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DNA-Controlled Ligations – Chemistry for Genetic Analyses

Christian Dose and Oliver Seitz

Institut für Chemie der Humboldt-Universität zu Berlin, Fachinstitut für Organische und Bioorganische Chemie, Brook-Taylor-Straße 2, 12489 Berlin, Germany

E-Mail: * oliver.seitz@chemie.hu-berlin.de

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Abstract

DNA-directed chemical ligations enable the highly sequence specific analysis of mutations in DNA. The full diagnostic potential of DNA-directed chemistry can be harvested when DNA-analogues that provide new opportunities such as improved affinity and selectivity in DNA-binding and/or ease and accuracy of detection are employed. It is shown that peptide nucleic acid (PNA) conjugates, non-ionic biostable DNA analogues, can be ligated by using native chemical ligation. This reaction proceeds as rapidly and more selective than T4-ligase mediated oligonucleotide ligations. The selectivity is higher than 3000-fold in discriminating matched from single mismatched DNA. This high selectivity is the result of a particular ligation architecture which involves an unpaired DNA-base opposite to the ligation site. It is suggested that the high sequence specificity of this so-called abasic ligation architecture facilitates the analysis of early cancer onset. As an example it is shown that as little as 0.2% of single-base mutant in presence of 99.8% wild-type DNA can be detected by mass-spectrometric analysis of the PNA-native chemical ligation. The PNA-ligation chemistry can also be applied to double stranded DNA-templates produced by PCR. In this case, auxiliary PNA needs to be added in order to help binding of the rather short PNA ligation probes to DNA. One drawback of using chemical methods for ligation of oligonucleotides and analogues is product inhibition. Usually the products of ligation bind to the template with higher affinity than the probes before ligation. This prevents catalytic turnover, which, how-

ever, is desired if targets are present at low concentration. An approach to overcome product inhibition in PNA ligation is presented. The approach is based on native chemical reactions involving isocysteine rather than cysteine. Ligations at isocysteine succeed through the intermediacy of a ligated thioester that rearranges via chain-extension. This step increases the flexibility of the final product, thereby reducing its affinity to the DNA template. To facilitate real-time monitoring of product formation a fluorescence resonance energy transfer (FRET)-based detection method was established. By using the FRET technique single base mutations can be detected within minutes and with perfect sequence selectivity at optimized conditions.

Introduction

The ability to detect specific nucleotide sequences is of interest in various fields from genetics, microbiology, oncology, haematology, immunology to food technology and forensics. An important issue is the sequence fidelity of the detection method, particularly when aiming for the detection of somatic nucleotide mutations. For example, to evaluate early stages of cancer, sophisticated methods are needed which can distinguish mutated gene sequences at low concentrations over the background of predominant wild-type sequences. One of the most accurate means of analysing DNA single base mutations is the oligonucleotide ligation assay (OLA) which is based on the discriminative ability of DNA ligases [1]. Herein, the presence of enzymes such as T4 and *Tth*-ligases induces ligation reactions which occur more than 1000-fold faster on match DNA-templates than on single mismatched sequences [2]. These enzymes are markedly less efficient on RNA targets [3]. The restricted tolerance to even small modifications of probes as well as the fact that ligases are unlikely to be used in living cells motivated chemists to investigate alternative non-enzymatic detection methods. One particularly successful strategy has been developed by Kool and coworkers who described a DNA-template controlled ligation of 5'-iodo oligonucleotides with 3'-phosphorothioates that proceeded 180-fold faster in rate on matched DNA than on single mismatched sequences [4, 5]. In general, the selectivity of a given chemical ligation method is governed by the hybridization of the probes to be ligated. We reckoned that enhancements of ligation selectivity should be achieved when using DNA-analogues that bind DNA targets with higher affinity and sequence selectivity. These features might be helpful in applications targeting double-stranded DNA, wherein short-length ligation probes have to compete with reannealing of long oligonucleotides. As an alternative to DNA we used PNA, an uncharged molecule in which the nucleobases are attached to repeating *N*-(2-aminoethyl)glycine units via methylen carbonyl linkers (Fig. 1) [6, 7]. This artificial scaffold leads to features such as high discriminative power against single base mismatches combined with strong DNA-affinity. Moreover, PNA is stable against both chemical and biological degradation.

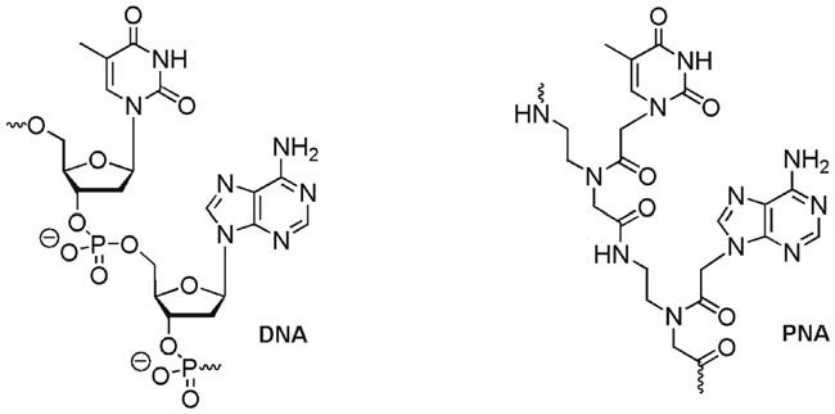
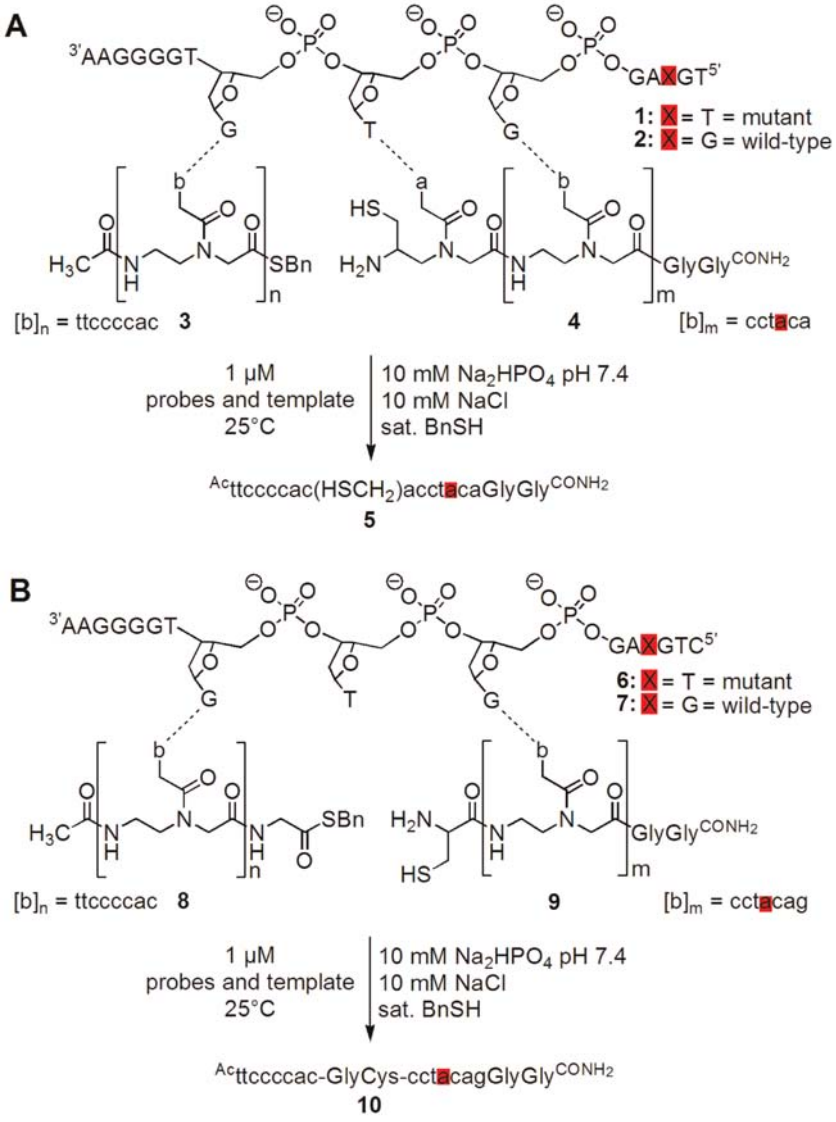


Figure 1. Structure of deoxyribo nucleic acid (DNA) and peptide nucleic acid (PNA).

Design of a Ligation Method

The probes have to be equipped with reactive groups that confer efficient coupling reactions in water. PNA comprises a pseudopeptide scaffold, hence it was considered to establish an amide forming ligation reaction. The native chemical ligation (NCL) reaction has been demonstrated as a powerful method for joining unprotected peptides in aqueous solution and at physiologically relevant conditions [8]. This reaction is induced by a reversible thiol exchange step in which a thioester conjugate is linked to a cysteine containing segment by forming a thioester intermediate. A subsequent $S \rightarrow N$ acyl-shift occurs spontaneously to furnish the final ligation product containing a natural amide bond.

DNA-directed chemical ligation systems are usually designed as nick ligations wherein the two fragments to be ligated align on the template by involving a contiguous segment of the template in base pairing. However, we surmised that a ligation, in which the template base opposite to the ligation site remains unpaired, would lead to enhancements of sequence fidelity due to omission of a less selective base stacking term. It was assumed that the application of a reaction method during which an abasic-site is formed at the ligation junction may lead to enhancements of sequence selectivity. Also, it is conceivable that the higher flexibility of the reactive groups in such an "abasic ligation" architecture results in the reduction of the effective molarity thereby diminishing rate accelerations exerted by the template. To test this proposition, it was necessary to explore two distinct ligation architectures. In a paradigm study we examined the native chemical ligation of two modified PNA probes targeted against the carcinogenic G12V mutation of *ras*-gene segments (Scheme 1).

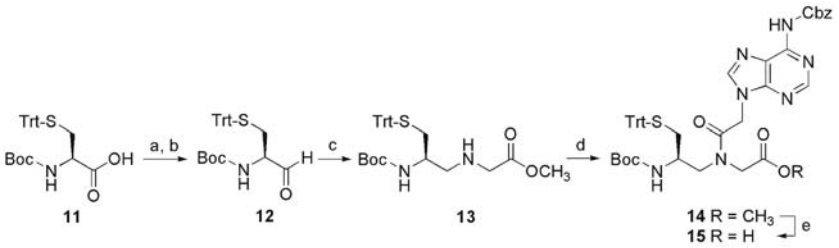


Scheme 1. Reaction of the A) nick and B) abasic Native Chemical PNA-Ligation.

Evaluation of the Native Chemical PNA-Ligation Reactions

To put the nick PNA ligation into practice a PNA-monomer is required which contains a 1,2-aminothiol structure. The desired Boc/Trt/Cbz-protected PNA adenine monomer **15** was synthesized from commercially available Boc/Trt-Cysteine **11** and incorporated in

solid- phase PNA synthesis (Scheme 2) [9]. The abasic ligation format was realized by allowing a PNA-glycine thioester **8** to react with a cysteinyl-PNA **9** (Scheme 1B). The central glycine-cysteine dipeptide served as isosteric replacement of the omitted adenine monomer.



Scheme 2. Synthesis of protected PNA-Monomer **15**. a) $HNCH_3(OCH_3)$, $ClCO_2iBu$, NMM, DCM, RT, 98%; b) LAH, THF, 0 °C, 100%; c) Gly-OCH_3, $NaBH_3CN$, CH_3OH, RT, 72%; d) 6-*N*-(Cbz)-9-(carboxymethyl)adenine, pivaloylchloride, NMM, DMF/CH_3CN (1:1), RT, 78%; e) LiOH, THF, 0 °C, 95%.

The ligation probes used in nick and abasic Native Chemical PNA-Ligation reactions **3**, **4**, **8**, and **9**, respectively, were synthesized via solid-phase PNA synthesis. Ligations were commenced by allowing PNA-nucleophiles **4** and **9** to react with PNA-thioesters **3** and **8**, respectively, in the presence of perfectly matched DNA, single mismatched DNA, or in the absence of DNA. HPLC analysis revealed that bimolecular nick ligation (Fig. 2A) and abasic ligation (Fig. 2B) were slow in the absence of DNA (< 0.2% yield after 60 min). In contrast, addition of complementary templates **1** and **6**, respectively, resulted in a significant increase in ligation rates and product yields. The abasic ligation on matched DNA **6** reached the 50% stage within 10 min and provided 75% yield after 60 min. For reasons of comparison with alternative ligation methods an apparent second order rate constant k_{app}= 2470 $M^{-1}\cdot s^{-1}$ was determined. Interestingly, nick ligation of **3** and **4** proceeded even faster than abasic PNA-ligation, as evidenced by the 1.6-fold enhanced second order rate constant k_{app}= 3973 $M^{-1}\cdot s^{-1}$. Hence, the Native Chemical PNA-Ligation reactions are amongst the fastest DNA-controlled ligation reactions [10 – 12]. Most noticeable were the results of the ligation experiments on single mismatched templates. While abasic ligation proved inefficient (0.2% yield after 60 min) on template **7**, nick ligation still proceeded remarkably well on mismatched DNA **2**. The initial rates of ligation indicated that abasic ligation of **8** and **9** on matched DNA **6** occurred 3450-fold faster than on single mismatched DNA **7**. This fidelity exceeds the sequence selectivity obtained with alternative chemical ligation methods [4] and rivals the speed and match/mismatch discrimination reported for enzymatic-based detection techniques [2]. Only 7-fold rate differences were determined for nick ligation of **3** with **4** on matched and single mismatched DNA-sequences **1** and **2**, respectively. These observations demonstrate clearly that the ligation architecture is of critical importance for the sequence selectivity of the Native Chemical PNA-Ligation reaction [13, 14].

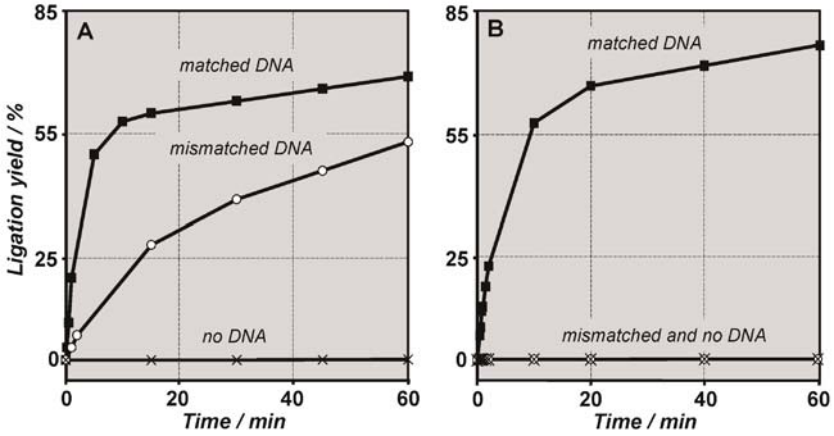


Figure 2. Reaction time course of A) nick ligation of **3** and **4** and B) abasic ligation of **8** and **9** in the presence of matched DNA, single mismatched DNA, and in the absence of DNA. Reaction conditions as shown in Scheme 1.

Detection of Mutated DNA Sequences as Minor Compounds

The previous results demonstrated high sequence selectivity and rate accelerations of the abasic PNA-ligation reaction. These features should allow the design of a very precise and rapid detection assay, wherein single base mutations are identified in minority compounds over the background of wild-type sequences. To emulate such early cancer stages matched and mismatched *ras*-gene segments were mixed in varying ratios in the presence of abasic ligation probes **8** and **9**. After 20 minutes reaction time the ligations were analysed by MALDI-TOF mass spectrometry. Indeed, Fig. 3A illustrates that single-nucleotide specific ligation products are detectable in spite of high backgrounds of wild-type DNA. The formation of mutant ligation product **10** ($m/z = 4260$) and, hence, occurrence of mutant DNA was detected when present as a mixture of wild-type/mutant DNA in 9:1 and 99:1 ratios. Moreover, even 0.2 % of mutant DNA was still detectable (signal-to-noise ratio = 32) in the presence of 99.8 % of wild-type DNA (ratio 499:1) while virtually no signal appeared when pure wild-type DNA (signal-to-noise ratio < 3) was present.

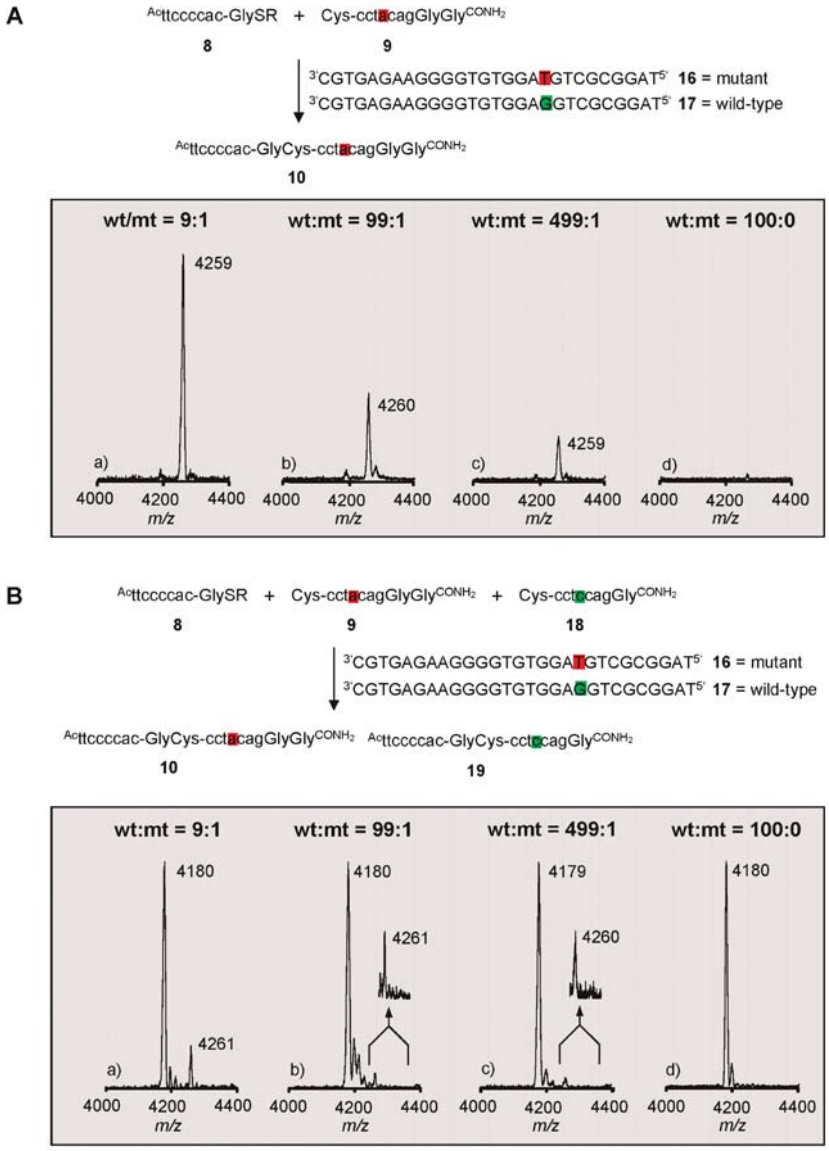


Figure 3. MALDI-TOF/MS analysis of abasic ligation in the presence of different match/mismatch DNA-ratios. Peak heights are calibrated on signal-to-noise ratios. Reaction conditions: 1 µM probes, sum of templates = 1 µM, 2 w/v sodium 2-mercaptoethanesulfonate, 10 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, 25 °C, t = 20 min, R = $(CH_2)_2SO_3^-$ (*m/z*: 4260 = **10**, 4179 = **19**).

In a duplex assay the formation of a wild-type specific ligation product can serve as an internal standard. Thus, reactions were carried out with mixtures of wild-type and mutant specific PNA-probes **9** and **18**, respectively. Figure 3B shows the ligation products formed in a reaction of thioester **8** with competing mutant thiol probe **9** and wild-type thiol probe **18**. Wild-type DNA **17** was the major component of the template mixture and indeed wild-type specific ligation product **19** (*m/z*= 4179) was the dominating peak in all instances. The smallest detectable proportion of the mutant template was 0.2% (signal-to-noise ratio = 6) which gives testimony to the high selectivity of the Native Chemical PNA-Ligation reaction [14].

Native Chemical PNA-Ligation on Single and Double Stranded PCR-DNA

Most published ligation reactions have been performed on single-stranded synthetic DNA templates. However, in practical applications of molecular diagnostics the challenge arises to perform such ligation chemistry on DNA from biological sources. Typically, the DNA segment of interest is extracted by PCR. It is hence mandatory that double-stranded DNA can be used as template. However, the reannealing of long complementary sequences after thermal denaturation, hinders hybridization of the short-length ligation probes. In order to compete against reannealing of the amplified DNA strands, probes have to be long enough to form stable probe/target duplexes. Unfortunately, the design criteria for high sequence fidelity demands short probes that usually can not compete with reannealing of the two amplified DNA-strands. As a solution to this problem a ligation system was designed wherein the combined use of a medium-size PNA-thioester **22** and a 12mer PNA-oligomer **23**, acting as a reannealing blocker, should facilitate hybridization of the short-length mutant and wild-type specific cysteine-PNA conjugates **9** and **18**, respectively (Fig. 4). After thermal denaturation of the 167mer double-stranded DNA at 80 °C in presence of probes **9**, **18** and **22** as well as the reannealing blocker **23** a subsequent rapid cooling to 20 °C allowed sequence selective hybridization of the probes to the template which, indeed, promoted template-controlled product formation as confirmed by MALDI-TOF mass spectrometry analysis. As expected, after two hours reaction time no product signal was detectable in the absence of dsDNA (Fig. 4A). The ligation on dsMT-DNA **20** delivered a mutant-specific ligation product with *m/z*= 5303 (Fig. 4B). On dsWT-DNA **21** wild-type related product with *m/z*= 5222 was detected (Fig. 4C) [15].

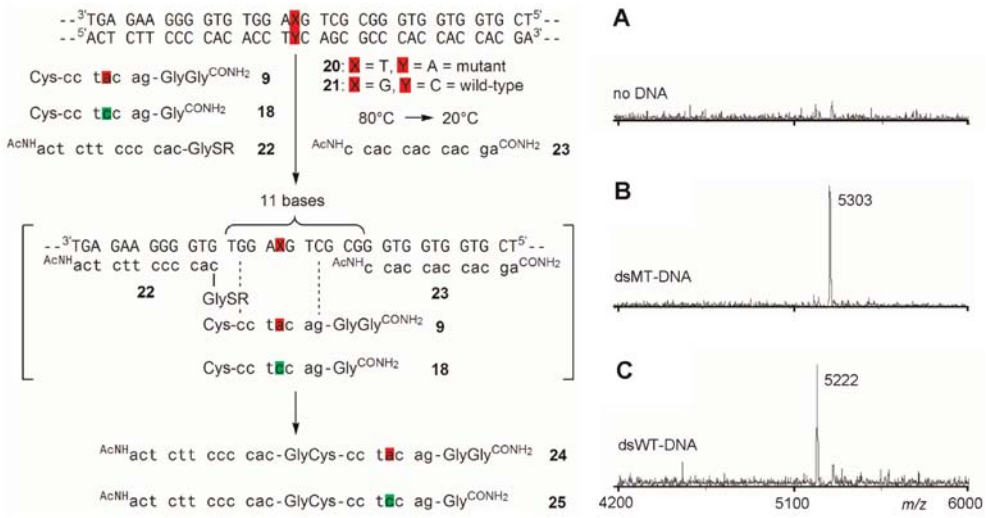
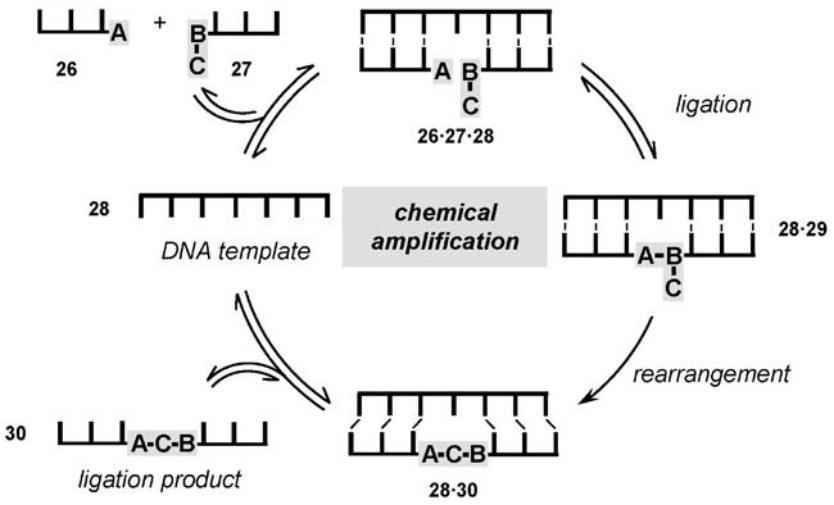


Figure 4. Sequence selective Native Chemical PNA-Ligation on double-stranded PCR-DNA. Reaction conditions: 1 µM probes and reannealing blocker, 500 nM dsDNA, 2 w/v sodium 2-mercaptoethanesulfonate, 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, 25 °C, t = 2 h, R = $(CH_2)_2SO_3^-$ (*m/z*: 5303 = **24**, 5222 = **25**).

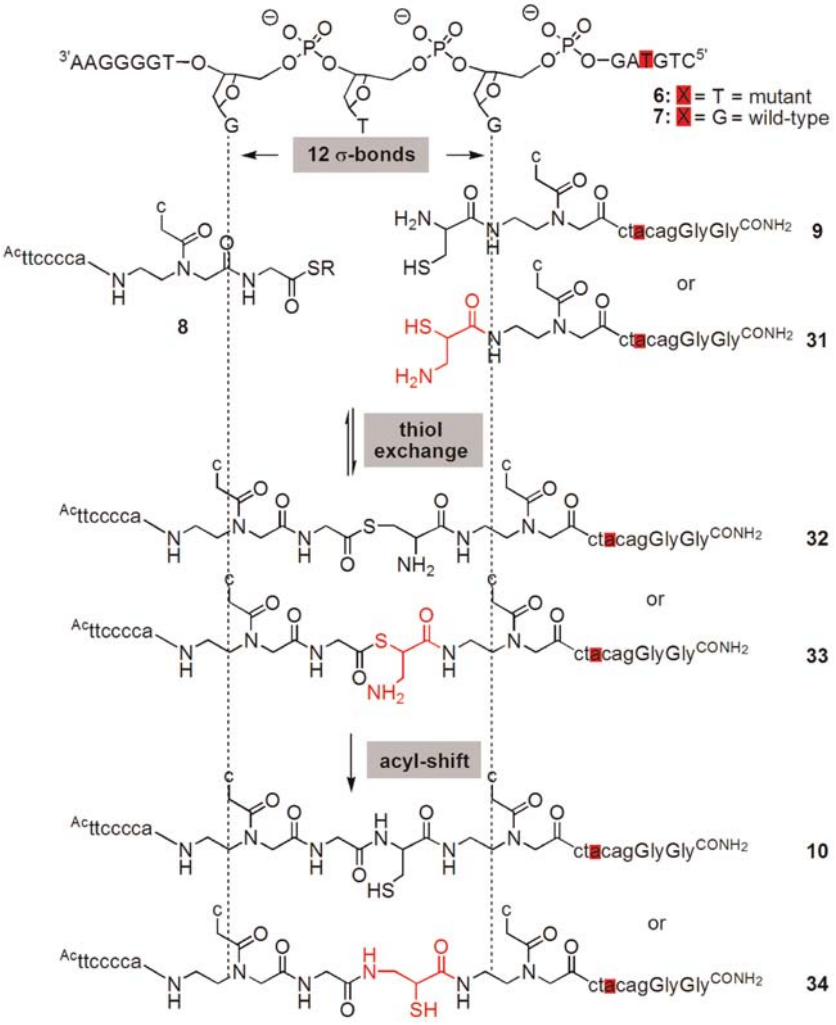
Design of a Template-Catalysed Chemical PNA-Ligation

In contrast to enzymatic reactions, chemical ligation strategies rarely provide multiple ligation products when the target is present at lower than stoichiometric concentrations. Turnover is usually inhibited by stronger binding of the ligation product to the template compared to the non-ligated probes. However, amplification of the product signal is desired in situations where DNA targets have to be detected at low concentrations. To achieve multiple reaction cycles in a chemical ligation format the challenge arises to decrease the DNA-affinity of the ligation product without affecting the DNA-affinity of the probes before ligation. As a potentially general solution to the problem of product inhibition in template controlled ligation reactions, a reaction cycle was designed which comprises a two-step ligation-rearrangement reaction as central event. As shown in Scheme 3 binding of the ligation probes **26** and **27** to the template triggers a chemical ligation reaction which forms intermediate **28**·**29**. A subsequent spontaneous rearrangement step (B→C) leads to an alteration of the scaffold in **29** providing the rearranged product **30**. We envisioned that the rearrangement facilitates dissociation of the rearranged product/template duplex **28**·**30**.



Scheme 3. General scheme of ligation-rearrangement reactions for reducing product inhibition in DNA-template controlled ligations.

A powerful reaction that contains a rearrangement step during the linkage of two modified ligation probes is the previously described Native Chemical PNA-Ligation [14, 15]. In this reaction PNA-thioester conjugate **8** is linked by the cysteine (Cys)-containing PNA-oligomer **9** whereby formation of reaction intermediate **32** proceeds (Scheme 4). A subsequent $S \rightarrow N$ acyl-shift leads to contraction of the main chain which is expected to align the nucleobases in product **10** in an interbase distance of 12 σ-bonds, more favourably than in thioester intermediate **32** (interbase distance = 13 σ-bonds). On the contrary, reaction of thioester **8** with isocysteine (*i*Cys)-PNA **31** proceeds via main chain extension providing an interbase distance of 13 σ-bonds in ligation product **34**. It was assumed that the product from the *i*Cys-mediated Native Chemical PNA-Ligation **34** should have lower affinity to template **6** than the product of Cys-mediated ligation **10**. The required Boc/Trt-protected isocysteine building block was synthesized starting from thiomalic acid. As key event of the 5-step synthesis the curtius-rearrangement enabled the convenient introduction of the Boc-protecting group [16]. The Boc/Trt-protected isocysteine was subsequently used in solid-phase PNA-synthesis to obtain PNA-probe **31** targeting the carcinogenic mutation G12V of a 16mer *ras*-gene segment. Next considered was the influence of the structural variation between Cys and *i*Cys containing ligation products **10** and **34**, respectively, on the stability of the corresponding DNA·PNA duplexes. Melting temperature measurements revealed that the duplex **6·34** containing the *i*Cys-related ligation product (T_M= 53 °C) dissociated easier than the Cys-containing duplex **6·10** (T_M= 58 °C).



Scheme 4. Reaction geometries of the Cys and *i*Cys-mediated chemical PNA-ligation on DNA templates.

The reactivity of *i*Cys and Cys was compared by studying template independent ligation reactions of **9** and **31** with PNA-thioester **8**. At 100 µM probe concentration the Cys-mediated ligation proceeded smoothly providing 77% yield of product **10** after 6.5 h (Fig. 5A). In contrast, the *i*Cys-mediated chemical ligation was less efficient leading to 49% yield after identical reaction time. The comparison of the initial rates revealed that the bimolecular *i*Cys-mediated PNA-ligation proceed 4.4-fold slower than the corresponding Cys-mediated reaction. This result can be explained by the lower nucleophilicity of the sterically hindered thiol group in *i*Cys. The lower reactivity was considered useful in template-controlled reactions, as it could result in lower levels of off-template ligations.

Indeed, at 1 µM probe concentration the *i*Cys-mediated ligation reaction was extremely slow and provided less than 0.2 % ligation yield after 60 minutes. The addition of perfectly matched DNA-template **6** triggered the *i*Cys-mediated ligation, which proceeded as fast as the template-controlled Cys-mediated chemical ligation (Fig. 5B, k_{app}= 2470 $M^{-1}\cdot s^{-1}$). Furthermore, *i*Cys ligation also delivered a similar match/mismatch discrimination (3450:1). It can be concluded that the replacement of Cys by *i*Cys reduced the template independent background reaction while speed and sequence fidelity of the template-controlled reaction were unaffected.

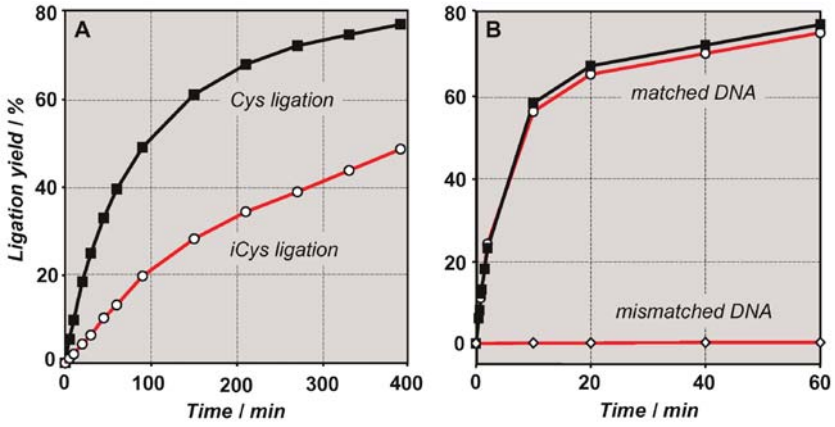


Figure 5. Time courses of the Cys (■) and *i*Cys-mediated (○) PNA-ligation. A) At 100 µM probe (reaction buffer: 100 mM Na_2HPO_4 sat. BnSH, pH 7.4, 25 °C) and B) at 1 µM probe in the presence of matched DNA **6** or single mismatched DNA **7** (♦, only *i*Cys-mediated ligation shown, reaction buffer: 10 mM Na_2HPO_4, 10 mM NaCl, sat. BnSH, pH 7.4, 25 °C).

The *i*Cys ligation was designed with the aim to allow signal amplification in scenarios where less than stoichiometric amounts of template are available. Both reactions were performed at different template/probe ratios using 100 – 10.000 equivalents of probes per equivalent target DNA. It was expected that a large excess of ligation probes over the template would facilitate the displacement of the reaction product. Turnover numbers were determined by counting the number of equivalents of ligation product caused by one equivalent template. To ensure that the measured ligation yield was the result of the template dependent reaction product yields in the absence of target DNA were subtracted. HPLC-analysis revealed that after 24 h incubation at 25 °C both reactions furnished significant turnover numbers at low template/probe ratios (Fig. 6). For example, at 1 µM concentration of probes **8** and **31** and 10 nM concentration of template **6** *i*Cys ligation yielded 3.5 turnovers which improved to 43 turnovers at 0.1 nM target. In contrast, the Cys-mediated ligation of **8** and **9** proceeded less efficiently providing 1.6 and 14 turnover numbers at 10 nM and 0.1 nM, respectively, target concentrations.

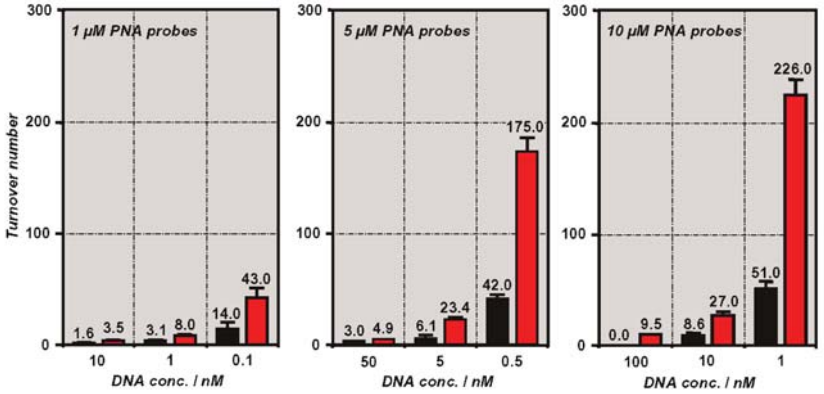


Figure 6. Turnover numbers in Cys (black bars) and *i*Cys-mediated (red bars) PNA-ligations after 24 h reaction time (reaction buffer: 10 mM Na_2HPO_4, 150 mM NaCl, 10 mM MESNa, pH 7.4, 25 °C).

An increase of the ligation probe concentration leads to higher turnover numbers at any given template/probe ratio. For example, signal amplification in Cys-mediated ligation reaction reached 42- and 51-fold at 5 µM and 10 µM probe concentration, respectively. Even higher turnover numbers were observed in the *i*Cys reaction. As shown, highest signal amplification was achieved with 226 turnovers at 10 µM ligation probe and 1 nM DNA target concentration [17]. This significantly exceeds the turnover numbers achieved with alternative chemical ligation methods [18].

Detection of the Native Chemical PNA-Ligation by Fluorescence Resonance Energy Transfer

Fluorescence based methods offer probably the most facile and sensitive means of detecting biomolecules in complex environments. In a chemical ligation reaction the formation of the ligation product should trigger the emission of a specific fluorescence signal. Fluorescence resonance energy transfer (FRET) is commonly used to report the proximity of two labelled molecules 19]. We chose an internal labelling approach to allow for convenient screening of various donor-acceptor distances. Internal labelling was made available via a new PNA-monomer that featured a lysine side chain. Carboxyfluoresceine (FAM) was attached to PNA-thioester **37** and tetramethylrhodamine (TMR) to *i*Cys-containing probe **38**.

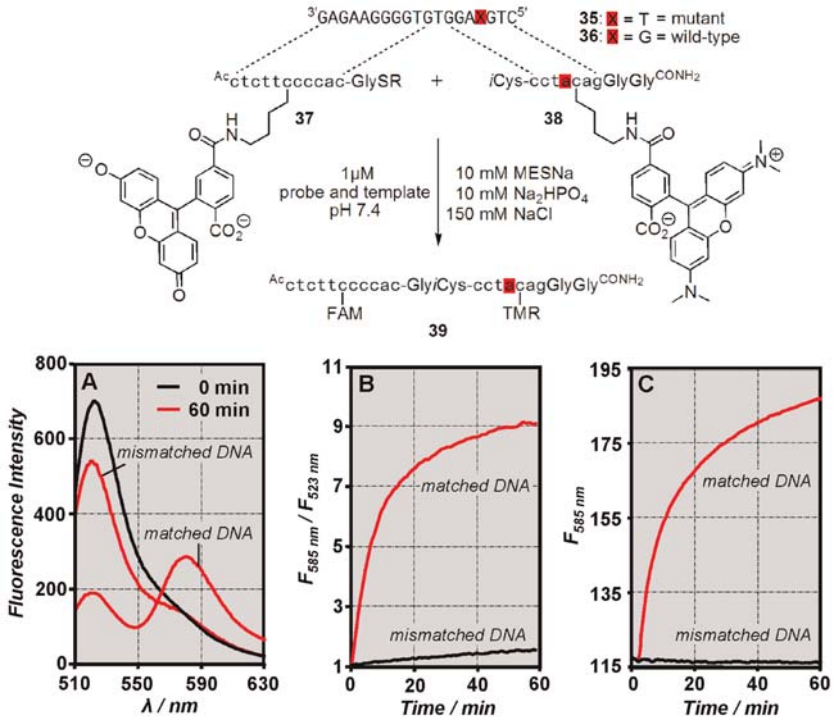


Figure 7. Reaction and fluorescence monitoring of the *i*Cys-mediated PNA-ligation of **37** and **38** on matched DNA **35** and single mismatched DNA **36**. A) Fluorescence spectra for reactions before addition of DNA and 60 min after addition. B) Ratio of fluorescence intensities at 585 nm and 523 nm measured for reactions at 25 °C. C) Fluorescence intensity at 585 nm for reactions at 37 °C, R =$(CH_2)_2SO_3^-$.

Figure 7 shows the results of reactions monitored in real-time by fluorescence spectroscopy. The fluorescence spectrum of the probes **37** and **38** in the absence of template is dominated by FAM emission at 523 nm (Fig. 7A, black). The addition of matched template **35** triggered ligation and resulted in a marked change of the fluorescence spectrum characterized by an increase of TMR emission (585 nm) and a decrease of FAM emission (523 nm). On the contrary, mismatched DNA template **36** was considerably less efficient in inducing fluorescence changes (Fig. 7A, red). The formation of the ligation product can be monitored by means of the ratio of fluorescence intensities F_{585}/F_{523} (Fig. 7B) or by directly following TMR emission at 585 nm (Fig. 7C). Either method generates a positive signal and was suited to reveal the high sequence selectivity of the ligation. Comparison of the initial rates confirmed that fluorescence was produced 127-fold faster on matched template **35** than on mismatched template **36**. Moreover, a perfect sequence selectivity was observed by performing the reaction at 37 °C.

Conclusion

In summary, we have introduced the Native Chemical PNA-Ligation reaction which can be applied to detect single base mutations in DNA-templates. It was shown that the template-controlled ligation reaction proceeds with higher sequence fidelity when performed opposite to unpaired template-nucleobases. This so-called "abasic PNA-ligation" was shown to occur as fast and as sequence selective as ligase-mediated reactions. The exceptional discriminative power of the PNA-ligation was used for detecting single base mutations by MALDI-TOF/MS in PCR amplified double-stranded DNA and in single-stranded DNA templates present as minority compound in different mutant/wild-type ratios. It was shown that 0.2 % single base mutant DNA over the background of 99.8 % wild-type sequence were clearly detectable. We also showed an approach to overcome product inhibition, which usually prevents catalytic turnover. This approach is based on reactions that succeed via ligation and a subsequent flexibility enhancing rearrangement. The native chemical ligation at isocysteine proceeds via such a chain-extending rearrangement and it was the isocysteine-mediated ligation that provided highest turnover numbers at low DNA template concentrations. To facilitate monitoring of product formation we established a fluorescence resonance energy transfer (FRET)-based detection method which allows real-time measurements. The FRET-signal is specific for product formation and provides for positive signalling. Speed and selectivity of the FRET-ligation were high. This account demonstrates the high potential of the Native Chemical PNA-Ligation for analysing mutated DNA sequences at single nucleotide resolution.

References

[1] Landegren, U., Kaiser, R., Sanders, J., Hood, L. (1988) A ligase-mediated gene detection technique. *Science* **241:**1077 – 1080.

[2] Luo, J., Bergstrom, D.E., Barany, F. (1996) Improving the fidelity of Thermus thermophilus DNA ligase. *Nucleic Acids Res.* **24:**3071 – 3078.

[3] Sekiguchi, J., Shuman, S. (1997) Ligation of RNA-containing duplexes by vaccinia DNA ligase. *Biochemistry* **36:**9073 – 9079.

[4] Xu, Y., Kool, E.T. (1999) High sequence fidelity in a non-enzymatic DNA autoligation reaction. *Nucleic Acids Res.* **27:**875**-**881.

[5] Xu, Y.Z., Karalkar, N.B., Kool, E.T. (2001) Nonenzymatic autoligation in direct three-color detection of RNA and DNA point mutations. *Nature Biotechnol.* **19:**148 – 152.

[6] Nielsen, P.E., Egholm, M., Berg, R.H., Buchardt, O. (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* **254:**1497 – 1500.

[7] Uhlmann, E., Peyman,A., Breipohl, G.,Will, D.W. (1998) PNA-synthetic polyamide nucleic acids with unusual binding properties. *Angew. Chem. Intl Ed.* **37:**2797 – 2823.

[8] Dawson, P.E., Muir, T.W., Clark-Lewis, I., Kent, S.B.H. (1994) Synthesis of proteins by native chemical ligation. *Science* **266:**776 – 779.

[9] Dose, C., Seitz, O. (2005) Convergent synthesis of peptide nucleic acids by native chemical ligation. *Organic Lett.* **7:**4365 – 4368.

[10] Saito, I., Miyauchi, Y., Saito, Y., Fujimoto, K.(2005) Template-directed photoreversible ligation of DNA via 7-carboxyvinyl-7-deaza-2'-deoxyadenosine. *Tetrahedron Lett.* **46:**97-99.

[11] Gryaznov, S.M., Schultz, R., Chaturvedi, S.K., Letsinger, R.L. (1994) Enhancement of selectivity in recognition of nucleic acids via chemical autoligation. *Nucleic Acids Res.* **22:**2366-2369.

[12] Sokolova, N.I., Ashirbekova, D.T., Dolinnaya, N.G., Shabarova, Z.A. (1988) Chemical-reactions within DNA duplexes-cyanogen-bromide as an effective oligodeoxyribonucleotide coupling agent. *FEBS Lett.* **232:**153-155.

[13] a) Mattes, A., Seitz, O. (2001) Sequence fidelity of a PNA-based ligation reaction. *Chem. Comm.* 2050 – 2051; b) Mattes, A., Seitz, O. (2001) Mass-spectrometrical monitoring of a PNA-based ligation reaction for the multiplex detection of DNA single base mutations. *Angew. Chem. Intl Ed.* **40:**3178-3181.

[14] Ficht, S., Dose, C., Seitz, O. (2005) As fast and selective as enzymatic ligations: unpaired nucleobases increase the selectivity of DNA-controlled native chemical PNA-ligation. *ChemBioChem* **6:**2098 – 2103.

[15] Ficht, S., Mattes, A., Seitz, O. (2004) Single nucleotide specific PNA-peptide ligation on synthetic and PCR DNA templates. *J. Am. Chem. Soc.* **126:**9970-9981.

[16] Dose, C., Seitz, O. (2004) New isocysteine building blocks and chemoselective peptide ligation. *Org. Biomol. Chem.* **2:**59 – 65.

[17] Dose, C., Ficht, S., Seitz, O. (2006) Reducing product inhibition in DNA-template controlled ligation reactions. *Angew. Chem. Intl Ed.* DOI: 10.1002/anie.200600464.

[18] Abe, H., Kool, E.T. (2004) Destabilizing universal linkers for signal amplification in self-ligating probes for RNA.*J. Am. Chem. Soc.* **126:**13980 – 13986.

[19] Stryer, L. (1978) Fluorescence energy transfer as a spectroscopic ruler. *Ann. Rev. Biochem.* **47:**819 – 846.