Electronic Supplementary Material (ESI) for Chemical Communications

Synthesis of squaraine dyes under mild conditions: Applications for labelling and sensing of biomolecules.

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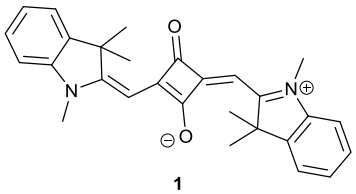
Supporting Information

1. General Information

Unless otherwise noted, all solvents and reagents were obtained from commercial sources and used without further purification. ^{1}H NMR spectra were recorded on a Bruker AVANCE 400 III HD spectrometer. Chemical shifts are reported on the δ scale in ppm using the residual peak solvent as the internal standard. Coupling constants (*J*) are reported in Hz. Microwave-assisted reactions on solid phase were performed on a Biotage initiator + SPWave Synthesiser. PNA and peptide solid-phase syntheses were carried out on ChemMatrix resins with rink amide linker. Fluorescence emission spectra were recorded on a Fluorolog instrument (Horiba). Purifications by flash chromatography were performed using Merck silica gel 60 (230-400 mesh). MALDI-TOF spectra were recorded on a MALDI micro MX instrument using sinapinic acid as a matrix. Gilson HPLC System was used for analysis equipped with 321 pump, 155 detector and 234 Autoinjector and ThermoScientific Hypersil GOLD C8 column (100x4.6mm, and particle size 5μm). The gradient which was used for the dye reactions analysis is of 5 to 95% MeOH in water (0.1%TFA) in 7 min, kept at 95% MeOH for 6 min then dropped back to 5% for 4 min. The gradient used for the purification of the PNA and peptides is of 5 to 95% ACN in water (0.1%TFA) in 10 min, kept at 95%ACN for 5 min then dropped to 5% for 4 min.

2. Synthesis of symmetrical squaraine dye 1 in solution.



1

To a suspension of squaric acid (30 mg, 0.263 μmol, 1 equiv.) in DCM (or any solvent described in Table 1, total volume 0.5 to 1mL) were successively added 2 equiv. of carbodiimide (DIC, EDCI or DCC) and 2 equiv. of 1,3,3-trimethyl-2-methyleneindoline (84 μL, 0.526 μmol). The reaction mixture was then stirred overnight in a sealed tube protected from light. Selected reactions (entries d-f) were purified over silica gel column chromatography (eluent: DCM to DCM: EtOH 95:5) to afford a green blue powder (yields 9-10%, see Table 1).[1] ^{1}H NMR ($CDCl_3$) δ= 7.38-7.32 (m, 4H), 7.18 (t, J=7.2 Hz, 2H) 7.03 (d, J=7.0 Hz, 2H), 5.99 (s, 2H), 3.61 (s, 6H), 1.79 (s, 12H).

All reactions described in table 1 (entries a-l) were analysed by HPLC. Reaction mixtures were diluted to 5 mL with DCM and an aliquot of 50 μL was taken and diluted in 450 μL of MeOH. Samples were then analysed by HPLC using the gradient described in the general information. An analytically pure sample of squaraine dye **1** was prepared according to Matsui *et al.*[1] and used as an internal standard to calculate reaction yields. Seven titrated solutions of dye **1** in MeOH were analysed by HPLC and the resulting calibration curve was used to estimate the yields of the different reactions summarised in table 1. A good agreement was obtained between the calculated yields and the purified yields measured for three of the reactions, thus confirming the reliability of our method.

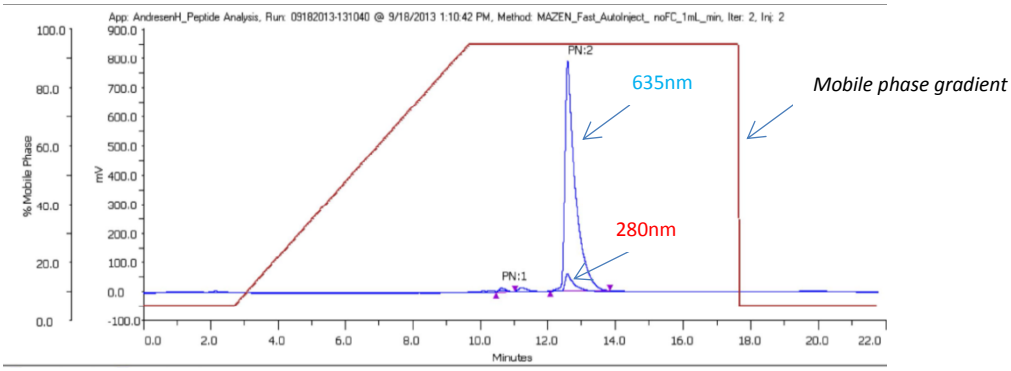


Figure S1. HPLC trace of a solution of analytically pure squaraine dye **1** used for the preparation of a calibration curve (Channels 1 and 2 were recorded at 635nm and 280nm, respectively).

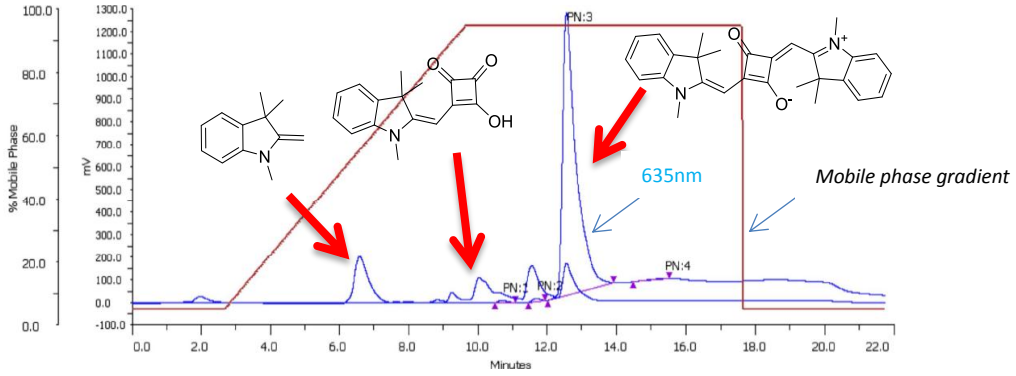
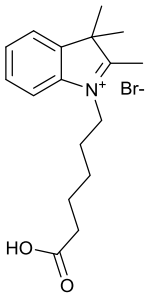


Figure S2. Example of HPLC trace obtained for one of the reaction mixtures (Table 1, entry J). Channels 1 and 2 were recorded at 635nm and 280nm, respectively. Peaks corresponding to the 1,3,3-trimethyl-2-methyleneindoline (starting material), the semi-squaraine dye and the symmetrical squaraine dye **1** have been indicated with red arrows.

3. Synthesis of semi-squaraine dyes

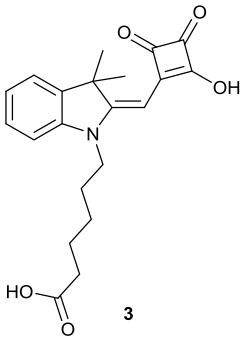


2

Compound **2** was synthesis by adapting a protocol previously reported by Kim *et al.*[2] Briefly, a mixture of 2,3,3-trimethyl-3H-indole (0.5g, 3.14mmol) and 6-bromohexanoic acid (0.73g, 3.77mmol, 1.2eq.) was irradiated for 1h at 120°C. The solution was cooled to room temperature, and the residue obtained was sonicated with acetone

to yield a pink/white powder after filtration (0.75 g, 67%).[2] ^{1}HNMR (DMSO-d_6): δ= 7.93–7.97 (1H, m), 7.79–7.87 (1H, m), 7.56–7.64 (2H, m), 4.43 (2H, t, J = 7.7 Hz), 2.82 (3H, s), 2.19 (2H, t, J = 7.2 Hz), 1.81 (2H, m), 1.52 (2H, m), 1.50 (6H, s), 1.35 (2H, m).

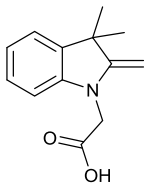
Compound **3**:



3

Compound **2** (0.15g, 0.73mmol) was refluxed with diethyl squarate (0.12g, 0.73mmol) in a mixture of EtOH (3ml) and Et_3N (0.30ml, 2.19mmol) for 3h. Solvents were evaporated and the crude residue was refluxed in a solution of 10% $NaOH_{aq}$ (0.5ml) and EtOH (3.5ml) for 10min, followed by neutralisation with a 2N solution of HCl. Solvents were evaporated and the residue was purified over silica gel (EtOAc to EtOAc:MeOH 9:1) to yield the desired semi-squaraine dyes as a yellow solid (0.10g, 72%).[3] ^{1}HNMR (400 MHz, MeOD) δ=7.32 (dd, J = 7.4, 1.2Hz, 1H), 7.26 (dd, J=7.7, 1.2Hz, 1H), 7.08 – 7.01 (m, 2H), 5.65 (s, 1H), 3.93 (t, J = 7.2Hz, 2H), 2.34 – 2.27 (t, J = 7.3Hz, 2H), 1.81 – 1.73 (m, 2H), 1.63(s, 6H), 1.60 – 1.47 (m, 4H).

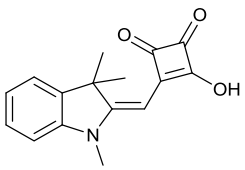
Compound **4**:



4

Compound **4** was prepared as previously reported by us.[4] ^{1}HNMR (DMSO-d_6) δ = 9.46 (d, J= 8.3 Hz, 1H), 7.88-7.82 (m, 1H), 7.37 (d, J= 7.3 Hz, 1H), 7.19 (td, J= 7.8, 1.1 Hz, 1H), 6.98-6.93 (m, 2H), 5.81 (dd, J= 14.2, 8.3 Hz, 1H), 5.62 (d, J= 12.6 Hz, 1H), 4.70 (s, 2H), 4.14 (q, J= 7.3 Hz, 2H), 1.59 (s, 6H), 1.19 (t, J= 7.3 Hz, 3H) ppm.

Compound **5**:



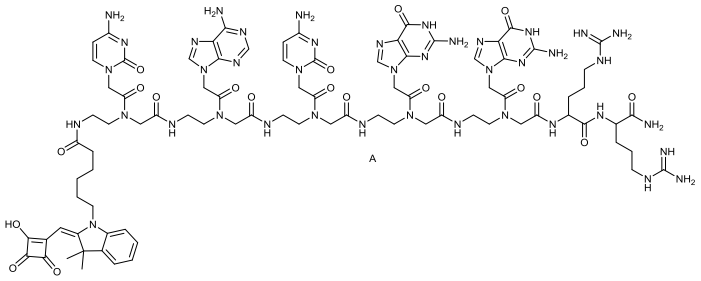
Compound **5** was prepared following a procedure previously reported in the literature.[5] ^{1}HNMR ($CDCl_3$) δ= 7.38 (t, J= 7.3 Hz, 1H), 7.25 (t, J= 7.3 Hz, 1H), 7.11 (t, J= 7.3 Hz, 1H), 7.01 (d, J= 7.3 Hz, 1H), 5.45 (s, 1H), 3.34 (s, 3H), 1.55 (s, 6H).

4. Peptide (and PNA) synthesis on solid-phase

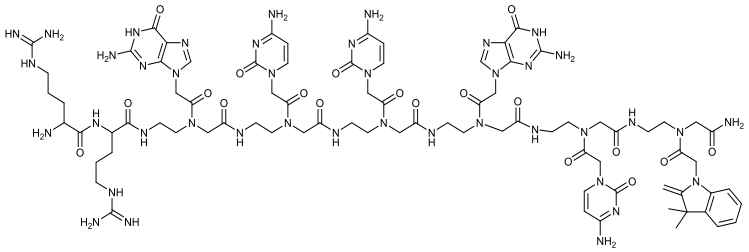
Coupling of Fmoc-protected amino acids and PNA monomers was achieved by adding a mixture of 5 equiv. of monomer, 5 equiv. of oxyma and 5 equiv. of DIC in DMF onto the resin and heating under vortex at 75ºC for 20 min. Coupling of **2**, **3** and **4** on resin was achieved by using HATU/DIPEA (5eq:5eq:10eq) in DMF at room temperature over 45min. Fmoc deprotection was done by mixing the resins with a solution of 20% Piperidine in DMF and vortexing for 10min at room temperature. For the functionalisation of PNAs at their C-terminus, Fmoc-PNA(Aloc)-OH was used to couple compound **4** by selective deprotection of the Alloc group as previously described by us.[6] The resins were washed in two cycles of DMF, MeOH and DCM after each reaction. Cleavage of the PNA sequences was done by addition of a solution of TFA:TIS:H_2O (95:2.5:2.5) and shaking the resins for 2h at room temperature, followed by precipitation of the crude product in Et_2O. Crudes were used without further purification.

The structures of the PNAs and Peptides synthesised are given below:

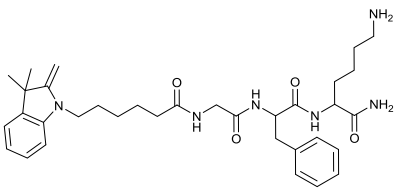
PNA1: Semi-squaraine-C6-cacgg-RR-NH_2



PNA2: NH_2-RR-gccgc-PNA(C_2-indole)-NH_2



Peptide: Indole-C6-GFK-NH_2:



5. Synthesis of squaraine dyes on solid-phase

Peptide labelling: To 50 mg of Indole-C6-GFK-resin (estimated loading ~0.5 mmol/g) was added a solution of **5** (1 to 5 equiv.) and DIC (1 to 5 equiv.) in DCM (1mL). The suspension was then shaken at RT for 6h then washed and cleaved following the general procedure. The crude products were then analysed by HPLC and MALDI-TOF (Figures S3 and S4).

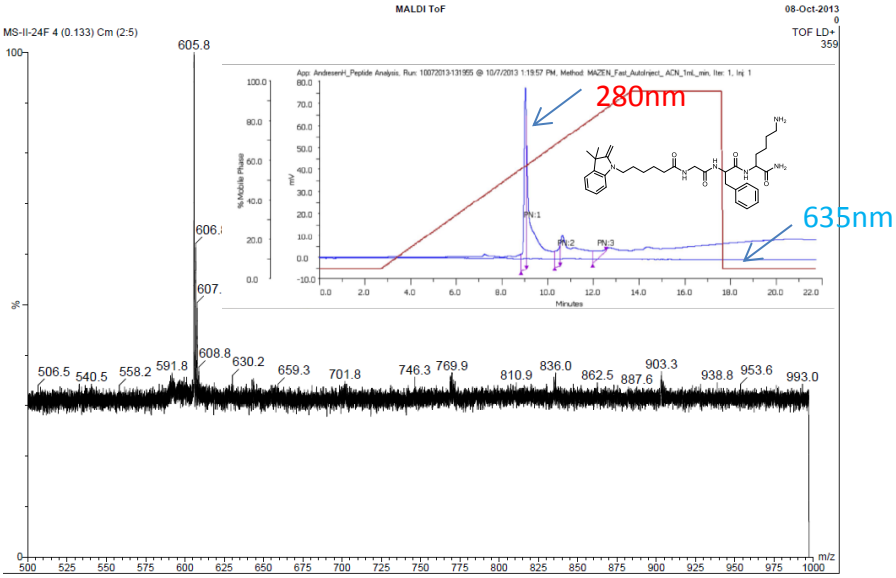


Figure S3. MALDI spectrum of the crude unlabelled peptide showing the starting peptide-Indole conjugate and (Insert) HPLC trace corresponding to the same crude mixture (λ = 635nm, 280nm). Labelling was achieved using 5equiv. of activated **5**.

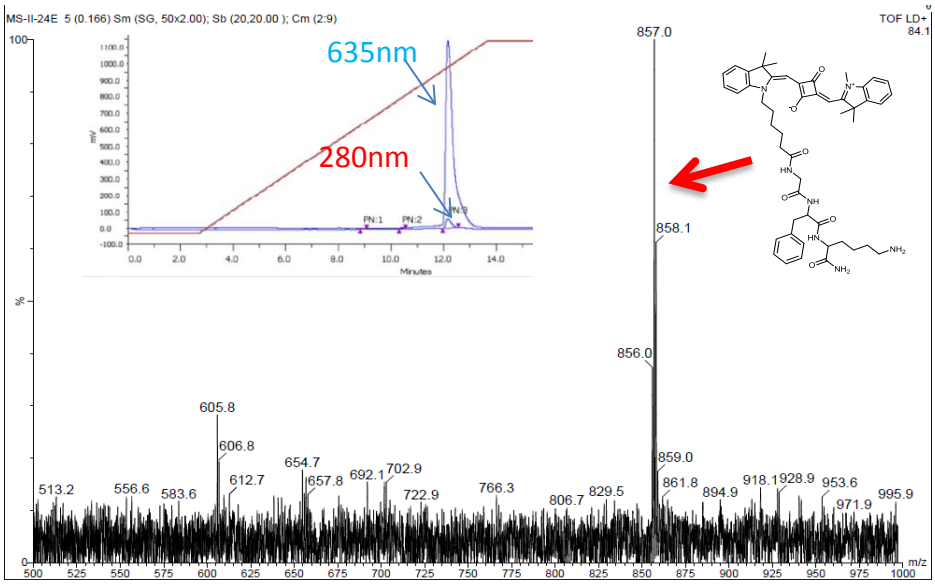


Figure S4. MALDI spectrum of a crude mixture of labelled peptide showing near-total conversion of the starting Peptide-Indole conjugate and (Insert) HPLC trace corresponding to the same crude mixture. Labelling was achieved using 5equiv. of activated **5**.

PNA labelling: A suspension of **5** (40mg, 3equiv.) in DCM (1mL) was mixed with DIC (23 μl) and added to the PNA-indole resin (100mg, 0.5mmol/g) and the suspension was shaken overnight at RT. The resin was then washed thoroughly and cleaved following the general procedure (Figures S5 and S6)

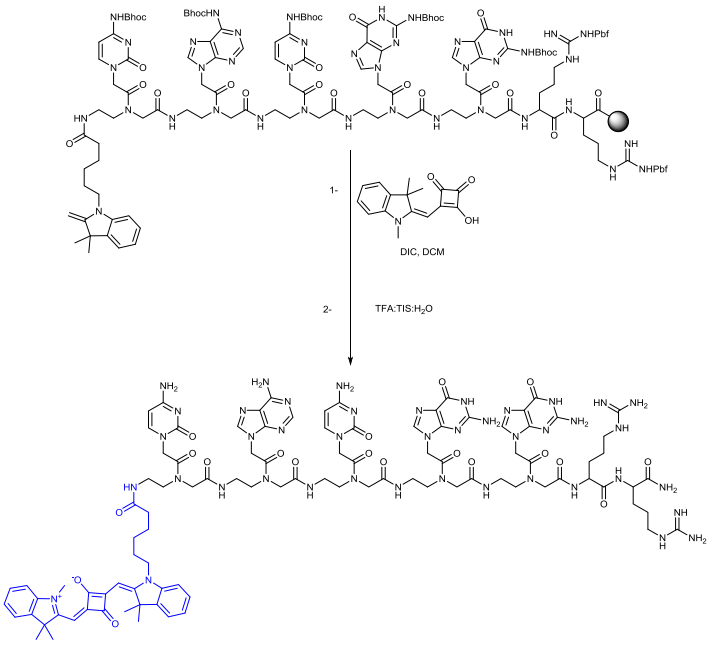


Figure S5. Solid-phase synthesis of a PNA-squaraine dye conjugate. (top) followed by MALDI-TOF and HPLC traces of the starting PNA-indole conjugate showing the unllabelled product (bottom).

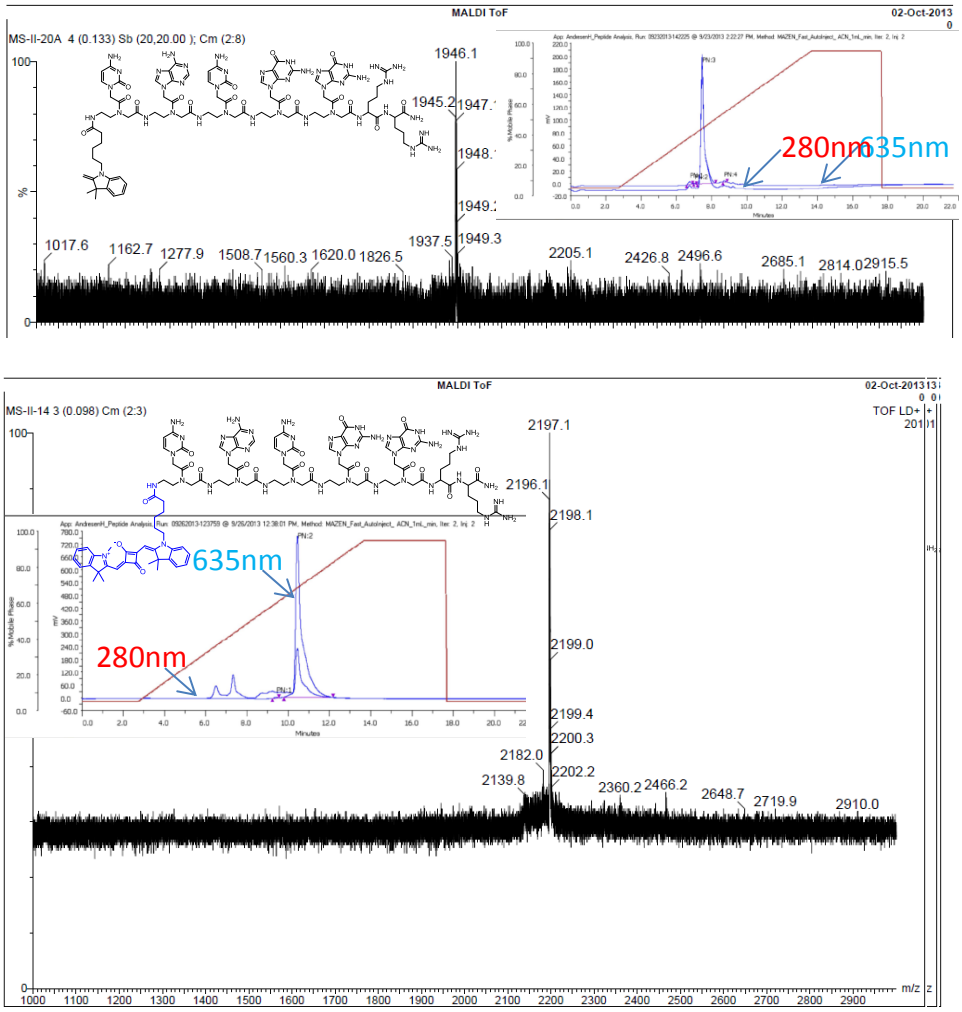


Figure S6. MALDI-TOF and HPLC traces of the starting PNA-indole conjugate showing the unlabelled product (top). MALDI-TOF and HPLC trace of the crude reaction mixture showing up to 70% conversion into the desired fluorescently labelled product (bottom).

6. DNA-templated synthesis of squaraine dyes.

DNA sequence was purchased from Sigma (5'-CCGTGTAAGCGGC-3')

PNA1: Semi-squaraine-C6-cacgg-RR-NH_2

PNA2: NH_2-RR-gccgc-PNA(C_2-indole)-NH_2

50µl of DNA (100 µM) were mixed with 9.5µl of potassium phosphate buffer (0.1M, pH= 7.4), 5µl of PNA 1(1mM), 0.5µl PNA 2 (10mM) and 5µl EDC (10mM). The solution was incubated at 40ºC, then diluted by adding 330µl of water and fluorescence emission spectra were recorded from 630 to 750nm (λ_{exc} = 610nm, slits = 5nm). Non-templated controls were prepared in the same manner by replacing the DNA solution with water.

7. Characterisation of the PNA1-squaraine dye-PNA2 product

The product of the OTR was characterized by MALDI, by adapting a procedure from Bowler *et al.*[7]

OTR: 5µl of DNA (1mM) were mixed with 10 µl of Q sepharose beads suspended in PBS (0.1M, pH= 7.4) and 5µl of PNA 2 (1mM). 5µl PNA 1 (1mM) and 5µl EDC (10mM) were premixed separately and then added to the mix. The solution was incubated at 40ºC for 3h in a thermomixer (300 RPM).

Control: A non-templated control was prepared in the same manner as above by replacing the DNA solution with water.

The reaction tubes were centrifuged and the supernatant removed, then the Q Sepharose beads were washed with 3% acetonitrile in water (3 x 200 µL). Sinapinic acid matrix (10 µL) was added to the resin, and the mixture was spotted (1 µL in duplicate) onto a stainless steel MALDI plate.

While the PNA1-squaraine-PNA2 conjugate could be clearly identified by MALDI in the DNA-templated reaction mixture (Figure S7), no trace of the product could be detected in the control experiment lacking the DNA template.

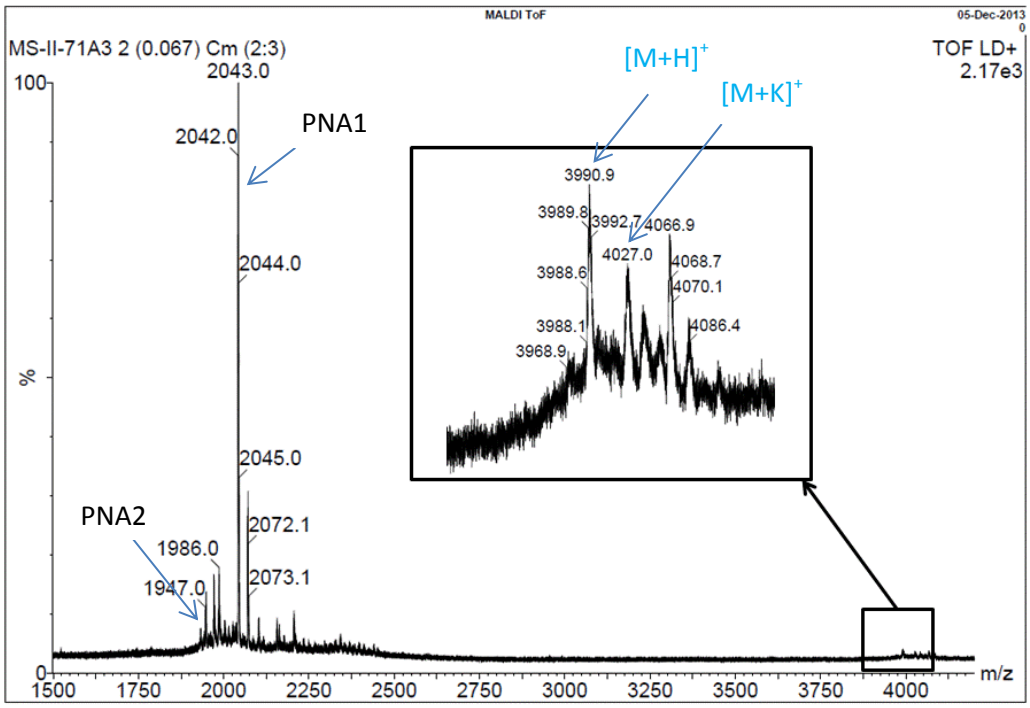


Figure S7. Product of a typical DNA-templated reaction analysed by MALDI-TOF.

8. References

[1] M. Matsui, M. Fukushima, Y. Kubota, K. Funabiki, M. Shiro, *Tetrahedron*, 2012, **68**, 1931.

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[7] F. R. Bowler, J. J. Diaz-Mochon, M. D. Swift, M. Bradley, *Angew. Chem. Int. Ed.*, 2010, **49**, 1809.