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ARTICLE *in* ANALYTICAL CHEMISTRY · OCTOBER 2008

Impact Factor: 5.64 · DOI: 10.1021/ac801336q · Source: PubMed

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Multiplex Mass Spectrometric Genotyping of Single Nucleotide Polymorphisms Employing Pyrrolidinyl Peptide Nucleic Acid in Combination with Ion-Exchange Capture

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A new ion-exchange capture technique is introduced for label-free sample preparation in single nucleotide polymorphism (SNP) genotyping. The DNA sample is hybridized with a new pyrrolidinyl peptide nucleic acid (PNA) probe and treated with a strong anion exchanger. The complementary PNA·DNA hybrid is selectively captured by the anion exchanger in the presence of noncomplementary or unhybridized PNA, allowing direct detection of the hybridization event on the anion exchanger by MALDI-TOF mass spectrometry after simple washing. The high specificity of the pyrrolidinyl PNA allows simultaneous multiplex SNP typing to be carried out at room temperature without the need for enzyme treatment or heating. Exemplary applications of this technique, in the identification of meat species in feedstuffs and in multiplex SNP typing of the human *IL-10* gene promoter region are demonstrated, clearly suggesting the potential for much broader applications.

Determination of the sequence of a specific region of DNA samples has a broad range of applications ranging from medical and forensic to agriculture and food sciences. Because of its importance, a number of techniques have been developed for such purposes.[1,2] For certain applications, such as the typing of single nucleotide polymorphisms (SNP), it is necessary to be able to analyze the samples with perfect accuracy, inexpensively, and in a high-throughput fashion. These requirements are not always possible with traditional sequencing or other gel-based assays. Among many new techniques being constantly developed, mass spectrometric analysis of DNA samples, either directly or in the presence of a probe, offers an attractive advantage from a high-throughput perspective;[3−6] however, the polyanionic and fragile nature of DNA usually results in poor sensitivity and complicates their mass spectrometric analysis.[3−6]

Peptide nucleic acid (PNA, **1**) is a neutral-backbone analogue of DNA that shows strong affinity and high specificity in binding to complementary DNA.[7] PNA has been extensively used as a probe for DNA sequence determination, including SNP genotyping.[8] PNA is ideal for use in combination with mass spectrometric based analysis of DNA sequences, since PNA is more resistant to fragmentation and less prone to the formation of alkali metal adducts than DNA.[4,9] In 1997, two research groups reported the use of PNA in combination with MALDI-TOF mass spectrometry in DNA sequence determination, including SNP typing.[10−12] Due to the more efficient ionization of PNA in the positive ion mode compared to DNA, it is possible to attain the selective detection of the PNA mass without the need to separate the PNA probe from the DNA target after hybridization. The success of such analysis, however, relied on a complete separation of the hybridized PNA from the unhybridized PNA probe. This had traditionally been carried out using biotinylated DNA samples in combination with streptavidin-coated magnetic beads, which adds to the cost (and time) of sample preparation. Furthermore, applications of such PNA-based approaches in multiplex SNP analysis were

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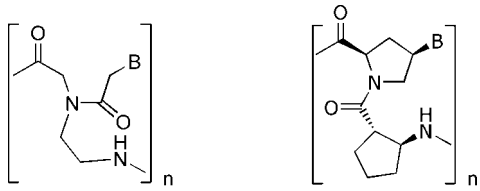
10.1021/ac801336q CCC: $40.75
Published on Web 09/27/2008



1 2

B = A, T, C, G

Figure 1. Structures of PNA.

limited by the variable thermal stabilities of the PNA·DNA hybrids bearing different base sequences.[4,12] Apart from the recent uses of PNA in DNA fingerprinting[13] and in the determination of CpG methylation levels,[14] no other major breakthrough in the field has been reported. Although many other indirect uses of PNA in combination with mass spectrometry for SNP typing have been demonstrated,[15−17] these techniques, however, require specially modified PNA probes and complicated reaction conditions, which may not be suitable for high-throughput applications.

We have recently introduced a new conformationally rigid pyrrolidinyl PNA (**2**) (Figure 1), which can hybridize to DNA with exceptionally high affinity and specificity.[18,19] Here, we examine the suitability of this new PNA system as a probe for DNA sequence analysis, especially in mass spectrometric-based SNP typing. To attain reliable high throughput analysis, we also demonstrate a new and simplified sample preparation technique that is based on the intrinsic charge difference between PNA and its hybrid with DNA. Absorption of DNA by insoluble positively charged polymers is very well-known and has often been used for DNA purification.[20−22] Since the PNA backbone is uncharged, it cannot be absorbed by a strong anion exchanger unless first hybridized with its complementary DNA target to form a negatively charged PNA·DNA hybrid. The support-bound hybridized PNA probe can easily be separated from the unhybridized probe and other impurities in the sample matrix, such as proteins and salts by simple washing (Figure 2). The presence of the hybridized PNA on the support can then be analyzed directly by MALDI-TOF mass spectrometry. Such anion-exchange capture technique should greatly simplify and reduce the cost of sample preparation because anion exchangers are inexpensive and the crude nonbiotinylated PCR products can be used without prior purification. Finally, we demonstrate the application of this technique in two relatively diverse applications; namely, the identification of meat species in feedstuffs, and in multiplex SNP typing of the human *IL-10* gene promoter region, the success of which suggests the potential applicability and benefits of this method may be widespread.

EXPERIMENTAL SECTION

Synthesis of PNA Probes. PNA oligomers were synthesized according to the previously reported Fmoc solid-phase peptide synthesis protocol.[18,19] After removal of the final Fmoc group and the nucleobase side-chain protection, end-capping was carried out using an appropriate modifier [Fmoc-Arg(Mtr)OH/HATU/DIEA for arginine or 4-carboxybutylphosphonium bromide *N*-hydroxysuccinimide ester/DIEA for phosphonium labeling]. The crude PNA oligomer was cleaved from the solid support by treatment with trifluoroacetic acid (TFA) and purified using reversed-phase HPLC with UV detection at 260 nm. A Varian Polaris C_{18} (3 μm particle size 4.6 × 50 mm) analytical HPLC column was used and eluted with a gradient of 0.1% TFA in acetonitrile and 0.1% TFA in deionized water. The purified product was collected and characterized by MALDI-TOF mass spectrometry (Table 1).

T_m Measurement. The melting temperatures of hybrids between PNA probes and complementary or single mismatched ssDNA (Bioservice Unit, National Science and Technology Development Agency, Bangkok) were measured on a CARY 100 Bio UV−visible spectrophotometer (Varian) equipped with a thermal melt system. An equimolar mixture of PNA and DNA (1.0 μM each) in sodium phosphate buffer pH 7.0 (10 mM) was placed in a 1 cm path length cuvette. The A_{260} was recorded in steps from 20 to 90 °C in two heating and one cooling cycles (20−90−20−90) with a temperature ramp of 1.0 °C/min and a hold time of 10 min at the beginning and the end of each cycle. The temperature was corrected for the difference between the block temperature and the built-in temperature probe and data was taken from only the last heating cycle and normalized by dividing the absorbance at each temperature by the initial absorbance. The T_m was obtained from the first derivative plot after smoothing using KaliedaGraph 3.6 (Synergy Software) and Microsoft Excel 2000. The T_m values from three independent experiments were accurate to within ±0.5 °C. The T_m data for relevant PNA·DNA hybrids are shown in Table 2.

Isolation of DNA and Preparation of PCR Products. *(a) DNA Samples for Meat Species Identification.* DNA from 14 meat and feedstuff samples obtained from commercial sources (Table 3) was extracted as described in the DNA Blood Kit manufacturer's (Qiagen) protocol starting from 300 mg of meat samples. The design of the primers and probes for detection of cytochrome *b* polymorphisms was based on the aligned sequences of mitochondrial DNA for cytochrome *b* of bovine and porcine species using Clustal W2 and BLASTN analysis (Figure S1 of the Supporting Information) of the NCBI database.[23,24] A target region of 53 bp (nucleotides 628−680) containing a single diagnostic T/C difference at nucleotide position 654 was PCR-amplified using 5′-GGAATCTCMTCAGAC-3′ and 5′-TTAATAGTGTAGTA-3′ as primers. PCR reactions (20 μL), containing 1 μL of DNA, 2 units of Taq DNA polymerase (Promega), 2.5 mM $MgCl_2$, 0.2 mM of each

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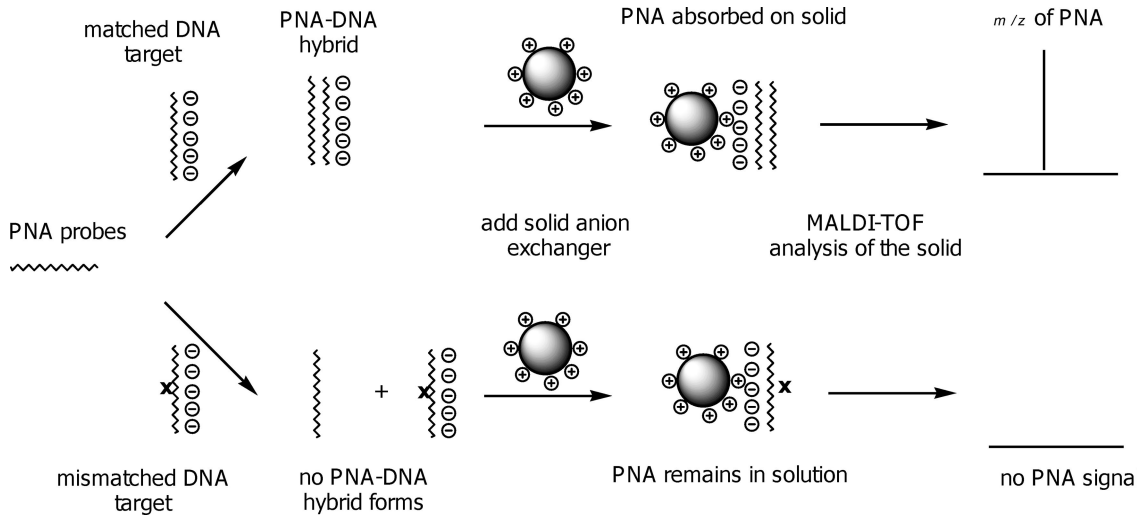

Figure 2. Schematic diagram showing the concept of ion-exchange capture of PNA in combination with MALDI-TOF mass spectrometry in DNA sequence determination.

Table 1. PNA Sequences Used in This Study

code	sequence[a]	m/z[b] (calcd)	m/z (found)	note
P1	Ac-TTTTTTTTT-LysNH$_2$	3179.4	3179.7	test probe
P2	Ac-GTAGATCACT-SerNH$_2$	3517.8	3517.6	test probe
P3a	Ac-TGTACGTCACAACTA-LysNH$_2$	5208.6	5209.4	test probe
P3b	Ac-TGTACGTAACAACTA-LysNH$_2$	5232.6	5232.4	test probe
P4a	Ac-Arg-AAAATCCCATT-SerNH$_2$	3961.2	3961.5	bovine probe
P4b	Ac-Arg-AAAATTCCATT-SerNH$_2$	3976.2	3976.4	porcine probe
P5a	P-TTCCCCCTCCCAA-SerNH$_2$	4667.8	4668.0	*IL-10*−1082G probe
P5b	P-TTCCCCTTCCCAA-SerNH$_2$	4682.8	4683.3	*IL-10*−1082A probe
P6a	P-ATGTAACATCTCT-SerNH$_2$	4785.9	4785.8	*IL-10*−819C probe
P6b	P-ATGTAATATCTCT-SerNH$_2$	4800.9	4801.4	*IL-10*−819T probe
P7a	P-GCCTGTCCTGTAG-SerNH$_2$	4818.9	4818.6	*IL-10*−592C probe
P7b	P-GCCTGTACTGTAG-SerNH$_2$	4842.9	4843.0	*IL-10*−592A probe

[a] P: 4-carboxybutylphosphonium ($Ph_3P^+CH_2CH_2CH_2CH_2CO$-) charge tag. [b] Av mass: M + H$^+$ for **P1**−**P4**, M$^+$ for **P5**−**P7**.

dNTP and 2 μM of each primer, were carried out under the following conditions: 3 min at 94 °C; 35 cycles of 30 s at 94 °C; 1 min at 50 °C; 40 s at 73 °C; and finally, 10 min at 73 °C. All samples were evaluated with the new method and independently confirmed by both PCR−RFLP analysis and the use of an electrochemical biosensor.[25,26]

(b) DNA Samples for Human IL-10 *SNP Identification.* Genomic DNA samples from 10 healthy individuals were extracted from buffy coat by salting out as described.[27] A 550 bp region that contains three diagnostic SNP sites (−1082, −819, and −592; see Figure S2 of the Supporting Information) of the *IL-10* gene promoter was amplified by using 5′-CACTACTAAGGCTTCTTTGG-3′ and 5′-CCCTTCCATTTTACTTTCC-3′ primers. PCR reactions (5 μL), consisting of 25 ng DNA, 0.25 μM of each primer, 3 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.125 unit of Taq DNA polymerase (Promega) with 1× reaction buffer, were performed at 2 min at 94 °C, followed by 35 cycles of 20 s at 94 °C; 50 s at 60 °C; 20 s at 72 °C; and finally, 7 min at 72 °C. All samples were blindly sent to test with the new method and independently verified by automated sequencing.

MALDI-TOF Mass Spectrometry. MALDI-TOF mass spectra were obtained on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics). One microliter of the sample was mixed with 10 μL of the matrix solution consisting of α-cyano-4-hydroxycinnamic acid (CCA) in 0.1% TFA in acetonitrile/water (1:2) solution. One microliter of this sample mixture was spotted onto the target, allowed to dry, and analyzed in positive ion linear time-of-fight mode with an accelerating voltage of +20 kV. All spectra were processed by averaging between 20 and 30 individual laser shots. External mass calibration was performed using three standard PNAs of known molecular weight covering the mass range between 1850.0 and 5208.6 Da.

PNA·DNA Hybridization and Ion-Exchange Capture Experiments. *(a) Single-Stranded (Synthetic) DNA.* The strong anion exchanger Q-Sepharose (GE Healthcare) was washed with deionized water and twice with binding buffer (10 mM sodium phosphate, pH 7.0) and resuspended in the same buffer. Equimolar quantities of the PNA probe (or probe mixture) and DNA sample (1−10 pmol each) were mixed in 30 μL of binding buffer and incubated at room temperature (30 °C) for 20 min. The pre-equilibrated anion exchanger (3 μL, ∼3 mg) was then added to the PNA·DNA hybrid solution, incubated for 20 min at room temperature, and then centrifugally washed with 3 × 100 μL of deionized water or aqueous acetonitrile (3−15% v/v) at room temperature. Finally, 1 μL aliquots of the washed anion exchanger

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Table 2. T_m data of PNA·DNA hybrids relevant to this study

code	sequence	PNA target	T_m (°C)[a]	ΔT_m (°C)[b]
D1	5′-AAAAAAAAA-3′	**P1**, complementary	74.9	
D2a	5′-AGTGATCTAC-3′	**P2**, complementary	54.8	
D2b	5′-AGTGAACTAC-3′	**P2**, single mismatch	37.0	−17.8
D2c	5′-AGTGAGCTAC-3′	**P2**, single mismatch	34.0	−20.8
D2d	5′-AGTGACCTAC-3′	**P2**, single mismatch	39.0	−15.8
D3a	5′-TAGTTGTGACGTACA-3′	**P3a**, complementary	78.6	
		P3b, single mismatch	60.5	−18.4
D3b	5′-TAGTTGTAACGTACA-3′	**P3a**, single mismatch	62.9	−15.7
D3c	5′-TAGTTGTCACGTACA-3′	**P3a**, single mismatch	63.1	−15.5
D3d	5′-TAGTTGTTACGTACA-3′	**P3a**, single mismatch	61.1	−17.5
		P3b, complementary	78.9	
D4a	5′-AATGGGATTTT-3′	**P4a**, complementary	63.1	
		P4b, single mismatch	37.8	−28.1
D4b	5′-AATGGAATTTT-3′	**P4a**, single mismatch	38.7	−24.4
		P4b, complementary	65.9	
D5a	5′-TTGGGAGGGGGAA-3′	**P5a**, complementary	63.8	
		P5b, single mismatch	32.8	−35.1
D5b	5′-TTGGGAAGGGGAA-3′	**P5a**, single mismatch	32.4	−31.4
		P5b, complementary	67.9	
D6a	5′-AGAGATGTTACAT-3′	**P6a**, complementary	72.6	
		P6b, single mismatch	43.9	−31.9
D6b	5′-AGAGATATTACAT-3′	**P6a**, single mismatch	52.8	−19.8
		P6b, complementary	75.8	
D7a	5′-CTACAGGACAGGC-3′	**P7a**, complementary	75.6	
		P7b, single mismatch	51.5	−16.2
D7b	5′-CTACAGTACAGGC-3′	**P7a**, single mismatch	44.5	−31.1
		P7b, complementary	67.7	

[a] Conditions: 10 mM sodium phosphate buffer pH 7.0, [PNA] = [DNA] = 1.0 μM. [b] $\Delta T_m = T_m$(complementary) − T_m (mismatched) (with respect to the same PNA probe).

Table 3. The Results of DNA Samples Obtained from Meat Products and Feedstuffs of Different Origins Analyzed by This PNA Method and Conventional Methods

sample	description	observed m/z (error, ppm)	results	independent analysis[a]
D4a	bovine positive control (synthetic)	3961.1 (−25.2)	bovine	bovine
D4b	porcine positive control (synthetic)	3976.5 (+75.4)	porcine	porcine
FS-1	cloned target DNA (bovine positive)	3961.4 (+50.4)	bovine	bovine
FS-2	soybean genomic DNA	none	negative	negative
FS-3	nontemplate control	none	negative	negative
FS-4	poultry meat (Thailand)	none	negative	negative
FS-5	ruminant meat (Thailand)	3961.5 (+75.7)	bovine	bovine
FS-6	pork meat (Thailand)	3976.3 (+25.1)	porcine	porcine
FS-7	porcine bone meal (Netherlands)	3976.5 (+75.4)	porcine	porcine
FS-8	hydrolyzed feather (Australia)	none	negative	negative
FS-9	poultry blood meal (Australia)	none	negative	negative
FS-10	poultry meal (France)	none	negative	negative
FS-11	pet food paste 7% protein (bovine and poultry, Thailand)	3961.4 (+50.4)	bovine	bovine
FS-12	pet food paste 8% protein (bovine and poultry, France)	3961.3 (+25.2)	bovine	bovine
FS-13	Actipro 95PHS (porcine and poultry, Netherlands)	3975.9 (−75.4)	porcine	porcine
FS-14	pet food bar (poultry, Thailand)	none	negative	negative

[a] Based on PCR−RFLP and electrochemical biosensor.[14,15]

was removed for analysis by MALDI-TOF mass spectrometry as described above. The analysis was carried out in duplicate for each experiment.

(b) Double-Stranded Synthetic DNA and PCR Samples. The anion exchanger was pre-equilibrated in binding buffer as described above. Double-stranded DNA (1−10 pmol for synthetic DNA or 5 μL for PCR samples containing the same amounts of DNA) in 30 μL of binding buffer was denatured by heating in a boiling water bath for 10 min and rapidly cooled down to 0 °C, and the pre-equilibrated anion exchanger (3 μL) was immediately added and mixed. After incubation at room temperature (30 °C) for 20 min, the anion exchanger was washed with 10 mM phosphate buffer pH 4.0 (2 × 200 μL), followed by binding buffer (2 × 200 μL). For hybridization, the PNA probe or probes mixture (1−10 pmol each) was added to the support-bound DNA suspended in 30 μL of the binding buffer. Incubation, washing, and MALDI-TOF MS analysis was then performed as described above.

RESULTS AND DISCUSSION

Validation of the Concept. To validate the anion-exchange capture concept shown in Figure 2, two very different model PNA probes (**P1** and **P2**, 100 pmol each; see Table 1 and 2 for sequences of PNA and DNA used in this study) were mixed in 30 μL of binding buffer and hybridized with the DNA target (**D1** or **D2a**, 200 pmol), followed by addition of a silica-based quaternary ammonium-type anion exchange support (BondElute

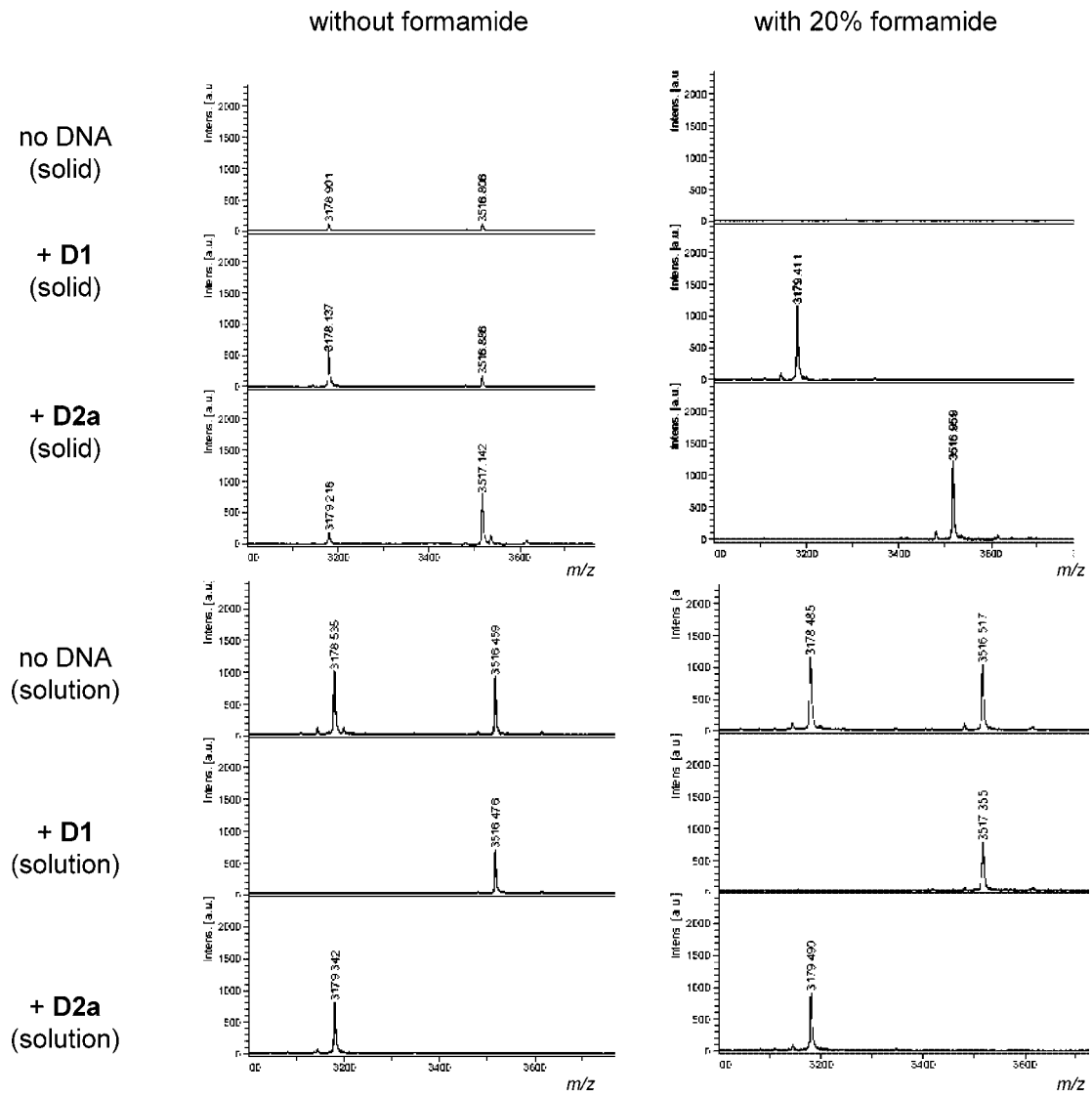

Figure 3. MALDI-TOF analysis of a mixture of two PNA probes, **P1** and **P2**, after hybridization with the DNA target **D1** or **D2a**, followed by addition of the anion exchanger (SAX) in the absence (left) or presence (right) of formamide (20% v/v). Experiments from top to bottom: no DNA (solid), +**D1** (solid), +**D2a** (solid), no DNA (solution), +**D1** (solution), +**D2a** (solution). Hybridization conditions: PNA 100 pmol and DNA 200 pmol in 30 μL binding buffer at 30 °C.

SAX, Varian). MALDI-TOF analysis of the support after washing with deionized water revealed mainly the signal of the PNA probe that is complementary to the DNA target, revealing the specificity of the hybridization (Figure 3, left). No signal of the DNA target was observed, which is most likely due to the much better ionization of PNA than DNA under the MALDI-TOF analysis conditions.[11] Nevertheless, substantial background absorption of the noncomplementary probes on the ion-exchange support was also observed, even in the absence of any DNA, possibly through hydrophobic interactions. Inclusion of 20% (v/v) formamide in the hybridization buffer essentially removed nonspecific binding, yielding a clear differentiation between complementary and noncomplementary DNA targets (Figure 3, right). Analysis of the remaining solutions revealed that the complementary PNA probes were quantitatively absorbed, leaving the noncomplementary probes in the solution.

Optimization of Conditions To Detect Single Base Mismatches in Single-Stranded DNA Samples. A more practical approach to avoid such nonspecific absorption is to use a less hydrophobic anion exchanger. When Q-Sepharose was used as the ion exchange support,[28] the addition of formamide was no longer necessary, which simplified the experimental set up even further. With a C-terminal-lysine-labeled PNA as the probe, it was possible to reduce the quantity of the PNA probes and DNA samples to 10 pmol while a reasonable signal-to-noise ratio was still obtained (data not shown). These conditions were tested with the more stringent discriminating between single base pair mismatched probes and DNA samples. Although simple aqueous washing was generally sufficient to remove any nonspecifically bound PNA probes, it was advantageous to wash the support after hybridization with 3−15% (v/v) aqueous acetonitrile when longer probe sequences (e.g., **P3a**) were used. In all cases, satisfactory discrimination of single-base mismatches was achieved at *room temperature* (30 °C) with very low background levels after the

(28) Other hydrophilic anion exchangers successfully employed with low nonspecific absorption include DEAE-Sepharose, DEAE cellulose, and chitosan. Q-Sepharose was preferred on the basis of its high ionic capacity and broad working pH range.

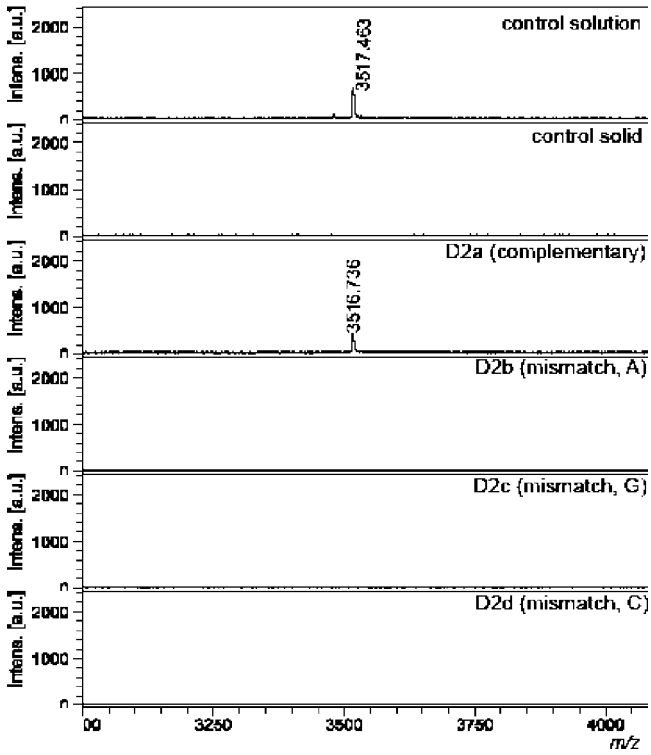

Figure 4. Discrimination between complementary and single-mismatched DNA targets by the 10-mer PNA probe **P2** using Q-Sepharose as the anion exchange support. Hybridization conditions: PNA 10 pmol and DNA 10 pmol in 30 μL binding buffer at 30 °C. Experiments from top to bottom: no DNA target (solution, positive control), no DNA target (solid, negative control), +**D2a** (complementary), +**D2b** (A·A mismatch), +**D2c** (A·G mismatch), **D2d** (A·C mismatch).

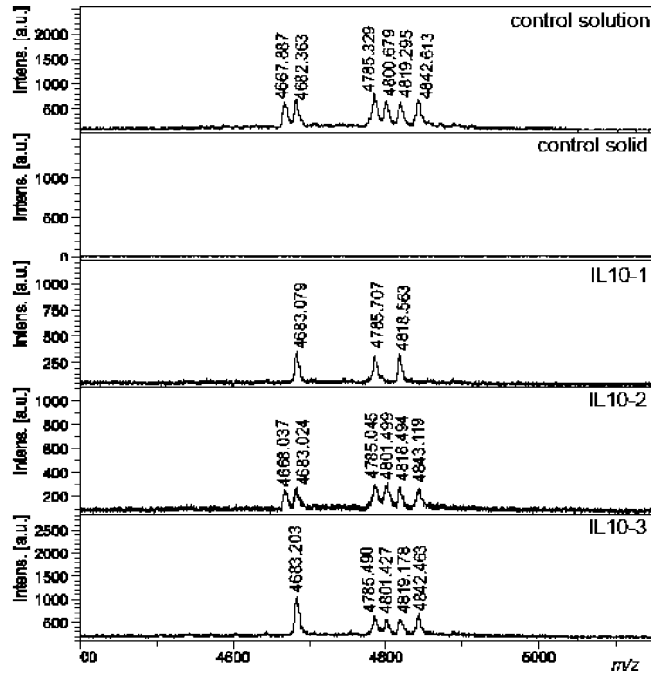

Figure 5. Simultaneous multiplex typing of the human *IL-10* gene promoter at SNP positions −1082, −819, and −592 using a combination of six PNA probes. Hybridization conditions: PNA 1 pmol each and DNA sample (from PCR) ~5 pmol in 30 μL binding buffer at 30 °C. Experiments from top to bottom: no DNA target (solution, positive control), no DNA target (solid, negative control), +**IL10-1** (−1082 A/A, −819 C/C, −592 C/C), +**IL10-2** (−1082 G/A, −819 C/T, −592 A/C), +**IL10-3** (−1082 A/A, −819 C/T, −592 A/C).

washing. A representative example, demonstrating the absolute specificity of the 10-mer PNA probe (**P2**) in distinguishing between complementary and various types of single-mismatch DNA, is shown in Figure 4. A similarly high level of specificity was also observed with the 15-mer PNA probes (e.g., **P3a**, Figure S3 of the Supporting Information). In all cases, PNA probes were detected when their sequences were complementary to that of the DNA targets, with specificity against all single-base-mismatched targets.

Although the stability difference between the complementary and single-mismatched PNA·DNA hybrids is high (ΔT_m 16−21 °C, Table 2), the high level of specificity observed seems surprising, considering the appreciable stability of the single-mismatch hybrids (T_m single-mismatch hybrids of **P3a**·DNA at ~61−63 °C is 31−33 °C above the hybridization and washing temperature). We assume that washing removes the nonspecifically bound probes or destroys any mismatched PNA·DNA hybrids that might have formed.[29,30] Importantly, all previously reported related works[10−12,14] require the hybridization or washing steps be performed at an elevated temperature, even when relatively short PNA probes (9−15-mers) were used. Furthermore, the optimal hybridization and washing conditions had to be determined for each experiment, depending on the length and sequence of the PNA. As an alternative to high temperature, single-strand specific nucleases have been used to increase the specificity of single-mismatch detection involving PNA probes,[31−34] allowing the experiments to be carried out at the same temperature, but requiring that the conditions are carefully controlled to avoid overdigestion of the complementary PNA·DNA hybrids. Furthermore, the cost of the enzyme increases the cost of the assay as well as increases the time and reduces the throughput of samples. The ability to distinguish a single-mismatched base at room temperature without enzymes, as shown in this case, is a significant advantage of this new PNA system over the conventional PNA. Of course, heating or enzymatic digestion may be ultimately required to achieve the required high level of specificity with very long probe sequences, but the requirement of PNA probes longer than 17 bases for general SNP typing in the human genome is expected to be rare.

For the purpose of SNP typing, it is preferable to use a combination of two PNA probes, each specific for each type of SNP, to provide self-validation when for a given homozygous sample, only one signal from one specific PNA probe is detected. For heterozygous samples, signals of both probes should be detected with approximately equal intensities. This slightly more exacting criteria was evaluated using two 15-mer PNA probes

(29) The stability of DNA·DNA hybrids is known to decrease in the presence of organic solvents. See Fiandaca, M.; Oliveira, K.; Stender, H. Patent Application WO2005/121373 A2; December 22, 2005.

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Table 4. Results of the Multiplex Analysis of the Three SNP Sites in the Human *IL-10* Promoter Region in DNA Samples Obtained from 10 Different Individuals

sample	observed m/z (error, ppm)	results	independent analysis[a]
IL10-1	4683.1 (+64.0)	−1082 A/A	−1082 A/A
	4785.7 (−41.7)	−819 C/C	−819 C/C
	4818.6 (−62.2)	−592 C/C	−592 C/C
IL10-2	4667.7 (−21.4), 4683.0 (+42.7)	−1082 G/A	−1082 G/A
	4785.2 (−125.4), 4801.5 (+124.1)	−819 C/T	−819 C/T
	4819.2 (+62.9), 4843.1 (+41.2)	−592 A/C	−592 A/C
IL10-3	4683.2 (+85.4)	−1082 A/A	−1082 A/A
	4785.5 (−83.5), 4801.4 (+104.1)	−819 C/T	−819 C/T
	4819.2 (+62.2), 4842.5 (−82.5)	−592 A/C	−592 A/C
IL10-4	4682.7 (−42.7)	−1082 A/A	−1082 A/A
	4800.5 (−83.3)	−819 T/T	−819 T/T
	4843.1 (+41.2)	−592 A/A	−592 A/A
IL10-5	4683.3 (+106.7)	−1082 A/A	−1082 A/A
	4785.5 (−83.5),4800.3 (−104.1)	−819 C/T	−819 C/T
	4819.3 (+83.0), 4843.0 (+20.6)	−592 A/C	−592 A/C
IL10-6	4683.3 (+106.7)	−1082 A/A	−1082 A/A
	4800.7 (−41.6)	−819 T/T	−819 T/T
	4842.5 (−82.5)	−592 A/A	−592 A/A
IL10-7	4682.5 (−64.0)	−1082 A/A	−1082 A/A
	4801.2 (+62.4)	−819 T/T	−819 T/T
	4842.7 (−41.2)	−592 A/A	−592 A/A
IL10-8	4668.0 (+42.8), 4683.0 (+42.7)	−1082 A/G	−1082 A/G
	4786.0 (+20.8), 4801.4 (+104.1)	−819 C/T	−819 C/T
	4818.5 (−83.0), 4843.1 (+41.2)	−592 A/C	−592 A/C
IL10-9	4667.4 (−85.6), 4683.0 (+42.7)	−1082 A/G	−1082 A/G
	4785.7 (−41.7), 4801.2 (+62.4)	−819 C/T	−819 C/T
	4819.3 (+83.0), 4843.1 (+41.2)	−592 A/C	−592 A/C
IL10-10	4682.6 (+42.7)	−1082 A/A	−1082 A/A
	4785.7 (−41.7)	−819 C/C	−819 C/C
	4818.5 (−83.0)	−592 C/C	−592 C/C

[a] Based on automated sequencing.

containing one mismatch position in the middle of the strands (**P3a** and **P3b**), and clearly demonstrated that the technique could discriminate between complementary and single-mismatched DNA targets with high fidelity (Figure S4 of the Supporting Information).

Optimization of Conditions To Detect Single Base Mismatches in Double-Stranded DNA Samples. These studies were performed with synthetic ssDNA, whereas native DNA or DNA samples derived from standard PCR are double-stranded, requiring an appropriate means to dissociate the duplex into single-stranded DNA. One quick but reliable approach is heating of the DNA sample, followed by fast cooling prior to the hybridization, in which the higher stability of PNA·DNA hybrids under low salt conditions, compared to the corresponding DNA·DNA hybrid, should facilitate this process. The applicability of this method to dsDNA samples was evaluated using synthetic double-stranded 15-mer DNA based on the **D3a** (ds**D3a**) and **D3d** (ds**D3d**) sequences. Following the usual protocol, except with the additional heating step followed by fast cooling just before hybridization with the PNA probe **P3a**, it was possible to distinguish between the complementary (ds**D3a**) and single-mismatched (ds**D3d**) dsDNA sequences with results comparable to the corresponding single-stranded DNA (see Figure S5 of the Supporting Information). The order of hybridization and absorption steps could be reversed without any observable difference. With dsDNA obtained from PCR amplifications, it was preferable to first heat the dsDNA and immediately trap the ssDNA by the anion exchange support before hybridization with the PNA probe, since this allowed extensive washing of the support-bound DNA to remove any absorbed proteins and other impurities which could complicate subsequent analyses. Typically, this washing step was carried out at pH 4.0 to convert any negatively charged proteins to their neutral or protonated forms to facilitate their removal from the anion exchanger. In effect, the DNA sample was purified in situ on the basis of the charge differences between the DNA and the impurities without the need for biotin−streptavidin affinity capture or other (expensive) commercial purification kits. Subsequent hybridization of the support-bound DNA with the PNA probe took place as efficiently as in solution, as judged by the similar intensities of the mass signals of the absorbed PNA probe in both cases (data not shown). This suggested that ssDNA is still recognized by the PNA probe after immobilization on the anion exchange support.

Applications in Identification of Meat Samples. With dsDNA sequence detection established, we investigated the suitability of this technique to solve real problems. One such application is the identification of meat species in feedstuffs to ensure safety due to the risk of bovine spongiform encephalopathy according to Directive 98/88/EC and Decision 2000/766/EC.[35,36] Detection of bovine species in feedstuffs is based on DNA sequence analysis using a fragment of the mitochondrial cytochrome *b* gene from bovine (*Bos taurus*) and porcine (*Sus scrofa*) (Figure S1 of the Supporting Information). The standard process of PCR-RFLP[25] was replaced by the detection of a diagnostic, between bovine and porcine, SNP in a fragment of the cytochrome *b* gene using PNA probes after PCR amplification, using the arginine-labeled PNA probes, **P4a** and **P4b**, specific for bovine and porcine species, respectively. As a control, the ability of each

probe to recognize their cDNA was confirmed using the synthetic oligonucleotides **D4a** and **D4b**. MALDI-TOF analysis of the ion exchange support after treatment with an equimolar mixture of **P4a** and **P4b** and **D4a**, according to the standard protocol for ssDNA, revealed only the signal of **P4a**. Similar treatment but with **D4b** instead of **D4a** resulted in the detection of only the signal of **P4b** (Figure S6 of the Supporting Information). Thus, the signal of **P4a** and **P4b** would indicate the presence of bovine and porcine DNA, respectively, in the sample. Following the protocol for analysis of dsDNA, a total of 14 DNA samples from meats and feedstuffs were analyzed. The results were in complete agreement with the species of origin of the samples and with two independent analysis techniques[25,26] (Table 3 and Figure S6 of the Supporting Information). With a single external calibration at the beginning of the experiment, a mass error range of <100 ppm was obtained, more than sufficient to unambiguously distinguish between the two PNA probes in the whole set of experiments.[37] Reproducibility between runs was also excellent (standard deviation for 10 independent measurements = 0.246 Da). The detection of animal meat origins here is only an example and could easily be applied to, for example, detection of transgenic crops and so on.

Applications in SNP Typing of *IL-10* Promoter Region. Another application of the present method is demonstrated in the simultaneous multiplex SNP typing of three different positions in the promoter region of the human interleukin-10 (*IL-10*) gene (−1082G/A, −819T/C, and −592A/C; see Figure S2 of the Supporting Information for DNA sequences). Multiple SNPs within the promoter of *IL-10* gene have been reported that are both correlated with different expression levels of this gene and associated with, and thus markers for, autoimmune diseases such as systemic lupus erythematosus[38] and psoriasis.[39] A low-cost and sensitive genotyping method that can haplotype these SNP sets within one assay should be very useful and could be applied to clinical use. Six PNA probes, **P5a**, **P5b**, **P6a**, **P6b**, **P7a** and **P7b**, corresponding to these three SNPs, were synthesized and labeled with a permanent charge tag (carboxybutyltriphenylphosphonium) to increase the sensitivity of the detection further.[13] By labeling of the PNA probe with the phosphonium charge tag, the amount of PNA probe and DNA sample required for one hybridization experiment (which was sufficient for several mass spectrometric analyses) could be reduced to 10 fmol/30 μL reaction mixture while a clear signal was still observed (S/N = 17.6; see Figure S7 of the Supporting Information). This is orders of magnitude more sensitive than the arginine and lysine-labeled probes previously employed. In practice, considerably larger amounts of probes and samples (1−5 pmol/30 μL) were purposely used to ensure high-quality spectra. Even in such cases, the amounts of PNA and DNA used compares favorably with other reported methods.[10−12] Indeed, since 1 nmol of the PNA probe is sufficient for the analysis of ∼1000 DNA samples, the cost of the PNA probes will contribute very little to the cost of the analysis.

Initially, two out of three SNP sites (−1082 and −819) were investigated separately with both real DNA samples and, as a control, synthetic DNA standards. All singleplex experiments revealed a clear and unambiguous signal corresponding only to the expected PNA probe. Testing with two real human DNA samples from two homozygous individuals with known genotypes (coded **N2** and **S64**) also resulted in attained PNA signals that were in accord with the sequencing results (Figures S8 and S9 of the Supporting Information), supporting the applicability of this technique. The possibility to perform the more challenging but useful simultaneous multiplex analysis was further investigated. The success of such multiplex analysis depends upon the relative stabilities of all complementary and single mismatched PNA·DNA hybrids involved. With six different PNA probes, 12 combinations (6 complementary and 6 single-mismatched pairs) are possible (Table 2). Because the thermal stabilities of conventional PNA·DNA hybrids are highly sequence-dependent, simultaneous multiplex SNP typing under one general condition is difficult, and previous attempts proved only marginally successful,[10,14] resulting in such analyses being carried out in separate reactions instead.[12] On the other hand, simultaneous PNA-based multiplex analysis of DNA samples which differ by more than one base were usually straightforward.[11,14] Unlike the conventional PNA (**1**), the stability of PNA·DNA hybrids derived from the new pyrrolidinyl PNA (**2**) are not influenced very much by the base composition, and therefore, this new PNA should be particularly suitable as multiplex-compatible probes.[40] The six fully complementary hybrids with a G·C content ranging from 23 to 61% had a T_m in the range of 64−76 °C, compared with 32−53 °C for the six single-mismatched hybrids (Table 2). The difference in T_m between the complementary hybrid with lowest stability and the single-mismatched hybrid with highest stability was ∼11 °C, which should be sufficient to allow simultaneous multiplexing. The six PNA probes were combined and hybridized with 550-bp DNA targets obtained from PCR amplification of genomic DNA employing two primers designed to cover all three SNP sites. The results, exemplified in Figure 5, clearly show that reliable multiplex SNP analysis is feasible. Hetero- and homozygous samples were unambiguously distinguished by the presence of one or two signals, respectively, in each SNP region. The intensities of the signals from homozygous samples were roughly twice that of heterozygous signals, which suggested possibilities of applying the present technique in quantitative applications,such as determination of the extent of CpG methylation[14] or population genetics of polyploidal species or multiple gene copies that are not subject to molecular drive. As in previous experiments, the mass error range of <150 ppm obtained by means of external calibration was sufficient to allow unambiguous identification of all six PNA

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probes. Analysis of DNA samples from 10 individuals gave results that are in perfect agreement with those obtained from automated sequencing (Table 4).

CONCLUSION

In conclusion, a new mass spectrometric method for DNA sequence analysis using PNA probes has been developed. The method features the use of a simple and inexpensive ion-exchange capture technique for the isolation of PNA·DNA hybrids from unhybridized PNA probes. Furthermore, the use of the new conformationally restricted pyrrolidinyl PNA (**2**) as a probe allows facile differentiation between complementary and single-base-mismatched 9−15-mer DNA targets at room temperature after simple washing. Applications in unpurified and unlabeled DNA samples obtained from standard PCR mixtures have been demonstrated, including the very challenging simultaneous multiplex SNP typing. Comparable sensitivity to other PNA-based mass spectrometric genotyping was achieved. This method should be useful for rapid, inexpensive, and high-throughput SNP typing and related applications, since all experiments are carried out at room temperature without the need for biotinylation or enzymatic digestion.

ACKNOWLEDGMENT

This work is financially supported by The Thailand Research Fund and the Commission on Higher Education (RMU4880030 to T.V.); the Radchadapiseksompoj Endowment (to OSRU and LRU); and the National Center of Excellence for Petroleum, Petrochemicals, and Advanced Materials (NCE-PPAM).

SUPPORTING INFORMATION AVAILABLE

DNA sequences, detailed mass spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review June 30, 2008. Accepted August 25, 2008.

AC801336Q