Detection of DNA base variation and cystosine methylation at a single nucleotide site using a highly sensitive fluorescent probe

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Supplementary Information

General Experimental Information:

Unless otherwise stated, solvents and reagents were obtained from commercial suppliers and used without further purification. Anhydrous solvents were dried by the usual procedures and used directly. Preparations of all target compounds were performed under an atmosphere of dry nitrogen, apart from the phosphitylation procedure, which was performed under argon. Column chromatography was carried out using silica gel (Merck, grade 60) or alumina (basic, Brockman activity I).

Automated DNA synthesis of probe sequences was performed either at Queens University, Belfast on an Expedite 8909 DNA synthesizer or at the University of Birmingham on an Applied Biosystems ABI 394 synthesizer. Millipore pure H_2O was used in all syntheses and studies of oligonucleotides.

Mass spectra of the oligo sequences were determined on a Waters LCT ESI-TOF mass spectrometer.

HPLC purification was carried out at Queen's University, Belfast using a Merck Hitachi Interface D-7000, pump L-7100, with a LaChrom diode array detector L-7455 or at the University of Birmingham using a Dionex system with Summit P580 pump and Summit UVD 170s UV/VIS Multi-Channel Detector with prep flow cell. Phenomenex Clarity Oligo-RP columns, 150 mm x 4.60 mm 5 micron and 150 mm x 10 mm 5 micron were used for analytical and preparative HPLC respectively.

DNA melting temperatures were determined on a Varian Cary 5000 with a peltier heating accessory on a range of 15 to 85 °C with a heating rate of 0.5 °C /min. The value of the T_m was calculated from the first derivative of the melting curve using Varian software. Unless stated otherwise, all samples were monitored at 260 nm.

UV/Vis spectra were recorded at the University of Birmingham using a Varian Cary 5000 or Varian Cary 50 spectrometer. Quantum yields and fluorescence titrations were carried out on a Shimatzu RF-5301 PC spectrofluorimeter.

Phosphoramidite synthesis:

2-(Anthracen-9-yloxy)-*N*-((2*S*,3*S*)-1,3-dihydroxybutan-2-yl)acetamide, 2a
2-(Anthracen-9-yloxy)acetic acid, **1a** (3.35 g, 13.0 mmol) was dissolved in anhydrous DMF (20 mL). HOBt (2.15 g, 13.0 mmol) was then added, followed by DIPC (2.08 mL, 13.0 mmol) and the solution then stirred under N_2 at room temperature in the absence of light for 15 mins. D-Threoninol (1.38 g, 13.0 mmol) and DIPEA (2.28 mL,

13.0 mmol) were added and the reaction left to stir at 40 ˚C for 40 hours. The solution was diluted in MeOH/DCM (1:2 100 mL) and washed with H_2O (3 x 50 mL) and dried over $MgSO_4$. The solvent was removed in *vacuo* and subsequent purification by silica column chromatography (DCM with 5% MeOH) gave the desired product as a pale yellow solid (1.82 g, 41%). (R_f = 0.58 in DCM with 10% MeOH); M.p. 178-182 ˚C; CHN found: C, 69.81; H, 6.12; N, 4.41%; $C_{20}H_{21}NO_4.0.25H_2O$ requires C, 69.85; H, 6.30; N, 4.07%); δ_H (300 MHz, CD_3CN) 8.33 (1H, s, Ar*H*), 8.23 (2H, dd, *J* = 8.3 and 1.6 Ar*H*), 8.04 (2H, dd, *J* = 6.5 and 3.3, Ar*H*), 7.65 (1H, s, N*H*), 7.33-7.62 (4H, m, Ar*H*), 4.66 (2H, d, *J* = 3.9, OCH_2CO), 4.16-4.36 (1H, m, CH_3CHCH_2OH), 3.95-4.02 (3H, m, C*H*NH and CHCH_2OH), 3.82 (2H, t, *J* = 5.3, CHCH_2OH), 3.38 (1H, d, *J*= 4.0, CH_3CHCH_2OH), 3.26 (1H, t, *J* = 5.2, $CHCH_2OH$), 1.29 (3H, d, *J* = 6.3, CH_3CHCH_2OH); δ_C (100 MHz, 1:1 CD_3CN:$CDCl_3$) 169.1, 149.5, 132.7, 128.9, 126.4, 126.2, 124.5, 123.6, 121.7, 74.1, 67.5, 63.8, 55.6, 20.7; *m/z* (ES+) calcd for $C_{20}H_{21}NO_4$ ($M+Na^+$) 362.1368, found 362.1357.

5-(Anthracen-9-yloxy)-*N*-((2*S*,3*S*)-1,3-dihydroxybutan-2-yl)pentanamide, 2b

5-(Anthracen-9-yloxy) pentanoic acid (2.95 g, 9.3 mmol) **1b** was dissolved in anhydrous DMF (20 mL). HBTU (3.54 g, 9.3 mmol) was added to the solution, which was stirred under N_2 at room temp. in the absence of light for 15 mins. D-Threoninol (0.97 g, 9.3 mmol) and DIPEA (1.6 mL, 9.3 mmol) were added and the reaction left to stir at 40 ˚C for 40 hours. The soln. was diluted in MeOH/DCM (1:2 100 mL) and washed with H_2O (3 x 50 mL) and dried over $MgSO_4$. The solvent was removed in *vacuo* and subsequent purification by silica column chromatography (DCM with 5% MeOH) gave the desired product as an oily yellow solid (2.2 g, 58%). (R_f =0.47 in DCM with 5% MeOH); M.p. 125-131 ˚C; δ_H (400 MHz, $CDCl_3$) 8.24 (2H, dd, *J* = 7.9 and 5.8, Ar*H*), 8.18 (1H, s, Ar*H*), 7.90-7.98 (2H, m, Ar*H*), 7.42-7.51 (4H, m, Ar*H*), 6.57 (1H, d, *J* = 8.5, N*H*), 4.05-4.19 (3H, m, OCH_2CH_2CH_2O and $CH(CH_3)$C*H*OH), 3.86 (1H, ddd, *J* = 16.9, 9.1 and 6.6, C*H*NH), 3.72-3.81 (2H, m, CHCH_2OH), 2.41 (2H, t, *J* = 6.6, CH_2CO), 2.06 (4H, m, $CH_2CH_2CH_2CH_2CO$), 1.18 (3H, d, *J* = 6.4, CH_3CHOH); δ_C (100 MHz, $CDCl_3$) 173.7, 151.1, 132.2, 128.4, 125.4, 125.1, 124.6, 122.2, 122.1, 75.6, 68.6, 64.9, 54.6, 36.6, 30.1, 22.7, 20.5; *m/z* (ES+) calcd. for $C_{23}H_{27}O_4NNa$ ($M+Na^+$) 404.1838, found 404.1835.

2-(Anthracen-9-yloxy)-*N*-((2*S*,3*S*)-1-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-hydroxybutan-2-yl)acetamide, 3a

2a (0.73 g, 5.3 mmol) was dissolved in anhydrous pyridine (30 mL). Dimethoxytritylchloride (0.72 g, 5.3 mmol) was added to the solution, followed by DMAP (0.038 g, 0.5 mmol) and the reaction left to stir under N_2 at room temp. in the absence of light for 24 hours. The reaction mixture was poured onto H_2O (50 mL), extracted with DCM (2 x 50 mL) and dried over $MgSO_4$. Column chromatography on silica (Hex/EtOAc/TEA, 40:59:1) afforded the desired compound as a pale yellow crystalline solid (1.52 g, 43%). (R_f = 0.38 in DCM with 5% MeOH); M.p. 82-85˚C CHN found: C, 75.9; H, 6.1; N, 2.1%; $C_{41}H_{39}NO_6.0.25$EtOAc requires C, 76.0; H, 6.2; N, 2.1%); δ_H (500 MHz, CD_3CN) 8.37 (1H, s, Ar*H*), 8.25 (2H, dt, *J* = 8.7 and 2.2, Ar*H*), 8.06 (2H, d, *J* = 8.5, Ar*H*), 7.53 (1H, d, *J* = 8.7, N*H*), 7.48-7.52 (4H, m, Ar*H* and DMT*H*), 7.42 (2H, m, Ar*H*), 7.37 (4H, m, DMT*H*), 7.30 (2H, dd, *J* = 10.5 and 4.8, DMT*H*), 7.20-7.26 (1H, m, DMT*H*), 6.84 (4H, dd, *J* = 1.8 and 8.7, DMT*H*), 4.69 (2H, d, *J* = 3.2, OCH_2CO), 4.15 (1H, m, C*H*NH), 4.11 (1H, m, CHC*H*OH), 3.72 (6H, d, *J* = 2.3, 2 x OCH_3), 3.20-3.37 (2H, m, CH_2ODMT), 1.2 (3H, d, *J* = 6.2, CH_3CHOH); δ_C (125 MHz, CD_3CN) 169.3, 159.7, 150.2, 146.2, 137.2, 137.0, 13.4,

131.0, 129.5, 129.0, 128.8, 127.8, 126.9, 126.8, 125.1, 124.2, 122.6, 114.1, 87.0, 74.7, 67.3, 64.4, 55.8, 55.3, 20.8; *m/z* (ES+) calcd. for $C_{41}H_{39}O_6NNa$ ($M+Na^+$) 664.2675, found 664.2675.

5-(Anthracen-9-yloxy)-*N*-((2*S*,3*S*)-1-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-hydroxybutan-2-yl)pentanamide, **3b**

2b (2.2 g, 5.8 mmol) was dissolved in anhydrous pyridine (20 mL). Dimethoxytritylchloride (1.95 g, 5.8 mmol) was added to the soln. followed by DMAP (0.08 g, 0.6 mmol) and the reaction left to stir under N_2 at room temp. in the absence of light for 24 hours. The reaction mixture was poured onto H_2O (50 mL), extracted with DCM (2 x 50 mL) and dried over $MgSO_4$. The solvent was removed in *vacuo* and subsequent column chromatography on silica (Hex/EtOAc/TEA, 40:59:1) afforded the desired compound as a pale yellow crystalline solid (1.36 g, 35%). (R_f = 0.48 in DCM with 5% MeOH); M.p. 77-79 ˚C; (found: C, 76.77; H, 6.55; N, 1.84%. $C_{44}H_{45}NO_6.0.25H_2O$ requires C, 76.78; H, 6.66; N, 2.03%); ν_{max}/cm^{-1} 3338 (OH), 2942 (N-H), 1651 (C=O) δ_H (400 MHz, CD_3CN) 8.28 (1H, s, Ar*H*), 8.23-8.27 (2H, m, Ar*H*), 8.03 (2H, dd, *J* = 6.5 and 2.9, Ar*H*), 7.40-7.49 (6H, m, Ar*H* and DMT*H*), 7.26-7.31 (6H, m, DMT*H*), 7.18 (1H, t, *J* = 7.3, DMT*H*), 6.82 (4H, d, *J* = 8.8, DMT*H*), 6.48 (1H, d, *J* = 8.8, N*H*), 4.16 (2H, t, *J* = 6.1, OCH_2), 3.90-3.99 (2H, m, NHCHC*H*OH and NHC*H*CHOH), 3.69 (6H, s, 2 x OCH_3), 3.05-3.19 (3H, m, CH_2ODMT and CH$_2$O*H*), 2.37 (2H, t, *J* = 7.1, OCH$_2$CH$_2$CH$_2$CH_2CO), 1.93-2.07 (4H, m, OCH$_2$CH_2CH_2CH$_2$CO), 1.05 (3H, d, *J* = 6.2, CH_3CHOH); δ_C (75 MHz, CD_3CN) 173.8, 159.5, 152.2, 146.1, 137.0, 133.4, 130.9, 129.3, 129.0, 128.7, 127.7, 126.6, 126.3, 125.5, 123.1, 122.9, 113.9, 86.8, 76.6, 67.2, 64.6, 55.7, 55.2, 36.7, 30.8, 23.4, 20.5; *m/z* (ES+) calcd for $C_{44}H_{45}NO_6$ (M^++Na) 706.3145, found 706.3143.

(2*S*,3*S*)-3-(2-(Anthracen-9-yloxy)acetamido)-4-(bis(4-methoxyphenyl)-(phenyl)methoxy)butan-2-yl 2-cyanoethyl diisopropylphosphoramidite, 4a

3a (0.15 g, 0.24 mmol), pre-dried overnight over P_2O_5, was dissolved in dry DCM (10 mL) and stirred under argon. DIPEA (0.17 mL, 0.96 mmol, 4 eq.,) was added followed by *2*-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.05 mL, 1.1 eq.), added dropwise *via* a syringe and the reaction stirred for 30 mins. Solid supported BnOH was added (0.3 g, 0.78 mmol) and left to stir for 1 hour in the absence of light. The solution was filtered, diluted with EtOAc (10 mL) and then washed with 2 M Na_2CO_3 (a.q.) soln. (2 x 50 mL), H_2O (1 x 50 mL) and brine (1 x 50 mL) and dried over Mg_2SO_4. The EtOAc was then removed in *vacuo* and the solid dissolved in 50:50 Hex:EtOAc. The compound was then columned through activated alumina in 49:50:1 EtOAc:Hex:TEA. The desired fractions were collected and the solvent removed in *vacuo*. Co-evaporation in *vacuo* with acetonitrile three times gave the phosphoramidite as a yellow solid (0.05g, 27%); (R_f =0.6 50:50 Hex:EtOAc); δ_H (300 MHz, CD_3CN) 8.39 (1H, s, An*H*), 8.22 (2H, d, *J* = 8.7, Ar*H*), 8.08 (2H, d, *J* = 8.5, Ar*H*), 7.45-7.56 (5H, m, Ar*H*, DMT*H* and N*H*), 7.36-7.45 (4H, m, Ar*H* and DMT*H*), 7.27-7.35 (2H, m, DMT*H*), 7.20-7.26 (1H, m, DMT*H*), 6.81-6.92 (4H, m, DMT*H*), 4.70 (2H, d, *J* = 2.6, OCH_2CO), 4.27-4.63 (2H, m, C*H*NH and CHC*H*OP), 3.73 (6H, s, OCH_3), 3.33-3.58 (6H, m, CH_2ODMT, POCH_2 and PNC*H*), 2.44-2.62 (2H, m, CH_2CN), 0.98-1.07 (12H, m, CH_3), 0.79 (3H, d, *J* = 6.8, CH_3CHOP); δ_P (121 MHz, CD_3CN) 148.3, 146.0; *m/z* (ES+) calcd. for $C_{50}H_{56}O_7N_3PNa$ ($M+Na^+$) 864.3754, found 864.3751.

(2*S*,3*S*)-3-(5-(anthracen-9-yloxy)pentanamido)-4-(bis(4-methoxyphenyl) (phenyl)methoxy)butan-2-yl (2-cyanoethyl) diisopropylphosphoramidite, 4b

5-(Anthracen-9-yloxy)-*N*-((2*S*,3*S*)-1-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-hydroxybutan-2-yl) pentamide (0.38 g, 0.56 mmol) **3b** was placed in a 25 mL round-bottomed flask with a stirrer bar and a septum. The flask was evacuated and filled with argon three times and the solid dissolved in anhydrous DCM (15 mL). DIPEA (0.4 mL, 2.1 mmol, 4.1 eq.) was added and the solution stirred at room temp. in the absence of light. *2*-Cyanoethyl N,N-diisopropylchlorophosphoramidite (0.13 mL, 0.61 mmol, 1.1. eq.) was added dropwise *via* a syringe and the reaction stirred for 1 hr. The soln. was then transferred to a 25 mL round-bottomed flask containing a stirrer bar and solid-supported BnOH (0.05 g, 0.13 mmol) and left to stir for 1 hr in the absence of light. The soln. was diluted with EtOAc (10 mL), filtered and then washed with 2 M Na_2CO_3 (a.q.) soln. (2 x 50 mL) and brine (1 x 50 mL) and dried over Mg_2SO_4. The soln. was then columned through activated basic alumina with EtOAC:Hex:TEA 49:50:1 and the filtrate evaporated in *vacuo* to give a yellow powdery solid (0.36 g, 59%); (R_f. =0.63 50% pet ether 50% DCM) δ_H (300 MHz, CD_3CN) 8.32 (1H, s, Ar*H*), 8.30-8.25 (2H, *m*, Ar*H*), 8.07 (2H, dd, *J* = 6.3 and 3.3, Ar*H*), 7.57-7.42 (4H, m, Ar*H* and DMT*H*), 7.39-7.26 (6H, m, Ar*H* and DMT*H*), 7.21 (3H, td, *J* = 7.2 and 5.0 DMT*H*), 6.84 (4H, ddd, *J* = 6.9, 5.0 and 2.6, DMT*H*), 6.26 (1H, dd, *J* = 16.6 and 9.2, N*H*), 4.22 (2H, dt, *J* = 12.1 and 6.2, $OCH_2CH_2CH_2$), 3.80-3.63 (3H, m, C*H*NH and $CH(CH_3)CHOH$), 3.73 (6H, s, 2 x OCH_3), 3.43-3.60 (4H, m, $POCH_2$ and PNC*H*), 3.06-3.21 (2H, m, CH_2ODMT), 2.52 (2H, dt, *J* = 33.9, *J* = 6.1, CH_2CN), 2.41 (2H, t, *J* = 6.0, $OCH_2CH_2CH_2CH_2CO$), 2.02-2.11 (4H, m, $OCH_2CH_2CH_2CH_2CO$), 1.29-1.05 (12H, m, CH_3), 0.98 (3H, d, *J* = 6.8, CH_3CHOP); δ_P (121 MHz, CD_3CN) 147.6, 147.0; *m/z* (ES+) calcd. for $C_{54}H_{64}O_7N_3NaP$ ($M+Na^+$) 906.4223, found 906.4225.

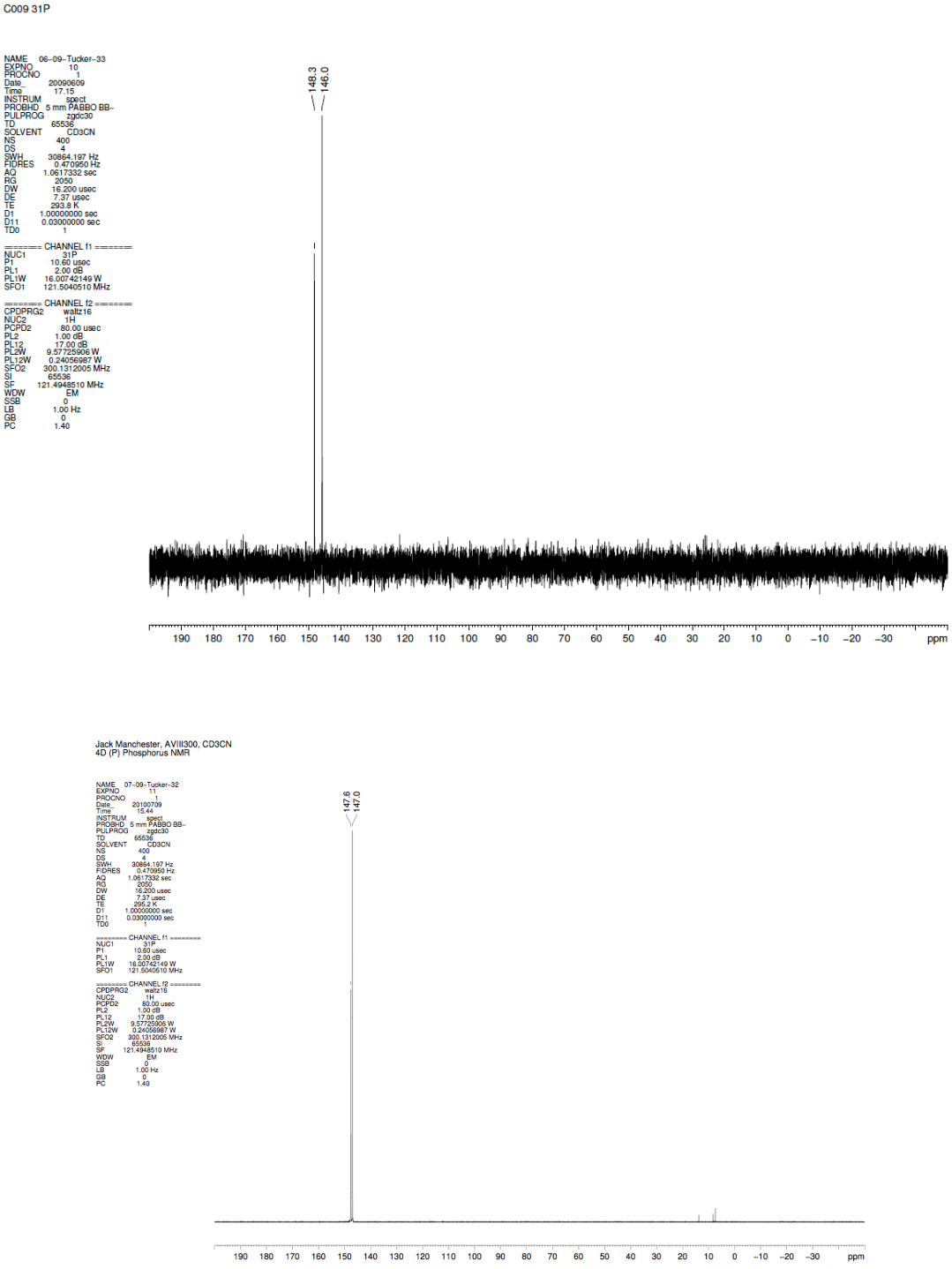


Fig. S1 ^{31}P NMR spectra of **4a** (top) and **4b** (bottom)

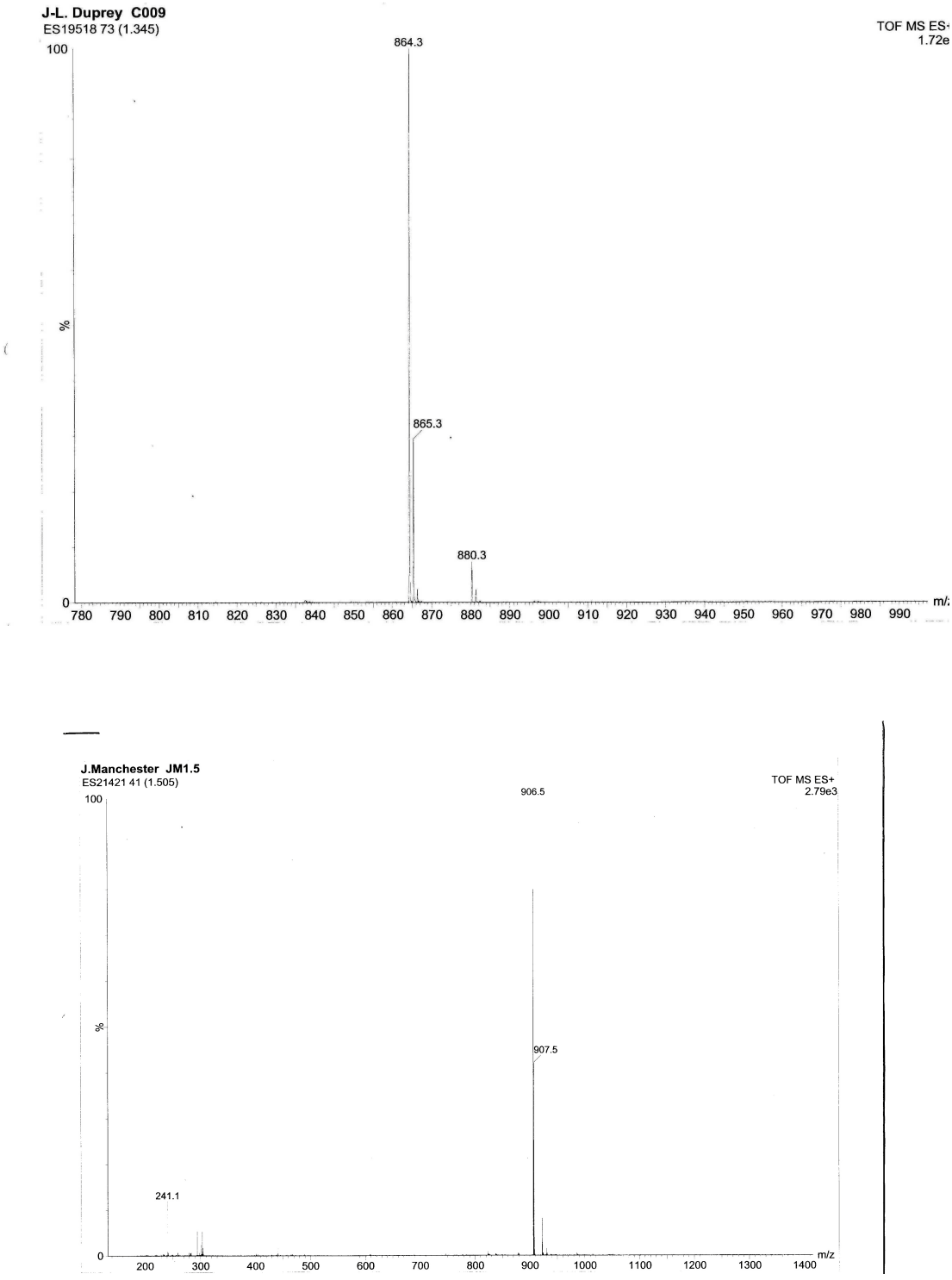


Fig. S2 ES+ mass spectra of **4a** (top) and **4b** (bottom)

Oligo characterisation by HPLC (analytical):

Oligo No	Sequence	retention time/mins
5 (224)	TGGACTC-**a**-CTCAATG	49.118
6 (276)	TGGACTC-**b**-CTCAATG	52.483
(282)	TGGACTC-**T**-CTCAATG	31.251
(293)	TGGACTC-**abasic**-CTCAATG	31.013
7 (294)	CATTGAG-**A**-GAGTCCA	23.028
8 (313)	CATTGAG-**C**-GAGTCCA	23.255
9 (A3)	CATTGAG-**T**-GAGTCCA	29.764*
10 (A5)	CATTGAG-**G**-GAGTCCA	28.115*
11 (316)	CATTGAG-**MeC**-GAGTCCA	25.143

HPLC conditions:
Solvent system **A**: 5% MeCN/0.1M TEAA pH 7.0; Solvent system **B**: 15% MeCN/0.1M TEAA pH 7.0; Solvent system **C**: MeCN

Gradient (linear increase):
0 - 35 mins, 30% B - 50% B; 35 - 38 mins, 50% B hold; 38 - 48 mins, 50% B - 100% B; 48 - 58 mins, 100% B - 100% C; 58 - 68 mins, 100% C hold; 68 - 73 mins, 100% C - 30% B

30 µL oligo sample, *ca* 70 µM was auto injected. Flow rate, 1.0 ml/min, monitored at 260 nm.

* A slightly shorter 60 mins programme was used.

Oligo characterisation by Mass Spectrometry (ES-):

Oligo No	Sequence	Calc. Mass	Obs. Mass
5 (224)	TGGACTC-**a**-CTCAATG	4640.076	4640.0
6 (276)	TGGACTC-**b**-CTCAATG	4682.123	4682.0
282	TGGACTC-**T**-CTCAATG	4543.020	4542.0
293	TGGACTC-**abasic**-CTCAATG	4418.990	4418.0
7 (294)	CATTGAG-**A**-GAGTCCA	4601.070	4600.0
8 (313)	CATTGAG-**C**-GAGTCCA	4577.046	4577.0
9 (A3)	CATTGAG-**T**-GAGTCCA	4592.058	4592.0
10 (A5)	CATTGAG-**G**-GAGTCCA	4617.071	4617.0
11 (316	CATTGAG-**MeC**-GAGTCCA	4591.073	4591.0

20 µL oligo sample *ca.* 70 mM was mixed with 50 ml Buffer (50% 1% TEA in water/acetonitrile), 10 µl of which was injected.

The nine mass spectra are displayed on pages S9-S13 in the order they appear in the table.

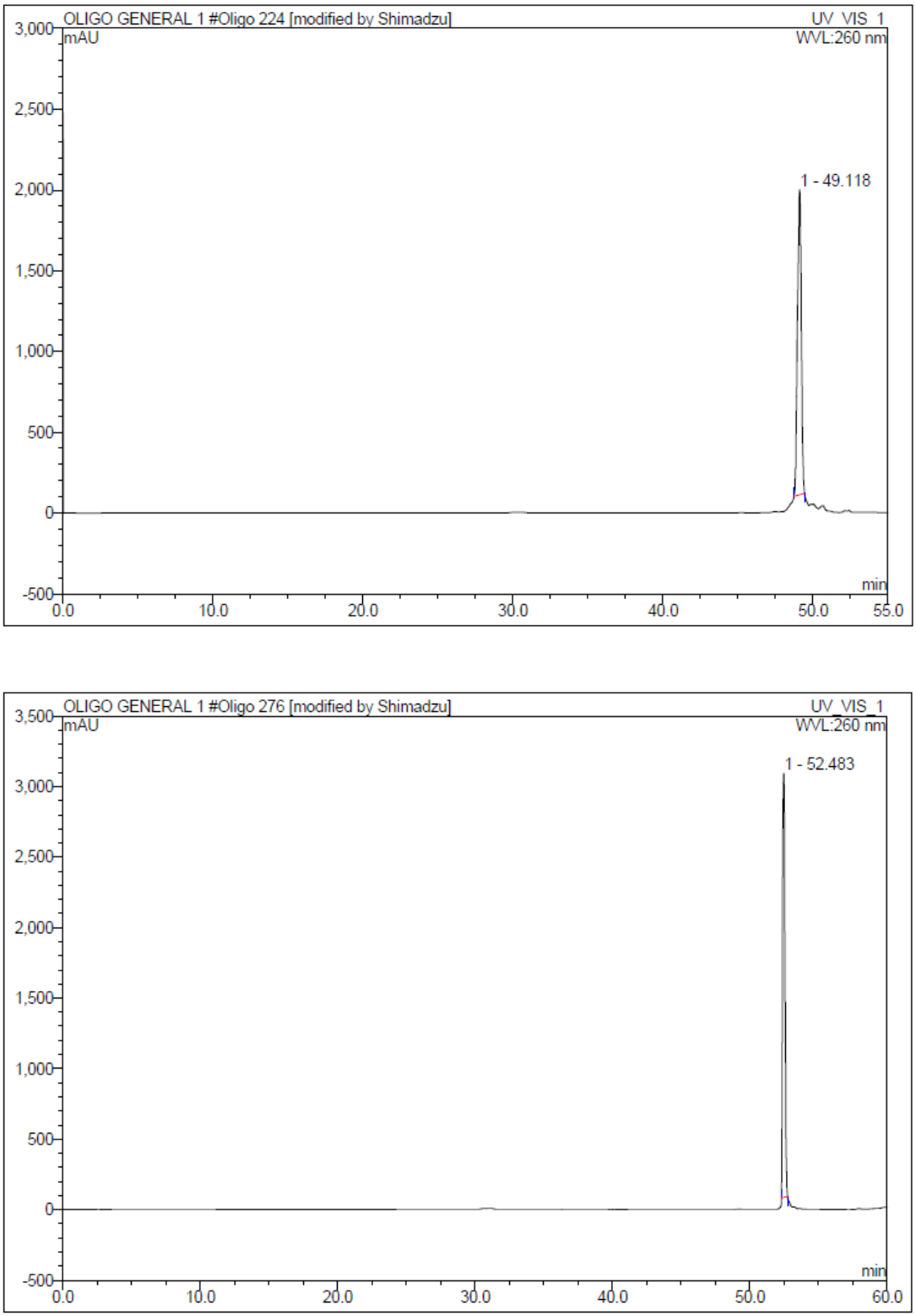
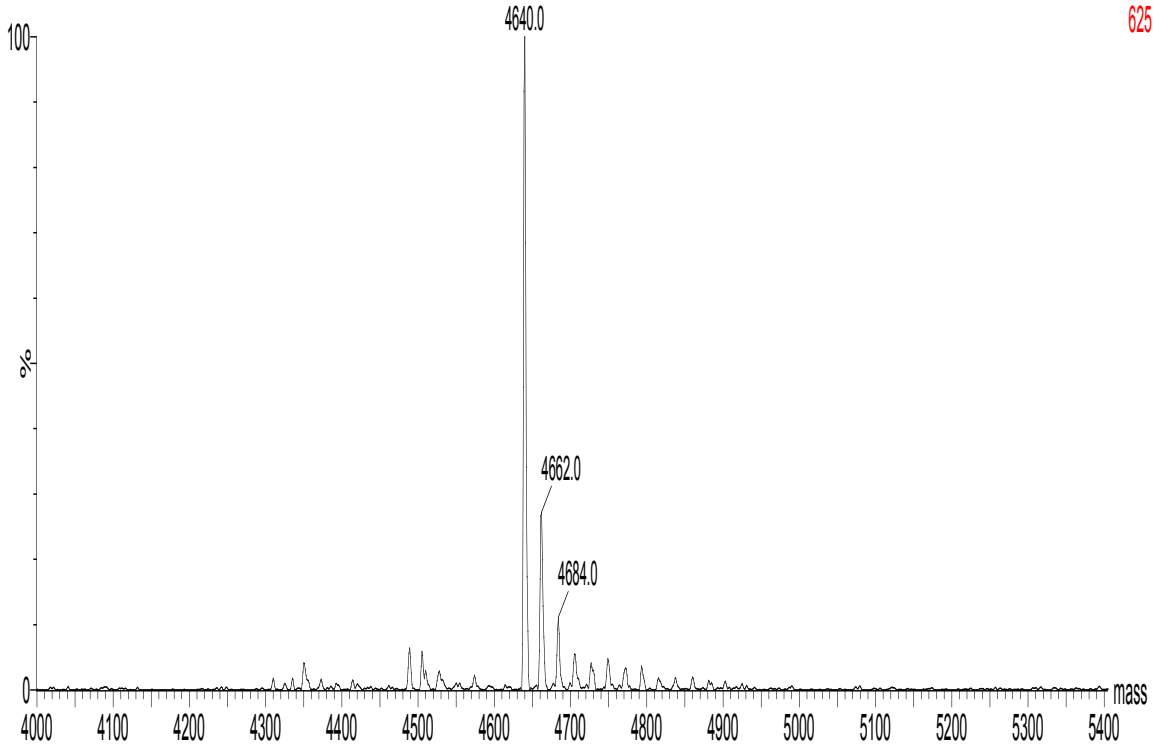


Fig S3 Analytical HPLC traces of probes **5** (top) and **6** (bottom)

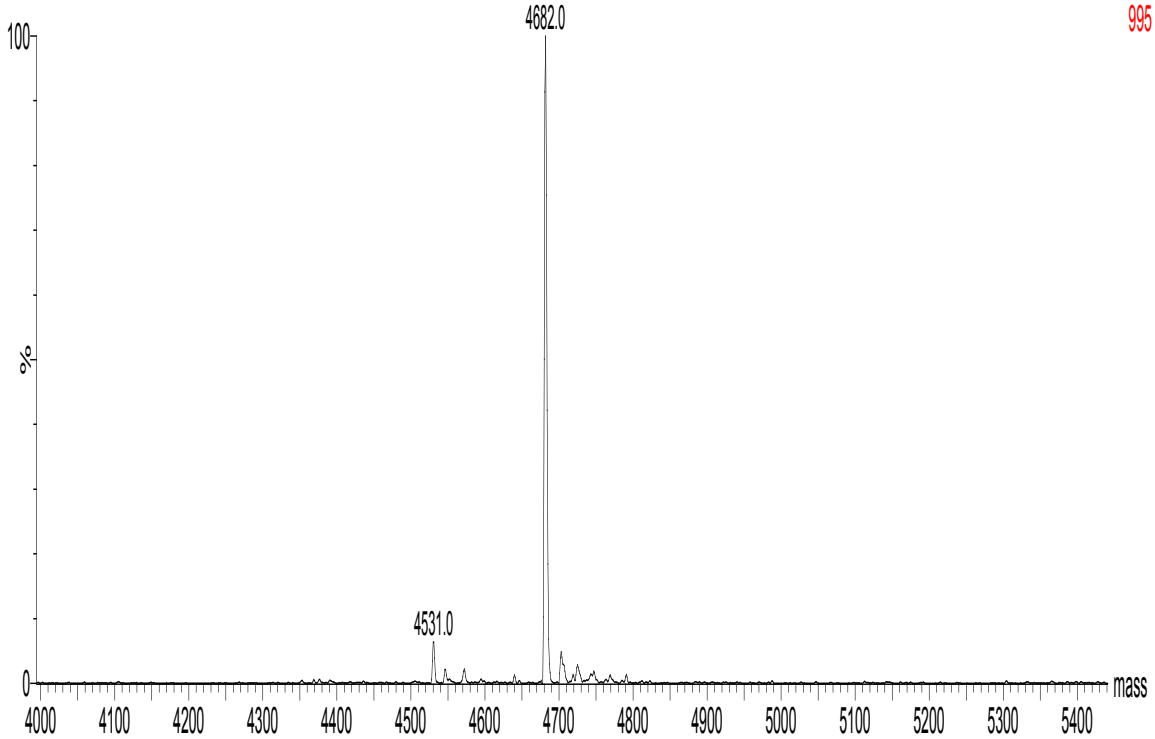
John Zhao Oligo 224

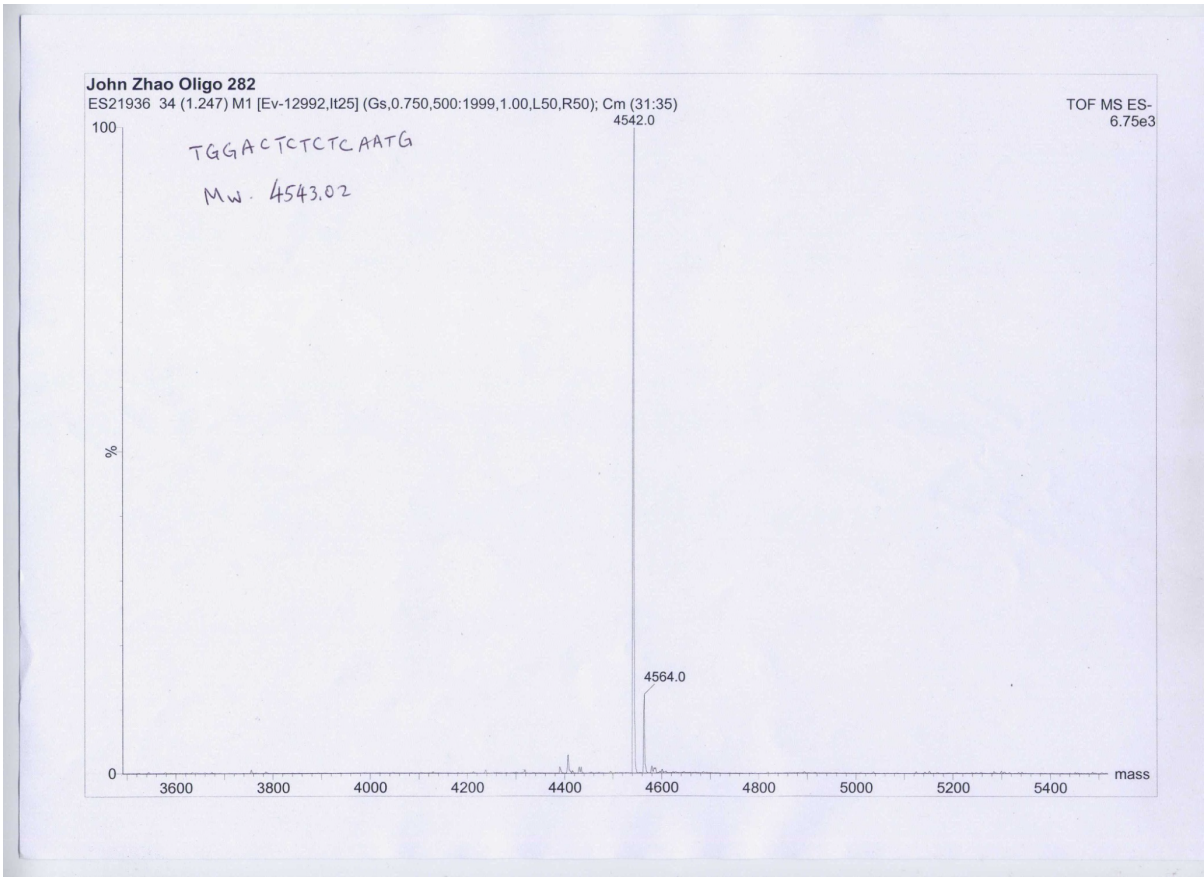
ES24686 15 (0.526) Sm (SG, 2x2.00); M1 [Ev-3479,It18] (Gs,0.750,400:2000,1.00,L25,R25); Cm (13:15) TOF MS ES-625

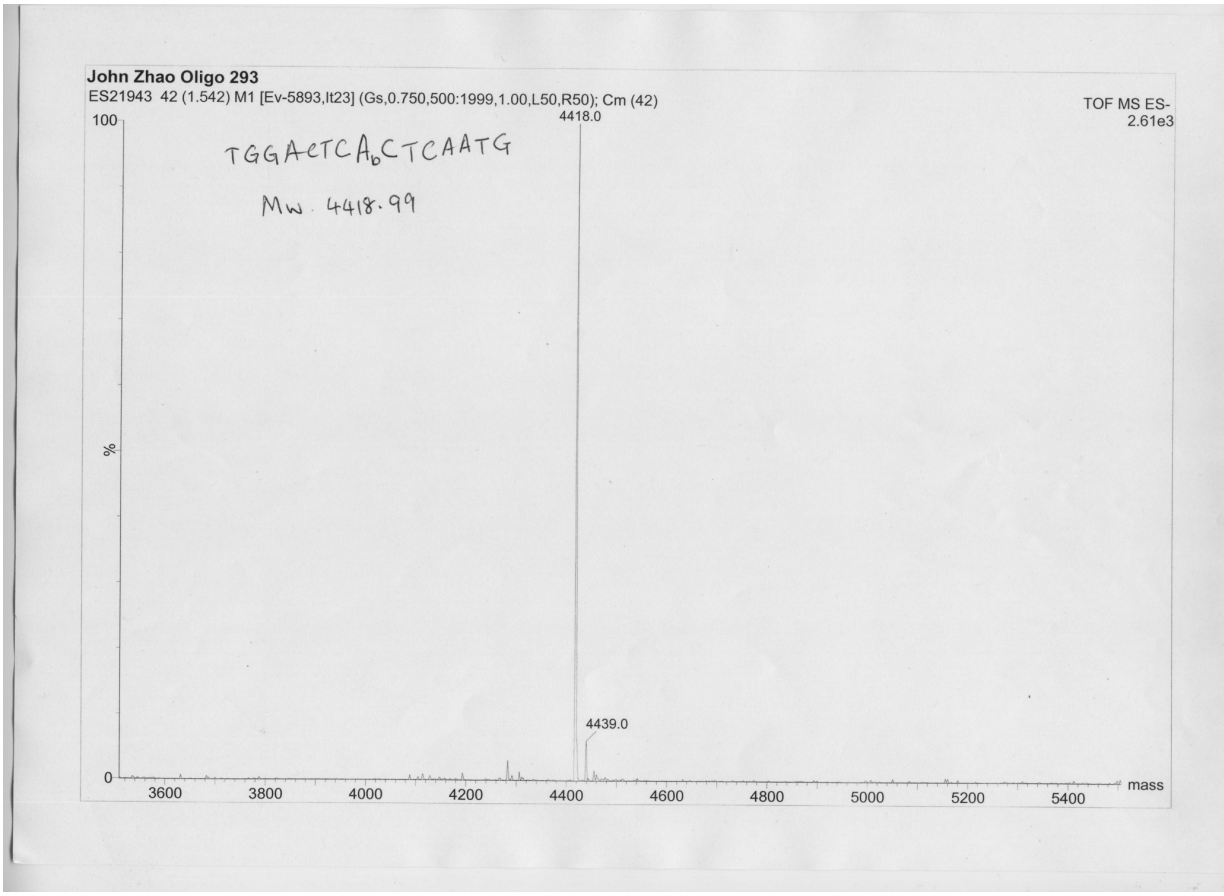


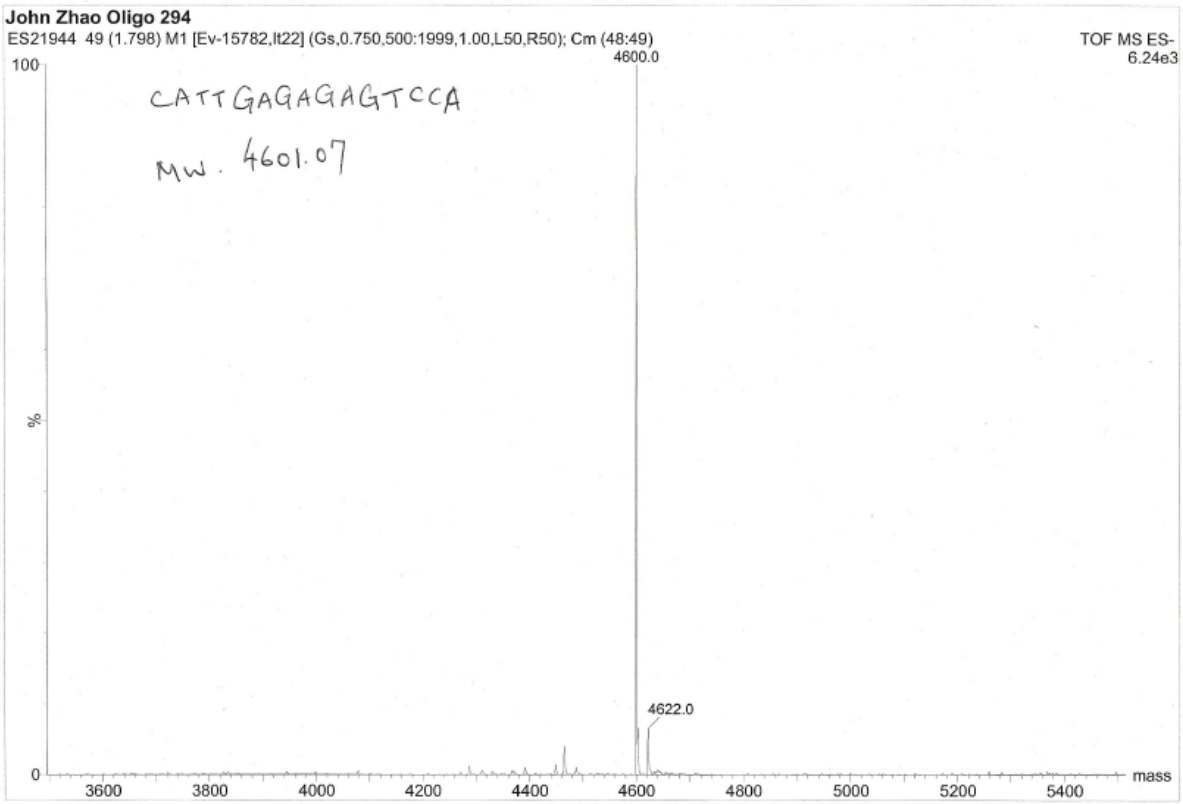
John Zhao Oligo 276

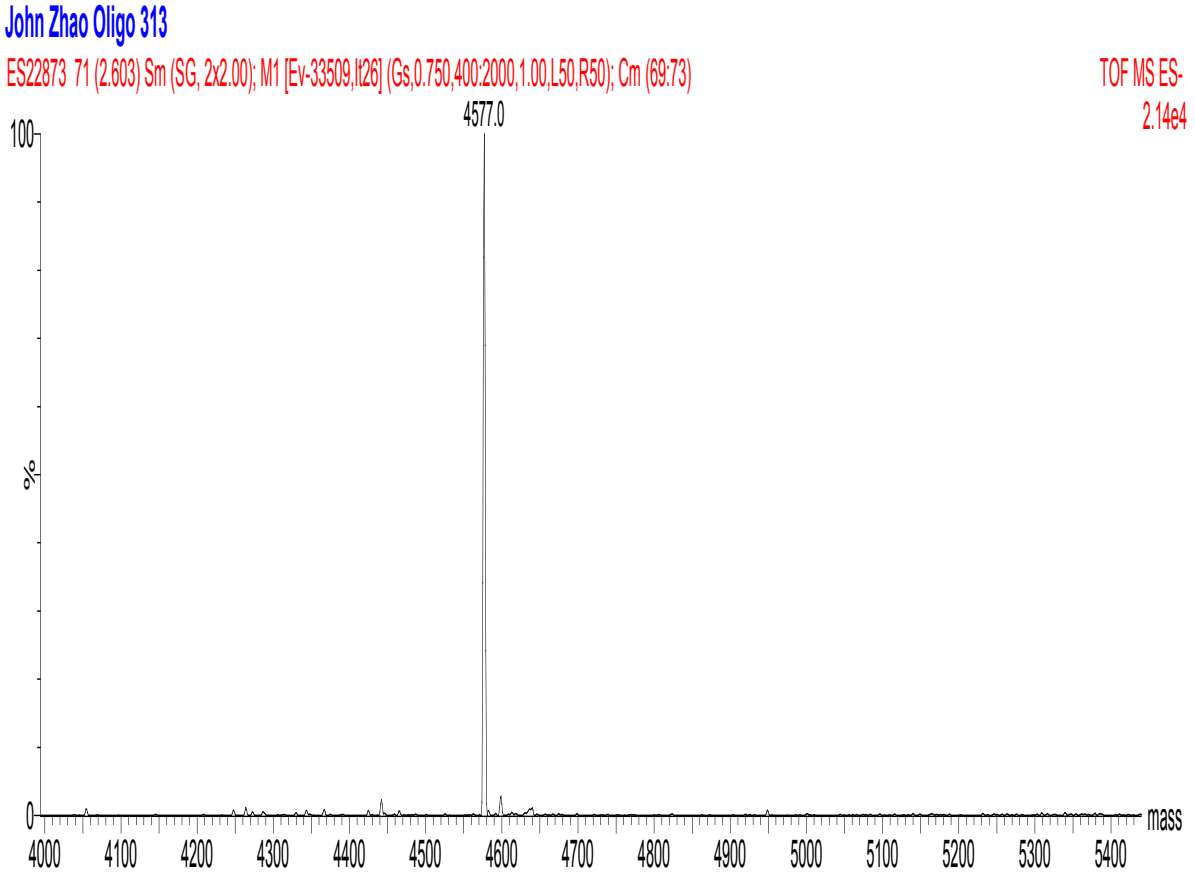
ES24687 13 (0.457) Sm (SG, 2x2.00); M1 [Ev-2020,It19] (Gs,0.750,400:1999,1.00,L25,R25); Cm (11:15-3:7) TOF MS ES-995



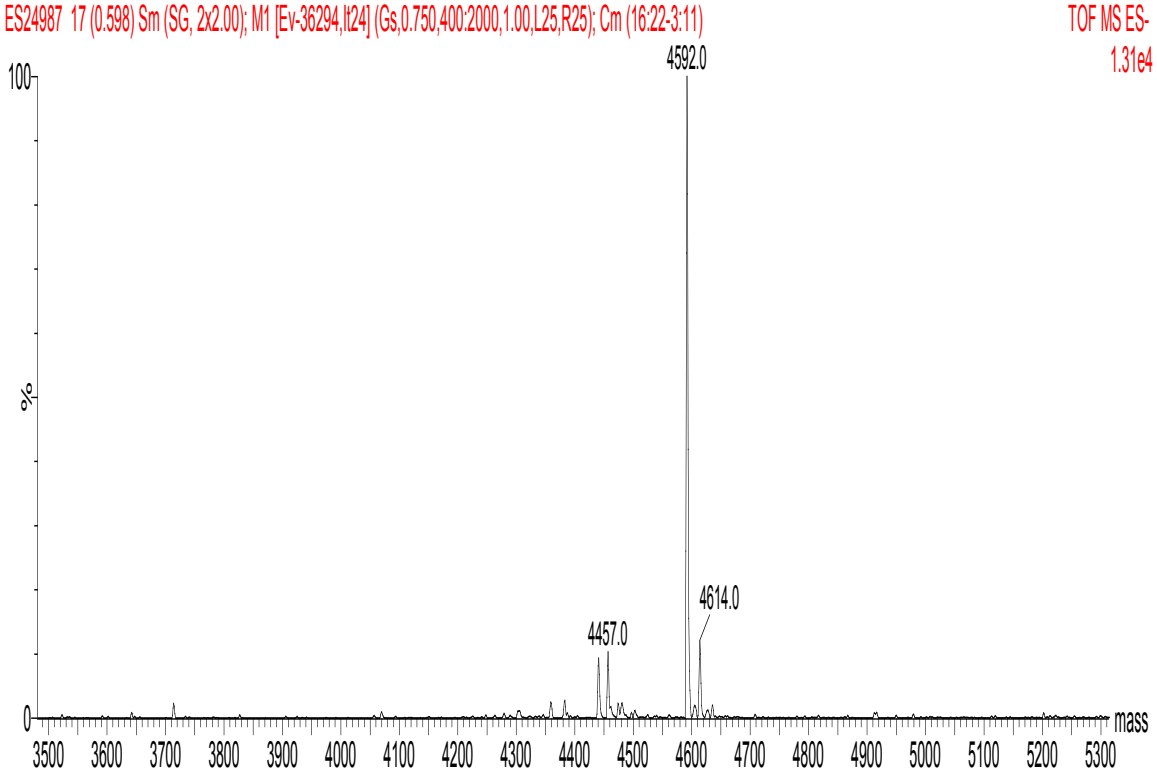

John Zhao Oligo 282
ES21936 34 (1.247) M1 [Ev-12992,It25] (Gs,0.750,500:1999,1.00,L50,R50); Cm (31:35)
TOF MS ES-
6.75e3
4542.0
100
TGGACTCTCTCAATG
Mw. 4543.02
%
4564.0
0
3600
3800
4000
4200
4400
4600
4800
5000
5200
5400
mass


John Zhao Oligo 293
ES21943 42 (1.542) M1 [Ev-5893,It23] (Gs,0.750,500:1999,1.00,L50,R50); Cm (42)
TOF MS ES-
2.61e3
4418.0
100
TGGACTCAbCTCAATG
Mw. 4418.99
%
4439.0
0
3600
3800
4000
4200
4400
4600
4800
5000
5200
5400
mass

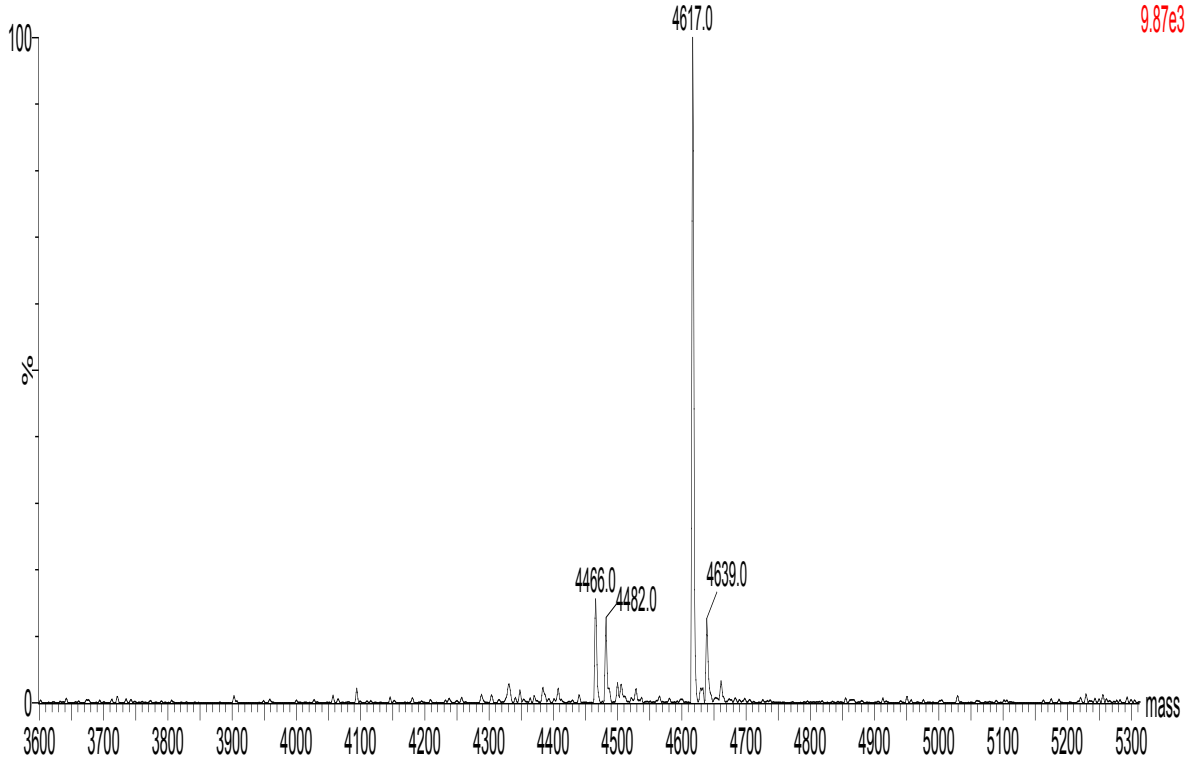

John Zhao Oligo 294
ES21944 49 (1.798) M1 [Ev-15782,It22] (Gs,0.750,500:1999,1.00,L50,R50); Cm (48:49)
TOF MS ES-
6.24e3
4600.0
CATTGAGAGAGTCCA
MW. 4601.07
100
%
0
4622.0
3600
3800
4000
4200
4400
4600
4800
5000
5200
5400
mass

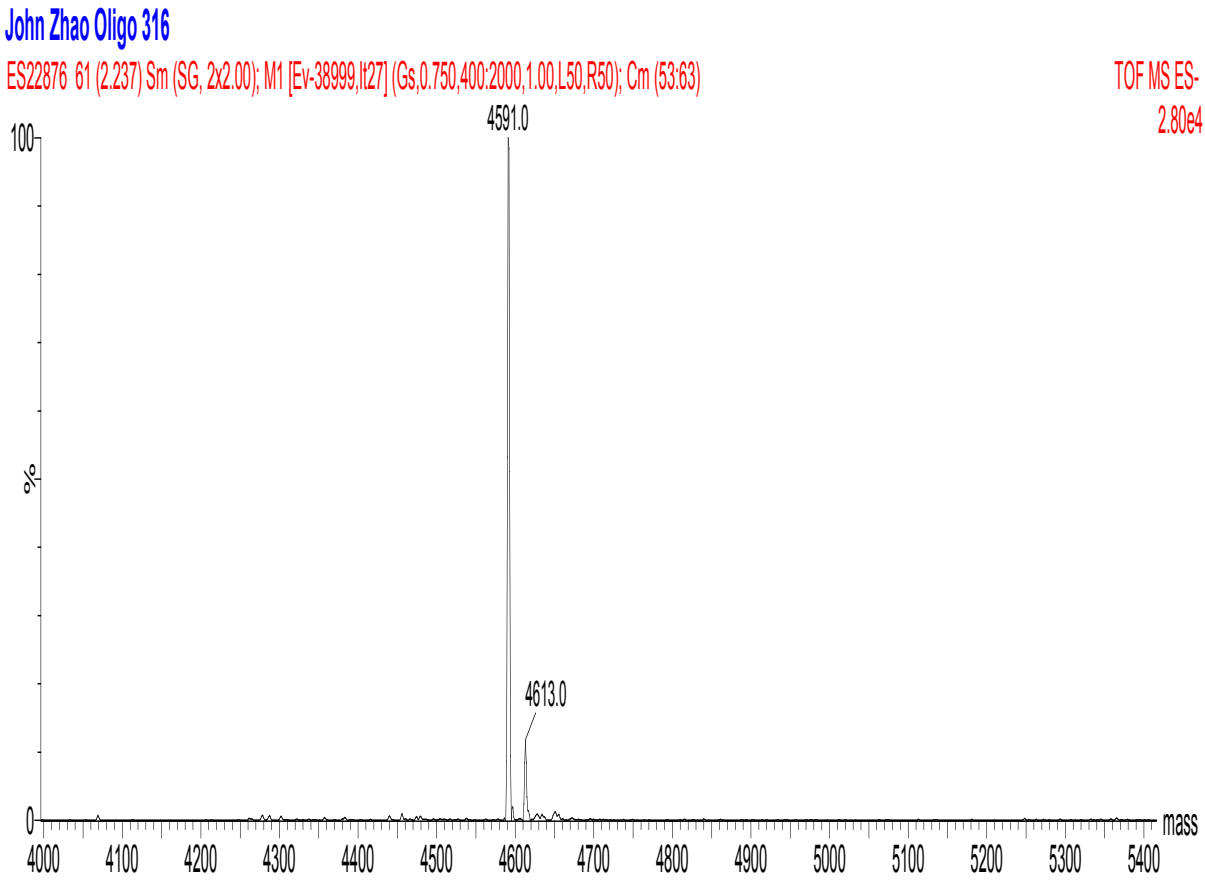

John Zhao Oligo 313
ES22873 71 (2.603) Sm (SG, 2x2.00); M1 [Ev-33509,It26] (Gs,0.750,400:2000,1.00,L50,R50); Cm (69:73)
TOF MS ES-
2.14e4
4577.0
100
%
0
4000
4100
4200
4300
4400
4500
4600
4700
4800
4900
5000
5100
5200
5300
5400
mass

John Zhao Oligo A3



John Zhao Oligo A5




John Zhao Oligo 316
ES22876 61 (2.237) Sm (SG, 2x2.00); M1 [Ev-38999,It27] (Gs,0.750,400:2000,1.00,L50,R50); Cm (53:63)
TOF MS ES-
2.80e4
4591.0
4613.0
100
%
0
mass
4000
4100
4200
4300
4400
4500
4600
4700
4800
4900
5000
5100
5200
5300
5400

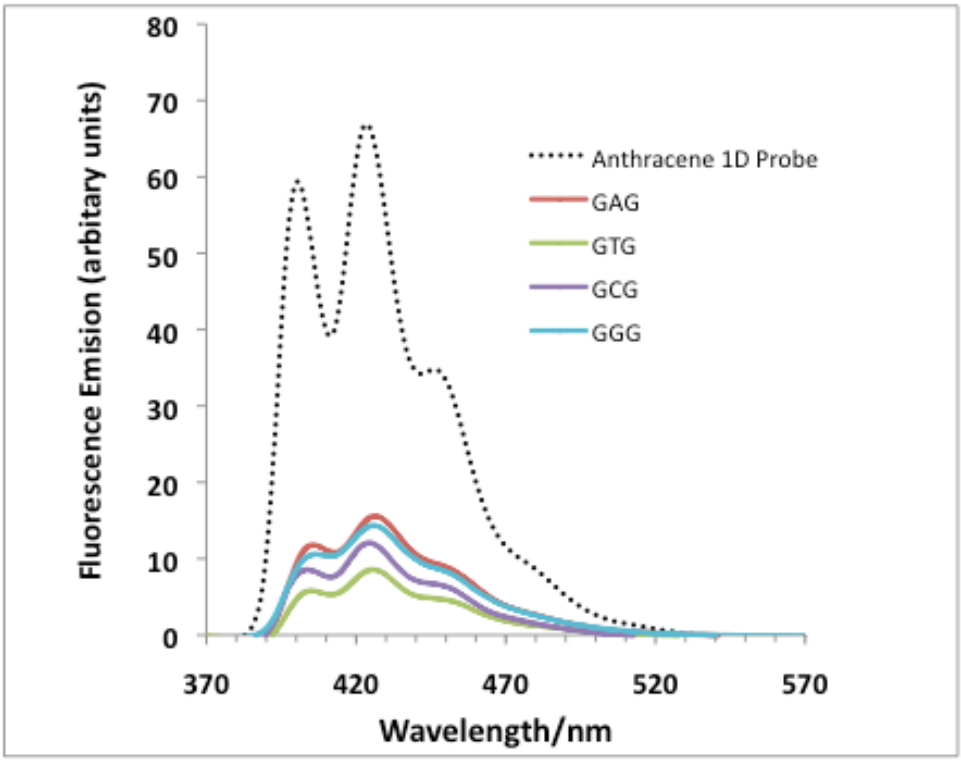


Fig S4 Overlaid fluorescence spectra of **5** (dotted line) and duplexes **5•7** (red), **5•9** (blue), **5•8** (purple), and **5•10** (green) (pH = 7, 10 mM phosphate buffer, 100 mM NaCl, *ca*. 293 K, λ_{ex} = 360 nm).

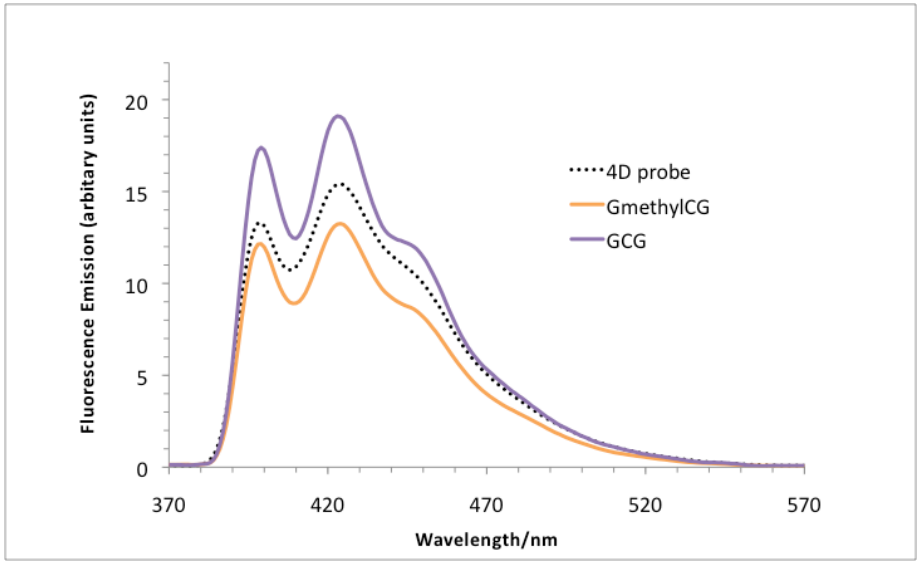


Fig S5 Overlaid fluorescence spectra of **6** (dotted line) and **6•8** (purple), and **6•11** (orange) (pH = 7, 10 mM phosphate buffer, 100 mM NaCl, *ca*. 293 K, λ_{ex} = 360 nm).