Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Rapid Chemical Ligation of Oligonucleotides by the Diels-Alder Reaction

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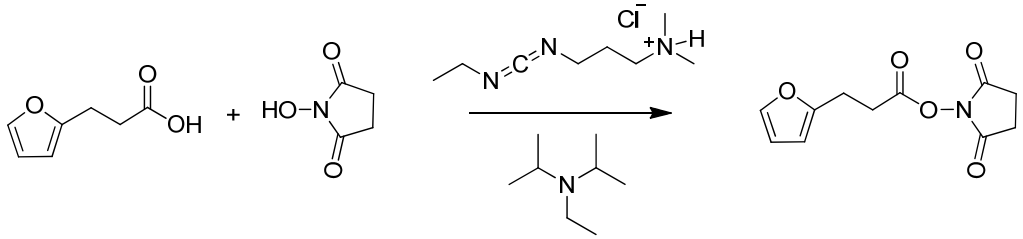
S1 Experimental

All reagents were purchased from Aldrich, Avocado, Fluka, Proligo, Applied Biosystems or Link Technologies and used without purification with the exception of DCM and di-isopropylethylamine (distilled over calcium hydride). Chemical transformations were carried out under an atmosphere of argon using oven-dried glassware. NAP gel-filtration columns were purchased from GE Healthcare and used according to the manufacturer's instructions. Column chromatography was carried out under pressure using Fisher Scientific DAVISIL 60A (35-70 microns) silica. Thin layer chromatography was performed using Merck Kieselgel 60 F_{24} (0.22mm thickness, aluminium backed). Compounds were visualized using ultraviolet light and staining with potassium permanganate solution. ^{1}H NMR spectrum was measured at 400 MHz, and ^{13}C NMR spectra were measured at 100 MHz on a Brüker DPX 400 spectrometer. Chemical shifts are given in ppm relative to tetramethylsilane, and *J* values are given in Hz, and are correct to within 0.5 Hz. All spectra are internally referenced to the appropriate residual undeuterated solvent signal. Multiplicities of ^{13}C signals were determined using DEPT spectral editing technique. Low-resolution mass spectra were recorded in acetonitrile (HPLC grade) using electrospray technique on a Fisons VG platform instrument. High-resolution mass spectrum was recorded in acetonitrile using electrospray technique on a Brüker APEX III FT-ICR mass spectrometer. MALDI-TOF MS were recorded using a ThermoBioAnalysis Dynamo MALDI-TOF mass spectrometer in positive ion mode using oligonucleotide dT standards.

3-(2-furnyl) propanoic acid NHS ester, 2.[1]



N-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC.HCl) (1.71 g, 8.9 mmol) and *N*-hydroxysuccinimide (1.03 g, 8.9 mmol) were added with stirring to a suspension of 3-(2-furyl) propanoic acid (0.50 g, 3.6 mmol) and *N,N'*-diisopropylethylamine (DIPEA) (1.55 mL, 8.9 mmol) in DCM (10 mL). The reaction mixture left to stir at room temperature for 4 h. The suspension was diluted with DCM (35 mL), and washed with dilute HCl (20 mL, 1 M), distilled water (20 mL) and brine

(20 mL). The organic layer was separated, dried over anhydrous $MgSO_4$, filtered and the solvent was removed in *vacuo*. The crude product was purified by column chromatography (DCM) to give the title compound as a white solid (0.65 g, 76%). δ_H (400 MHz, $CDCl_3$) 7.58 (1H, d, *J*=1.1, O-C*H*=CH), 6.42 (1H, dd, *J*=3.3, 1.1, O-CH=C*H*), 6.25 (1H, d, *J*= 3.3, O-C=CH-C*H*), 3.06 (4H, m, 2CH_2), 2.86 (4H, s, 2CH_2); δ_C (100 MHz, $CDCl_3$) 169.0, 167.7, 152.5 (*C*), 141.6, 110.3, 106.1 (*C*H), 29.7, 25.6, 23.1 (CH_2); *m/z* LRMS [ES^+, MeCN] found 260.2, $C_{11}H_{11}NNaO_5$ required 260.2; HRMS: found 260.0530, $C_{11}H_{11}NNaO_5$ required 260.0529.

Oligonucleotides Synthesis and HPLC purification

Standard DNA phosphoramidites, solid supports and additional reagents including the C7-aminoalkyl cpg and 5'-aminolink phosphoramidite monomer were purchased from Link Technologies or Applied Biosystems Ltd. All oligonucleotides were synthesized using standard 0.2 or 1.0 μmole phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by an automated trityl cation conductivity monitoring facility and in all cases were >98.0%. All β–cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time was 25 s for A,G,C and T monomers and 360 s for the 5'-aminolink phosphoramidite monomer. 3'-Aminoalkyl oligonucleotides were synthesized starting from C7-aminolink cpg. Cleavage of the oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 ^oC.

Oligonucleotides were purified by reversed-phase HPLC (Gilson 7.12), using an ABI Aquapore column (C8), 8 mm × 250 mm, pore size 300 Å. Protocol of the HPLC purification is as follows; Gradient (time in mins (% buffer B)): 0.0 (0), 3.0 (0), 15.0 (35), 16 (100), 17.0 (100), 17.5 (0), 19.9 (0), 20.0 (0). Elution buffers: (A) 0.1 M ammonium acetate, pH 7.0; (B) 0.1 M ammonium acetate with 35% acetonitrile, pH 7.0. Elution of oligonucleotides was monitored by ultraviolet absorption at 295 nm. After HPLC purification, oligonucleotides were desalted using NAP-10 Sephadex columns (GE Healthcare), aliquot into Eppendorf tubes and stored at -20 °C.

Maleimide Labelling of Oligonucleotides

Coupling of maleimide to the 5'- or 3'-end of amino oligonucleotides was carried out by incubating the oligonucleotide (0.2 μmol) in 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.5, 70 μL) and the 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (4 μmol) in DMSO (30 μL) for 1 h at room temperature. The crude oligonucleotide was desalted by gel filtration using NAP-10 columns according to the manufacture's instructions (GE Healthcare), and characterized by MALDI-TOF mass spectrometry. To avoid deactivation of the maleimide by reaction with water, the labelled oligonucleotides were freeze-dried immediately after gel filtration and stored at -20 ^oC.

Synthesis of Furan–Labelled Oligonucleotides

Coupling of furan NHS ester at either the 5'- or the 3'-end of amino oligonucleotide was carried out by mixing the oligonucleotide (0.2 μmol) in 0.5 M $Na_2CO_3/NaHCO_3$ buffer (pH 8.75, 80 μL) and 3-(2-furyl) propanoic acid NHS ester (5 μmol) in DMSO (80 μL) for 4 h at room temperature. The crude oligonucleotides were desalted using NAP-10 columns according to the manufacturer's instructions (GE Healthcare), purified by reversed-phase HPLC, desalted again (NAP-10) and characterized by MALDI-TOF mass spectrometry.

Synthesis of Dual-Furan–Labelled Oligonucleotide

The 5', 3'-amino oligonucleotide was dual labelled with 3-(2-furyl) propanoic acid NHS ester by incubating the oligonucleotide (0.2 μmol) in 0.5 M $Na_2CO_3/NaHCO_3$ buffer (pH 8.75, 80 μL) in the presence of the furan active ester (12 μmol) in DMSO (80 μL) for 4 h at room temperature. The crude oligonucleotide was desalted by gel filtration using NAP-10 columns, purified by reversed-phase HPLC, desalted again on a NAP-10 column and characterized by MALDI-TOF mass spectrometry.

Template-Mediated Diels-Alder Reaction to form Type I Diels-Alder Ligated Oligonucleotides

5'-Maleimide-labelled MO1 (9 nmol) was added to a solution of 3'-furan-labelled FO2 (9 nmol) and their complementary template TO3 (9 nmol) in 10 mM Na_2HPO_4/NaH_2PO_4, 0.2 M NaCl buffer (pH 6.2, 150 μL). The reaction mixture was

kept at room temperature and aliquots were taken at specific time points, desalted on a NAP-10 column (GE Healthcare), immediately freeze dried then analyzed by 20 % denaturing polyacrylamide gel electrophoresis. The resulted ligated oligonucleotide was characterized by MALDI-TOF mass spectrometry [($M+H^+$) calcd, 10352; found, 10353.2].

Type I Diels-Alder ligated LO4 was also prepared using 10 mM Na_2HPO_4/NaH_2PO_4, 0.2 M NaCl buffer (pH 7.0).

Template-Mediated Diels-Alder Reaction to form Type II Diels-Alder Ligated Oligonucleotides

3'-Maleimide-labelled MO5 (9 nmol) was added to a solution of 5'-furan-labelled FO6 (9 nmol) and their complementary template TO7 (9 nmol) in 10 mM Na_2HPO_4/NaH_2PO_4, 0.2 M NaCl buffer (pH 6.2, 150 μL). The reaction mixture was kept at room temperature and aliquots were taken at the desired time, desalted on a NAP-10 column (GE Healthcare), immediately freeze dried then analyzed by 20 % denaturing polyacrylamide gel electrophoresis. The resulted ligated oligonucleotide was characterized by MALDI-TOF mass spectrometry [($M+H^+$) calcd, 10352; found, 10353.9].

Type II Diels-Alder ligated LO8 was also prepared using 10 mM Na_2HPO_4/NaH_2PO_4, 0.2 M NaCl buffer (pH 7.0).

Template-Mediated Diels-Alder Ligation to form Type III Diels-Alder Ligated Oligonucleotides

A mixture of the 5'- and 3'-dual-furan-labelled FFO10 (6 nmol) with its complementary template TO11 (6 nmol) was annealed by heating at 80 °C for 5 min then allowed to cool to ambient temperature slowly (2 hr) in 10 mM Na_2HPO_4/NaH_2PO_4, 0.2 M NaCl buffer (pH 6.2, 300 μL). The solution was then added to the lyophilized 5'-maleimide-labelled MO1 (6 nmol) and 3'-maleimide-labelled MO5 (6 nmol). The reaction mixture was kept at room temperature and aliquots were taken at the desired time, desalted on a NAP-10 column (GE Healthcare), immediately freeze dried then analyzed by 20 % denaturing polyacrylamide gel electrophoresis.

For the non-annealing reaction conditions, the above procedure was repeated without heating, i.e. the mixture of the 5'- and 3'-dual-furan-labelled FFO10 and its

complementary template TO11 were set aside at room temperature in buffer for 2 hr instead of heating/cooling.

After running the gel, the desired bands were excised, crushed, and incubated in 2 mL of sterile water at 37 °C for 18 hr. The tubes were then vortexed, centrifuged then the supernatant was desalted using NAP-25 column (GE Healthcare).

The resulted ligated oligonucleotide was characterized by electrospray mass spectrometry [$(M+H^+)$ calcd, 15946; found, 15946].

Non-Templated Diels-Alder Ligation to form Type I and II Diels-Alder Ligated Oligonucleotides

Synthesis of Type I and II Diels-Alder ligated ODNs were repeated as described above in 10 mM Na_2HPO_4/NaH_2PO_4, 0.2 M NaCl buffer (pH 6.2) without their complement splint. The reaction mixture was kept at room temperature for the desired time then analysed by 20% polyacrylamide gel electrophoresis.

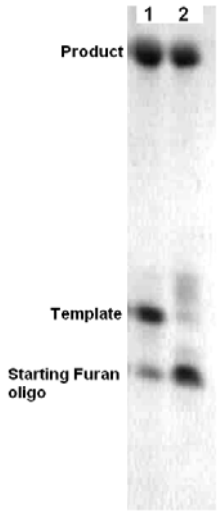


Figure S1: 20 % polyacrylamide/7 M urea gel at constant power of 20 W using 0.09 M Tris-borate-EDTA buffer (pH 8.0). Type II Diels-Alder reaction. Lane 1, templated, 1 hr. Lane 2 , non templated , 20 hr.

S2: Ultraviolet melting studies

UV melting studies were performed using 1 μM oligonucleotide concentration in 10 mM Na_2HPO_4/NaH_2PO_4, 0.2 M NaCl buffer (pH 7) on a Varian Cary 400 scan UV-visible spectrophotometer. Hellma SUPRASIL synthetic quartz 10 mm path length cuvettes were used and T_m values were calculated using Cary Win UV thermal application software.

ODN ID	Sequence of ODNs (5`- 3`), and their complem entary template (3` - 5`)	T_m (°C)
LO8 + TO7	CTTTCCTCCACTGTTGC*M+F*GCGATCAATCAGACG GTGACAACG---CGCTAGTTA	57.91
LO4 + TO7	CTTTCCTCCACTGTTGC*F+M*GCGATCAATCAGACG GTGACAACG---CGCTAGTTA	58.96
CO9 + TO7	CTTTCCTCCACTGTTGCGCGATCAATCAGACG GTGACAACGCGCTAGTTA	67.10

Table S1 UV melting of type I and type II Diels-Alder ligated oligonucleotides

S3: Mass Spectrometric Oligonucleotide Analysis Protocol

Mass Spectra of oligonucleotides were recorded by negative mode electrospray on a Fisons VG platform mass spectrometer in acetonitrile/water (HPLC grade), on a Bruker micrOTOF^{TM} II focus ESI-TOF MS instrument or by MALDI-TOF using a ThermoBioAnalysis Dynamo MALDI-TOF mass spectrometer in positive ion mode with oligo-dT standards.[2]

Entry	Sequences (5' – 3')	Calc. mass	Found mass
MO1	***M***GCGATCAATCAGACG	4958	4959
FO2	CTTTCCTCCACTGTTGC***F***	5394	5394
TO3	TCTGATTGATCGCGCAACAGTGGAGG	nd	nd
LO4	CTTTCCTCCACTGTTGC***F***+***M***GCGATCAATCAGACG	10352	10353
MO5	CTTTCCTCCACTGTTGC***M***	5465	5467
FO6	***F***GCGATCAATCAGACG	4887	4887
TO7	ATTGATCGCGCAACAGTG	nd	nd
LO8	CTTTCCTCCACTGTTGC***M***+***F***GCGATCAATCAGACG	10352	10353
CO9	CTTTCCTCCACTGTTGCGCGATCAATCAGACG	nd	nd
FFO10	***F***GAACTCTACGACGTAG***F***	5523	5523
TO11	TTTTTTTTCGTCTGATTGATCGCCTACGTCGTAGAGTTCGCAACAGTGGAGGAAAGT TTTTTTT	nd	nd
LO12	CTTTCCTCCACTGTTGC***M***+ ***F***GAACTCTACGACGTAG***F***+***M***GCGATCAATCAGACG	15946	15946

Table S2: List of oligonucleotides involved in Diels-Alder reactions and molecular ions.

M = maleimide, ***F*** = furan, + = Diels-Alder linkage. MO = maleimide ODN, FO = furan ODN, TO = template ODN, CO = complementary ODN. nd = not done for unmodified oligonucleotides.

S4: Buffer Stability Studies on Maleimide-Labelled Oligonucleotides

5'-Maleimide-labelled MO1 and 3'-maleimide-labelled MO5 were separately dissolved in 10 mM Na_2HPO_4/NaH_2PO_4, 0.2 M NaCl buffer (pH 6.2 or pH 7). The reaction mixture were left at room temperature for 1 h, and then desalted on a NAP-10 column (GE Healthcare), and characterized by MALDI-TOF mass spectrometry. MO1 and MO5 were also dissolved in 1X Tris-borate-EDTA buffer and incubated at 35 °C for 2 h (conditions for the polyacrylamide gel) before they were desalted on a NAP-10 column (GE Healthcare), analyzed by capillary electrophoresis, and characterized by ESI or MALDI-TOF mass spectrometry. The mass spectra showed only the correct mass for the 5'- and 3'-maleimide in phosphate buffer (pH 6.2 and 7). In the case of the 1X TBE buffer it showed the correct mass in addition to $M^+ +18$ (hydrolysis) as shown below.

A) B)

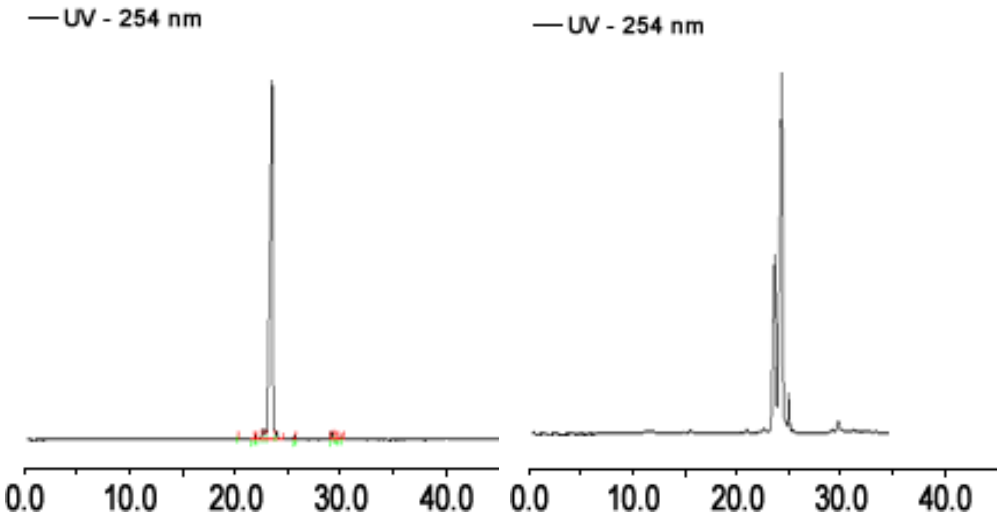
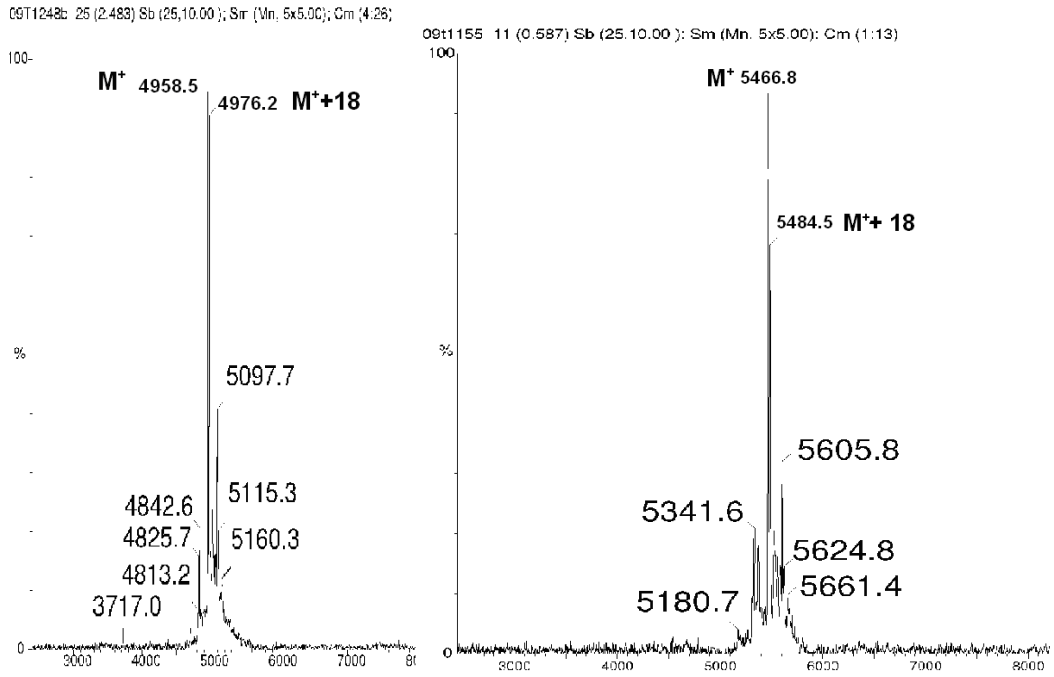
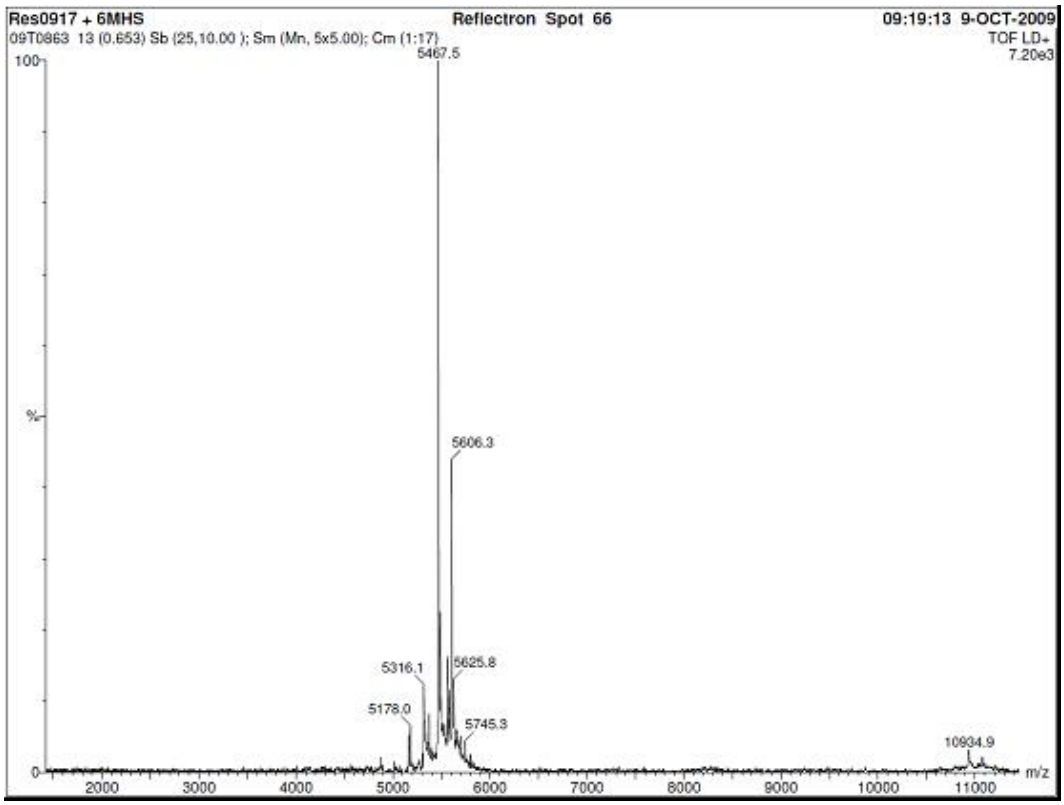


Figure S1: Capillary electrophoresis (CE), of 5'-maleimide-labelled oligonucleotide MO1 before (A) and after (B) incubation in 1X TBE buffer at 35 °C, 2 h respectively. X-axis is time (min), Y-axis is UV absorbance at 254 nm. Oligonucleotides (0.4 OD/100 µL) were injected into a ssDNA 100-R Gel using Tris-Borate-7 M Urea (Kit No 477480) on a Beckman Coulter P/ACE^{TM} MDQ Capillary Electrophoresis System using 32 Karat software. UV-254 nm, inject-voltage 10.0 kv and separate-voltage 9.0 kv (45.0 min duration).

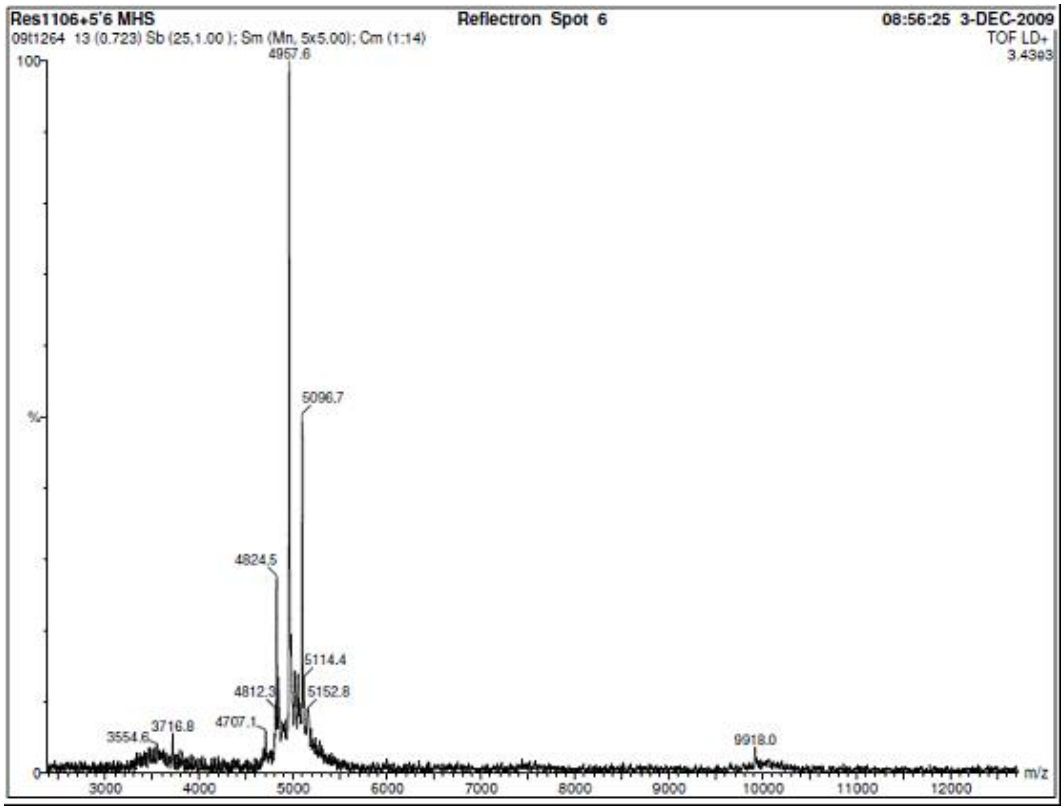


MALDI-TOF mass spectra of thermal stability study showing [M^+] peak, and [M^++18] peak which is indicative of hydrolysis of maleimide-labelled ODN in 1X TBE buffer (left) 5'-maleimide-labelled ODN-5 in 1X TBE buffer at 35 °C, 2 h. (right) 3'-maleimide-labelled ODN-2 in 1X TBE buffer at 35 °C, 2 h

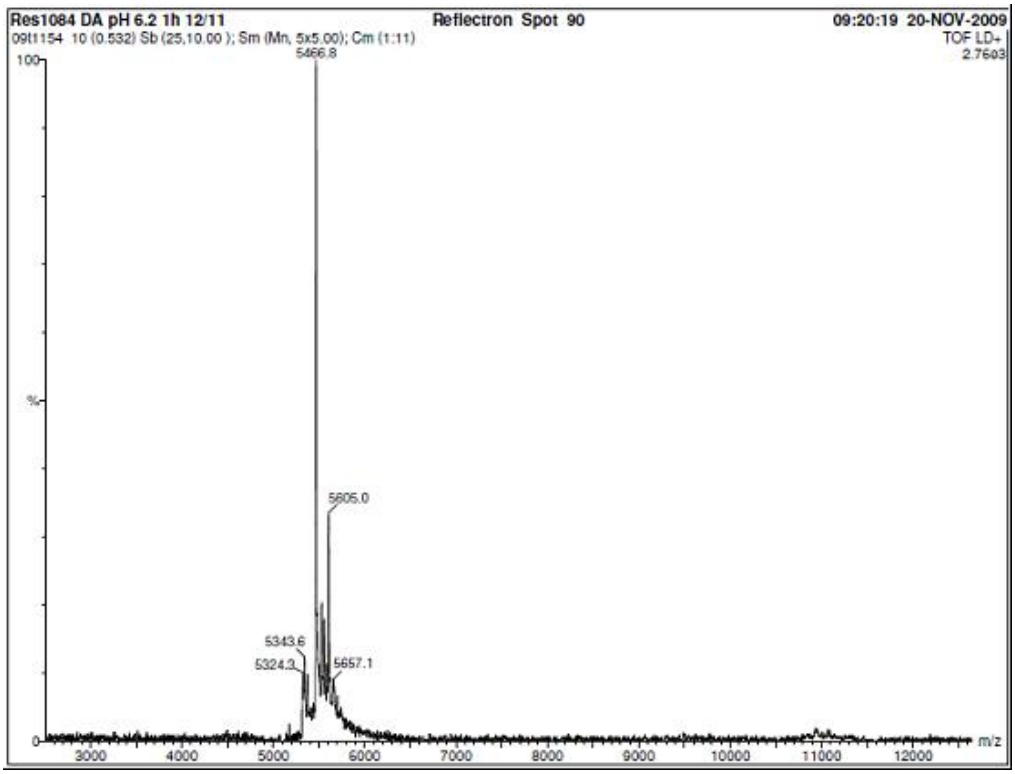
MALDI-TOF mass spectrum of 3'-Maleimide-labelled MO5



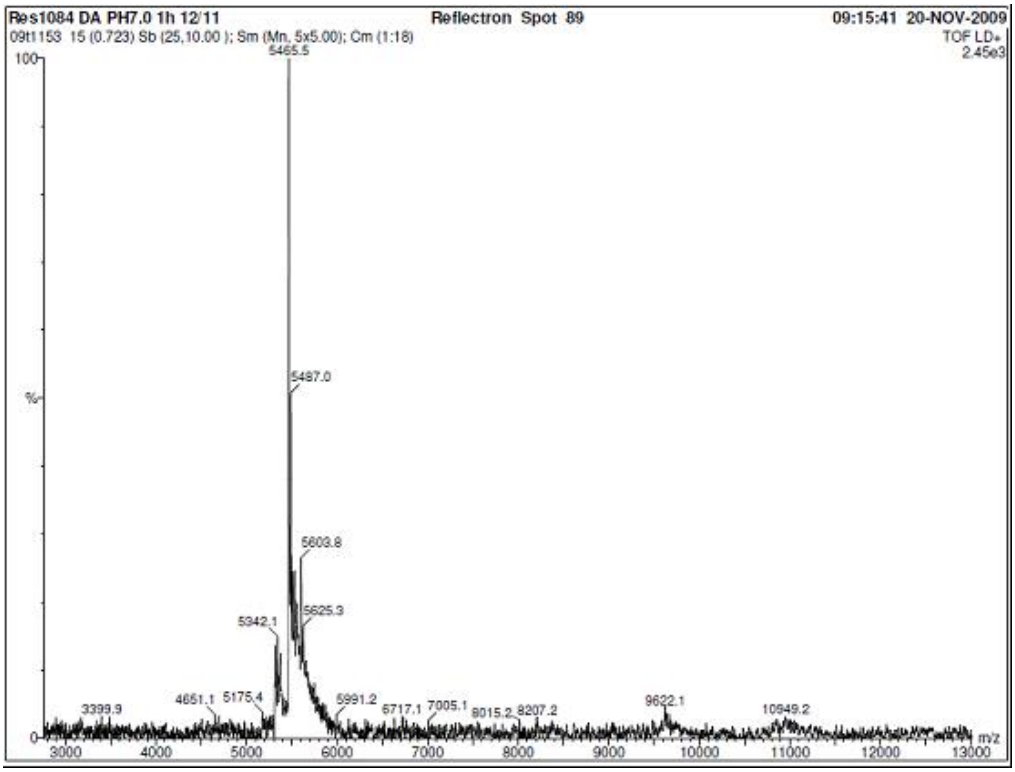
MALDI-TOF mass spectrum of 5'-Maleimide-labelled MO1



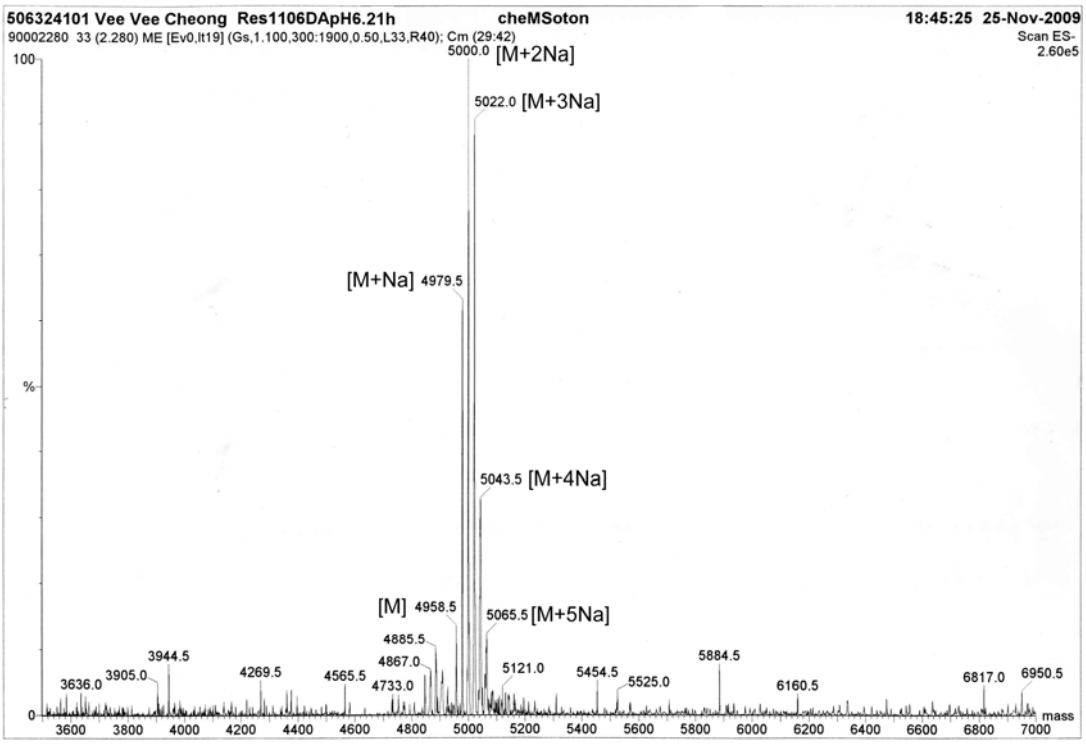
MALDI-TOF mass spectrum of 3'-Maleimide-labelled MO5 in 10 mM NaH_2PO_4/Na_2HPO_4, 0.2 M NaCl buffer, pH 7.0, 1 h



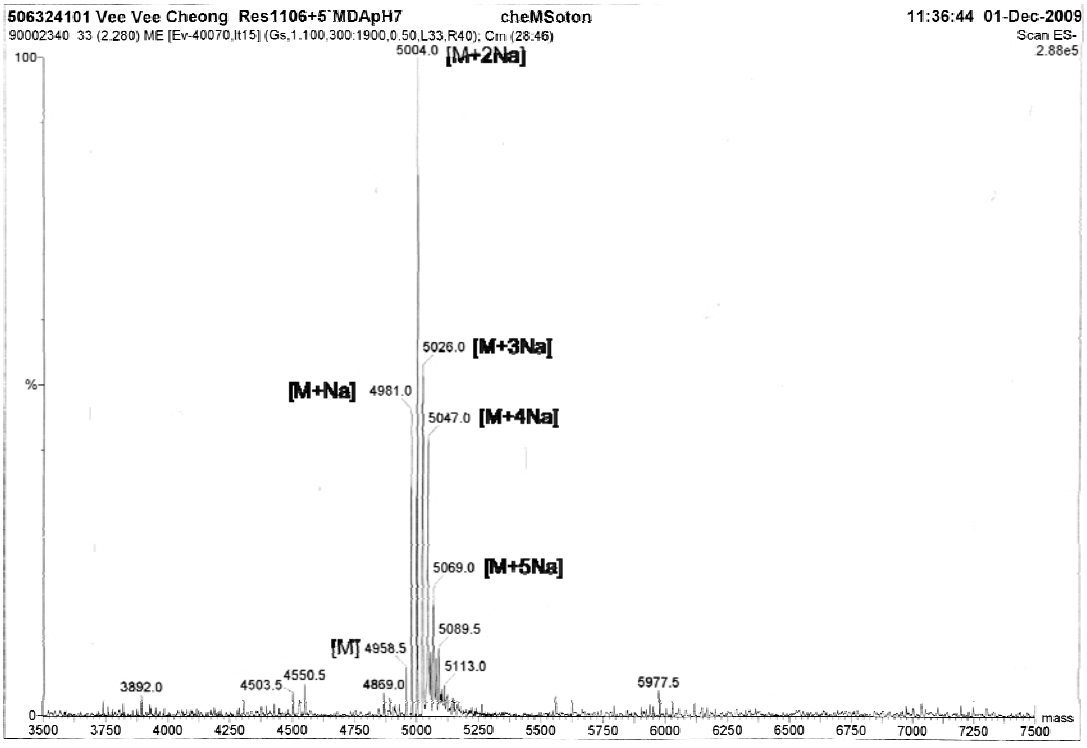
MALDI-TOF mass spectrum of 3-'Maleimide-labelled MO5 in 10 mM NaH_2PO_4/Na_2HPO_4, 0.2 M NaCl buffer, pH 6.2, 1 h



Electrospray mass spectrum of 5'-Maleimide-labelled MO1 in 10 mM NaH_2PO_4/Na_2HPO_4, 0.2 M NaCl buffer, pH 6.2, 1 h



Electrospray mass spectrum of 5'-Maleimide-labelled MO1 in 10 mM NaH_2PO_4/Na_2HPO_4, 0.2 M NaCl buffer, pH 7.0, 1 h



S5: References

1. L. S. Huang and R. J. Kerns, *Bioorg. Med. Chem.*, 2006, **14**, 2300-2313.
2. G. J. Langley, J. M. Herniman, N. L. Davies and T. Brown, *Rapid Commun. Mass Spectrom.*, 1999, **13**, 1717-1723.