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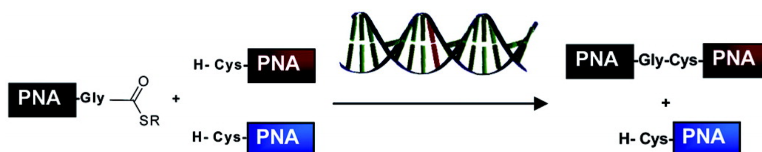

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Single-Nucleotide-Specific PNA–Peptide Ligation on Synthetic and PCR DNA Templates

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Abstract: DNA-directed chemical synthesis has matured into a useful tool with applications such as fabrication of defined (nano)molecular architectures, evolution of amplifiable small-molecule libraries, and nucleic acid detection. Most commonly, chemical methods were used to join oligonucleotides under the control of a DNA or RNA template. The full potential of chemical ligation reactions can be uncovered when nonnatural oligonucleotide analogues that can provide new opportunities such as increased stability, DNA affinity, hybridization selectivity, and/or ease and accuracy of detection are employed. It is shown that peptide nucleic acid (PNA) conjugates, nonionic biostable DNA analogues, allowed the fashioning of highly chemoselective and sequence-selective peptide ligation methods. In particular, PNA-mediated native chemical ligations proceed with sequence selectivities and ligation rates that reach those of ligase-catalyzed oligodeoxynucleotide reactions. Usually, sequence-specific ligations can only be achieved by employing short-length probes, which show DNA affinities that are too low to allow stable binding to target segments in large, double-stranded DNA. It is demonstrated that the PNA-based ligation chemistry allowed the development of a homogeneous system in which rapid single-base mutation analyses can be performed even on double-stranded PCR DNA templates.

Introduction

DNA and RNA act as templates which can organize substrates such that subsequent ligation reactions are facilitated.[1–9] The demand for highly selective detection chemistries in gene diagnostics has stimulated the development of sequence-selective ligation reactions that proceed under the control of specific nucleic acid templates.[10–12] In this scenario, the product of such a sequence-specific ligation serves as an indicator for the presence of the complementary nucleic acid template. Enzymatic ligation assays allow single-mismatch-specific DNA detection. For example, DNA segment couplings catalyzed by enzymes such as the T4 ligase used in the oligonucleotide ligation assay (OLA) occur 100-fold faster on complementary templates than on single-mismatched templates.[13] With the help of *Tth* ligase single-mismatch selectivities can reach orders of 450-fold.[14]

Despite their usefulness, enzymatic ligations are by no means generally applicable. Ligases show markedly reduced activities on RNA templates.[15,16] A further limitation stems from the restricted substrate acceptance which hampers the use of modified oligonucleotides such as labeled or biostable oligonucleotides or analogues thereof.[17,18] On the contrary, chemical ligation strategies[19] offer nearly unlimited substrate tolerance with fascinating applications such as fabrication of defined molecular architectures,[20–24] evolution of amplifiable small-molecule libraries,[25] and nucleic acid detection even within cells.[26] In this regard, many chemistries for achieving a nonenzymatic oligonucleotide ligation have been reported including DNA-directed formation of pyrophosphates,[27,28] phos-

(1) Orgel, L. E. *Nature* **1992**, *358*, 203.
(2) von Kiedrowski, G. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 932.
(3) Sievers, D.; von Kiedrowski, G. *Nature* **1994**, *369*, 221.
(4) Orgel, L. E. *Acc. Chem. Res.* **1995**, *28*, 109.
(5) Joyce, G. F. *Cold Spring Harbor Symposia on Quantitative Biology*; Cold Spring Harbor Press: New York, 1987; Vol. 52, pp 41–51.
(6) Wintner, E. A.; Conn, M. M.; Rebek, J. *Acc. Chem. Res.* **1994**, *27*, 198.
(7) Anderson, S.; Anderson, H. L.; Sanders, J. K. M. *Acc. Chem. Res.* **1993**, *26*, 469.
(8) Kanavarioti, A. *J. Org. Chem.* **1998**, *63*, 6830.
(9) Kurz, M.; Göbel, K.; Hartel, C.; Göbel, M. W. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 842.
(10) Xu, Y.; Kool, E. T. *Nucleic Acids Res.* **1999**, *27*, 875.
(11) Gryaznov, S. M.; Schultz, R.; Chaturvedi, S. K.; Letsinger, R. L. *Nucleic Acids Res.* **1994**, *22*, 2366.
(12) Summerer, D.; Marx, A. *Angew. Chem., Int. Ed.* **2002**, *41*, 89.
(13) Landegren, U.; Kaiser, R.; Sanders, J.; Hood, L. *Science* **1988**, *241*, 1077.
(14) Luo, J.; Bergstrom, D. E.; Barany, F. *Nucleic Acids Res.* **1996**, *24*, 3071.
(15) Sekiguchi, J.; Shuman, S. *Biochemistry* **1997**, *36*, 9073.
(16) Sriskanda, V.; Shuman, S. *Nucleic Acids Res.* **1998**, *26*, 3536.
(17) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Anti-Cancer Drug Des.* **1993**, *8*, 53.
(18) Hanvey, J. C.; Peffer, N. J.; Bisi, J. E.; Thomson, S. A.; Cadilla, R.; Josey, J. A.; Ricca, D. J.; Hassman, C. F.; Bonham, M. A.; Au, K. G.; Carter, S. G.; Bruckenstein, D. A.; Boyd, A. L.; Noble, S. A.; Babiss, L. E. *Science* **1992**, *258*, 1481.
(19) Naylor, R.; Gilham, P. T. *Biochemistry* **1966**, *5*, 2722.
(20) von Kiedrowski, G.; Eckardt, L. H.; Naumann, K.; Pankau, W. M.; Reimold, M.; Rein, M. *Pure Appl. Chem.* **2003**, *75*, 609.
(21) Eckardt, L. H.; Naumann, K.; Pankau, W. M.; Rein, M.; Schweitzer, M.; Windhab, N.; von Kiedrowski, G. *Nature* **2002**, *420*, 286.
(22) Lovrinovic, M.; Seidel, R.; Wacker, R.; Schroeder, H.; Seitz, O.; Engelhard, M.; Goody, R. S.; Niemeyer, C. M. *Chem. Commun.* **2003**, 822.
(23) Winssinger, N.; Harris, J. L.; Backes, B. J.; Schultz, P. G. *Angew. Chem., Int. Ed.* **2001**, *40*, 3152.
(24) Gothelf, K. V.; Thomsen, A.; Nielsen, M.; Clo, E.; Brown, R. S. *J. Am. Chem. Soc.* **2004**, *126*, 1044.
(25) Gartner, Z. J.; Kanan, M. W.; Liu, D. R. *J. Am. Chem. Soc.* **2002**, *124*, 10304.
(26) Sando, S.; Kool, E. T. *J. Am. Chem. Soc.* **2002**, *124*, 9686.

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phodiesters,[28,29] phosphorothioates,[10,30−34] phosphoroselenoates,[35] phosphoamides,[28,36] secondary amines,[37−39] amides,[40] salenes,[41] and metal complexes[42] and various other thiol and amine alkylations and [2+2]-cycloadditions.[43] Through the impressive work carried out by Liu and co-workers the reaction scope of DNA-directed synthesis has been extended further as illustrated by conjugate additions, Wittig reactions, and [2+3]-cycloadditions.[25,44,45]

Comparatively little data has been reported about the sequence specificity of template-directed reactions, a critical issue as far as gene diagnostic applications are concerned. Letsinger described the sequence-selective reaction of an oligonucleotide phosphorothioate with a bromoacetamido oligonucleotide.[11] Mismatch selectivities of greater than 100-fold rate difference were achieved by Kool and co-workers by coupling 5′-iodo oligonucleotides with 3′-phosphorothioate or 3′-phosphoroselenoate oligonucleotides.[31,35] These methods led to the development of elegant homogeneous DNA detection techniques based on fluorescence resonance energy transfer.[31,32,46] In the work mentioned above, chemical methods were used to join oligonucleotides under the control of a DNA or RNA template. However, the full potential of chemical ligation reactions can be uncovered when nonnatural oligonucleotide analogues that can provide new opportunities such as increased biostability, hybridization selectivity, or ease and accuracy of detection are employed. There are only a few examples of template-directed ligations of oligonucleotide analogues, which include contributions from Orgel[40,47−49] and Eschenmoser.[50,51] None of these studies addressed issues relevant to diagnostic applications such as sequence selectivity and usage of nonsynthetic DNA templates. Previously, we explored the sequence selectivity of a carbodiimide-mediated fragment condensation of modified peptide nucleic acid (PNA)[52,53] segments, a nonionic biostable DNA analogue that binds complementary nucleic acids with high affinity and selectivity.[54,55] Herein we describe the fashioning of a highly selective ligation method that supports single-nucleotide-specific PNA ligations on nonsynthetic duplex DNA templates.

The use of double-stranded DNA targets presents perhaps the biggest challenge to the application of nonenzymatic nucleic acid ligation reactions. Usually, sequence-specific ligation can only be achieved by employing short-length segments. This inevitably results in relatively modest DNA-binding affinities, which complicate the use of double-stranded templates since, after denaturation, reannealing competes with hybridization of the oligonucleotides to be ligated. There is, to the best of our knowledge, only one report in which the use of a mixed-sequence double-stranded DNA template has been demonstrated.[31] It has been shown that affixing of PCR DNA templates to nylon membranes in a heterogeneous slot-blot format rendered double strands accessible to the ligation probes. Ligation of triplex-forming oligonucleotides or, as recently described, ligation of minor-groove-binding polyamides may present an alternative.[56,57] However, the low occurrence of triplex-forming sequences or the as yet low sequence selectivity of the ligation of minor-groove-binding polyamides limits the usefulness as far as diagnostic applications are concerned.[58] The present investigation addressed the complications of using double-stranded templates. It will be shown that the use of PNA and a peptide-based native chemical ligation chemistry allowed the development of a homogeneous system in which a rapid sequence analysis can be performed even on double-stranded PCR DNA templates.

Results

Design of a PNA Ligation System. When human DNA segments are targeted, usually oligonucleotide probes longer than 16 base pairs are required to provide a unique sequence.[59] Probes of this length are, however, relatively unselective DNA binders. A simple hybridization event alone might therefore not suffice to unambiguously detect a single-base mutation. This particularly holds true for long PNA probes, which bind complementary DNA with very high affinity. In contrast, short-length PNA oligomers feature a high discrimination against single-base mismatches combined with a sufficiently high DNA affinity. Strategies that utilize the ligation of two short oligomers take advantage of both the longer and the shorter probes in that a unique and therefore comparably long oligonucleotide can be analyzed with a high selectivity that approaches that of short oligomers. PNA is not a substrate of any of the known ligases, and any attempt to ligate PNA oligomers has to rely solely on chemical methods.

For the application of PNA in a DNA-directed ligation, both reactants **1** and **2** have to be equipped with reactive groups A and B which support coupling reactions in an aqueous milieu. PNA is a pseudopeptide, and therefore, it was considered straightforward to employ amide-bond-forming reactions be-

(27) von Kiedrowski, G.; Wlotzka, B.; Helbing, J. *Angew. Chem., Int. Ed.* **1989**, *28*, 1235.
(28) Sokolova, N. I.; Ashirbekova, D. T.; Dolinnaya, N. G.; Shabarova, Z. A. *FEBS Lett.* **1988**, *232*, 153.
(29) Rohatgi, R.; Bartel, D. P.; Szostak, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 3332.
(30) Xu, Y. Z.; Kool, E. T. *Tetrahedron Lett.* **1997**, *38*, 5595.
(31) Xu, Y. Z.; Karalkar, N. B.; Kool, E. T. *Nat. Biotechnol.* **2001**, *19*, 148.
(32) Sando, S.; Kool, E. T. *J. Am. Chem. Soc.* **2002**, *124*, 2096.
(33) Gryaznov, S. M.; Letsinger, R. L. *J. Am. Chem. Soc.* **1993**, *115*, 3808.
(34) Herrlein, M. K.; Nelson, J. S.; Letsinger, R. L. *J. Am. Chem. Soc.* **1995**, *117*, 10151.
(35) Xu, Y. Z.; Kool, E. T. *J. Am. Chem. Soc.* **2000**, *122*, 9040.
(36) Dolinnaya, N. G.; Sokolova, N. I.; Gryaznova, O. I.; Shabarova, Z. A. *Nucleic Acids Res.* **1988**, *16*, 3721.
(37) Goodwin, J. T.; Lynn, D. G. *J. Am. Chem. Soc.* **1992**, *114*, 9197.
(38) Zhan, Z. Y. J.; Lynn, D. G. *J. Am. Chem. Soc.* **1997**, *119*, 12420.
(39) Li, Z. Y.; Zhang, Z. Y. J.; Knipe, R.; Lynn, D. G. *J. Am. Chem. Soc.* **2002**, *124*, 746.
(40) Koppitz, M.; Nielsen, P. E.; Orgel, L. E. *J. Am. Chem. Soc.* **1998**, *120*, 4563.
(41) Czlapinski, J. L.; Sheppard, T. L. *J. Am. Chem. Soc.* **2001**, *123*, 8618.
(42) Brunner, J.; Mokhir, A.; Kraemer, R. *J. Am. Chem. Soc.* **2003**, *125*, 12410.
(43) Fujimoto, K.; Matsuda, S.; Takahashi, N.; Saito, I. *J. Am. Chem. Soc.* **2000**, *122*, 5646.
(44) Gartner, Z. J.; Liu, D. R. *J. Am. Chem. Soc.* **2001**, *123*, 6961.
(45) Gartner, Z. J.; Kanan, M. W.; Liu, D. R. *Angew. Chem., Int. Ed.* **2002**, *41*, 1796.
(46) Sando, S.; Abe, H.; Kool, E. T. *J. Am. Chem. Soc.* **2004**, *126*, 1081.
(47) Bohler, C.; Nielsen, P. E.; Orgel, L. E. *Nature* **1995**, *376*, 578.
(48) Schmidt, J. G.; Christensen, L.; Nielsen, P. E.; Orgel, L. E. *Nucleic Acids Res.* **1997**, *25*, 4792.
(49) Kozlov, I. A.; Zielinski, M.; Allart, B.; Kerremans, L.; Van Aerschot, A.; Busson, R.; Herdewijn, P.; Orgel, L. E. *Chem.—Eur. J.* **2000**, *6*, 151.
(50) Bolli, M.; Micura, R.; Eschenmoser, A. *Chem. Biol.* **1997**, *4*, 309.
(51) Bolli, M.; Micura, R.; Pitsch, S.; Eschenmoser, A. *Helv. Chim. Acta* **1997**, *80*, 1901.
(52) Nielsen, P. E.; Berg, M. E. R.; Buchardt, O. *Science* **1991**, *254*, 1497.
(53) Uhlmann, E.; Peyman, A.; Breipohl, G.; Will, D. W. *Angew. Chem., Int. Ed.* **1998**, *37*, 2797.
(54) Mattes, A.; Seitz, O. *Chem. Commun.* **2001**, 2050.
(55) Mattes, A.; Seitz, O. *Angew. Chem., Int. Ed.* **2001**, *40*, 3178.
(56) Poulin-Kerstien, A. T.; Dervan, P. B. *J. Am. Chem. Soc.* **2003**, *125*, 15811.
(57) Li, T. H.; Weinstein, D. S.; Nicolaou, K. C. *Chem. Biol.* **1997**, *4*, 209.

(58) Dervan and co-workers explored the sequence selectivity of the ligation of six-ring hairpin polyamides in the presence of double-helical DNA. The ligation on matched templates occurred twice as fast as ligations on single-mismatched templates.

(59) Liu, Q.; Segal, D. J.; Ghiara, J. B.; Barbas, C. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 5525.

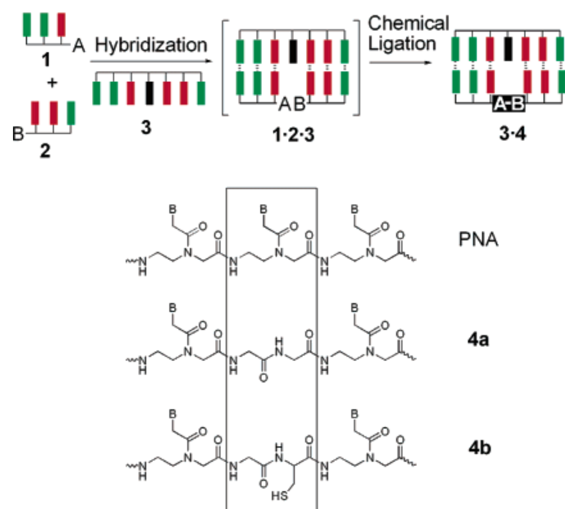


Figure 1. Two PNA−amino acid conjugates, **1** and **2**, are ligated when bound to a complementary ssDNA template, **3**. The ligation of two PNA−amino acid conjugates leads to the formation of a PNA hybrid in which a central PNA monomer is isosterically replaced by a dipeptide such as **4a** or **4b** (B = nucleobase).

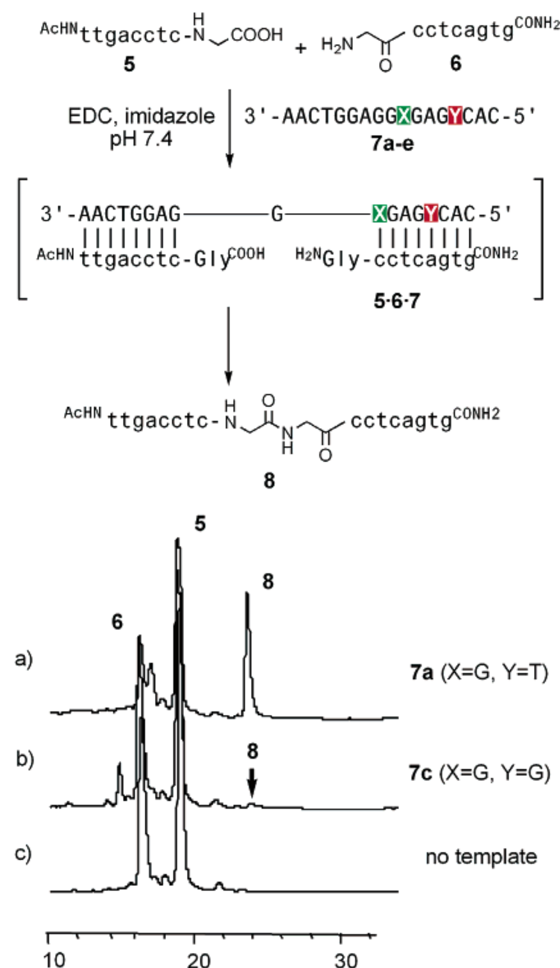


Figure 2. Carbodiimide-mediated ligation reaction of PNA−glycine conjugates **5** and **6**. The ssDNA templates **7** differ in positions X and Y (**7a**, X = G, Y = T; **7b**, X = T, Y = T; **7c**, X = G, Y = G; **7d**, X = G, Y = C; **7e**, X = G, Y = A). HPLC analysis of ligation reactions in (a) the presence of matched template **7a**, (b) the presence of single-mismatched template **7c**, and (c) the absence of template. Conditions: 10 μM probes and templates, 0.3 M EDC·HCl, 0.6 M imidazole (pH 7.4) at 25 °C (EDC·HCl = *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride).

tween appended amino acids (Figure 1). In this particular architecture the ligation takes place opposite an unpaired nucleobase. We speculated that the presence of this abasic site disrupts the cooperativity of "unselective" base-stacking interactions within the evolving duplex. As a result, the contribution of the more selective Watson−Crick hydrogen-binding may become more important, which ultimately may lead to an enhanced ligation fidelity. Indeed, previous work suggested that an unselective ligation reaction can be rendered selective after incorporation of an abasic site.[55] The abasic site can be readily integrated by replacing a central PNA monomer by an isosteric dipeptide which will be formed upon ligation of two PNA−amino acid conjugates.

Optimization of Ligation Fidelity. Orgel and co-workers have shown that a template-controlled ligation of PNA is feasible by using a water-soluble carbodiimide in imidazole-containing buffers.[60] These conditions were tested in the DNA-directed ligation of PNA−glycine conjugates **5** and **6** (Figure 2). Particular emphasis was placed on the issue of ligation fidelity on matched and single-mismatched templates.[61,62] PNA−glycine conjugates **5** and **6** were allowed to react in the absence and presence of matched ssDNA template **7a** and single-mismatched ssDNA templates **7b**−**e**. Table 1 lists the ligation yields as determined by HPLC analyses of aliquots taken after 6 h of reaction time.

No product was formed in the absence of template, indicating that template-independent "background" reactions did not occur at 10 μM concentrations of both starting material and templates (Figure 2). The addition of the fully complementary ssDNA **7a** (X = T, Y = T) led to the formation of ligation product **8** in 25% yield. To determine the sequence selectivity, the ligation reaction was carried out on ssDNA templates **7b**−**e**, which contained single-base mismatches at positions X and Y. It was found that template **7b** containing a single mismatch in the immediate vicinity of the ligation site was as effective in promoting the ligation as complementary template **7a**. On the contrary, templates **7c**−**e**, all of which contained a mismatched base at a central rather than a terminal position, proved to be unproductive, and only traces of the ligation product were detected by HPLC analysis (Figure 2). It can be concluded that template **7b** with the mismatch at the ligation site was able to align the ligation probes and enhance the effective molarity of the reaction partners while mismatches at a central position of probe **6** prevented binding. Indeed, melting analyses of duplex **6·7** confirmed that the influence of base mismatches on duplex

Table 1. Yields of Ligation Product **8** Formed in Reactions of **5** and **6** in the Absence and Presence of ssDNA Templates[a]

template	X	Y	yield of **8**/%
none			0
7a	G	T	25
7b	T	T	25
7c	G	G	3
7d	G	C	3
7e	G	A	2

[a] For the conditions see Figure 2.

(60) Harada, K.; Orgel, L. E. *J. Mol. Evol.* **1994**, *38*, 558.
(61) The sequence of the DNA template **7** was taken from the cystic fibrosis transmembrane regulator gene.
(62) Kerem, B.-s.; Rommens, J. M.; Buchanan, J. A.; Markiewicz, D.; Cox, T. K.; Chakravarti, A.; Buchwald, M.; Tsui, L.-C. *Science* **1989**, *245*, 1059.

Table 2. Melting Temperatures of Duplexes of PNA Probe **6** with the ssDNA Templates **7a**−**e**[a]

DNA **7a-e** 3'-AACTGGAGGXGAGYCAC-5'
PNA **6** H_2NGly-cctcagtg^{CONH_2}

template	X	Y	T_M/°C
7a	G	T	46
7b	T	T	39
7c	G	G	<15
7d	G	C	<15
7e	G	A	<15

[a] Conditions: 1 μM probe and template, 100 mM NaCl, 10 mM NaH_2PO_4 (pH 7.0). Denaturation curves were collected from 15 to 85 °C at a rate of 0.5 °C/min.

stability was more pronounced at central rather than terminal positions. Both the T_M = 39 °C measured for the mismatched PNA−DNA duplex (template **7b**) and the T_M = 46 °C for the matched PNA−DNA duplex (template **7a**) exceeded the 25 °C reaction temperature (Table 2). Hybridization of PNA probe **6** with mismatched templates **7c**−**e** failed to give sigmoid melting curves, which suggests that formation of duplexes with a central mismatched base (Y = A, C, G) is less favorable than formation of the duplex containing a mismatched base at a terminal postion.

In comparison with the single-mismatched single-stranded templates **7c**−**e**, complementary single-stranded template **7a** enhanced product formation by a factor of 8−13. Since a template-independent background reaction was not observed, it appears most likely that the little but noticeable product formation (2−3%) on mismatched templates occurred due to nonselective hybridization under the reaction conditions.[63] Reduction of probe and target concentrations resulted in enhanced selectivities (vide infra). The initial model experiments allowed the conclusion that a PNA-based ligation system has the potential to distinguish between matched and single-mismatched ssDNA templates when the mutation site is located at a central rather than terminal position. The ligation yields provided by the carbodiimide ligation were moderate. Nevertheless, the possibility of considering product formation as a means for DNA detection would not require high ligation yields as long as enough product is formed to be detectable.

Detection by MALDI-TOF Mass Spectrometry. The majority of the commonly applied DNA detection methods employ fluorescent probe molecules. Numerous powerful assays have been reported including TDI assays,[64] TaqMan assays,[65] molecular beacons,[66,67] scorpion probes,[68] adjacent probes,[69,70] FIT probes,[71] and Invader assays.[72] A drawback is the need for cost-intensive labeling and the comparatively small number of spectrally resolved fluorophores, which sets limitations to the design of multiplex assays. In contrast, mass spectrometry offers high resolution and has been demonstrated to enable genotyping of single-nucleotide polymorphisms.[73−75] PNA as opposed to DNA is not a polyanion, which greatly facilitates its mass spectrometric detection, e.g., by MALDI-TOF/MS measurements.[76,77] Fragmentations as observed with DNA[78,79] only occur at high laser intensities with PNA, peak widths are smaller, and peak intensities are higher.

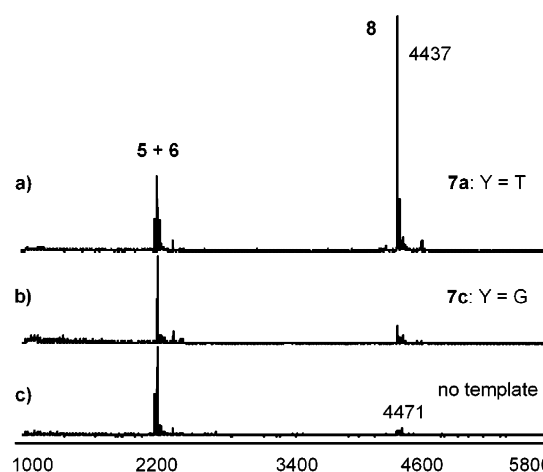


Figure 3. MALDI-TOF/MS analysis of the ligation of **5** and **6** in the presence of ssDNA templates **7a** (a) and **7c** (b) and in the absence of any DNA template (c) as described schematically in Figure 2 ($[M + H]^+$ *m*/*z* 2237, **5**; 2219, **6**; 4437, **8**). Matrix: sinapinic acid.

Figure 3 shows normalized positive ion MALDI-TOF mass spectra of the ligations described in Figure 2. In the absence of the ssDNA template, only the PNA probes **6** and **5** with an *m*/*z* ratio of 2219 and 2237 were detectable. A noncovalent dimer aggregate of **5** with an *m*/*z* ratio of 4471 appeared in low intensity. The MALDI-TOF analysis of aliquots from the ligation reactions revealed that the matched ssDNA **7a** (Y = T) was the only template that led to the occurrence of an additional signal of highest intensity with an *m*/*z* ratio of 4437 for the ligation product.

To further assess the general applicability, the ligation was performed on a different ssDNA template with competing ligation probes. It was the aim to explore the ligation fidelity in response to a G → T transition in DNA segment **11** (Figure 4). Two nucleophilic PNA−glycine conjugates **10a** and **10b** probed the mutation site X of ssDNA targets **11a** and **11b**. In addition, **10c** was added to explore the possibility of the formation of SNP-unrelated ligation products. The three ligation probes were allowed to compete for the reaction with the acyl donor segment **9**. In principle, this ligation system can produce three possible ligation products, **12a**−**c**. MALDI analysis revealed that in the presence of the mutant ssDNA **11a** (X =

(63) The melting curves suggested selective hybridization; however, note that oligonucleotide concentrations were 5 times lower during melting analyses. In this model study relatively high concentrations (10 μM in ligation probes and templates) were used to facilitate HPLC analysis. Enhanced ligation selectivities were achieved by lowering template concentrations.
(64) Chen, X.; Kwok, P. Y. *Nucleic Acids Res.* **1997**, *25*, 347.
(65) Holland, P. M.; Abramson, R. D.; Watson, R.; Gelfand, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7276.
(66) Tyagi, S.; Kramer, F. R.; Lizardi, P. M. PCT-WO 95/13399, 1995.
(67) Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303.
(68) Whitcombe, D.; Theaker, J.; Guy, S. P.; Brown, T.; Little, S. *Nat. Biotechnol.* **1999**, *17*, 804.
(69) Morrison, L. E.; Akin, C.; Heller, M. J.; Prevatt, W. D. EP 0070685 A2, 1983.
(70) Cardullo, R. A.; Agrawal, S.; Flores, C.; Zamecnik, P. C.; Wolf, D. E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8790.
(71) Köhler, O.; Seitz, O. *Chem. Commun.* **2003**, 2938.
(72) Mein, C. A.; Barratt, B. J.; Dunn, M. G.; Siegmund, T.; Smith, A. N.; Esposito, L.; Nutland, S.; Stevens, H. E.; Wilson, A. J.; Phillips, M. S.; Jarvis, N.; Law, S.; de Arruda, M.; Todd, J. A. *Genome Res.* **2000**, *10*, 330.
(73) Tost, J.; Gut, V. G. *Mass Spectrom. Rev.* **2002**, *21*, 388.
(74) Ross, P.; Hall, L.; Smirnov, I.; Haff, L. *Nat. Biotechnol.* **1998**, *16*, 1347.
(75) Griffin, T. J.; Smith, L. M. *Trends Biotechnol.* **2000**, *18*, 77.
(76) Ross, P. L.; Lee, K.; Belgrader, P. *Anal. Chem.* **1997**, *69*, 4197.
(77) Griffin, T. J.; Tang, W.; Smith, L. M. *Nat. Biotechnol.* **1997**, *15*, 1368.
(78) Nordhoff, E.; Karas, M.; Cramer, R.; Hahner, S.; Hillenkamp, F.; Kirpekar, F.; Lezius, A.; Muth, J.; Meier, C.; Engels, J. W. *J. Mass Spectrom.* **1995**, *30*, 99.
(79) Zhu, L.; Parr, G. R.; Fitzgerald, M. C.; Nelson, C. M.; Smith, L. M. *J. Am. Chem. Soc.* **1995**, *117*, 6048.

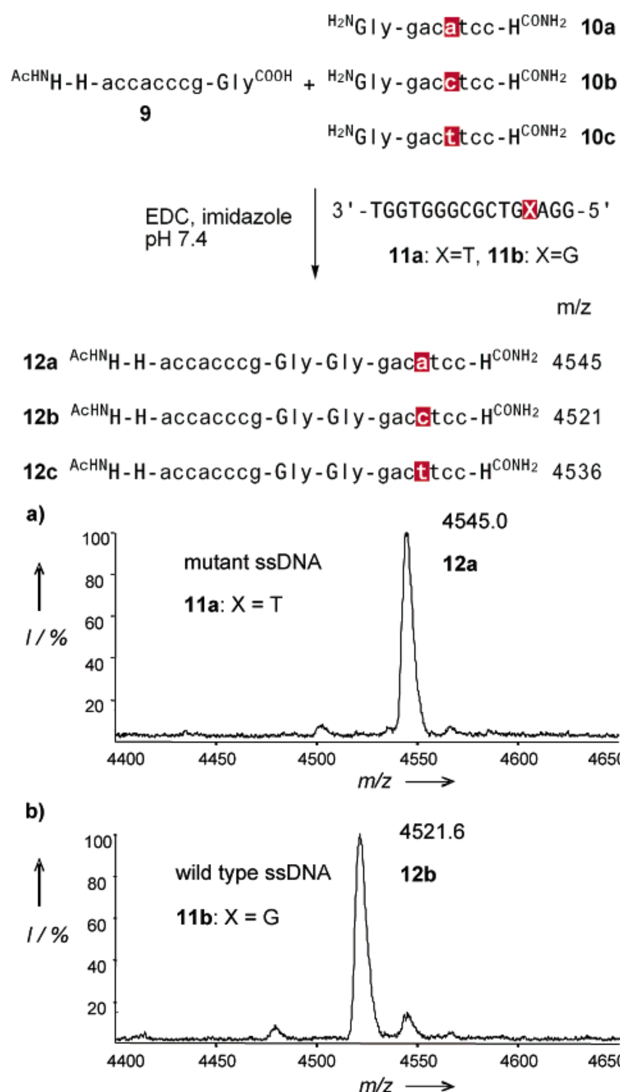


Figure 4. Competing probes ligation. Three variable PNA−glycine probes, **10a**−**c**, were treated with one common PNA−glycine probe, **9**, in the presence of either mutant ssDNA **11a** (X = T) or wild-type ssDNA **11b** (X = G). MALDI-TOF/MS analysis of the ligation mixtures after 2 h of reaction time in the presence of (a) ssDNA **11a** and (b) ssDNA **11b** ($[M + H]^+$ *m/z* 4545.0, **12a**; 4521.6, **12b**). Matrix: sinapinic acid. Conditions: 10 μM probes and templates, 0.3 M EDC, 0.6 M imidazole (pH 7.4) at 25 °C (H = histidine).

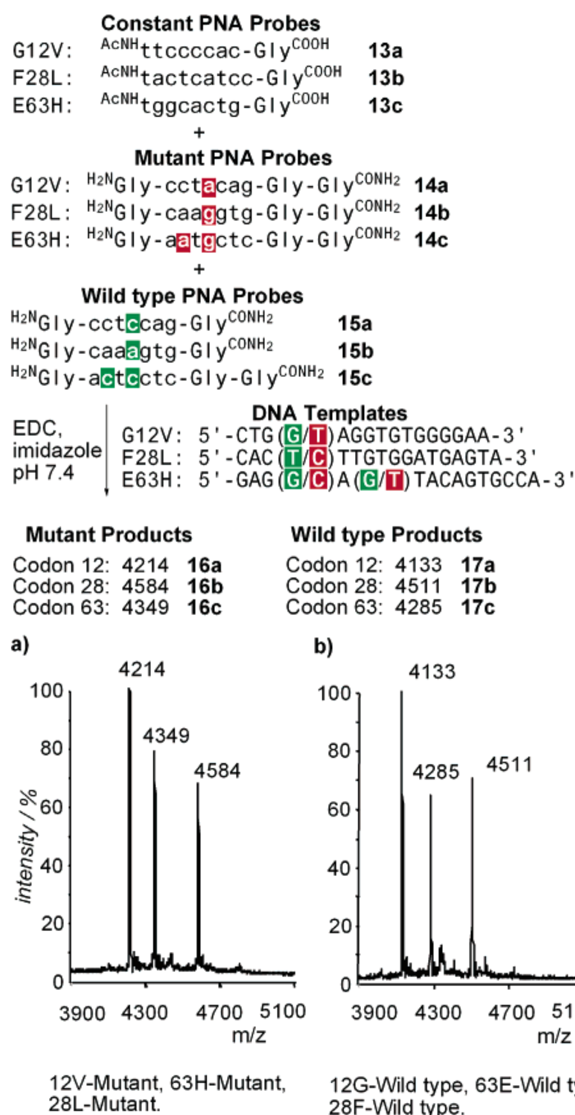


Figure 5. Multiplex ligation. Three electrophilic PNA probes, **13a**−**c**, were allowed to react with six nucleophilic PNA−glycine conjugates, **14a**−**c** and **15a**−**c**. PNA conjugates **14a**−**c** probed the mutant (marked in red), and **15a**−**c** probed the wild-type (marked in green). MALDI-TOF/MS analysis after 2 h of reaction time in the presence of (a) three mutant ssDNA targets and (b) three wild-type ssDNA targets. Conditions: 1 μM probes, 500 nM templates, 0.3 M EDC, 0.6 M imidazole (pH 7.4) at 25 °C. Matrix: sinapinic acid.

T) only one ligation product was formed (Figure 4a). The *m/z* ratio of 4545 corresponded to product **12a**, indicating that only the adenine-containing probe **10a** was converted. The presence of wild-type ssDNA **11b** (X = G) led to a predominant formation of the cytosine-containing product **12b** with a molecular mass of 4521 (Figure 4b). In none of the MALDI spectra was a signal of the thymine-containing product **12c** detected, which gives further testimony to the high ligation fidelity.[80]

As an extension of the competing ligation format depicted in Figure 4, the applicability of a multiplexed ligation system in which a set of competing ligation probes would react on multiple template sequences was investigated. The goal was to simultaneously specify three carcinogenic mutations of *ras* gene segments, the G12V (G → T), F28L (T → C), and E63H (G → C, G → C/T) mutations. For the exploration of each potential mutation site, competing wild-type and mutant PNA−glycine probes were allowed to react with one common acyl donor. In total, the multiplexed ligation was comprised of six PNA−glycine nucleophiles and three acyl donor segments potentially giving rise to six specific ligation products (three wild-types and three mutants) and twelve cross-ligation products (Figure 5). Overlapping product signals were avoided by fashioning specific glycine patterns onto the C-terminus of the nucleophilic PNA−glycine probes. It was assumed that a decrease in the concentration of ligation probes from 10 to 1 μM would aid in preventing undesired ligation reactions. First the multiplex ligation was performed on mutant ssDNAs. Of the 18 ligation products that could form only 3 were observed (Figure 5a). By comparison with the calculated masses for the possible ligation

(80) Careful MALDI/MS analysis revealed, however, also the formation of adenine-containing ligation product **12a** albeit to a moderate extent. It can be concluded that a ligation probe that led to the formation of a G−A mismatch was converted while T−C, T−T, and G−T mismatches were not tolerated, at least within the studied sequence context.

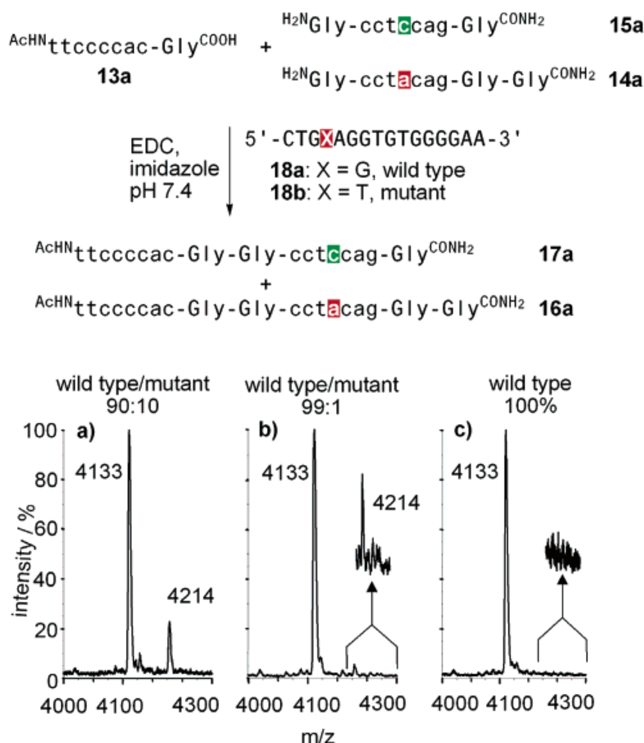


Figure 6. Ligation with competing probes on target mixtures. PNA−glycine conjugate **13a** and competing PNA probes **15a** and **14a** were allowed to react in the presence of ssDNA target mixtures. MALDI-TOF mass spectra were recorded after 2 h of ligation reaction in the presence of (a) 90:10 and (b) 99:1 wild-type (**18a**)/mutant (**18b**) mixtures and (c) pure wild-type ssDNA **18a** (*m/z* 4133, **17a**; 4214, **16a**). Conditions: 1 μM probes, 500 nM templates, 0.3 M EDC, 0.6 M imidazole (pH 7.4) at 25 °C. Matrix: sinapinic acid.

products **16a**−**c** and **17a**−**c**, it became apparent that ligation on mutant templates produced only mutant ligation products **16**. Likewise, MALDI analysis revealed that the presence of wild-type ssDNA led to the predominant formation of the three wild-type ligation products **17a**−**c** (Figure 5b).[81] It can be concluded that the fidelity of the PNA-based ligation system enabled a clear distinction between the presence of three matched or three single-mismatched single-stranded templates within a single analysis.

The selectivity demands were further increased by allowing the ligation to take place with competing probes in the presence of competing templates. Both the probes **15a** and **14a** and the single-stranded templates **18a** and **18b** differed by one mismatched base only (Figure 6). In the event, the ligation reactions were carried out on mixtures of templates corresponding to codon 12 wild-type and mutant *ras* sequences. A 1:1 mixture of wild-type and mutant templates emulates a heterozygous state. The MALDI-TOF spectrum of the ligation products indicated that both wild-type and mutant ligation products were formed, which, in a diagnostic setting, would suggest that both templates were present in similar amounts. Figure 6 shows the product spectra as measured after addition of a 9:1 or 99:1 ratio of wild-type and mutant templates. These situations mimic the presence of somatic mutations where the challenge arises to detect a certain mutation despite a high background of wild-type sequences. In a 90:10 mixture of wild-type and mutant DNA, the latter was easily detected by the observation of the corresponding ligation product **16a** (*m/z* 4215) (Figure 6a). The mutant signal still exceeded the noise level when the ligation reaction was carried out on a 99:1 mixture of wild-type/mutant templates (Figure 6b). In the presence of pure wild-type template, it was impossible to detect ligation product (Figure 6c). These experiments substantiate the power of PNA−DNA recognition and of the ligation system and suggest that it is possible to detect rare single-base mutations on a high background of wild-type ssDNA. Such properties could prove useful when the aim is the detection of acquired single-base mutations as needed in early cancer diagnosis.

(81) Careful inspection of the product spectrum exposes a low-intensity peak around $m/z = 4340$. This peak is likely to arise from cross-ligation between **13c** and **15b**. The origin of this background reaction is not known yet. We note, however, that the undesired cross-ligation led to the union of two PNA oligomers that display the highest purine content within this series.

Carbodiimide-Mediated Ligations at Elevated Temperature. The initial experimentation was focused on the use of synthetic single-stranded DNA templates to facilitate the evaluation and optimization of the ligation fidelity. From the collected data it can be concluded that the PNA-based ligation system enables the specific detection of single-base mutations if desired in a multiplex format. For practical applications the DNA templates would need PCR amplification first, before the ligation method is applied. PCR products are usually double-stranded. Denaturation of PCR products is, hence, a necessary step to ensure accessibility of probes to the segment under scrutiny.

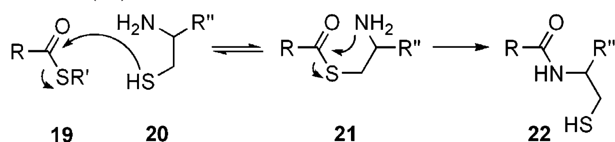
To evaluate the utility of the ligation system, the use of double-stranded DNA templates was explored. The PNA−glycine conjugates **14a** and **15a** were allowed to react with the acyl donor segment **13a** in the presence of the corresponding duplex DNA. It was attempted to enable access to the template by denaturation and subsequent cooling to 25 °C. However, HPLC analysis failed to indicate any product formation. For assessing the compatibility of EDC to elevated temperatures, the ligation reaction was performed at 40 °C by using single-stranded template **18b**. While template **18b** was found to promote the ligation of **14a** and **13a** at 25 °C (see Figure 6), only 4% ligation product was formed at 40 °C, which is well below the T_m of PNA−DNA duplex **14a·18b**. It was furthermore noted that the reaction mixture turned yellow and that subsequent cooling to 25 °C proved likewise inefficient in conferring product formation. Presumably, the elevated temperature led to accelerated hydrolysis of the condensing agent EDC.

Native Chemical Ligation on Synthetic DNA Templates. Although the template-controlled ligation of PNA−glycine conjugates via EDC activation met the fidelity demands, the lack of the option of using higher temperatures is a limitation. In seeking a ligation chemistry that tolerates high temperatures required to denature double-stranded templates, the utility of the native chemical ligation was explored. This reaction involves a thioester fragment, **19**, and a cysteinyl conjugate, **20** (Scheme 1).

The versatility of the native chemical ligation has been demonstrated in numerous examples and has been successfully employed to achieve the ligation of peptide segments[82−85] and

(82) Wieland, T.; Bokelmann, E.; Bauer, L.; Lang, H. U.; Lau, H. *Liebigs Ann. Chem.* **1953**, *583*, 129.
(83) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.
(84) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923.
(85) Lee, D. H.; Granja, J. R.; Martinez, J. A.; Severin, K.; Ghadiri, M. R. *Nature* **1996**, *382*, 525.

Scheme 1. Native Chemical Ligation Is Initiated by a Thiol-Exchange Reaction of a Cysteine Conjugate (**20**) with a Thioester (**19**)[a]



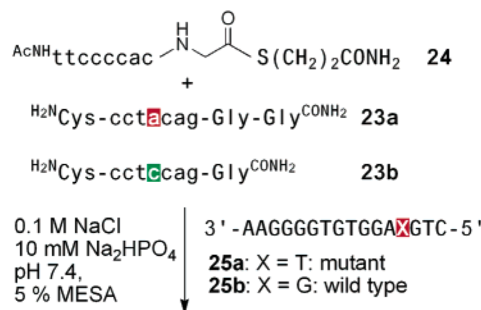
[a] The formed thioester intermediate **21** is subject to a spontaneous S−N−acyl shift, which establishes the α-peptide bond in product **22** (R, R″ = peptide, peptide nucleic acid; R′ = alkyl chain).

to conjugate peptides with oligonucleotides.[22,86] Due to the high rate constants of native chemical ligation reactions, special care has to be taken as far as template-independent background reactions are concerned.[87] We surmised that background reactions should be disfavored at a 1 μM concentration. To test this hypothesis, two nucleophilic PNA probes, **23a** and **23b**, were allowed to compete for the acyl donor segment **24** (transthioesterificated by MESA) in the presence either of the mutant ssDNA **25a** or the wild-type ssDNA **25b** or in the absence of DNA (Figure 7). In principle, two ligation products, **26a** and **26b**, with molecular masses of 4259 and 4179 could have been formed. Aliquots taken after a 120 min reaction time were analyzed by MALDI-TOF/MS. In the absence of DNA template, no product was formed, which indicated that a template-independent background reaction was slow (Figure 7c). The MALDI-TOF/MS analysis revealed that in the presence of the mutant ssDNA **25a** only the desired mutant product **26a** with the mass 4258 was formed (Figure 7b). The addition of wild-type ssDNA led to the exclusive formation of the wild-type ligation product **26b** as evidenced by a single peak at m/z = 4179 (Figure 7a).

For a more precise assessment of the ligation fidelity, HPLC measurements were performed (Figure 8). The two ligation probes **23a** and **24** were allowed to react in the presence of matched and mismatched DNA templates and without any added DNA. It became apparent that on the matched template **25a** the ligation product was formed in 70% yield (Figure 8a). The addition of single-base-mismatched template **25b** or the absence of template had little effect (Figure 8b,c). The ligation product **26a** was formed in less than 0.5% yield, which translates into a mismatch selectivity of greater than 140-fold difference in ligation yield. It can be concluded that PNA ligation via native chemical ligation is superior to EDC-mediated ligations in terms of both practicability and ligation yield. The observed match/mismatch discrimination is similar to that determined for enzymatic ligations of oligonucleotides and is expected to suit the demands of DNA diagnostic applications.

As outlined earlier, it would be desirable if the ligation chemistry was tolerant of elevated reaction temperatures. The EDC-mediated ligation proved inefficient at 40 °C possibly due to accelerated hydrolysis of the activating reagent. In contrast native chemical ligation reactions rely on the intrinsic electrophilicity of thioesters, and thioesters are hence the species that would be depleted by hydrolysis. We monitored thioester hydrolysis under ligation conditions at 20, 30, and 40 °C. After 120 min of reaction time, thioester **24** was hydrolyzed in 3.5%, 5.5%, and 8.5% yield, respectively (data not shown). The relatively low level of hydrolysis suggests that native chemical ligation reactions can be performed at temperatures above ambient temperature.

(86) Stetsenko, D. A.; Gait, M. J. *J. Org. Chem.* **2000**, *65*, 4900.
(87) Previous experiments indicated that template-independent PNA ligation occurred at a 160 μM concentration.



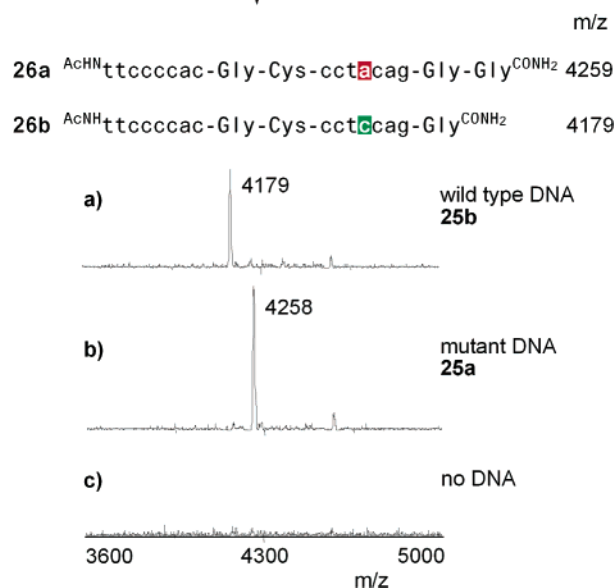


Figure 7. Native chemical ligation with competing probes. PNA−thioester conjugate **24** was allowed to react with the two competing PNA−cysteine probes **23a**,**b**. MALDI-TOF/MS analysis of the competition ligation in (a) the presence of wild-type ssDNA **25b**, (b) the presence of mutant ssDNA **25a**, and (c) the absence of template (m/z 4259, **26a**; 4179, **26b**). MALDI-TOF mass spectra were recorded after 2 h of reaction time. Conditions: 1 μM probes, 500 nM templates, 3% MESA, 100 mM NaCl, 10 mM NaH_2PO_4 (pH 7.4) at 25 °C (MESA = 2-mercaptoethanesulfonic acid). Matrix: sinapinic acid.

Native Chemical Ligation on PCR DNA Templates. The previous ligation experiments were performed on single-stranded synthetic DNA templates. Next it was investigated whether single-stranded PCR DNA would serve as a template. For facilitating comparability, it was chosen to explore the use of short-length PCR DNA. Plasmids encoding the wild-type pig H-*ras* sequence and its codon 12 G → T single-base mutant were obtained. Both sequences were confirmed by DNA sequencing. Short-length mutant DNA was prepared by PCR of the mutant plasmid involving a biotinylated primer strand and subsequent treatment with restriction endonuclease *Ear*I. The resulting 28 bp duplex was immobilized on streptavidin-coated magnetic beads, washed, and treated with 100 mM NaOH to release the single-stranded 28-mer **29**. This short-length PCR fragment proved able to promote the chemical ligation of PNA probes **27** and **28a** (Figure 9). MALDI-TOF/MS analysis revealed that addition of mutant DNA **29** led to predominant formation of mutant product **30a** (Figure 9a). No product formation was observed without added template, which indicated that the reaction indeed proceeded on DNA **29** and not in a

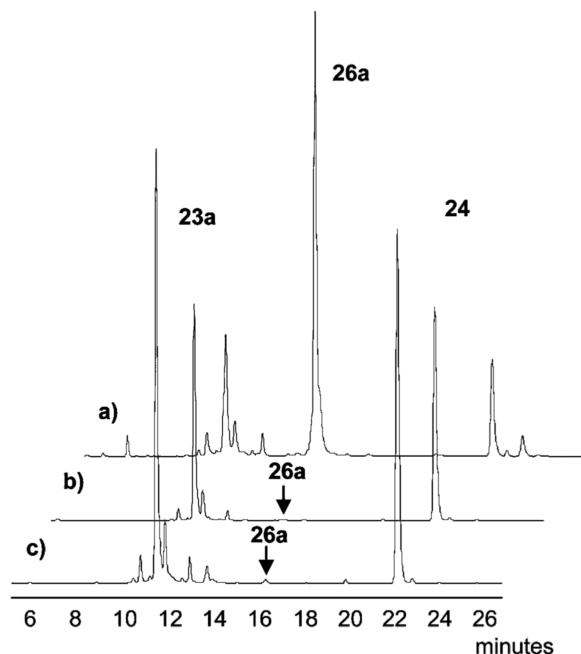


Figure 8. HPLC analysis of the ligation of PNA−thioester conjugate **24** with PNA−cysteine probe **23a** in (a) the presence of mutant ssDNA **25a**, (b) the presence of wild-type ssDNA **25b**, and (c) the absence of template. HPLC analysis was performed after 2 h of reaction time and at $\lambda = 280$ nm. Conditions: 1 μM probes, 1 μM template, saturated benzyl mercaptan, 10 mM NaCl, 10 mM NaH_2PO_4 (pH 7.4) at 25 °C.

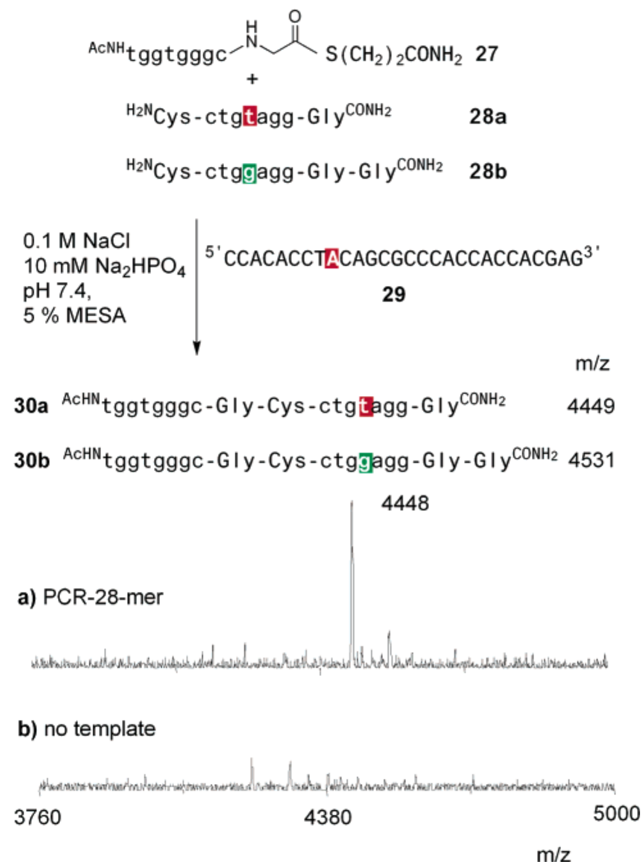


Figure 9. MALDI-TOF/MS analysis of the ligation of PNA−thioester conjugate **27** with the two competing PNA−cysteine probes **28a**,**b** in (a) the presence of mutant PCR-amplified ssDNA **29** and (b) the absence of template (m/z 4449, **30a**). Conditions: 1 μM probes, 500 nM template, 3% MESA, 100 mM NaCl, 10 mM NaH_2PO_4 (pH 7.4) at 25 °C. MALDI-TOF/MS spectra were recorded after 2 h of reaction time. Matrix: sinapinic acid.

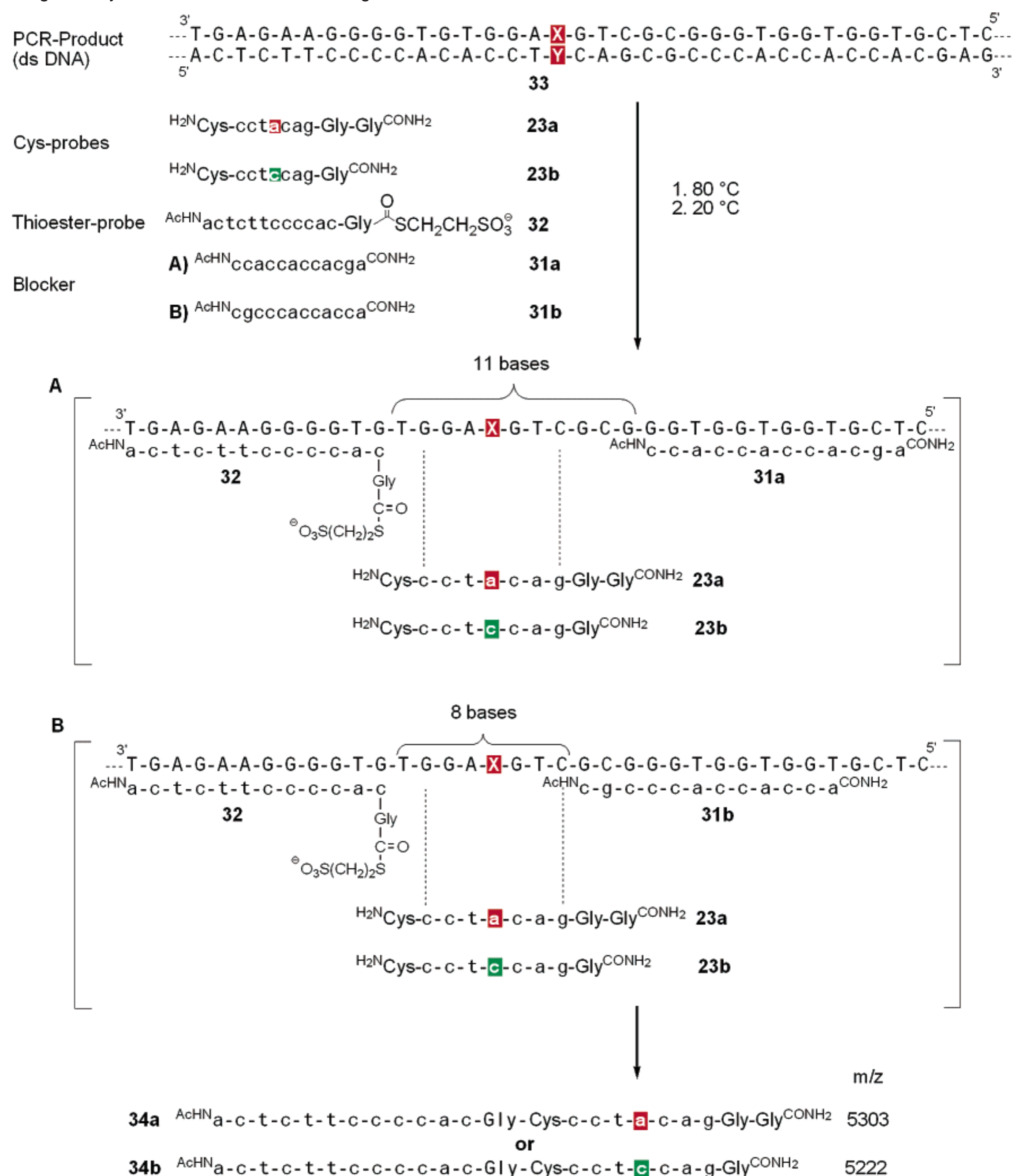
template-independent manner (Figure 9b). These results indicate that DNA from enzymic sources can act as a template.

A direct applicability of double-stranded PCR DNA would be desirable, particularly in DNA diagnostics. However, the problems associated with hybridization to double-stranded PCR DNA present a significant challenge. Most assays employ thermal denaturation as a means to separate the PCR strands followed by a rapid cooling step in which designed probe molecules hybridize to the target strand. This process draws upon the relatively fast kinetics of hybridization of short- to medium-length oligonucleotides when compared to the slow kinetics of reannealing. The probes have to be long enough to confer sufficiently low off-rates, which is essential to afford stable probe−target duplexes. On the contrary, the design criteria for single-mismatch-specific ligation reactions call for very short probes that normally cannot compete with reannealing of the two long DNA strands. One solution to this problem was presented by Kool and co-workers.[31] The PCR DNA template was denaturated first followed by affixing of the separated DNA strands to nylon membranes in a slot-blot format. The ligation reactions were performed on the nylon membrane. We surmised that a homogeneous ligation format should be feasible if reannealing to the area of interest was blocked by the aid of two flanking high-affinity DNA binders. In following this idea, it was realized that medium-length PNA possesses the desired binding affinity. A ligation system was fashioned in which a medium-length PNA thioester probe, **32**, and a PNA-based reannealing blocker, **31a**,**b**, enabled stable hybridization of a short-length PNA cysteine conjugate dedicated to probe the mutation site (Scheme 2). In one arrangement the 12-mer blocker PNA **31b** was designed to keep open an eight-base template segment for hybridization of the 7-mer ligation probes **23a** and **23b** (see part B). Hence, this format would have allowed contigous base pairing. In the arrangement shown in Scheme 1A, the blocker PNA **31a** left an unhybridized 11-base segment to reduce steric hindrance.

The double-stranded PCR DNA was thermally denaturated in the presence of ligation probes **23a**, **23b**, and **32** and blocker **31a** or **31b**, when added. Rapid cooling to 20 °C allowed hybridization and therefore commenced the template-directed ligation reaction. Product formation was analyzed by MALDI-TOF/MS after a 120 min incubation time. As expected, no ligation product signal was detected in the absence of DNA (Figure 10e). PCR DNA alone was also inefficient in promoting the ligation reaction, and addition of PNA blocker **31b** had little effect (Figure 10c,d). However, incubation in the presence of PNA blocker **31a** proved successful. The PCR DNA from mutant plasmids led to the exclusive formation of mutant ligation product **34a** with $m/z = 5303$ (Figure 10b). On wild-type PCR DNA the wild-type ligation product **34b** ($m/z = 5222$) was formed (Figure 10a), providing evidence for a high sequence selectivity in a competing probes format.

These data provide clear evidence that the use of PNA oligomers that block reannealing enables sequence-selective ligations to be performed on double-stranded PCR DNA in homogeneous solution. It became obvious that care has to be taken in choosing the blocker hybridization site to not interfere with hybridization of the short-length ligation probe. A distance of three base pairs between the C-terminus of ligation probe

Scheme 2. Ligation System for the Detection of Single-Base Mutations in Double-Stranded PCR DNA[a]



[a] Thermal denaturation separates the target DNA strands **33**. Rapid cooling in the presence of PNA−glycine thioester probe **32** and of PNA-Based Reannealing blockers **31a** and **31b** yields gapped duplexes that offer an (A) 11-base or (B) 8-base segment, respectively, for binding of the PNA−cysteine probes **23a**,**b**. X = T and Y = A for the mutant DNA sequence; X = G and Y = C for the wild-type DNA sequence.

23a or **23b** and the N-terminus of blocker **31a** was shown to provide sufficient flexibility to allow concomitant binding. This investigation presents, to the best of our knowledge, the first example of sequence-selective chemical ligations on double-stranded PCR DNA templates in a homogeneous system.

Discussion

PNA in DNA-Directed Synthesis. DNA-directed reactions have been explored with the aim of studying self-replication and molecular evolution, constructing and copying defined (nano)molecular architectures, endowing small synthetic molecules with amplifiable information, and developing ligation chemistries that allow sequence-specific detection of DNA. Its extraordinary recognition properties make PNA an interesting candidate in these applications where chemical reactivities are under the control of nucleic acid type hybridization processes.

In DNA-directed reactions, the DNA template aligns the reactive groups and, hence, increases the effective molarity of the ligation probes. The higher the binding affinity, the lower is the required ligation probe concentration and the less likely is ligation to occur in the absence of templates. PNA binds complementary nucleic acids with extremely high affinity and selectivity.[88] Indeed, template-independent "background reactions" were shown to be neglectible at probe concentrations at

(88) Geiger, A.; Lester, A.; Kleiber, J.; Orum, H. *Nucleosides Nucleotides Nucleic Acids* **1998**, *17*, 1717.

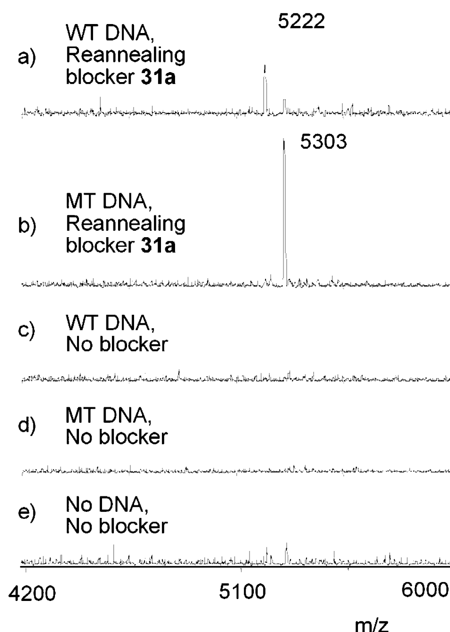


Figure 10. MALDI-TOF/MS analysis of the competition ligation of **23a** and **23b** with **32** in the presence of (a) PCR DNA from wild-type plasmids with blocker **31a**, (b) PCR DNA from mutant plasmids with blocker **31a**, (c) PCR DNA from wild-type plasmids without blocker, (d) PCR DNA from mutant plasmids without blocker, and (e) in the absence of DNA. Single peaks detected corresponded to ligation product **34a** (*m/z* 5303 ([M + H]$^+$) and ligation product **34b** (*m/z* 5222 ([M + H]$^+$)). Conditions: 500 nM DNA, 1 μM probes each, 2 h reaction time. Matrix: sinapinic acid.

or below 10 μM for carbodiimide-mediated reactions and 1 μM for native chemical ligation reactions.

The high affinity of PNA for complementary DNA sometimes requires that special care is taken during sample preparation. One example concerns HPLC analysis, in which sharp peaks can only be obtained if ligation product−target complexes had been fully dissociated. However, separation of the probe strand can be enforced by addition of TFA, which results in partial degradation of the DNA target.

Due to the enhanced chemical stability of PNA, such treatments are without risk of decomposition of the ligated product. The increased chemical stability can furthermore be of advantage when a suitable detection method is being chosen. The DNA-directed PNA ligations described throughout this paper have been analyzed by HPLC methods and MALDI-TOF mass spectrometry. Particularly the latter revealed advantages of using PNA since peak widths are smaller and peak intensities higher than with DNA due to less fragmentation and the reduced tendency to form salt adducts. MALDI-TOF mass spectrometry is a convenient and also accurate means of analysis. It offers unparalleled resolution when compared to HPLC- and fluorescence-based detection methods demonstrated best by the multiplex ligation described in Figure 5.

Moreover, the enhanced biostability is expected to confer significant advantages when the aim is applications inside live cells. In live cell analysis chemical ligation reactions must proceed in the absence of added activating agents. The described PNA-based fragment couplings required the addition of either reagents such as the carbodiimide used in the condensation of two PNA−glycine conjugates or additives such as the benzyl mercaptan that was added to maintain a reducing environment during native chemical ligation reaction. Nevertheless, the native chemical ligation in principle meets the demands set by live cells. It was recently shown that fluorescently labeled cysteine conjugates are able to ligate to protein thioesters inside a live cell.[89,90]

Sequence Selectivity. The data suggest that at constant temperature the ligation fidelity is influenced by two major factors, mismatch position and ligation probe concentration. At a ligation probe concentration of 10 μM, HPLC analysis had indicated inefficient but measurable ligation in the presence of mismatched templates (see Figure 2). The trace amounts of ligation products were even easier to detect by MALDI-TOF/MS analysis. A thorough analysis suggests that the power of match/mismatch discrimination is dependent upon the type of mismatch and probably also upon the sequence context. For example, an A−G mismatch within ligation system **9** and **10a**−**c** led to the wild-type DNA-induced formation of a mutant ligation product, while C−T, T−T, and T−G mismatches proved inefficient in inducing any ligation at all. Other mismatches have not been explored; however, sequence dependencies have been reported for other DNA-based ligation systems as well.[10] The ligation reactions were highly selective when performed at a 1 μM probe concentration (see Figure 5). For example, the native chemical ligation system shown in Figure 8 was selective against a mismatch by more than 140-fold in ligation yield. In previous work on enzymatic and nonenzymatic oligonucleotide ligation reactions, ligation rates rather than ligation yields have been compared. However, differences in ligation yield give a lower estimate of differences in ligation selectivity and represent the measured observable in a diagnostic setup. In most cases of PNA-based native chemical ligations, unspecific ligation products were not detectable at all.

Double-Stranded PCR DNA. Most ligation chemistries were performed on synthetic single-stranded DNA templates. However, applications in molecular diagnostics would require complex, double-stranded DNA to be used. The problem that arises is that stable access of low-affinity probes to a long DNA strand has to be assured in the presence of a long complementary strand. Kool and co-workers addressed this problem by developing a heterogeneous assay, in which double-stranded DNA template was denatured and immobilized on positively charged nylon membranes.[31] This treatment led to an affixing of separated strands and provided accessibility for the subsequent ligation reaction. It was the objective of this work to develop the first ligation method that avoids the need for target immobilization and subsequent washing steps. In a homogeneous system, however, strand separation has to occur in the presence of reactive ligation probes. Thermal denaturation is a convenient means of separating the two PCR-amplified strands. However, it became apparent that carbodiimide activation is not compatible with thermal denaturation. On the contrary, thioesters such as **32** have sufficient temperature stability. The feasibility to implement thermal denaturation was a necessary step but was not sufficient to allow ligation in the presence of double-stranded PCR DNA. It proved essential to add auxiliary PNA that blocked reannealing and thereby helped to keep access to an 11-base segment. It is interesting to note that very recently auxiliary oligonucleotides have been employed to allow DNA ligation reactions on folded RNA.[46]

(89) Yeo, D. S. Y.; Srinivasan, R.; Uttamchandani, M.; Chen, G. Y. J.; Zhu, Q.; Yao, S. Q. *Chem. Commun.* **2003**, 2870.
(90) Lue, R. Y. P.; Chen, G. J.; Hu, Y.; Zhu, Q.; Yao, S. Q. *J. Am. Chem. Soc.* **2004**, *126*, 1055.

We surmise that the use of reannealing blockers could enable native chemical ligations to be performed during the PCR process. Future work will examine this new option for quantitative PCR. Specially designed PNA probes are able to displace one strand in duplex DNA by a process termed strand invasion. In particular, the work performed by Frank-Kamenetskii[91,92] and Nielsen[93] showed that so-called PNA openers are able to unshield one strand of duplex DNA. It is a fascinating vista to apply this local, nonthermal denaturation in the fashioning of a tool for live cell DNA analysis.

Comparison with Other Ligation Methods. Enzymatic ligations exhibit highest selectivity at the ligation junction, where a precise alignment of the termini facilitates attack of the 3′-OH group at the 5′-phosphate.[13] However, under certain conditions ligases can accommodate mismatched bases at the ligation site.[94] The PNA-based chemical ligations described herein as well as the chemical oligonucleotide ligations described by Letsinger[11] and Kool[10] require the mutation site to be positioned at internal sites of a short probe to allow product formation specific for complementary targets. It hence appears as a common feature of chemical ligation methods that ligation fidelity is governed by the selectivity of ligation probe hybridization. PNA−amino acid ligation discriminated mismatched templates by 2 orders of magnitude in ligation yield, which exceeds the selectivity of T4 ligase-mediated ligations. The selectivity of the chemically induced peptide bond formation is similar to the ligation fidelity described for phosphothioate ligation of oligodeoxynucleotides.[10]

It remains to be seen whether the PNA native chemical ligation offers a true alternative to the commonly used enzymatic ligase reactions. One of the necessary requirements, the ability to use double-stranded PCR DNA in homogeneous solution, is fulfilled. In terms of practicability and product yield, there are no significant differences. A ligase reaction typically requires 2−4 h in standard assays. The PNA native chemical ligation reached 70% yield within 2 h and proceeds therefore more rapidly than phosphothioate- and phosphoselenoate-mediated reactions.[35] Kool and co-workers have provided a kinetic analysis of the latter two reactions which on the basis of initial rates allows an upper estimate of 16% and 54% yield after 2 h of phosphothioate and phosphoselenoate ligation, respectively, to be given.[35] In contrast, native chemical PNA ligation furnished 50% ligation product in 22 min.

Experimental Section

General Procedures. Analytical HPLC was run either on an Agilent 1100 instrument using RP-C18 columns CC 250/4 Nucleosil (100-5) HD and EC 250/4 Nucleosil (100-5) PPN (Macherey&Nagel, Düren, Germany) or on a Merck-Hitachi Elite LaChrom using an RP-C18-A5u "Polaris" column (PN 2000-250X046, Varian). Detection of the signals was achieved with a photodiode array detector at wavelengths $\lambda = 260$ nm and $\lambda = 280$ nm. Eluents A (0.1% TFA in water + 1% MeCN) and B (0.1% TFA in MeCN + 1% water) were used in a linear gradient at 50 °C with a flow rate of 1 mL/min. Gradient A: 1% B → 14% B in 30 min. Gradient B: 1% B for 2 min, 1% B → 15% B in 30 min. Gradient C: 3% B → 30% B in 30 min.

MALDI-TOF mass spectra were measured on a Voyager-DE Pro biospectrometry workstation of PerSeptive Biosystems. A 10% solution of sinapinic acid in MeCN/1% TFA (1:1) was used for generating the probe−matrix mixture. The concentrations of the stock solutions of the oligonucleotides were determined by measuring the optical density at $\lambda = 260$ nm on a Varian Carey 100. The specific absorption coefficients ϵ of the oligonucleotides were calculated using the nearest-neighbor method.[95]

Materials. DNA was purchased from MWG in HPSF quality. Water was taken from a Milli-Q ultrapure water purification system (Millipore Corp.). Taq DNA polymerase and PCR buffer were purchased from Quiagen and nucleotides from Carl Roth GmbH & Co. KG, Karlsruhe, Germany. The streptavidin-coated magnetic beads were obtained from Roche Diagnostics. Solid-phase synthesis of PNA and PNA conjugates and full characterization are described in the Supporting Information.

T_M Measurements. UV melting curves were measured at 260 nm by using a Varian Carey 100 spectrometer equipped with a peltier block. A degassed aqueous solution of 100 mM NaCl and 10 mM NaH_2PO_4 adjusted at pH 7.0 using 2 M NaOH was used as buffer. The oligonucleotides were mixed to 1:1 stoichiometry and the solutions adjusted to a final duplex concentration of 1 μM. Prior to analysis, the samples were heated to 95 °C and cooled within 3 h to a starting temperature of 15 °C. The samples were heated to 85 °C with a rate of 0.5 °C/min. T_m values were defined as the maximum of the first derivative of the melting curve.

Carbodiimide-Mediated Ligations. Aqueous stock solutions of the employed oligonucleotides were prepared with concentrations between 1 and 5 mM. The ligation buffer was comprised of an aqueous solution of either 0.6 M imidazole or 0.3 M EDC. Ligation buffers were freshly prepared prior to use. The pH was adjusted at 7.4 using a 2 M NaOH solution. The ligation buffer was placed in Eppendorf tubes, and the appropriate amounts of reactants and template were added and allowed to react by vortexing at 25 °C for 6 h.

Native Chemical Ligations. Aqueous stock solutions of the employed oligonucleotides were prepared with concentrations between 1 and 5 mM. According to requirements, the ligation buffer was comprised of an aqueous solution of either 100 mM NaCl and 10 mM NaH_2PO_4 (subsequent MALDI measurements) or 10 mM NaCl and 10 mM NaH_2PO_4 (subsequent HPLC measurements). Ligation buffers were freshly prepared prior to use. The pH was adjusted at 7.4 using a 2 M NaOH solution. Subsequent manipulation was carried out by avoiding unnecessary exposure to oxygen. The ligation buffer was placed in Eppendorf tubes, and the appropriate amounts of reactants and template were added together with 3% benzyl mercaptan (subsequent HPLC analysis) or 2-mercaptoethanesulfonic acid (subsequent MALDI measurements) and allowed to react by vortexing at 25 °C for 2 h.

HPLC Analysis. (1) Analysis of Carbodiimide-Mediated Ligations. The mixture was extracted with a water-equilibrated RP C18 Sep-Pak cartridge, washed with water, and eluted with water/MeCN (1:1). The solvent was removed in vacuo followed by the addition of 120 μL of 20% aqueous TFA. After 2 h of vortexing at 50 °C (to guarantee the complete hydrolosis of the DNA template), the mixture was characterized by HPLC (Agilent-HPLC instrument, column RP-C18 PPN, gradient A).

(2) Analysis of Native Chemical Ligations. Benzyl mercaptan was separated from the reaction mixture by centrifugation and removed. The reaction was then quenched by adding 10% TFA. After 30 min, the solvent was removed in vacuo, and the residue was dissolved in water containing 1% MeCN and 0.1% TFA and analyzed by HPLC (Merck-Hitachi-HPLC instrument, "Polaris" column, gradient C).

MALDI-TOF/MS Analysis. The reaction mixture was extracted with a water-equilibrated RP C18 ZipTip, washed with water, and eluted with water/MeCN (1:1). After addition of 20% aqueous TFA (25 μL),

(91) Kuhn, H.; Demidov, V. V.; Gildea, B. D.; Fiandaca, M. J.; Coull, J. C.; Frank-Kamenetskii, M. D. *Antisense Nucleic Acid Drug Dev.* **2001**, *11*, 265.
(92) Kuhn, H.; Demidov, V. V.; Coull, J. M.; Fiandaca, M. J.; Gildea, B. D.; Frank-Kamenetskii, M. D. *J. Am. Chem. Soc.* **2002**, *124*, 1097.
(93) Larsen, H. J.; Nielsen, P. E. *Nucleic Acids Res.* **1996**, *24*, 458.
(94) Alexander, R. C.; Johnson, A. K.; Thorpe, J. A.; Gevedon, T.; Testa, S. M. *Nucleic Acids Res.* **2003**, *31*, 3208.
(95) Puglisi, J. D.; Tinoco, I. *Methods Enzymol.* **1989**, *180*, 304.

the mixture was vortexed for 30 min to 2 h, subsequently evaporated to dryness, dissolved in 3 μL of matrix, and analyzed by MALDI/MS.

Ligations on PCR DNA. (1) DNA 29. Plasmids containing the pig H-*ras* oncogene mutation at codon 12 (GGA → GTA) and the wild-type pig H-*ras* were kindly donated by Dr. J. Kuhlmann.[96,97] A segment of 150 bp containing the G12V mutation was PCR-amplified using the primers Biot. 5′-CTC-GTG-GTG-GTG-GGC-GCT-G and 5′-GAT-GTC-CAG-CAG-GCA-CGT-CTC-CC. The resulting DNA was treated with the endonuclease *Ear*I to give the 28 bp biotinylated dsDNA. This dsDNA was immobilized on streptavidin-coated magnetic beads. Subsequent washing with 100 mM NaOH afforded the corresponding 28-base-long ssDNA. The product was quantified using the ssDNA quantification kit from Molecular Probes. The sequence was proved by Sanger sequencing (Genotype, Hirschhorn, Germany).

(2) DNA 33. A segment of 167 bp containing the G12V mutation was PCR-amplified using the primers 5′-ATG-ACG-GAG-TAT-AAG-CTC-GTG-G and 5′-AGG-ATG-TCC-AGC-AGG-CAC.

Aqueous stock solutions of the employed oligonucleotides were prepared with concentrations between 1 and 5 mM. The ligation buffer was comprised of an aqueous solution of 100 mM NaCl and 10 mM NaH_2PO_4 and was freshly prepared prior to use. The pH was adjusted at 7.4 using a 2 M NaOH solution. The so-obtained buffer was degassed and subsequent exposure to oxygen avoided. The ligation buffer was placed in Eppendorf tubes, and the appropriate amounts of reactants and template were added together with 3% 2-mercaptoethanesulfonic acid. The reaction mixture was first heated in a water bath with a temperature of 80 °C for 3 min, then cooled for 3 min using an ice bath, and vortexed at 25 °C for 20 min. This procedure was repeated four times.

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(97) Further information about the sequence: http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=6650729.
(98) Mayfield, L. D.; Corey, D. R. *Anal. Biochem.* **1999**, *268*, 401.

Conclusion

In concluding and as detailed above we have shown that the PNA-based chemical ligations and in particular native chemical ligation of PNA−amino acid thioesters with PNA−cysteine conjugates proceed with sequence selectivities and ligation rates that rival those of ligase-mediated reactions of oligodeoxyucleotides. The use of PNA in DNA-directed chemical ligation has not only facilitated the detection of ligation products by, for example, MALDI-TOF/mass spectrometry but also allowed ligation analysis to be performed in a multiplex format. The exceptional base-pairing properties have enabled the development of a homogeneous system, in which, for the first time, sequence-selective chemical ligation was achieved by using double-stranded PCR DNA templates. This research may open opportunities for single-base mutation analysis in high-throughput format, real-time applications, and live cell analysis. Current work is focused at ligation reactions that may allow template catalysis and the use of fluorescently labeled ligation probes for real-time PCR.

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Supporting Information Available: Synthesis of PNAs and PNA conjugates, characterization data for all new compounds, and reagent preparation for the synthesizer (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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