0.0473, $wR_2 = 0.0917$, max/min residual electron density 2.057/ −1.062 e Å^{-3}. Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication nos. CCDC-163342 (**1**) and CCDC-163343 (**2**). Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

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Mass-Spectrometric Monitoring of a PNA-Based Ligation Reaction for the Multiplex Detection of DNA Single-Nucleotide Polymorphisms**

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The genetic variation that occurs most frequently is the exchange of single nucleobases (single-nucleotide polymorphisms, SNP).[1, 2] These single-base mutations are associated with diseases, such as cystic fibrosis,[3] familial hypercholesterolemia,[4] cancer,[5] and many others. DNA diagnostic assays depend on a binding event between the target DNA and a complementary oligonucleotide probe. The hybridization alone, however, barely exhibits sufficient selectivity in distinguishing matched from single-base mismatched DNA targets. An enhancement of the discriminatory power is possible by accommodating a probe-modifying event such as oligonucleotide ligation[6] or primer extension.[7] There is an increasing demand for the development of assay systems in which a single analysis is able to report on more than one diagnostic event (multiplexing). Most commonly fluorescence-based techniques, such as fluorescent dye-terminator-extension,[7] TaqMan assays,[8] molecular beacons,[9, 10] or scorpion probes[11] are used. A drawback is the need for labor- and cost-intensive fluorescence labeling. More importantly, the degree of multiplexing that can be achieved is limited by the number of spectrally resolved fluorophores. In contrast, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass-spectrometry (MS) offers unparalleled resolution and has been demonstrated to enable a genotyping of single-base mutations.[12, 13] However, the fragmentation and the known tendency to form DNA metal-salt adducts can impede the resolving power of MALDI-based detection when oligonucleotides are used as probes.[14, 15] Herein, a novel approach for single-base-mutation detection is proposed that capitalizes upon the use of peptide nucleic acids (PNAs)[16]—a chemically stable and nonionic DNA analogue that combines superior base-pairing properties with ease and accuracy of detection.[17, 18] Contrary to previous reports on PNA-based MALDI-TOF genotyping[19, 20] it is a DNA-controlled chemical ligation of two short probes that is monitored rather than the hybridization of one long probe.[21]

PNA exhibits an unparalleled suitability for MALDI-TOF MS analysis in terms of molecular-weight resolution and accuracy.[19, 20] The discriminative power of PNA-hybridization itself, however, depends on base content, sequence, and particularly on the length of the oligomer. Oligomers that are longer than 16 base pairs are required to provide a unique sequence. However, the longer PNA segments are relatively unselective as DNA binders.[22] Conversely, short-length PNA oligomers exhibit a sufficiently high DNA affinity combined with a very good discrimination for single-base mismatches. We thought that a highly specific sequence analysis of a unique gene segment should be possible by employing a PNA-ligation strategy. Since PNA is not a substrate of any known ligase, any attempt to employ PNA as a ligation probe has to rely solely on chemical methods.

PNA is a pseudopeptide and ligation chemistry can easily be implemented by using amide-bond forming reactions.[21] For the development of a sequence-specific PNA-based ligation reaction we examined the all-PNA model system shown in Scheme 1 and the carbodiimide-mediated condensation of PNA-fragments **1a** and **2a** was investigated. It became apparent that the addition of the complementary template **3a** increased the rate and the yield of the ligation reaction (Figure 1 A). Surprisingly, the presence of template **3b**, which contained a single-base mismatch, gave an even higher increase in the ligation yield.

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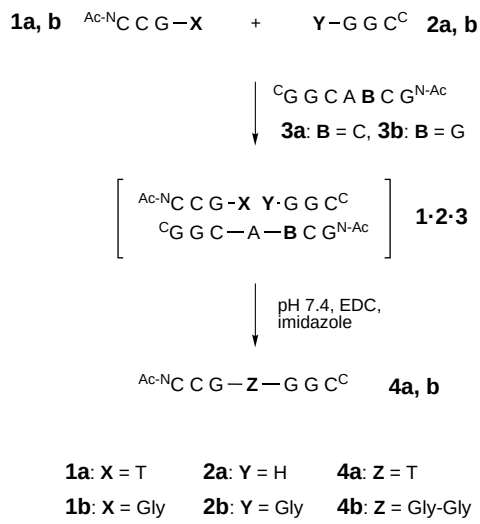


Scheme 1. PNA-fragment condensation of the tetramer **1a** and the trimer **2a** as well as the abasic-site ligation of PNA – glycine conjugates **1b** and **2b** on fully complementary PNA template **3a** and the single-mismatched template **3b** (the mutation site (B) is marked in bold). Concentration of ligation probes **1** and **2** and the PNA templates: 160 μM. (EDC = 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide-hydrochloride).

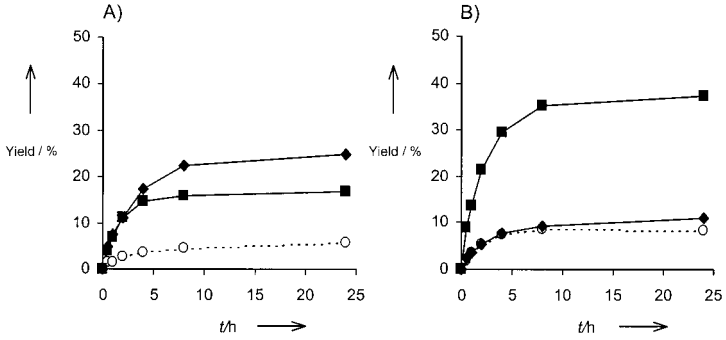
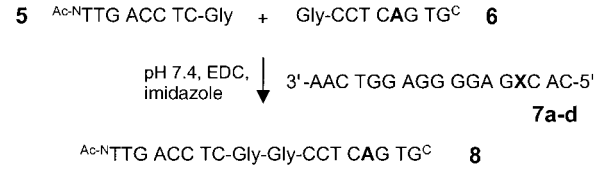


Figure 1. Kinetics of product formation in the presence of match template **3a** (■), mismatch template **3b** (◆), and in the absence of template (○). A) PNA-fragment ligation of **1a** and **2a**; B) abasic-site ligation of PNA – glycine conjugates **1b** and **2b**. Yields of ligation products were determined by subjecting aliquots to high-pressure liquid chromatography (HPLC) analysis.

A solution to the problem of poor template selectivity was found by performing the ligation reaction so that an abasic site was introduced. We speculated that the incorporation of an abasic site would disrupt the cooperative base stacking, thereby rendering the ternary complex **1**·**2**·**3** less tolerant to further disturbances.[23] The abasic site was integrated by replacing a central PNA monomer such that an isosteric dipeptide would be formed upon ligation of two PNA – glycine conjugates, such as **1b** and **2b**. The model experiments indicated the validity of this approach (Figure 1B). The addition of the complementary template **3a** greatly enhanced ligation efficiency and led to the formation of **4b**. More importantly, the ligation displayed a remarkable sequence selectivity: the single-mismatch template **3b** was ineffective in accelerating the formation of PNA – dipeptide hybrid **4b**.

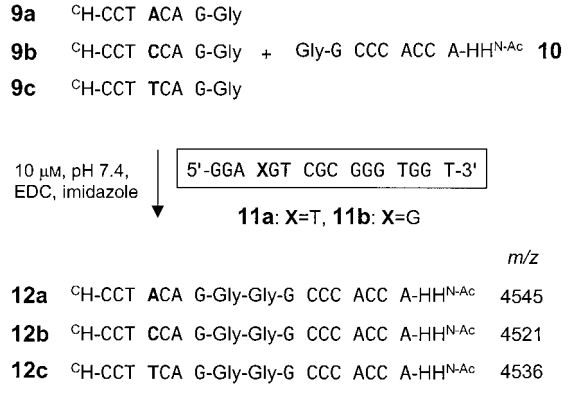
The conditions that enabled a highly sequence-selective ligation in the model system were employed for the MALDI-TOF-based detection of a single-base mutation in a 17mer DNA target.[24] We studied the ligation of the 8mer PNA – glycine conjugates **5** and **6** in the presence of the DNA targets **7a**–**d**. The oligonucleotides **7a**–**d** differ in one central position, each one contains a different nucleobase (Scheme 2). The reactions were performed at a 10 μM



Scheme 2. Abasic-site ligation of PNA – glycine conjugates **5** and **6**. The DNA targets **7** differ in one central position (highlighted in bold, X = T (**7a**), G (**7b**), C (**7c**); A (**7d**)). The PNA base opposite to the mutation site is also in bold. Concentration of ligation probes **5** and **6** and target DNA **7a**–**d**: 10 μM.

concentration of DNA and ligation probes, which completely suppressed the blank reaction. After 6 h the DNA templates were degraded by a treatment with trifluoroacetic acid (TFA). MALDI-TOF MS analysis indicated that only with the fully complementary DNA (X = T) **7a** was a significant peak for a ligation product **8** *m*/*z* 4437 observed (Figure 2). All other DNA templates (X = A, C, G) proved unproductive and only trace amounts of **8** were detected.

To assess the discriminative power of the abasic-site ligation strategy we examined a competition ligation reaction with three PNA – glycine conjugates that would compete with the template. The aim was to detect the G → T mutation in the synthetic DNA segment **11** (Scheme 3). Three nucleophilic



Scheme 3. Detection of a G → T mutation (marked in bold) in the DNA **11**. The three variable PNA – glycine probes **9a**–**c** (probing sites marked in bold) were treated with one common PNA – glycine probe **10** in the presence of either mutant DNA **11a** (X = T) or wild-type DNA **11b** (X = G). Concentration of ligation probes **9a**–**c** and **10** and target DNA **11a**, **b**: 10 μM. (H = histidine).

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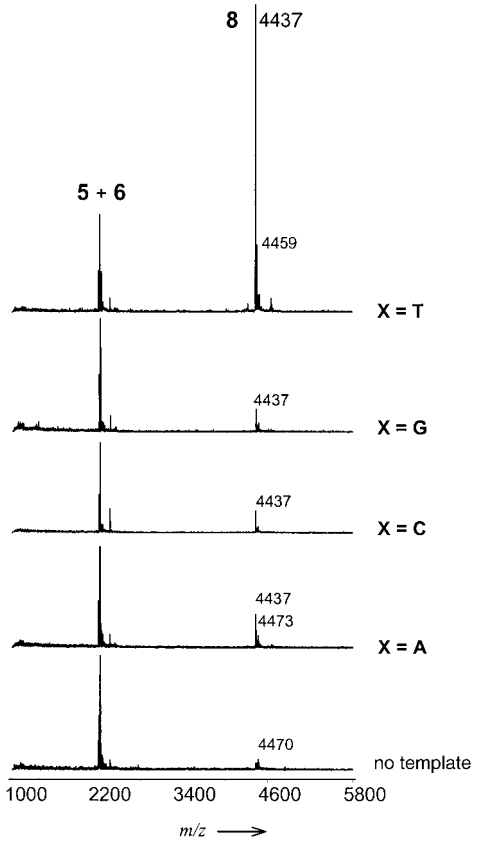

Figure 2. MALDI-TOF MS of the abasic-site ligation of **5** and **6** after 6 h in the presence of DNA templates **7a–d** (*m/z* $[M+H]^+$: 2237 (**5**); 2219 (**6**); 4437 (**8**)). Matrix: sinapinic acid.

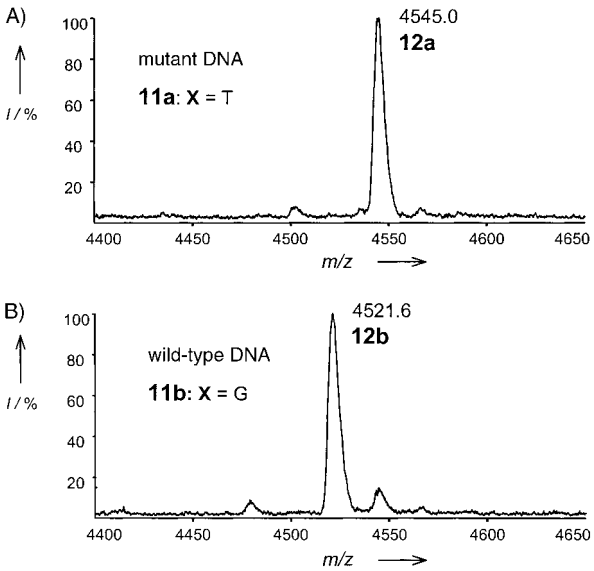

Figure 3. MALDI-TOF MS analysis of the competition ligation for the detection of a single-base mutation in the presence of A) mutant DNA **11a** (X = T) and B) wild-type DNA **11b** (X = G). Single peaks detected corresponded to the ligation product **12a** (*m/z*: 4545 $[M+H]^+$) and the ligation product **12b** (*m/z*: 4521 $[M+H]^+$). Matrix: sinapinic acid.

PNA–glycine conjugates (**9a–c**) served to probe the mutation site and were allowed to react with the acyl-donor segment **10**. The MALDI-TOF analysis revealed that in the presence of the mutant DNA **11a** (X = T) only one of the three possible ligation products was formed (Figure 3). The *m/z* ratio of 4545 corresponded to the molecular mass of the PNA–diglycine hybrid **12a**, which indicated that only the A-containing probe **9a** was involved in the ligation. When the reaction was carried out on the wild-type DNA target **11b** (X = G), predominantly the C-containing product **12b** was observed. The high selectivity of the ligation process was also demonstrated in that the T-containing PNA probe **9c** showed no sign of ligation.

The positive results obtained in the competition ligation encouraged us to investigate whether the high ligation fidelity and the accuracy of mass-spectrometric detection would enable a multiplexed ligation assay to be performed. The goal was to obtain information about three potential mutations in the pig-*ras* gene segment in a single experiment.[25] The mutations examined corresponded to the carcinogenic G12V (G→T), F28L (T→C), and the E63H (G→C, G→T) transformations. Each potential mutation site was probed by a ligation of a common PNA–glycine acyl donor with a wild-type or a mutant PNA–glycine conjugate (Figure 4A). In total, the multiplexed ligation comprised six nucleophilic PNA–glycine probes and three acyl-donor segments such that six specific ligation products and 12 cross-ligation products could form. The multiplex ligation was performed at a 500 nM concentration of DNA and ligation probes. After 4 h the ligation reactions were analyzed by MALDI-TOF MS. Of the 18 ligation products that could form only three were observed when the nine ligation probes were allowed to react with the mutant DNAs (Figure 4B). The ligation events were highly specific and a comparison with the calculated masses of the possible ligation products revealed that significant product formation was observed only for the three mutant ligation products. As a control the multiplex ligation was examined in the presence of the wild-type DNAs. Clearly, the ligation product spectrum showed the occurrence of the wild-type DNAs and the observed molecular masses matched those of the wild-type ligation products (Figure 4C).

To probe the sensitivity of the detection system, the ligation was performed on a mixture of templates corresponding to codon 12 wild-type and mutant *ras* sequences. The mutant DNA was easily detected when present as a mixture of wild-type/mutant-sequences in ratio of 9/1 and at a 500 nM total DNA concentration. The signal from the mutant DNA still exceeded the noise level when the ligation was carried out on a 99/1 mixture of wild-type/mutant-gene segments. On a pure wild-type template, formation of the mutant ligation product was not measurable. These preliminary results indicate that the ligation system may deliver reliable results at 5 nM concentrations of target DNA.

Starting from a simple model system we have devised a DNA-controlled PNA-ligation strategy that has been made single-mismatch specific by "engineering" an abasic site into the ligation site. PNA contrary to DNA is stable against acid hydrolysis. This acid stability was employed to remove DNA-based background signals, thereby eliminating the need to perform solid-phase based hybridization techniques. From the data acquired with six different sequences we deduce that the nucleobase opposite to the glycylglycine abasic site plays no important role since adenine, guanine, and thymine were

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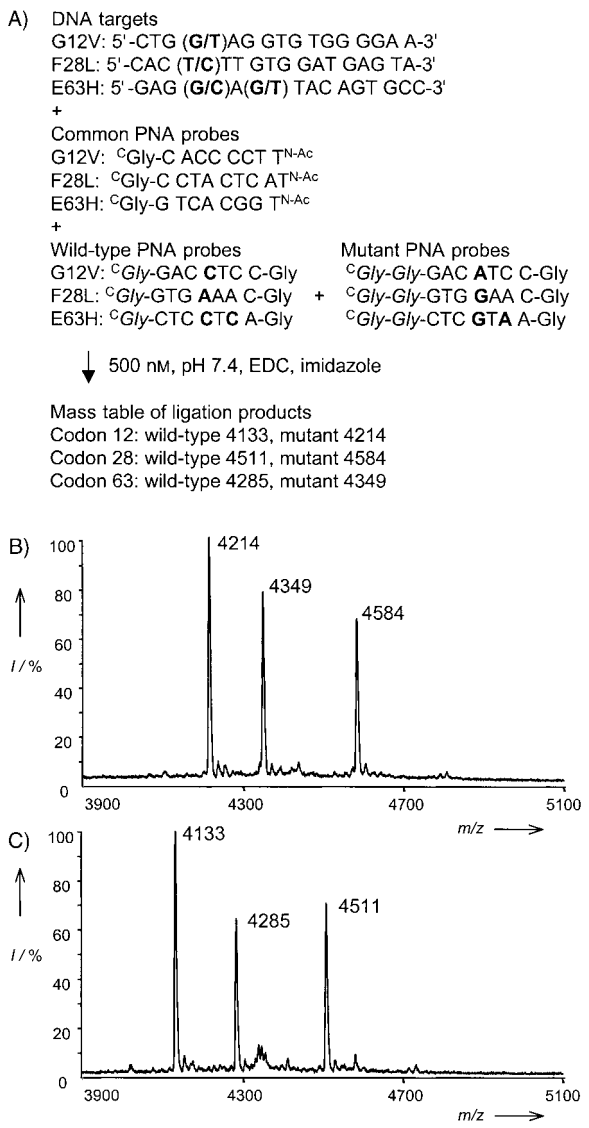

Figure 4. A) For the multiplex detection of three carcinogenic mutations in the pig-*ras* gene segment (G12V (G→T), F28L (T→C), E63H (G→C, G→T)) three common PNA probes were treated with three wild-type- and three mutant-PNA probes (probing sites marked in bold) in a single experiment. The glycine residues in italics were incorporated for mass-tagging. Concentration of ligation probes and target DNA: 500 nM. Positive-mode MALDI-TOF MS in the presence of B) the three mutant-DNA targets; C) the wild-type DNA targets. Matrix: sinapinic acid.

tolerated. It thus seems likely that the abasic site ligation is a general reaction which might allow for the multiplex detection of single-base mutations in homogeneous solution. In future studies we will apply the PNA-ligation assay to PCR-amplified DNA samples.

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