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Peptide nucleic acid probes with charged photocleavable mass markers

Towards PNA-based MALDI-TOF MS genetic analysis

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Halogen-labelled peptide organic acid (HPOA) monomers have been synthesised and incorporated into sequence-specific peptide nucleic acid (PNA) probes. Three different types of probe have been prepared; the unmodified PNA probe, the PNA probe with a mass marker, and the PNA probe with photocleavable mass marker. All three types of probe have been used in model studies to develop a mass spectrometry-based hybridisation assay for detection of point mutations in DNA.

Introduction

Peptide Nucleic Acid (PNA) is an analogue of DNA in which the sugar-phosphate backbone is replaced with a synthetic peptide composed of repeating *N*-(2-aminoethyl) glycine units.[1] Individual nucleobases are connected to each unit via methylene carboxamide linkers and the resulting PNA oligomers hybridise to their complementary nucleic acid, obeying the Watson-Crick base-pairing rules.[2] PNA hybridization probes have been used in combination with MALDI-TOF mass spectrometry for the detection of sequence polymorphisms in PCR-amplified DNA.[3-6] Photocleavable linkages, with or without mass tags, have also been used in conjunction with DNA probes for genetic analysis using MALDI techniques.[7-10] PNA shows excellent discrimination between wild-type and mutant DNA targets i.e., under appropriate conditions, it binds selectively to its fully complementary DNA target in the presence of an equivalent PNA sequence containing a single mismatched base. Additionally, PNA is quite stable in a range of environments and does not fragment during MALDI analysis.[11] In a previous study, to take advantage of these favourable properties of PNA, Griffin et al. attached a variable number of 8-amino-3,6-dioxaoctanoic acid units to PNA to enhance the mass separation between different probes for determination of tyrosine exon-4 polymorphisms.[12]

Here we describe the synthesis of PNA probes modified with halogen-labelled photocleavable mass markers, and the application of these probes in a model hybridization assay for the detection of point mutations by mass spectrometry (**Fig. 1**). The halogen tags, which are incorporated into the PNA during solid-phase PNA synthesis, provide characteristic isotope patterns that enable facile identification of the target DNA. Probes with and without photolabile linkers have been compared to explore the advantages of liberating the small molecule mass tags from the parent PNAs during the analysis. The effects of attaching positively charged sensitisers to the mass tags have also been investigated.

Results and Discussion

The synthesis of HPOA monomer. Previously, peptide organic acid (POA) monomers have been prepared for use as DNA intercalators by coupling the appropriate carboxylic acids to the Fmoc-protected PNA backbone. When incorporated into a PNA sequence, the aromatic rings are capable of stacking but are unable to hydrogen-bond in the PNA/DNA duplex.[13,14] In this study, a series of POA monomers were required as mass tags, so a simple, convenient synthetic procedure was required. Fukuyama and co-workers have described an efficient synthesis of secondary amines from nitrobenzenesulfonamides[15] and this method has been utilized (**Scheme 1**) to prepare the desired POA backbone **6**.[16,17] Glycine *tert*-butyl ester **3** was converted to sulfonamide **4**, which in turn was coupled to alcohol **2**,[18,19] under standard Mitsunobu conditions, to give the *N,N*-disubstituted 2,4-dinitrobenzene sulfonamide **5** in 82% yield. The deprotection of **5** to give backbone **6** as the hydrochloride salt was achieved in high yield, by treatment with excess mercaptoacetic acid in the presence of DIPEA. Using this method, the Fmoc-protected backbone **6** was prepared in 58% overall yield. This synthetic route requires minimal chromatographic separation, and despite being longer than the alternative procedure,[16,20] it is straightforward and reliable. With the required backbone in hand, the HPOA monomers were assembled. Reaction of the

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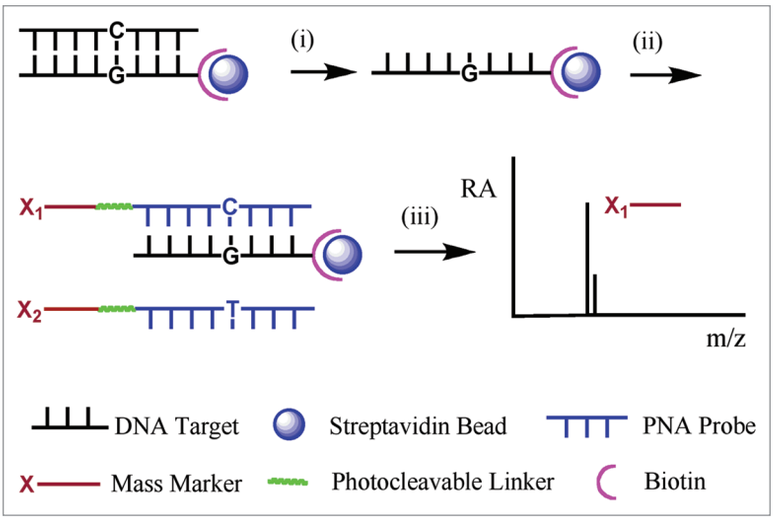
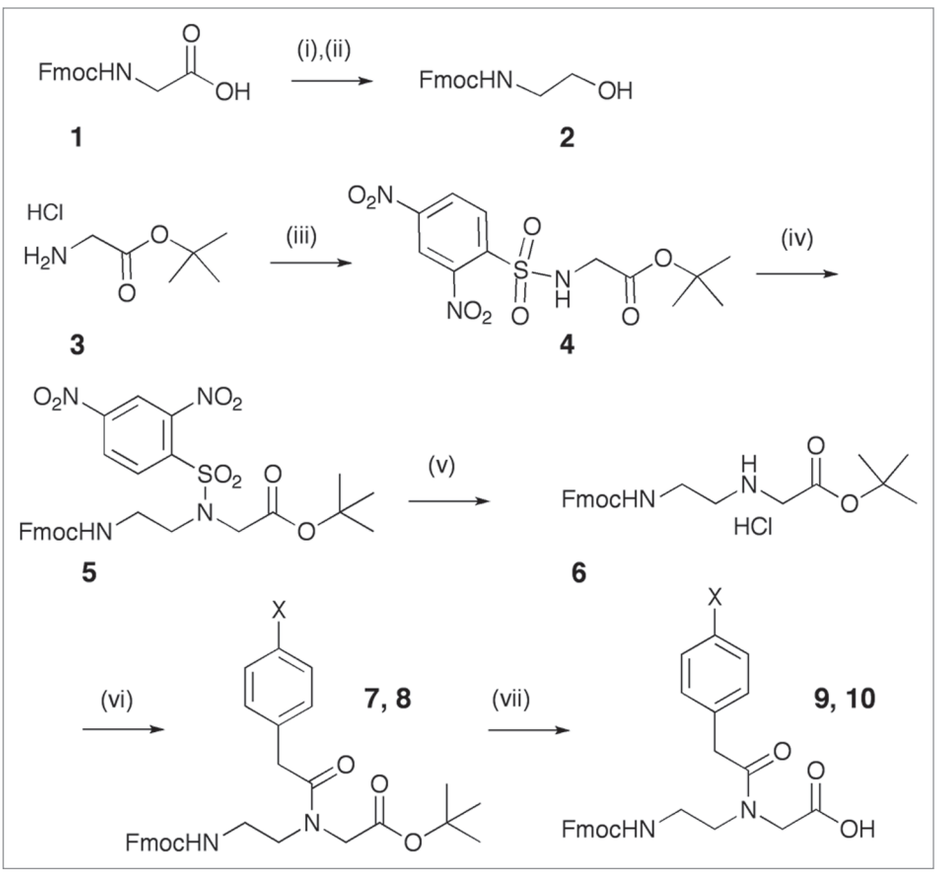


Figure 1. PNA hybridization assay. (i) The double-stranded biotinylated PCR product is immobilised on a streptavidin bead, denatured and the unbound strand is washed away. (ii) PNA probes complementary to the two possible alleles are added and the non-complementary probes are removed by heating and washing. (iii) The PNA/DNA/bead complex is analysed by MALDI-TOF MS. The genotype is determined by the quasi-molecular ion of the mass marker cleaved from the PNA probe.

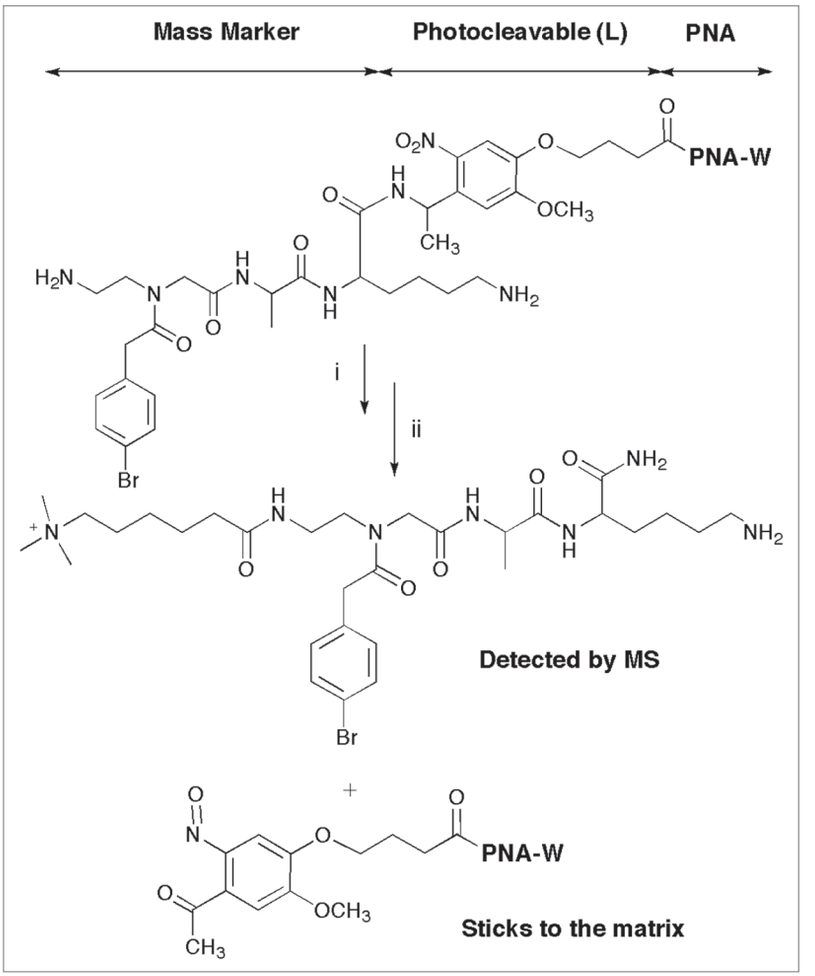
appropriate commercially-available halide-substituted phenylacetic acid derivatives with backbone **6**, in the presence of EDC, afforded the protected monomers **7** and **8**. Hydrolysis of the ester with trifluoroacetic acid gave the desired monomers **9** and **10**. The mass spectra showed the expected halogen isotope patterns; 1:1 (^{79}Br:^{81}Br) and 3:1 (^{35}Cl:^{37}Cl) for the bromo-**9** and chloro-monomers **10** respectively.

PNA probe design and synthesis. As proof of principle, 14-mer PNA probes were prepared to study a G to A transition mutation on the W1282X locus of the ABCC7 gene (GenBank; accession M28668, version M28668.1, mutation at position 3,978 and www.genet.sickkids.on.ca/cftr). The wild-type (W) and mutant (M) PNA probes were modified with a mass marker and also a combined photolabile linker (L) to provide each sequence with a unique molecular-weight identifier (**Scheme 2**). The mass marker was composed of lysine (to aid the solubility of the probe), a low molecular-weight amino acid and a halogen-labelled POA (HPOA) monomer, for unambiguous identification upon cleavage from the probe. The halogen isotope pattern enables the use of a proprietary software package, e.g., cluster analysis (MassLynx 4.0), which automatically identifies designated patterns, e.g., naturally occurring isotopes or synthetically designed isotope patterns. Isotope patterns enable facile identification of the mass marker when present in the mass spectrum at low levels. Using this method, mass marker ions can readily be distinguished from the matrix ions. The modifications were added to the *amino*-terminus of the PNA, under standard peptide-coupling conditions during solid-phase synthesis,[20-22] to make three different types of PNA probe for comparison in the hybridization assay (**Table 1**): Unmodified PNA probe (PNA-W and PNA-M), PNA probe with mass marker (BrQ-PNA-W and ClQ-PNA-M) and PNA probe with photocleavable mass (BrQ-L-PNA-W and ClQ-L-PNA-M). Detection of the G to A transition mutation at the W1282X locus requires a cytosine base at the site of mutation for the wild-type probe and thymine base for the mutant. Unfortunately the mutant probe was found to bind to both mutant and wild-type targets,



Scheme 1. Reagents and conditions. (i) *iso*-butyl chloroformate (1.0 eq), NMM (1.0 eq), DME, -15°C, 10 min. (ii) $NaBH_4$ (1.5 eq), H_2O, -15°C, 10 min, 85%. (iii) 2,4-DNsCl (1.0 eq), 2,6 lutidine, CH_2Cl_2, -15°C, 1 h, 91%. (iv) DIAD (1.5 eq), PPh_3 (1.5 eq), alcohol **2** (1.0 eq), CH_2Cl_2, rt, 1 h, 82%. (v) $HSCH_2CO_2H$ (2.0 eq), DIPEA (3.0 eq), CH_2Cl_2, rt, 1 h, 92%. (vi) 4-bromophenylacetic acid or 4-chlorophenylacetic acid (1.1 eq), EDC (1.5 eq), DIPEA, DMF, 2 h, **7** (X = Br), 54%, **8** (X = Cl), 67%. (vii) TFA:CH_2Cl_2 (1:1 v/v), rt, 1 h, **9** (X = Br), 95%, **10** (X = Cl), 96%.

complicating the mass spectrometric analysis. This is not surprising as the mutant probe, when bound to the mutant target, has the least stable Watson-Crick base pair at the mutation site (T:A). This is replaced by a G:T mismatch when this probe is bound to the wild-type target. The G:T mispair is very stable due to the presence of two inter-base imino-carbonyl hydrogen bonds that require only a small distortion of the phosphodiester backbone. Hydrogen bonding of two molecules of water to the carbonyl and amino groups of the G and T is thought to further contribute to duplex stability (**Fig. 2**), and might reduce the rate of probe-target dissociation (kinetic effect).[23-26] To circumvent this problem, and to demonstrate efficacy of the mass spectrometry assay, PNA probes were prepared to study a G to T transversion mutation at the W1282X locus. In this case the *"wild-type probe/wild-type target"* duplex contains a C:G base pair at the mutation site and competes favourably with the *"wild-type probe/mutant target"* duplex that contains a C:T mispair. Similarly, the (mutant probe/mutant target) duplex containing an A:T base pair at the mutation site competes favourably with a (mutant probe/wild-type target duplex) which contains an A:G mispair (**Table 1**).



Scheme 2. Sample preparation for MALDI-TOF: (i) derivitisation of BrQ-L-PNA-W probes to NHS-6-oxohexyl(trimethyl)ammonium bromide, 1M trimethylammonium bicarbonate pH 8.5, 5°C, 10 min, on MALDI plate; and (ii) products after irradiation with nitrogen laser in MALDI-TOF spectrometer at 337 nm (Complete photocleavage at 365 nm).[31]

UV-melting analysis. UV-melting experiments were carried out to investigate the ability of the probes to discriminate between fully-matched synthetic oligonucleotide targets and those containing a single-base mismatch. Analysis of these data sets showed excellent discrimination between fully-matched and single-base mismatch PNA/DNA duplexes with T_m values differing by up to 18.5°C (**Table 1**). Little variation was observed in the stability (melting temperatures) of the modified and unmodified PNA probe/DNA duplexes. This demonstrates that the photolabile linker and mass markers do not affect the binding and specificity of the PNA/DNA duplex, and as a consequence the same assay conditions can be used with the modified PNA probes as their unmodified counterparts.

MALDI assay. Experiments were carried out with PCR-amplified wild-type human genomic DNA. The sequence data for the W1282X locus was obtained from GenBank (accession M28668). The primers were designed to give amplicons of 100 bases. The forward primer was modified with a 5'-biotin (Primer 1) and the reverse primer was unmodified (Primer 2). PCR conditions were adapted from those described by Thelwell and co-workers.[27,28] The PCR products were purified using a Qiagen PCR purification kit and concentrated (10 μL from 50 μL reaction) prior to immobilisation on streptavidin-coated magnetic beads. Purification was necessary to ensure all unextended primers were removed before the immobisation step, as the free biotinylated PCR primers bind to the magnetic beads much more rapidly than the PCR product. The duplex was denatured to remove the unbiotinylated strand (complementary strand) and the beads were incubated at 80°C then cooled to room temperature in the presence of the fully-complementary and mismatched PNA probes. Washing with hybridization buffer removed unbound probes and the mixture was heated for a further 20 minutes at 45°C. The beads were then washed with hybridization buffer and water to remove non-complementary probes and detergents, then loaded directly on the MALDI target ready for MS analysis. Whilst the PCR product remained immobilised on the beads, the PNA probe was denatured from the DNA during application of the MALDI matrix. The genotype of the sample was determined from the quasi-molecular ion of the PNA probe, or the mass marker released photo-chemically during laser ablation (**Fig. 1**). The unmodified probes (PNA-W) and probes labelled with mass markers (BrQ-PNA-W) were readily detected from PCR-amplified genomic DNA (**Figs. 3 and 4** respectively). No signal was seen for the mass marker cleaved from the PNA probe, but the intact probe was observed (BrQ-L-PNA-W). To overcome

Table 1. The PNA, target DNA and primer sequences W1282 locus of ABCC & gene, MALDI MS and UV melting analysis

Oligo ID	Sequence	Mass (Da)		Yield	T_m°C	
		Observed	Calculated	μmole	(Probe:Target)	
PNA-W	CTTTCCTCCACTGT	3690.3	3690.6	0.50	54.0 (C:G)	35.5 (C:T)
PNA-M	CTTTCCTACACTGT	3714.5	3714.6	0.43	39.1 (A:G)	50.3 (A:T)
BrQ-PNA-W	**9**-Lys-Ala-CTTTCCTCCACTGT	4186.7	4187.0	0.37	52.0 (C:G)	33.8 (C:T)
ClQ-PNA-M	**10**-Lys-Phe-CTTTCCTACACTGT	4242.6	4242.7	0.32	37.6 (A:G)	50.4 (A:T)
BrQ-L-PNA-W	**9**-Lys-Ala-L-CTTTCCTCCACTGT	668.3	668.3	0.39	52.8 (C:G)	34.4 (C:T)
ClQ-L-PNA-M	**10**-Lys-Phe-L-CTTTCCTACACTGT	N.D	700.4	0.16	34.1 (A:G)	51.7 (A:T)
Target DNA-Wt	ACAGTGGAGGAAAG					
Target DNA-Mt	ACAGTGTAGGAAAG					
Primer 1	Biotin-GGCTAAGTCCTTTTGCTCAC					
Primer 2	ATGGTGTGTCTTGGGATTCA					

Mutation site indicated in red. L is photocleavage moiety (Scheme 2), **9** = bromo monomer, **10** = chloro monomer (Scheme 1), Lys, Lysine; Phe, Phenylalanine. Mass spectra were detected by linear and refectron MALDI-TOF-MS and the average chemical mass was used to calculate the mass of the protonated molecule $(M + H)^+$. Yields of PNA were calculated after RP-HPLC purification (from 2 μmol synthesis). T_m values were determined with synthetic DNA targets (Wt and Mt are wild-type and mutant respectively). Melting curves determined in 10 mM Tris, 1 M NaCl, pH 7. ND indicates not determined.

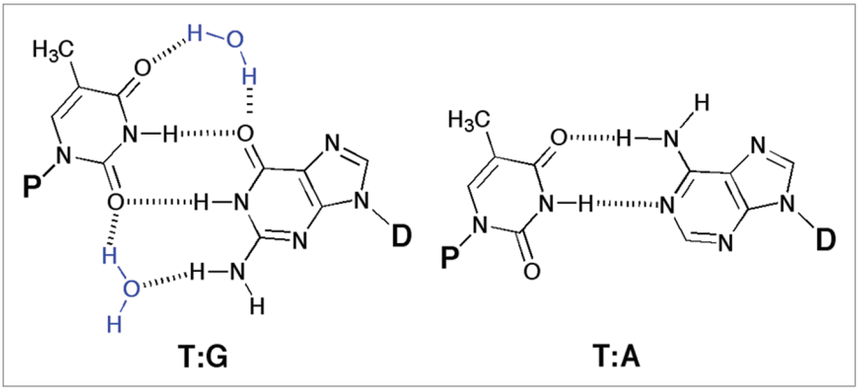


Figure 2. T:G mismatched base pair with putative water-binding cavities compared to T:A Watson-Crick base pair. p = PNA, D = DNA.

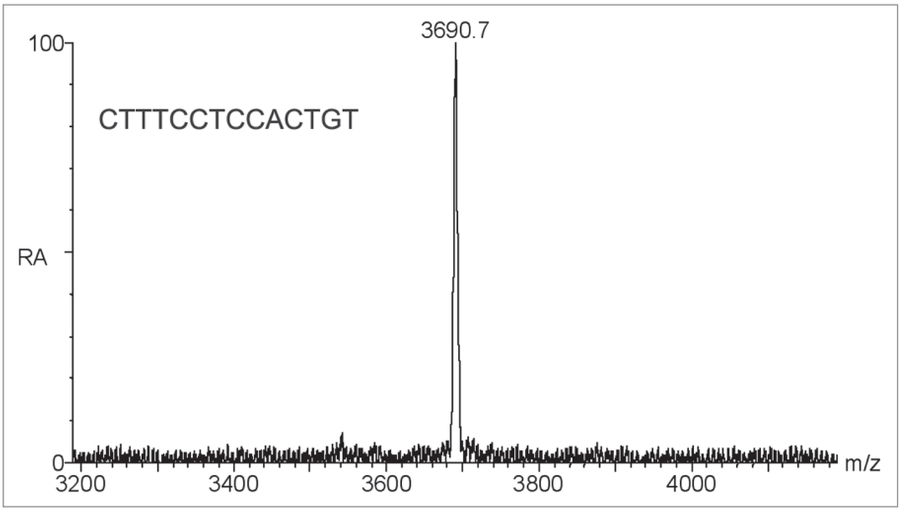


Figure 3. MALDI-TOF MS of the wild-type probe (PNA-W) released from the immobilised PCR product. Expected mass $(M + H)^+$ 3690.6.

this problem, the mass marker was derivatised with the NHS ester of 6-oxohexyl(trimethyl) ammonium bromide (C_5Q)[29] on the MALDI target prior to the addition of the matrix (**Fig. 5 and Scheme 2**). Addition of the positive charge allowed facile detection of the cleaved mass marker from the amplified DNA. Minor additional ions resulting from uncontrolled derivatisation were also observed i.e., extra C_5Q adduct at the lysine side chain (823.5 Da), fragment of $[2(C_5Q)\text{-}CH_3]$ (809.1 Da), $[2(C_5Q)\text{-}CH_2N(Me)_3]$ (764.0 Da) and $[C_5Q\text{-}Me_3]$ (624.8 Da). However, these fragments do not interfere with the interpretation of the assay. The ratio of PNA:C_5Q required to derivatise with mostly one functionality was found to be 5:1 (data not shown). Further optimisation of this derivatisation procedure would be beneficial.

Materials and Methods

General. All chemical reactions were carried out under argon using oven-dried glassware. Column chromatography was carried out under pressure using Fisher Scientific DAVISIL 60 Å (35–70 micron) silica. Compounds were visualised by irradiation at 254 nm or by staining with phosphomolybdic acid:ethanol (10% w/v). Thin layer chromatography was performed using Merck Kieselgel 60 F24 (0.22 mm thickness, aluminium backed) plates. Infra-red spectra were recorded on a BIORAD FT-IR using a Golden Gate adapter and BIORAD WIN-IR

software, or on a Satellite FT-IR using a Golden Gate adapter and WIN FIRST-lite software. Infra-red absorptions are described as strong (s), medium (m), broad (br) or weak (w). ^{1}H NMR spectra were measured at 400 MHz on a Bruker DPX400 spectrometer and ^{13}C NMR spectra were measured at 100 MHz on the same spectrometer. Chemical shifts are given in ppm relative to the residual solvent peak,[30] and *J* values are correct to within 0.5 Hz. Carbon multiplicities are indicated by 0 (C), 1 (CH), 2 (CH_2) and 3 (CH_3). Low-resolution mass spectra were recorded using electrospray ionisation on a Waters ZMD quadrupole mass spectrometer in methanol or water. High-resolution mass spectra were recorded on a Bruker APEX III FT-ICR mass spectrometer in methanol or water using an Apollo electrospray ionisation source. Reagents were purchased from Sigma-Aldrich, Avocado, Cruachem, Fisher Scientific, Fluka, Lancaster, Link Technologies Ltd., Novabiochem® or Rathburn, and were used without purification, with the exception of CH_2Cl_2 and DIPEA, which were purified by distillation over calcium hydride. 9*H*-9-Fluorenylmethyl *N*-(2-hydroxyethyl)carbamate **2** was synthesised according to the literature procedure.[18] The NHS ester of 6-oxohexyl(trimethyl) ammonium bromide was prepared according to the reported method.[29]

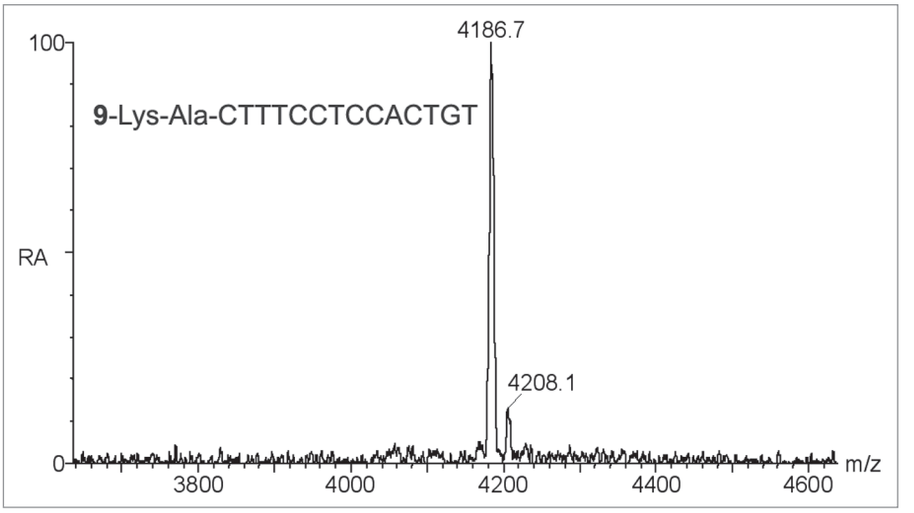


Figure 4. MALDI-TOF MS of the wild-type probe modified with a mass marker (BrQ-PNA-W) released from the immobilised PCR product. Expected mass $(M + H)^+$ 4186.9.

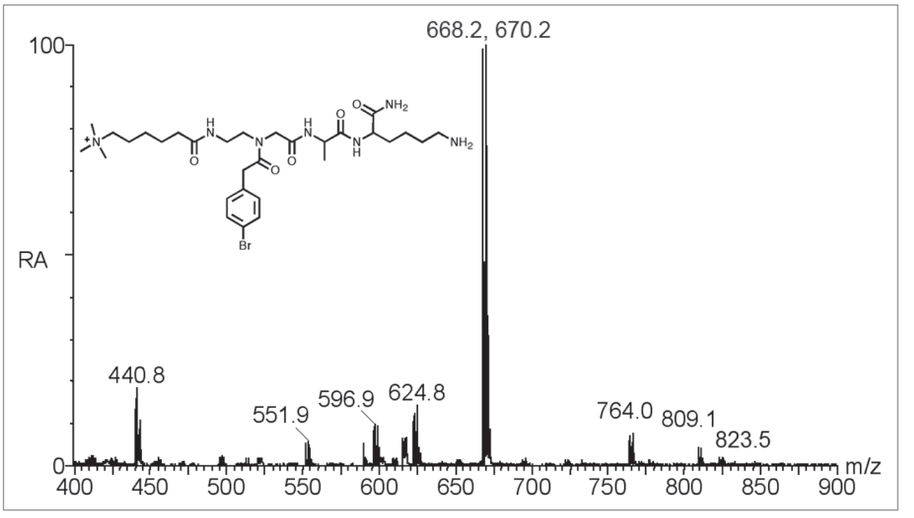


Figure 5. MALDI-TOF MS of the mass marker cleaved from the wild-type probe (BrQ-L-PNA-W) released from the immobilised PCR product. Expected mass $(M)^+$ 668.2 & 670.2 (bromine isotope pattern).

***tert*-Butyl 2-[(2,4-dinitrophenyl) sulfonyl]aminoacetate 4.** Following the procedure described by Fukuyama et al.[15] 2,4-dinitrobenzenesulfonyl chloride (5.54 g, 20.79 mmol) in CH_2Cl_2 (30 mL) was added to a solution of *tert*-butyl 2-aminoacetate hydrochloride, **3** (3.00 g, 20.79 mmol) in CH_2Cl_2 (40 mL) at 15°C (adjusted to pH 8 with 2, 6-lutidine). The reaction mixture was allowed to warm to room temperature and stirred for 1 hour, then diluted with CH_2Cl_2 (30 mL) and washed with aq citric acid (10%, 100 mL) and saturated aq KCl (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The resulting yellow residue was recrystallised from ethyl acetate/hexane to yield sulfonamide **4** as an orange crystalline solid (6.83 g, 91%). R_f = 0.28 (CH_2Cl_2) UV; mp = 123–125°C (ethyl acetate/hexane); υ_{max} (film, cm^{-1}) 3350 (w), 3112 (w), 2982 (w), 1739 (m), 1605 (w), 1540 (s), 1413 (w), 1350 (s), 1251 (m), 1156 (s), 1116 (w); δ_H (400 MHz, $CDCl_3$) 8.68 (1H, d, *J* = 2.0 Hz, Ar**H**), 8.45 (1H, dd, *J* = 2.0, 8.5 Hz, Ar**H**), 8.23 (1H, d, *J* = 8.5 Hz, Ar**H**), 6.11 (1H, t, *J* = 5.5 Hz, N**H**), 3.89 (2H, t, *J* = 5.5 Hz, C$\mathbf{H}_2$NH), 1.28 (9H, s, C(C$\mathbf{H}_3$)$_3$); δ_C (100 MHz, $CDCl_3$) 167.9 (0), 150.2 (0), 148.4 (0), 140.2 (0), 132.5 (1), 127.4 (1), 121.4 (1), 83.8 (0), 46.1 (2), 28.2 (3); LRMS (ES^+) *m/z* 384.0 $(M + Na)^+$ (100%); (ES^-) *m/z* 360.0 $(M - H)^-$ (100%); HRMS (ES^+) *m/z* 745.1052 $(2M + Na)^+$; $C_{24}H_{30}N_6O_{16}S_2Na$ requires 745.1052.

***tert*-Butyl 2-[[(2,4-dinitrophenyl)sulfonyl](2-[(9*H*-9-fluorenylmethoxy)carbonyl]aminoethyl)amino]acetate 5.** Following the procedure described by Fukuyama et al.[15] Diisopropylazodicarboxylate (1.68 mL, 8.30 mmol) in anhydrous CH_2Cl_2 (5 mL) was added to a solution of sulfonamide **4** (2.00 g, 5.54 mmol), triphenylphosphine (2.18 g, 8.30 mmol) and alcohol **2** (1.57 g, 5.54 mmol) in anhydrous CH_2Cl_2 (10 mL). The reaction mixture was stirred overnight at room temperature under an atmosphere of argon then preabsorbed directly onto silica gel and purified by silica gel column chromatography (90:10 hexane:ethylacetate) to yield sulfonamide **5** as an amorphous

yellow solid (2.86 g, 82%). R_f = 0.34 (1:1 ethyl acetate/hexane) UV and phosphomolybdic acid; υ_{max} (film, cm^{-1}) 1719 (m), 1538 (s), 1453 (w), 1369 (s), 1235 (s), 1685 (s), 1103 (w), 1017 (w); δ_H (400 MHz, $CDCl_3$), 8.36 (1H, dd, *J* = 2.0, 8.5 Hz, Ar**H**), 8.31 (1H, d, *J* = 2.0 Hz, Ar**H**), 8.18 (1H, d, *J* = 8.5 Hz, Ar**H**), 7.69 (2H, d, *J* = 7.5 Hz, Fmoc**H**), 7.51 (2H, d, *J* = 7.5 Hz, Fmoc**H**), 7.32 (2H, t, *J* = 7.5 Hz, Fmoc**H**), 7.24 (2H, t, *J* = 7.5 Hz, Fmoc**H**), 5.27 (1H, br.t, N**H**), 4.28 (2H, d, *J* = 7.5 Hz, C$\mathbf{H}_2$CH), 4.13 (1H, t, *J* = 7.5 Hz, C**H**), 3.89 (2H, s, COC$\mathbf{H}_2$N), 3.46 (2H, t, *J* = 6.0 Hz, NC$\mathbf{H}_2$$CH_2$NH), 3.35 (2H, t, *J* = 6.0 Hz, NCH_2C$\mathbf{H}_2$NH), 1.33 (9H, s, C(C$\mathbf{H}_3$)$_3$); δ_C (100 MHz, $CDCl_3$) 169.4 (0), 158.2 (0), 151.3 (0), 149.7 (0), 145.5 (0), 143.0 (0), 140.1 (0), 134.4 (1), 129.4 (1), 128.7 (1), 127.7 (1), 126.8 (1), 121.7 (1), 121.2 (1), 84.9 (0), 68.7 (2), 51.2 (2), 50.5 (2), 48.8 (1), 40.4 (2), 29.6 (3); LRMS (ES^+) *m/z* 649.3 $(M + Na)^+$ (100%); HRMS (ES^+) *m/z* 649.1552 $(M + Na)^+$; $C_{29}H_{30}N_4O_{10}SNa$ requires 649.1578.

***tert*-Butyl 2-[(2-[(9*H*-9-fluorenylmethoxy)carbonyl]aminoethyl)amino]acetate hydrochloride 6.** Following the procedure described by Fukuyama et al. Mercaptoacetic acid (0.95 g, 10.31 mmol) and DIPEA (2.6 mL, 15.46 mmol) in CH_2Cl_2 (5 mL) was added to a solution of sulfonamide **5** (3.23 g, 5.15 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was stirred for 20 minutes, diluted with CH_2Cl_2 (25 mL) and extracted with saturated aq $NaHCO_3$ (50 mL) followed by saturated aq KCl (50 mL) until the aqueous layer was clear. The organic layer was washed with aq HCl (1 M, 50 mL), dried over Na_2SO_4, filtered, and concentrated in vacuo to yield PNA backbone **6** as the hydrochloride salt, as an off-white amorphous powder (1.89 g, 92%). R_f = 0.55 (95:5 ethyl acetate/CH_3OH) UV and phosphomolybdic acid; υ_{max} (film, cm^{-1}) 2978 (w), 2934 (w), 2360 (w), 2338 (w), 1719 (s), 1691 (s), 1532 (w), 1477 (w), 1450 (m), 1395 (w), 1369 (m), 1249 (m), 1205 (s), 1154 (s), 1032 (w); δ_H (400 MHz, CD_3OD) 7.05 (2H, d, *J* = 7.5 Hz, Fmoc**H**), 6.95 (2H, d, *J* = 7.5 Hz, Fmoc**H**), 6.65 (2H, t, *J* = 7.5 Hz, Fmoc**H**), 6.60 (2H, t, *J* = 7.5 Hz, Fmoc**H**), 3.65 (2H, d, *J* = 6.5 Hz, C$\mathbf{H}_2$CH), 3.50 (1H, t, *J* = 6.5 Hz, C**H**), 2.65 (2H, s, NHC$\mathbf{H}_2$$CO_2C(CH_3)_3$), 2.55 (2H, t, *J* = 5.5 Hz, NHC$\mathbf{H}_2$$CH_2$NH), 2.05 (2H, t, *J* = 5.5 Hz, $NHCH_2$C$\mathbf{H}_2$NH), 0.75 (9H, s, C(C$\mathbf{H}_3$)$_3$); δ_C (100 MHz, CD_3OD) 171.5 (0), 158.9 (0), 145.2 (0), 142.5 (0), 128.7 (1), 128.0 (1), 126.1 (1), 120.8 (1), 82.8 (0), 67.7 (2), 51.1 (2), 49.4 (2), 48.4 (1), 40.7 (2), 28.2 (3); LRMS (ES^+) *m/z* 397.3 $(M + H)^+$ (100%), 419.4 $(M + Na)^+$ (10%); HRMS (ES^+) *m/z* 397.2120 $(M + H)^+$; $C_{23}H_{29}N_2O_4$ requires 397.2122.

***tert*-Butyl 2-[[2-(4-bromophenyl)acetyl]-(2-[(9*H*-9-fluorenylmethoxy)carbonyl]aminoethyl)amino]acetate 7.** 4-Bromo-phenylacetic acid (0.57 g, 2.65 mmol) was added to a solution of PNA backbone **6** (1.00 g, 2.52 mmol) and EDC (0.73 g, 3.78 mmol) (adjusted to pH 8 with DIPEA) in anhydrous DMF (30 mL). The reaction mixture was stirred for 2 hours at room temperature under an atmosphere of argon then diluted with CH_2Cl_2 (150 mL) and washed with saturated aq KCl (3 x 100 mL). The organic phase was dried over Na_2SO_4, filtered, preabsorbed directly onto silica gel and purified by silica gel column chromatography (1:1, ethyl acetate/hexane). Fractions containing the product were concentrated in vacuo to yield monomer **7** as a white foam (0.81 g, 54%). R_f = 0.62 (99:1 ethyl acetate/aq NH_3) UV and phosphomolybdic acid; υ_{max} (film, cm^{-1}) 3319 (br w), 3065 (w), 2979 (w), 2938 (w), 1718 (s), 1645 (s), 1518 (m), 1488 (m), 1449 (s), 1415 (m), 1405 (w), 1367 (m), 1318 (w), 1243 (s), 1180 (s), 1104 (w), 1071 (w), 1012 (m); δ_H (400 MHz, $CDCl_3$) (2:1 rotamers) 7.69 (6H, d, *J* = 7 Hz, Fmoc**H**), 7.53–7.50 (6H, m, Fmoc**H**), 7.30–7.36 (12H, m, Ar**H**, Fmoc**H**), 7.21–7.25 (6H, m, Fmoc**H**), 7.00–7.04 (6H, m, Ar**H**), 5.76 (2H, br s, N**H**), 5.39 (1H, br s, N**H**), 4.32–4.36 (4H, m, C$\mathbf{H}_2$CH), 4.25–4.28 (2H, m, C$\mathbf{H}_2$CH), 4.10–4.16 (3H, m, C**H**), 3.88 (4H, s, NC$\mathbf{H}_2$$CO_2C(CH_3)_3$), 3.82 (2H, s, NC$\mathbf{H}_2$$CO_2C(CH_3)_3$), 3.56 (4H, s, COC$\mathbf{H}_2$Ar), 3.47–3.52 (6H, m, $NHCH_2$C$\mathbf{H}_2$N, COC$\mathbf{H}_2$Ar), 3.37–3.43 (2H, m, $NHCH_2$C$\mathbf{H}_2$N), 3.26–3.35 (6H, m, NHC$\mathbf{H}_2$$CH_2$N), 1.46 (18H, s, C(C$\mathbf{H}_3$)$_3$), 1.41 (9H, s, C(C$\mathbf{H}_3$)$_3$); δ_C (100 MHz, $CDCl_3$) 172.7 (0), 172.1 (0), 170.2 (0), 169.3 (0), 157.4 (0), 144.8 (0), 144.6 (0), 142.2 (0), 134.5 (0), 134.1 (0), 132.62 (0), 132.5 (0), 131.6 (1), 131.5 (1), 128.6 (1), 128.5 (1), 127.9 (1), 127.9 (1), 125.9 (1), 125.8 (1), 121.9 (1), 121.7 (1), 120.8 (1), 120.8 (1), 84.0 (0), 83.2 (0), 67.8 (2), 67.6 (2), 61.2 (2), 52.4 (2), 50.7 (2), 50.4 (2), 49.0 (2), 48.1 (1), 48.0 (1), 40.8 (2), 40.2 (2), 28.9 (3), 28.8 (3); LRMS (ES^+) *m/z* 615.5, 617.2 $(M + Na)^+$ (Br isotope pattern 100%); HRMS (ES^+) *m/z* 615.1454 $(M + Na)^+$; $C_{31}H_{33}N_2O_5{}^{79}BrNa$ requires 615.1454.

***tert*-Butyl 2-[[2-(4-chlorophenyl)acetyl](2-[(9*H*-9-fluorenylmethoxy)carbonyl]aminoethyl)amino]acetate 8.** 4-Chlorophenylacetic acid (0.79 g, 4.63 mmol) was added to a solution of PNA backbone **6** (1.67 g, 4.21 mmol) and EDC (1.21 g, 6.32 mmol), (adjusted to pH 8 with DIPEA) in anhydrous DMF (30 mL). The reaction mixture was stirred for 1 hour at room temperature under an atmosphere of argon then diluted with CH_2Cl_2 (150 mL) and extracted with saturated aq KCl (3 x 100 mL). The organic phase was dried over Na_2SO_4, filtered, preabsorbed onto silica gel and purified by silica gel column chromatography (1:1, ethyl acetate/hexane). Fractions containing the product were concentrated in vacuo to yield monomer **8** as a white foam (1.56 g, 67%). R_f = 0.64 (95:5, ethyl acetate/CH_3OH) UV and phosphomolybdic acid; υ_{max} (film, cm^{-1}) 3311 (br w), 2977 (w), 2941 (w), 1720 (s), 1646 (s), 1515 (m), 1492 (m), 1449 (s), 1407 (w), 1367 (m), 1318 (w), 1231 (s), 1152 (s), 1090 (w), 1015 (w); δ_H (400 MHz, $CDCl_3$) (2:1 rotamers) 7.67–7.71 (6H, m, Fmoc**H**), 7.48–7.53 (6H, m, Fmoc**H**), 7.29–7.35 (6H, m, Fmoc**H**), 7.19–7.24 (6H, m, Fmoc**H**), 7.17 (6H, dd, *J* = 2.0, 9.1, Ar**H**), 7.08 (6H, dd, *J* = 2.0, 9.1, Ar**H**), 5.77 (2H, br t, N**H**), 5.39 (1H, br t, N**H**), 4.30–4.36 (4H, m, C$\mathbf{H}_2$CH), 4.25–4.28 (2H, m, C$\mathbf{H}_2$CH), 4.12–4.18 (3H, m, C**H**), 3.88 (4H, s, NC$\mathbf{H}_2$$CO_2C(CH_3)_3$), 3.83 (2H, s, NC$\mathbf{H}_2$$CO_2C(CH_3)_3$), 3.58 (4H, s, COC$\mathbf{H}_2$Ar), 3.48–3.53 (6H, m, $NHCH_2$C$\mathbf{H}_2$N, COC$\mathbf{H}_2$Ar), 3.38–3.45 (2H, m, $NHCH_2$C$\mathbf{H}_2$N), 3.27–3.35 (4H, m, NHC$\mathbf{H}_2$$CH_2$N), 3.23–3.27 (2H, m, NHC$\mathbf{H}_2$$CH_2$N), 1.42 (18H, s, C(C$\mathbf{H}_3$)$_3$), 1.39 (9H, s, C(C$\mathbf{H}_3$)$_3$); δ_C (100 MHz, $CDCl_3$) 170.5 (0), 169.8 (0), 167.9 (0), 167.0 (0), 155.1 (0), 142.4 (0), 142.3 (0), 139.8 (0), 131.6 (0), 131.5 (0), 131.3 (0), 128.9 (1), 128.8 (1), 127.3 (1), 127.2 (1), 126.2 (1), 126.2 (1), 125.6 (1), 125.5 (1), 123.2 (1), 123.5 (1), 118.5 (1), 118.4 (1), 81.6 (0), 80.6 (0), 65.5 (2), 65.3 (2), 58.8 (1), 50.1 (2), 48.4 (2), 48.1 (2), 46.7 (2), 45.7 (2), 45.7 (2), 38.4 (2), 38.0 (2), 26.5 (3), 26.5 (3); LRMS (ES^+) *m/z* 571.3, 573.3 (M +

Na)$^+$ (Cl isotope pattern 100%); HRMS (ES$^+$) *m/z* 571.1970 (M + Na)$^+$; $C_{31}H_{33}N_2O_5{}^{35}ClNa$ requires 571.1970.

2-[[2-(4-Bromophenyl)acetyl](2-[(9*H*-9-fluorenylmethoxy)carbonyl]aminoethyl)amino]acetic acid 9. Trifluoroacetic acid (10 mL) was added to a solution of compound **8** (1.00 g, 2.52 mmol) in CH_2Cl_2 (10 mL) and the mixture was stirred for 30 minutes at room temperature. The reaction mixture was concentrated in vacuo and Et_2O (10 mL) was added. A precipitate formed on cooling, which was washed with Et_2O (10 mL) then dried in vacuo to yield monomer **9** as a white amorphous solid (0.81 g, 54%). R_f = 0.42 (95:5, CH_2Cl_2/CH_3OH) UV and phosphomolybdic acid; υ_{max} (solid, cm^{-1}) 3286 (m), 3795 (w), 2891 (w), 17213 (s), 1672 (m), 1632 (s), 1592 (w), 1472 (m), 1445 (s), 1403 (m), 1360 (w), 1328 (m), 1275 (w), 1223 (m), 1191 (m), 1134 (m), 1095 (m), 1064 (m), 1009 (w); δ_H (400 MHz, d_6-DMSO) (2:1 rotamers) 12.85 (3H, s, CO_2**H**), 7.98–8.03 (6H, m, Fmoc**H**), 7.75–7.79 (6H, m, Fmoc**H**), 7.48–7.60 (14H, m, Ar**H**, Fmoc**H**, N**H**), 7.40–7.46 (6H, m, Fmoc**H**), 7.28 (1H, br t, N**H**), 7.24–7.29 (6H, m, Ar**H**), 4.42–4.46 (4H, m, C$\mathbf{H}_2$CH), 4.37–4.40 (2H, m, C$\mathbf{H}_2$CH), 4.30–4.36 (3H, m, C**H**), 4.29 (4H, s, NC$\mathbf{H}_2$COOH), 4.06 (2H, s, NC$\mathbf{H}_2CO_2$H), 3.80 (4H, s, COC$\mathbf{H}_2$Ar), 3.68 (2H, s, COC$\mathbf{H}_2$Ar), 3.35–3.56 (6H, m, NHCH$_2$C$\mathbf{H}_2$N), 3.24–3.34 (4H, m, NHC$\mathbf{H}_2$CH$_2$N), 3.20–3.23 (2H, m, NHC$\mathbf{H}_2$CH$_2$N); δ_C (100 MHz, d_6-DMSO) 171.6 (0), 171.3 (0), 171.2 (0), 170.8 (0), 156.8 (0), 156.6 (0), 144.3 (0), 141.2 (0), 135.6 (0), 135.6 (0), 132.1 (1), 131.9 (1), 131.4 (1), 131.4 (1), 128.1 (1), 127.5 (1), 125.6 (1), 125.5 (1), 120.6 (1), 120.0 (1), 65.9 (2), 48.3 (2), 47.9 (2), 47.2 (1), 47.2 (1), 47.0 (2), 39.3 (2), 38.9 (2), 38.4 (2); LRMS (ES$^+$) *m/z* 559.1, 561.1 (M + Na)$^+$ (Br isotope pattern 100%); (ES$^-$) *m/z* 535.2, 537.2 (M - H)$^-$ (Br isotope pattern 100%); HRMS (ES$^+$) *m/z* 559.0839 (M + Na)$^+$; $C_{27}H_{25}N_2O_5{}^{79}BrNa$ requires 559.0837.

2-[[2-(4-Chlorophenyl)acetyl](2-[(9*H*-9-fluorenylmethoxy)carbonyl]aminoethyl)amino]acetic acid 10. Trifluoroacetic acid (10 mL) was added to a solution of compound **9** (1.54 g, 2.80 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred for 60 minutes at room temperature then concentrated in vacuo. Et_2O (10 mL) was added and a precipitate formed on cooling. This was washed with Et_2O (10 mL) then dried in vacuo to yield monomer **10** as a white, amorphous solid (1.32 g, 96%). R_f = 0.44 (95:5, CH_2Cl_2/CH_3OH) UV and phosphomolybdic acid; υ_{max} (solid, cm^{-1}) 3282 (w), 2979 (w), 2949 (w), 2634 (w), 2565 (w), 1723 (s), 1673 (m), 1632 (s), 1596 (w), 1492 (w), 1471 (m), 1444 (s), 1404 (m), 1361 (w), 1329 (m), 1277 (w), 1224 (m), 1192 (m), 1134 (m), 1094 (m), 1064 (m), 1023 (w); δ_H (400 MHz, d_6-DMSO) (2:1 rotamers) 12.75 (3H, s, CO_2**H**), 7.98–8.01 (6H, m, Fmoc**H**), 7.7–7.80 (6H, m, Fmoc**H**), 7.50–7.54 (6H, m, Fmoc**H**), 7.40–7.45 (14H, m, Fmoc**H**, Ar**H**, N**H**), 7.28–7.34 (7H, m, Ar**H**, N**H**), 4.40–4.44 (4H, m, C$\mathbf{H}_2$CH), 4.38–4.40 (2H, m, C$\mathbf{H}_2$CH), 4.29–4.32 (3H, m, C**H**), 4.32 (4H, s, NC$\mathbf{H}_2CO_2$H), 4.06 (2H, s, NC$\mathbf{H}_2CO_2$H), 3.82 (4H, s, COC$\mathbf{H}_2$Ar), 3.69 (2H, s, COC$\mathbf{H}_2$Ar), 3.40–3.52 (6H, m, NHCH$_2$C$\mathbf{H}_2$N), 3.26–3.31 (4H, m, NHC$\mathbf{H}_2$CH$_2$N), 3.23–3.26 (2H, m, NHC$\mathbf{H}_2$CH$_2$N); δ_C (100 MHz, d_6-DMSO) 170.7 (0), 170.5 (0), 170.3 (0), 170.0 (0), 155.9 (0), 155.7 (0), 143.4 (0), 140.3 (0), 134.3 (0), 134.2 (0), 130.8 (1), 130.6 (1), 127.6 (1), 127.5 (1), 127.2 (1), 126.6 (1), 124.7 (1), 124.6 (1), 119.7 (1), 65.0 (2), 49.6 (2), 47.4 (2), 47.1 (2), 46.3 (1), 46.2 (1), 39.7 (2), 39.5 (2), 39.3 (2), 37.9 (2), 39.7 (2), 37.5 (2); LRMS (ES$^+$) *m/z* 515.11, 517.1 (M + Na)$^+$ (Cl isotope pattern 100%); (ES$^-$) *m/z* 491.1, 493.1 (M - H)$^-$ (Cl isotope pattern 100%); HRMS (ES$^+$) *m/z* 515.1344 (M + Na)$^+$; $C_{27}H_{25}N_2O_5{}^{35}ClNa$ requires 515.1351.

Synthesis, purification and analysis of PNA probes. PNA probes were synthesised on a PerSeptive Biosystems Expedite Nucleic Acid/PNA synthesiser modified for PNA chemistry. The synthesis was performed on a 2 μmole scale by the Fmoc strategy using standard PNA monomers and reagents purchased from Applied Biosystems. The Fmoc-protected amino acids and photolabile linker were purchased from Novabiochem®. The crude oligomers were purified by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC). Purification was carried out on a Gilson instrument using a Brownlee Prep 10 Octyl column (10 mm x 250 mm, particle size 20 μm). The system was controlled by Gilson 7.12 software and the following protocol was used: Run time 25 minutes, flow rate 4 mL min^{-1}, binary system, gradient: Time in minutes (% buffer B); 0 (0); 18 (40); 21 (95); 22 (95); 23 (0); 25 (0). Elution buffer A: water (0.1% TFA), buffer B: acetonitrile (0.08% TFA). Elution of PNAs was monitored by ultraviolet absorption at 300 nm. The optical density (OD) of each purified PNA was recorded at 260 nm on a Lambda 15 UV/Vis Spectrophotomer and was used to calculate their concentration. The molecular mass of the PNAs was verified by MALDI-TOF MS on a Waters TofSpec2E operating in linear mode equipped with a 337-nm nitrogen laser. Data were recorded in positive-ion mode, using delayed extraction and an initial accelerating voltage of 20 kV with α-cyano-4-hydroxycinnamic acid in acetonitrile/water (1:1, 0.1% TFA) using Dowex 50WX8-200 ion-exchange beads. The instrument was externally calibrated using a mixture of commercially available peptides. The yield and the MS are shown in **Table 1**.

Synthesis, purification and analysis of oligonucleotides.[9] All oligonucleotides were synthesised on an Applied Biosystems 394 automated DNA/RNA synthesiser using the standard 0.2 μmole phosphoramidite cycle of acid-catalysed detritylation, coupling, capping and iodine oxidation. Biotin was added with a biotin-TEG monomer purchased from Glen Research Inc., (www.glenresearch.com). The biotinylated oligonucleotides were purified by RP-HPLC prior to removal of the final trityl-protecting group using aqeous acetic acid (80%) at room temperature for 30 minutes. HPLC conditions were similar to those used for PNA purification except that a linear gradient of acetonitrile in 0.1 M ammonium acetate buffer (pH 7) was used.

UV-melting studies. The UV absorbance vs. temperature profiles were obtained simultaneously at 260 nm using an intracuvette temperature probe on a Varian Cary 400 UV-Visible spectrometer in Hellma® SUPRASIL synthetic quartz, 10 mm pathlength cuvettes. The PNA probe and oligonucleotide target were each at a concentration of 1.0 μM in 10 mM Tris, 1 M NaCl buffer (pH 7) in a volume of 1.5 mL. The samples were filtered with Kinesis regenerated cellulose syringe filters (13 mm, 0.45 micron) and rapidly heated from 15°C to 80°C at 10°C min^{-1} then cooled to 15°C at 0.5°C min^{-1} prior to analysis. The UV-melting

curves were recorded for three consecutive heat and cool cycles. The temperature was increased in increments of 0.1°C min^{-1} and the T_m values were determined from the maximum of the first-order derivative of the average of the three cycles.

PCR amplification of human DNA. The CFTR W1282X region was amplified using one 5'-biotinylated primer (Primer 1, **Table 1**) and one unmodified primer (Primer 2, **Table 1**). PCR amplifications were performed using a Progene thermal cycler (Techne). PCR reagents and buffers were obtained from Promega. Human genomic DNA was purchased from Novagen. PCR reactions in a final volume of 50 μL contained each primer (0.5 μM), dNTPs (200 μM each of dATP, dCTP, dTTP and dGTP), $MgCl_2$ (3 mM), Taq Polymerase (2.5 units) and 1x PCR Buffer. Human DNA template in water (5 ng μL^{-1}) or water (for no template control), was added to each reaction. Each tube was subjected to an initial activation (95°C, 5 minutes), and then subjected to 35 cycles of annealing (65°C, 30 seconds), extension (65°C, 30 seconds) and denaturing (74°C, 30 seconds), followed by a final extension phase (72°C, 10 minutes). After thermal cycling, each sample (5 μL) was electrophoresed on a agarose gel (2%) stained with ethidium bromide, and photographed under a UV-transilluminator. Samples were then individually purified using a Qiagen PCR purification kit and concentrated to 10 μL.

Derivatisation of PNA probes with quarternary ammonium ion. 1.0 μl of a cold solution (5°C) of NHS-6-oxohexyl(trimethyl) ammonium bromide (3.0 mg, 3.6 μmol) in aq trimethylammonium bicarbonate (0.1 M, pH 8.5, 0.5 mL) was added to the PNA/DNA bead complex on the MALDI plate. The mixture was left for 10 minutes at 5°C and samples were then dried in vacuo.

PNA hybridization assay. For immobilisation of the biotin to streptavidin bead, the purified PCR product (10 μL) was added to Dynal Biotech M-280 streptavidin beads (30 μg) in binding buffer (10 mM Tris, 1 M NaCl, pH 7, 20 μL). The mixture was allowed to bind at room temperature for 30 min. The beads were isolated and washed with 0.1 M aq NaOH (2 x 200 μL) and resuspended in 0.1 M aq NaOH (200 μL). After 5 minutes, the beads were isolated and washed with binding buffer (2 x 200 μL) and hybridization buffer (10 mM Tris, 0.1% BSA, 1 M NaCl, pH 7, 2 x 200 μL). For hybridization, a solution of PNA (30 pmol) in hybridization buffer (20 μL) was added to the resulting beads, and this mixture was left at room temperature for 15 minutes. The mixture was heated to 80°C for 1 hour and allowed to cool to room temperature over 1 hour. The beads were isolated and washed with hybridization buffer (2 x 200 μL) and binding buffer (2 x 200 μL), then were re-suspended in binding buffer (200 μL) and incubated at 45°C for 20 minutes. The beads were subsequently washed with more binding buffer (2 x 200 μL), wash buffer (10 mM Tris, 0.1% SDS, pH 7, 2 x 200 μL) and water (2 x 200 μL) then re-suspended in 1 μL of matrix (α-cyano-4-hydroxycinnamic acid in acetonitrile:water (1:1, 0.1% TFA)) and loaded directly on the MALDI plate with Dowex 50WX8-200 ion exchange beads. Spectra were collected on a Waters TofSpec2E as previously described.

Conclusions

Three different types of PNA probe have been prepared; the unmodified PNA probe, the PNA probe with halogen-labelled mass marker and the PNA probe with a photocleavable, halogen-labelled mass marker. The basic experimental protocol has been established to utilize these probes in a mass spectrometry-based hybridization assay. All three types of PNA probe have been successfully used for detection of point mutations. Mass markers are essential to improve the mass difference between similar PNA mass probes. The assay based on PNA probes linked to low molecular-weight charged photocleavable tags is promising and optimisation of this could yield a practical method of SNP/point mutation analysis.

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