 µL. Samples were incubated at 37 °C for 4 hours prior to addition of DNAse I (1 µL) and further incubation for 30 minutes. The mRNA was then purified using the GeneJet RNA Cleanup and Concentration Micro Kit (ThermoFisher) following the manufacturer's protocol and eluted in H_2O.

Modification of amine-containing oligonucleotides with uvLA-Biotin

To a 1.5 mL microcentrifuge tube was added the desired, amine-modified ASO (Table 3, 10 µM), NaHCO3 (100 mM) and PC-Biotin NHS Ester (Click Chemistry Tools, 5 mM) in a total volume of 50 µL and the reaction incubated at room temperature overnight. The solution was diluted to 50 µL with H_2O, washed with $CHCl_3$ (3x 200 µL), 50 µL DMSO added and transferred to a HPLC injection vial. The oligonucleotide was then purified by HPLC on an Agilent Polaris C18 column (150x4.5 mm), heated to 50 °C, using a gradient of 5-30% MeCN over 20 minutes with 10 mM TEAB pH 8.5 throughout. The resulting oligonucleotide was lyophilised, resuspended in H_2O and analysed by LCMS for purity.

Modification of amine-containing oligonucleotides with bLA-Biotin

To a 1.5 mL microcentrifuge tube was added the desired, amine-modified ASO (Table 3, 23.5 µM), MOPS pH 8.5 (119 mM) and PFP-Coumarin (as prepared previously,[1] 17.5 mM) in 59% DMF in a final reaction volume of 34 µL. The reaction was incubated in a Thermomixer (Eppendorf) at 37 °C and 800 RPM overnight. The solution was diluted to 50 µL with H_2O, washed with $CHCl_3$ (3x 200 µL), 50 µL DMSO added and transferred to a HPLC injection vial. The oligonucleotide was then purified by HPLC on an Agilent Polaris C18 column (150x4.5 mm), heated to 50 °C, using a gradient of 5-30% MeCN over 20 minutes with 10 mM TEAB pH 8.5 throughout. The resulting oligonucleotide was lyophilised, resuspended in H_2O and analysed by LCMS for purity.

Modification of 5'-amine-containing oligonucleotide with bLA-Biotin

To a 1.5 mL microcentrifuge tube was added the desired, amine-modified ASO (Table 3, Entry 5, 23.5 µM), MOPS pH 8.5 (119 mM) and PFP-Coumarin (as prepared previously,[1] 17.5 mM) in 59% DMF in a final reaction volume of 34 µL. The reaction was incubated in a Thermomixer (Eppendorf) at 37 °C and 800 RPM overnight. The solution was diluted to 100 µL with H_2O, washed with $CHCl_3$ (3x 200 µL), and purified using a NAP-5 desalting column (Cytiva). The oligonucleotide was then lyophilised, resuspended in H_2O and analysed by LCMS for purity.

Screening for antisense oligonucleotide sequences active against *mVenus*

A python script was used to identify potential antisense oligonucleotides which form a hairpin structure via complementary stem length of 4 nucleotides (nt). Similarly, the constraints were reduced, and oligonucleotides found with a step length of 1, 2 and 3. These oligonucleotides were then ordered and labelled by the starting nucleotide in the *mVenus* gene sequence, followed by the length of stem in brackets.

The 4-stem oligonucleotides were then screened against its target *mVenus* mRNA. 30 ng/µL (94 nM) of *mVenus* mRNA (prepared as above), 0.6 U/µL RNase H (recombinant *E. coli*, Takara), and either 0.1 ng/µL (14.34 nM) or 0.5 ng/µL (71.7 nM) of the ASO in a buffer system containing 30 mM HEPES pH 7, 100 mM KCl, 20 mM $MgCl_2$ and 2 mM DTT in a total of 10 µL were incubated for 1 hour at 37 °C. Similarly, for the lower constraint oligonucleotides, 30 ng/µL (94 nM) of *mVenus* mRNA (prepared as above), 0.3 U/µL RNase H (recombinant *E. coli*, Takara), and the ASO of interest at a concentration of 71.7 nM were incubated in the buffer mentioned above for 1 hour at 37 °C. RNA loading dye (NEB, B0363S) was then added, samples heated to 70 °C for 10 min and then analysed by agarose gel electrophoresis.

Illumination of samples

Samples were held in a PCR tube rack (StarLabs) over aluminium foil with open lids. Irradiation was then performed top-down with a ThorLabs 455 nm LED (M455L4) equipped with a collimator (COP4-A) from a distance of 30 cm at 64 $mW \cdot cm^{-1}$ or a ThorLabs 365 nm LED (M365L3) equipped with a collimator (COP5-A) from a distance of 34 cm at 1.06 $mW \cdot cm^{-1}$, both controlled by a ThorLabs driver (LEDD1B) set at 1 A maximum drive current.

RNase H assay with handcuffed ASOs

30 ng/µL (94 nM) of *mVenus* mRNA (prepared as above), 0.6 U/µL RNase H (recombinant *E. coli*, Takara), and a concentration of 71.7 nM of the bLA- or uvLA-ASO in a buffer system containing 30 mM HEPES pH 7, 100 mM KCl, 20 mM $MgCl_2$ and 2 mM DTT in a total of 10 µL were incubated for 1 hour at 37 °C. RNA loading dye (NEB, B0363S) was then added, samples heated to 70 °C for 10 min and then analysed by agarose gel electrophoresis.

Binding of Streptavidin to modified oligonucleotides

In a 0.5 mL DNA LoBind Tube, LA-Biotin-modified DNA was diluted to a final concentration of 0.2-0.3 µM with Dulbecco Phosphate Buffered Saline (Sigma-Aldrich). To this was then added 0.5 molar equivalents of wild-type Streptavidin (NEB, N7021S), unless otherwise indicated, the solution vortexed, covered in foil and incubated at 4 °C overnight.

DNase I stability assay

The amine-modified, 5'-bLA-Biotin modified and handcuffed bLA-229[1] oligonucleotides (without, or with 10 seconds of 455 nm illumination at either 17.54 or 64 $mW \cdot cm^{-1}$) were incubated with or without DNase I (0.2 U/µL, ThermoFisher) in a buffer consisting of 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ at pH 7.6 for 4 hours at 37 °C in a total volume of 10 µL in a 200 µL PCR tube. The samples were then analysed by denaturing PAGE (8%, 7 M Urea) and stained with GelRed.

Cell-free protein synthesis

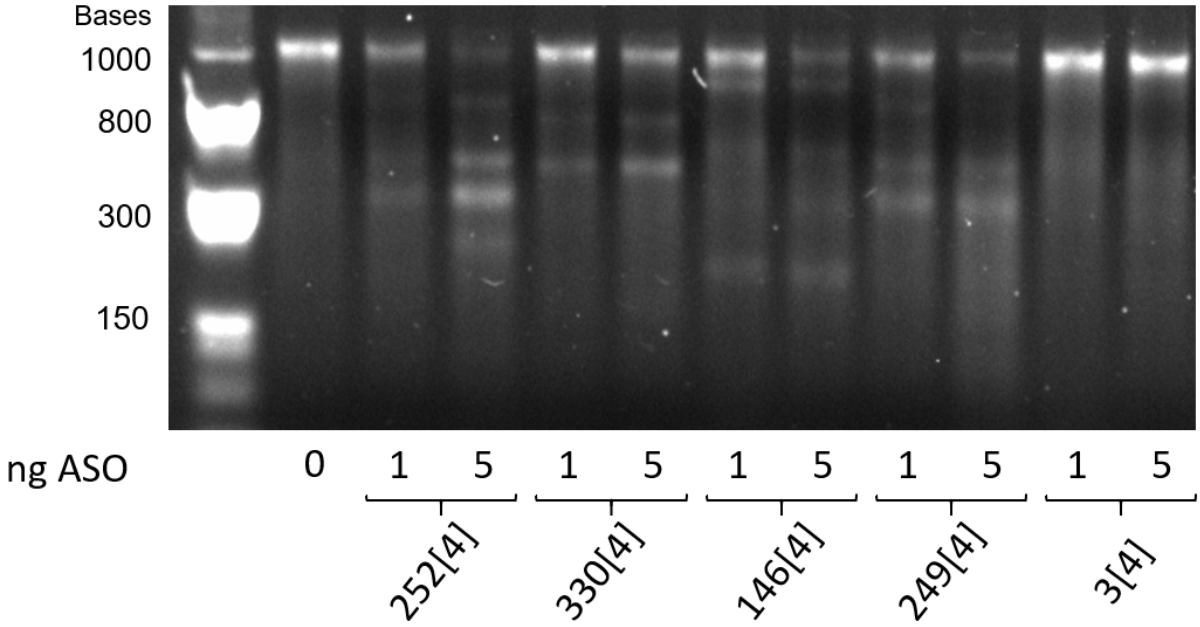
In a 200 µL PCR tube, 5 ng/µl of linear template DNA for mVenus were added to PURExpress® (NEB, E6800) with 0.6 U/µL RNase H (recombinant *E. coli*, Takara) and to this bLA-252[4] (4.34 ng/µL, 571 nM) or bLA-229[1] (0.5 ng/µL, 85 nM) were added. The resulting solutions were kept at room temperature in the dark and illuminated as required, before placing them in a thermocycler and incubated at 37 °C for 4 hours. 2 µL of each solution was then placed into 39 µL of H_2O and mixed by pipetting. 40 µL of the resulting solutions were then transferred into a 384 well plate and placed into a plate reader (Tecan Infinity M1000) and fluorescence measurements were taken for mVenus ($\lambda_{Ex/Em}$: 515/527 nm, gain 173).

Transfection of handcuff-oligonucleotide bLA-229[1]

229[1] + bLA-Biotin as well as bLA-229[1] were transfected into HEK293T cells using Lipofectamine 3000 following the manufacturer's protocol. In brief, ~10^5 cells in Dulbecco's Modfied Eagle Medium (120 µL) were plated in a 96-well plate with blackened sides (Corning 3603) and grown overnight in a 5% CO_2 incubator at 37 °C until they reached 70-90% confluency. 10 µL of a solution of Lipofectamine 3000 (0.15 µL, ThermoFisher) and 270 ng of either 229[1] + bLA-Biotin, the bLA-229[1] handcuff or no DNA in Opti-MEM was added to the cells, the plate gently titled to mix, and then incubated for 6 hours in a 5% CO_2 incubator at 37 °C. The medium was then gently aspirated and replaced by Opti-MEM without phenol red (120 µL) prior to imaging on a Leica DMi8 inverted epi-fluorescence microscope. Experiments were performed in duplicates.

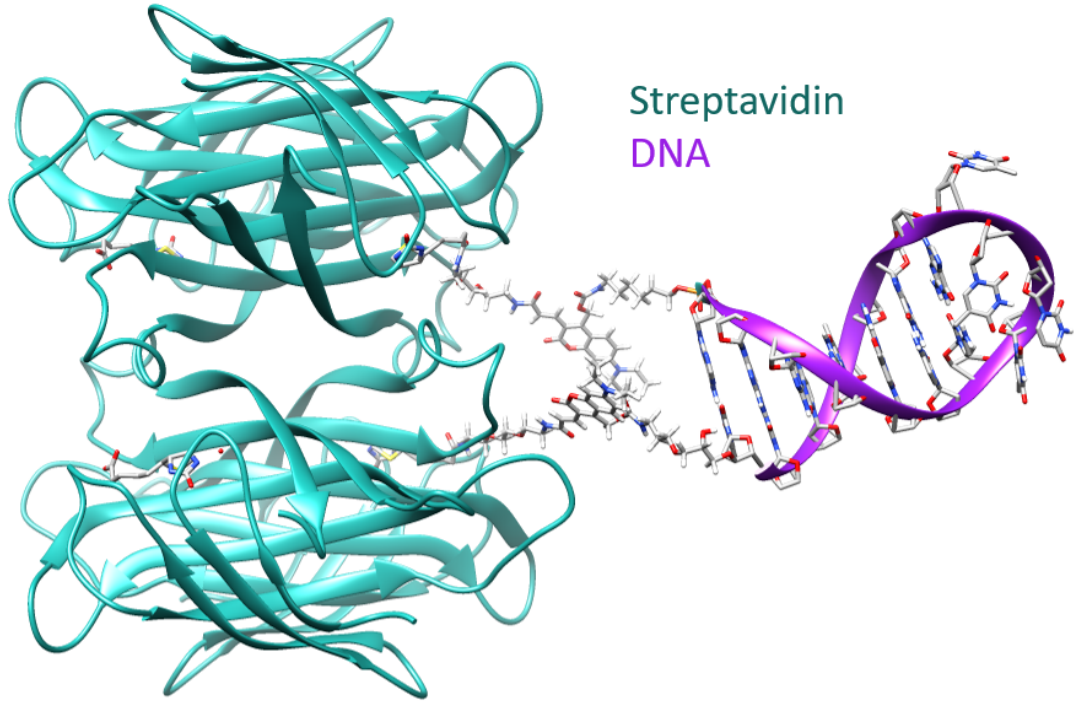
Supplementary Data

Screening for an antisense oligonucleotide sequence with a hairpin motif



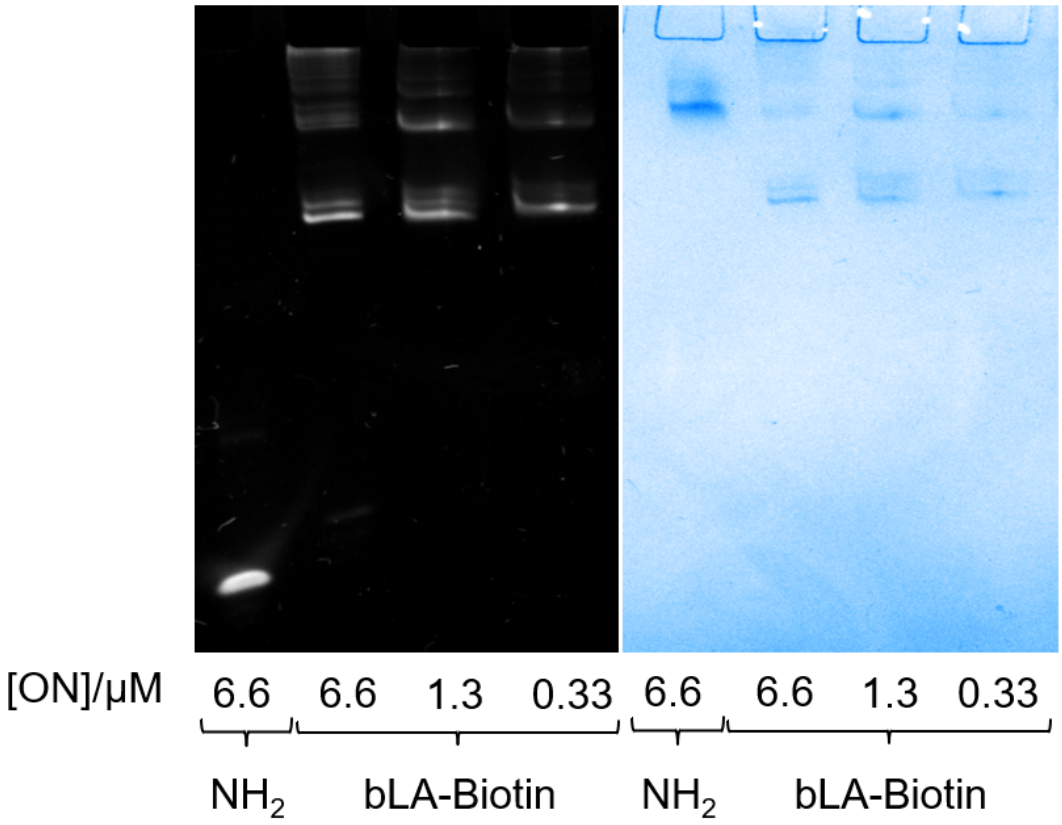
Supplementary Figure 1: Screening for a hairpin-forming antisense oligonucleotide against *mVenus*.

Chimera model of a DNA-Hairpin oligonucleotide bound to wild-type Streptavidin



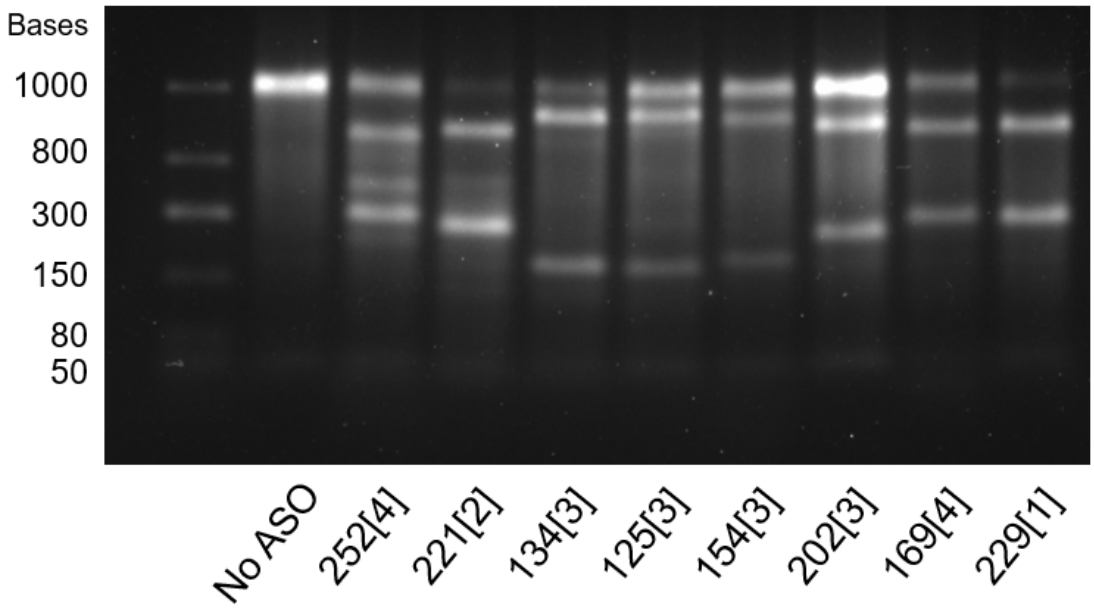
Supplementary Figure 2: Molecular model of a bLA-Biotin-modified DNA hairpin bound to wild-type streptavidin. Crystal structures from the RSPB PDB[2] were used to prepare this graphic. This includes the DNA hairpin[3,4] (PDB ID: 2VAI, DOI: 10.2210/pdb6j6j/pdb) and tetravalent Streptavidin[5,6] (PDB ID: 6J6J, DOI: 10.2210/pdb2VAI/pdb). Molecular graphics were prepared with UCSF Chimera.[7]

Binding of Streptavidin to bLA-Biotin-modified oligonucleotide 252[4] at different dilutions



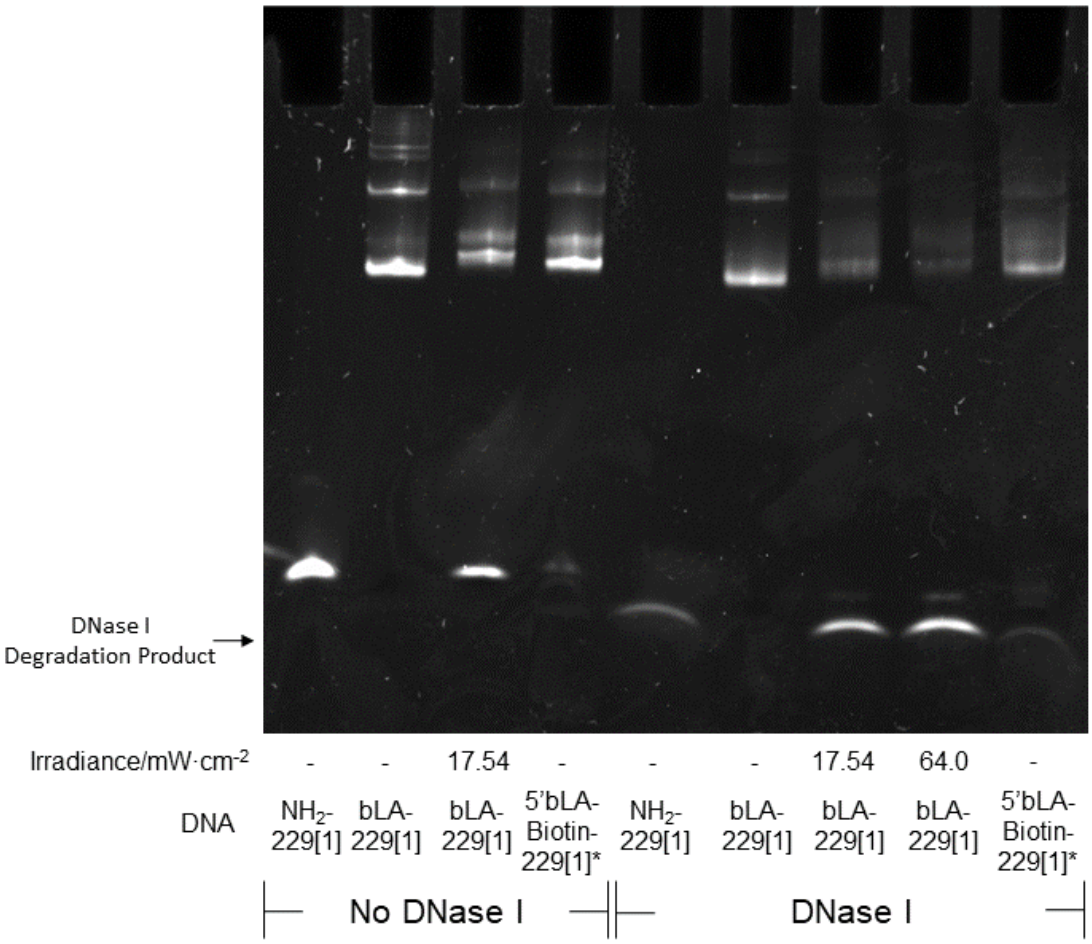
Supplementary Figure 3: Formation of DNA-wtSA 'handcuffs' at different dilutions/ON concentrations in Dulbecco's phosphate buffered saline (DPBS). 80 ng DNA were used per condition.

Screening for antisense oligonucleotide Sequences with lower constraints



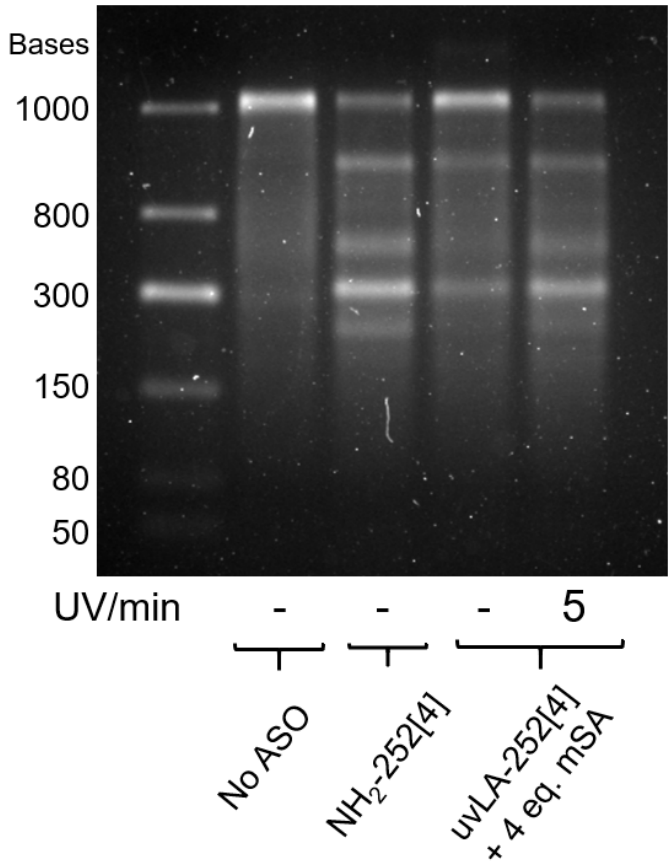
Supplementary Figure 4: Additional ONs with lower constraints screened for RNase H activity against mVenus mRNA.

DNase I stability assay with the bLA-229[1] handcuff



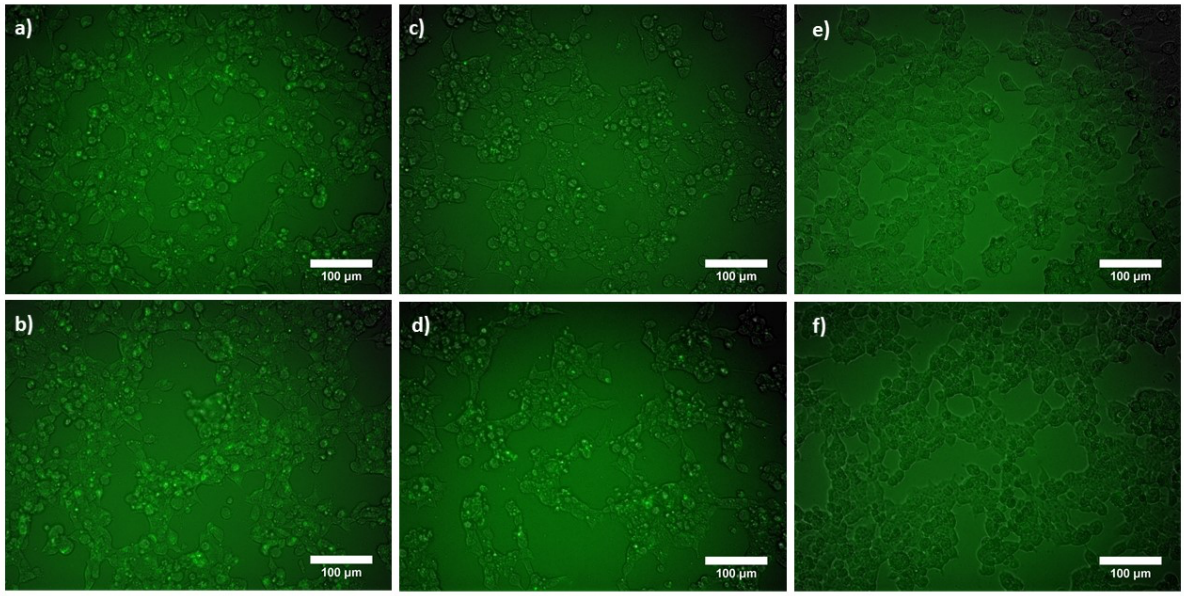
Supplementary Figure 5: DNase I stability assay of modified oligonucleotides. The handcuff structure showed no discernible DNase I degradation product, showing that the DNA cannot be effectively recognised in the handcuff structure. There is also a steric component to this from the wtSA, as was seen with the mono-Biotinylated ON incubated with 1 eq. of wtSA. * - Sample Incubated with 1 eq. of wtSA. Upon photocleavage, the bands corresponding to uncleaved product as well as partially cleaved products show DNase I degradation.

RNase H assay with mSA-caged uvLA-252[4]



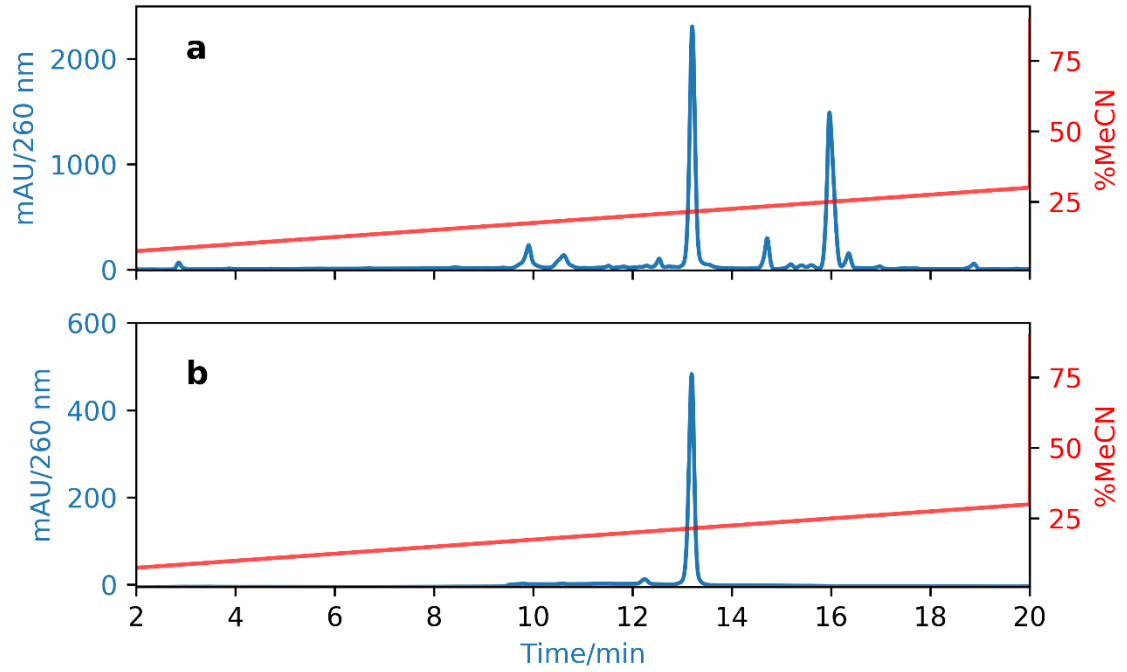
Supplementary Figure 6: RNase H-mediated degradation using a dumbbell-shaped uvLA-252[4] ON bound to monovalent Streptavidin (mSA, 4 eq. for incubation). Terminal mSA reduces, but does not fully abolish RNase H-mediated degradation of the complementary mRNA.

Transfection of bLA-229[1] into HEK293T



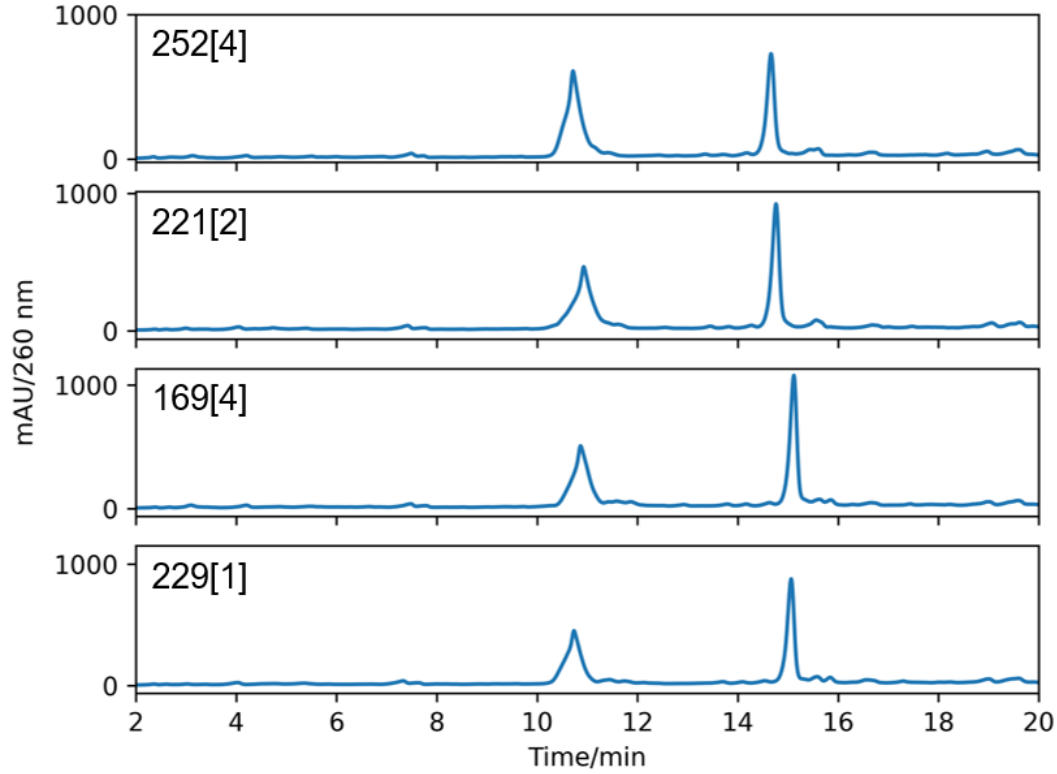
Supplementary Figure 7: Transfection of bLA-Biotin-modified 229[1] and handcuffed bLA-229[1] into HEK293T Cells with Lipofectamine3000. **a,b**) Transfection of bLA-Biotin-modified 229[1] ON. **c,d**) Transfection of handcuffed bLA229[1] ON. **e,f**) Transfection of Lipofectamine only. Experiments were performed in duplicates. It was found that both the DNA as well as the DNA/protein conjugate can be effectively transfected into HEK293T cells.

HPLC Trace of uvLA-Biotin-modified 252[4]



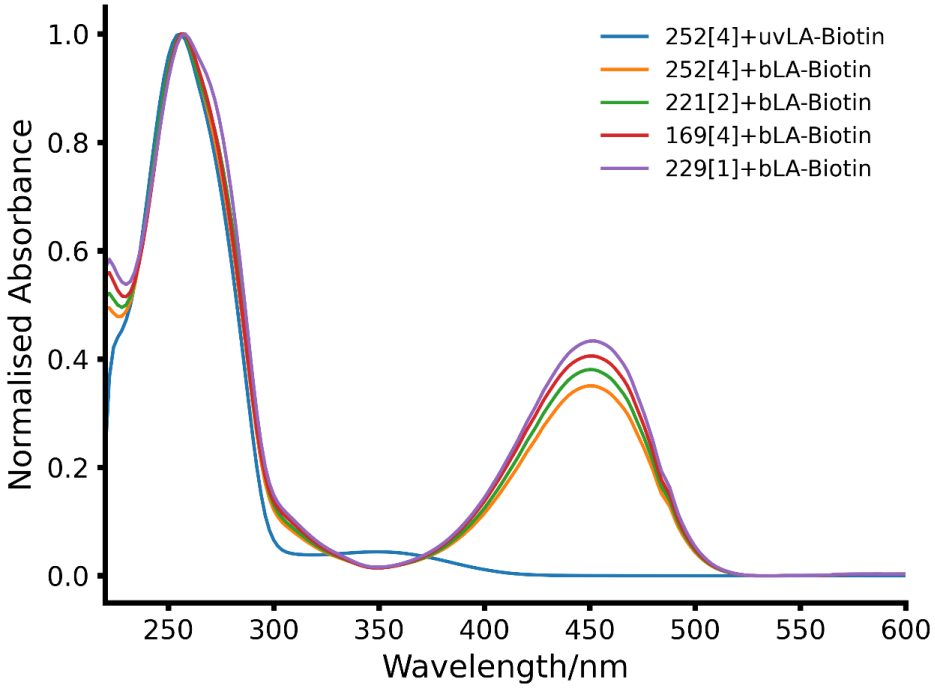
Purification of uvLA-Biotin modified 252[4] oligonucleotide. **a**) Crude reaction trace. **b**) Purified oligonucleotide trace.

Crude HPLC Traces of bLA-Biotin-modified Oligonucleotides



Crude Chromatograms of bLA-Biotin-modified oligonucleotides.

UV-Visible Absorbance Traces of LA-Biotin-modified Oligonucleotides



UV-Visible traces of PC-Biotin-modified oligonucleotides as recorded by HPLC.