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A Template-Mediated Click–Click Reaction: PNA–DNA, PNA–PNA (or Peptide) Ligation, and Single Nucleotide Discrimination

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A highly efficient chemical ligation was developed for quantitative conjugation of PNA with DNA (PNA or peptide) by using the copper-catalyzed azide–alkyne cycloaddition reaction. Whereas PNAs with an alkyne at the C-terminus and an azide at the N-terminus have been used, an efficient click–click reaction occurs. The PNA click ligation is sequence specific and capable of single nucleotide discrimination.

Introduction

Peptide nucleic acids (PNAs) are nuclease-resistant DNA mimics in which the sugar–phosphate backbone has been replaced by a pseudopeptide backbone.[1,2] PNA binding to complementary DNA or RNA shows higher stability, greater specificity, and a faster rate than DNA binding.[3–5] Due to these properties, PNAs are currently of considerable interest as potential reagents for antisense/antigene therapy, molecular diagnostics, biosensors, molecular biology, and nanotechnology.[6–8] However, the biological applications of PNAs are limited by their poor water solubility, tendency to self-aggregation, and low cell penetration ability. One of the approaches to improve their physicochemical and biological properties is to synthesize PNA–DNA[9] or PNA–peptide conjugates.[10] As the standard methods for the synthesis of DNA (or RNA) and PNA are incompatible, complex modifications of PNA monomers and synthetic protocols were required for the construction of the PNA conjugates. Furthermore, during chain elongation, aggregation of the growing oligomer chain can be caused by either intra- or intermolecular interactions. This can lead to low coupling efficiencies. Thus, postsynthetic conjugation (e.g., ligation) must be considered as a route to PNA conjugates or longer PNAs. Recently, native chemical ligation and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) mediated ligation have been developed for conjugating PNA with DNA, PNA, or peptides in the Seitz and Orgel laboratories.[11–13] PNA ligation has also been used for the detection of single nucleotide polymorphisms in ligation-based chemical approaches.[12,13] However, there is considerable scope for developing more efficient methods for PNA conjugation for its outstanding applications.

Ideally, the chemistry for postsynthetic modification would be clean, fast, high-yielding, and operate under mild conditions. The copper(I)-catalyzed Huisgen cycloaddition of azides and alkynes, the most prominent example of click chemistry,[14] appears to fulfil all the necessary requirements. It functions efficiently in aqueous media and has been proven to be a powerful method for postsynthetic DNA modification.[15] It has previously been used for the preparation of surface-immobilized DNA,[16] DNA–protein conjugates,[17] and cyclic peptide structures[18] and in DNA cross-linking and DNA labeling.[19,20] Recently, the click re-

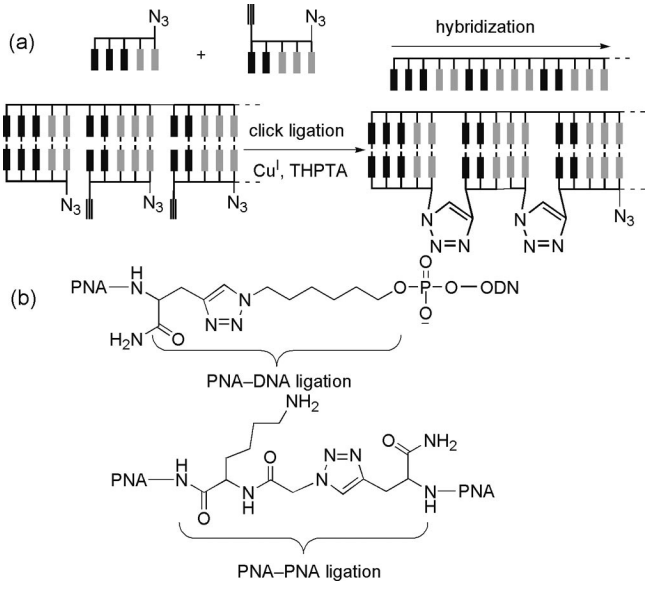

Figure 1. Template-directed click–click ligation of DNA/PNAs: (a) schematic structure and (b) chemical structure at ligation point.

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action has been used for template-directed DNA ligation and covalent intramolecular DNA circularization and catenation in the Brown laboratory.[21]

Here we report a convenient, versatile, and highly efficient method for sequence-specific conjugation of PNA with DNA by using template-directed click ligation, which is capable of single nucleotide discrimination (Figure 1). This method is also useful for PNA–PNA and PNA–peptide ligation. When a PNA was modified with an azide at the N-terminus and an alkyne at the C-terminus, an efficient click–click reaction occurs. This enables the preparation of extended PNA sequences. The ligation reaction is characterized by fast, clean, mild reaction conditions and by its tolerance to a broad range of functional groups.

Results and Discussion

Azide-containing oligodeoxynucleotides (ODN) were prepared by labeling 5′-bromo-modified ODN with NaN_3 in DMF in the presence of NaI.[22] PNAs containing an alkyne at the N-terminus were synthesized by using Fmoc-L-propargylglycine (Supporting Information). DNA template-directed ligations were performed by using a 3′-[^{32}P]-labeled DNA substrate (ODN-**1** or -**2**), a DNA template (ODN-**3** or -**4**), and a PNA substrate (PNA-**5** or -**6**; Table 1). All ligation reactions were carried out in 100 mM NaCl and 10 mM potassium phosphate buffer to ensure complete formation of the DNA/PNA duplex with the template. Duplex stability of PNA-**5**·ODN-**3** (T_m = 62.5 ± 1.5 °C) and PNA-**6**·ODN-**3** (T_m = 63.2 ± 1.3 °C) was determined by UV-melting analysis. Each ligation reaction was studied with 50 nM of 3′-[^{32}P]-labeled DNA substrate and different concentrations of PNA substrate in the presence or absence of DNA template.

Table 1. Oligonucleotide sequences used for this study.

Code	Sequences
ODN-**1**	5′-d$N_3$$(CH_2)_6$ GA TTG CGG TAG TGA TGG A
ODN-**2**	5′-d$N_3$$(CH_2)_6$GCA CGC GTC G
ODN-**3**	5′-dTCCATCACTACCGCAATCAGGCCAGATC
ODN-**4**	5′-dCGA CGC GTG C *AG GCC AGA TC*A GGC CAG ATC AGG CCA GAT CAG GCC AGA TC
PNA-**5**	H-(Lys)GATCTGGCCT(propargylglycine)
PNA-**6**	N_3CH_2CO(Lys)GATCTGGCCT(propargylglycine)
7[a]	ODN-**1**-**L**-PNA-**5**
Peptide-**8**	$N_3CH_2CO(Lys)_6$
9[a]	PNA-**5**-**L**-peptide-**8**
10[a]	ODN-**2**-(**L**-PNA-**6**)$_4$
PNA-**15**	H-(Lys)GGT CAG AG (propargylglycine)
16a–d	5′-dGGC GGC ATG ACTC*X*GACC (**a**: *X* = T; **b**: *X* = A; **c**: *X* = C; **d**: *X* = G)
ODN-**17**	5′-dCGA CGC GTG CAG GCC AGA TC

[a] **L**: Triazole linker.

The ligation conditions were optimized by using substrates ODN-**1**, PNA-**5**, and template ODN-**3**. The effect of the Cu^I catalyst/ligand, template, and concentration of PNA on the ligation reaction were investigated. The ligation product was not observed without the Cu^I catalyst (Figure 2, lane 1). In the presence of the Cu^I catalyst, 50% ligation yield was obtained. Unfortunately, considerable degradation of the DNA and DNA–PNA ligation products occurred. Indeed, we observed 72% DNA degradation within 30 min and 98% within 1 h (Figure 2, lanes 2 and 3; Supporting Information). This problem was encountered previously in other laboratories.[21,23] However, addition of the water-soluble tris(triazolylamine) Cu^I-binding ligand {e.g. tris[(hydroxypropyl)triazolyl]amine (THPTA)}[21,24] greatly reduced the degradation and increased the ligation yield (Figure 2, lanes 4 and 5). When a 10-fold excess of ligand was employed relative to the amount of Cu^I used, almost no decomposition was observed and near quantitative conversion to DNA–PNA conjugate **7** was observed by denaturing PAGE as well as by HPLC (Supporting Information). The ligation reaction did not depend on PNA concentration and was highly efficient using equimolar ratios of DNA and PNA substrates (50 nM). The ligation reaction is complete within 1 h at room temperature. The rate of conjugate **7** formation at 25 °C in the presence of template ODN-**3** followed first-order kinetics (k_{ICL} = $1.7 \pm 0.5 \times 10^{-3}$ s^{-1}; $t_{1/2}$ = 7 min; Figure 3A). DNA–PNA conjugate **7** was purified by gel electrophoresis and characterized by ESI MS (*m*/*z* = 8780.0 $[M + H]^+$, calcd. *m*/*z* = 8780.9; Supporting Information).

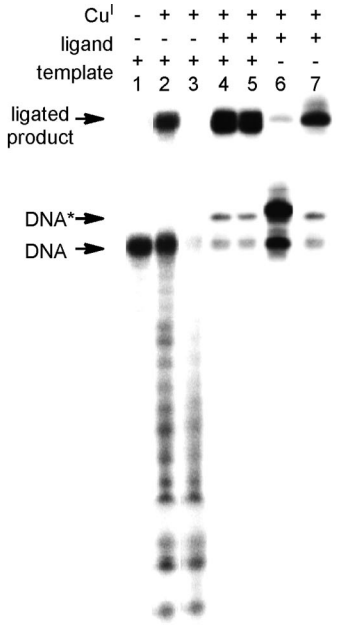


Figure 2. Phosphorimage autoradiogram of denaturing PAGE analysis of the click ligation reaction with ODN-**1** and PNA-**5** under different conditions (lanes 1, 3, and 5–7: the reactions were carried out at room temperature for 60 min; lanes 2 and 4: the reactions were carried out at room temperature for 30 min; lanes 1–5: [PNA] = [DNA substrate] = 50 nM; lane 6: [PNA] = 20 × [DNA substrate] = 1.0 μM; lane 7: [PNA] = 100 × [DNA substrate] = 5.0 μM).

In the absence of a template, no significant ligation (<2%) was observed when the PNA concentration was below 1.0 μM ([DNA] = 50.0 nM; Figure 2, lane 6). However, an increase in the PNA concentration enhanced the ligation yield. At high PNA concentration (>5.0 μM), 80% of DNA formed a PNA conjugate. The rate of click ligation between ODN-**1** and

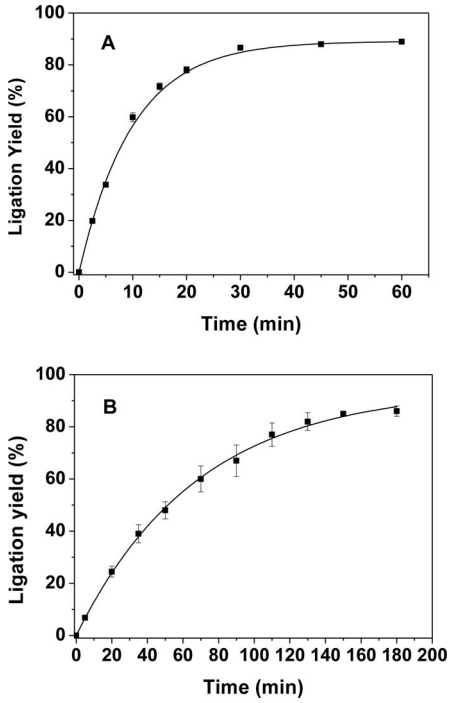
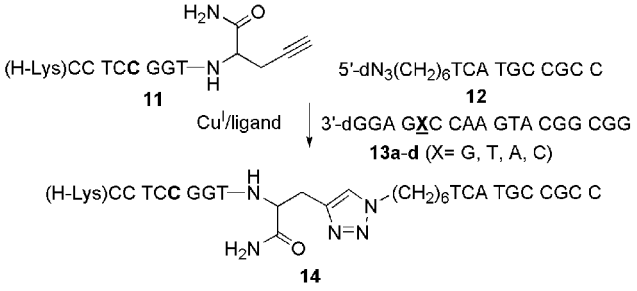


Figure 3. Rate of ligation product formation from ODN-**1** and PNA-**5** (A) in the presence of template ODN-**3** ([ODN-**3**] = [ODN-**1**] = [PNA-**5**] = 50 nM) or (B) in the absence of template ([ODN-**1**] = 50 nM, [PNA-**5**] = 5.0 μM).

PNA-**5** without a template is approximately one order of magnitude slower than that in the presence of a template ($k_{ICL} = 2.44 \pm 0.3 \times 10^{-4}$ s^{-1}; $t_{1/2}$ = 47 min; Figure 3B). Apart from the formation of DNA–PNA conjugates, a byproduct (DNA*) was observed during the click ligation reaction (Figure 2, lanes 4–7). This compound is most likely caused by the side reaction between DNA and the ligand, as it was not observed in the absence of the ligand (Figure 2, lanes 2 and 3). The nontemplate-directed click ligation was applied for the conjugation of PNA and peptide. The reaction of PNA-**5** and peptide-**8** in the presence of ligand/$CuSO_4$/Na-ascorbate yielded PNA–peptide conjugate **9**, which was characterized by ESI MS (Supporting Information).

The template-dependence of the DNA–PNA click ligation indicated that the efficiency of the click reaction depended on the formation of the duplex. Further investigation showed that this approach was capable of detecting DNA single-nucleotide polymorphism. To determine the sequence selectivity, the ligation reaction of PNA-**11** and ODN-**12** was carried out with DNA targets **13a**–**d** (Scheme 1). The template contained sequences from the region around codon 248 in exon 7 of the p53 gene that is often mutated in human cancer.[25] Hybridization of PNA-**11** and ODN-**12** to the fully complementary target **13a** followed by click ligation led to near quantitative formation of ligation product **14** (92%) within 1 h (Figure 4, lane 2). However, in the presence of mutant targets **13b**–**d**, all of which contain a mismatched base at a central position in the PNA complementary region, only trace amounts of the ligation product (<2%) were detected by denaturing PAGE (Figure 4, lanes 3–5). UV-melting analysis of PNA–DNA duplexes showed that the PNA–mismatched DNA hybrids were much less stable (T_m < 19 °C when X = T, A, or C vs. T_m = 49 °C when X = G, the matched base). We did observe a low level of product formation with the mismatched templates. However, because the same amount of ligation was observed in the absence of target (Figure 4, lane 1), it appears most likely that it was due to a template-independent background reaction. To assess the generality of the sequence specificity, the click ligation was performed with a different PNA probe (PNA-**15**), DNA templates **16a**–**d**, and ODN-**12**. Similar results were observed. An efficient ligation (90%) was obtained in the presence of fully complementary target **16a** and less than 2% of the ligation product was formed in the presence of mutant targets **16b**–**d** (Supporting Information). The sequence-selective reaction argues that a PNA-based click ligation system is a promising method for detecting DNA sequences capable of single nucleotide discrimination. The yields provided by the click reaction were almost quantitative. The reaction is characterized by its fast, clean, and mild reaction conditions and by its high selectivity.



Scheme 1. Single-nucleotide-specific click ligation of PNA-**11** and ODN-**12**. DNA targets **13a**–**d** differ in one central position (highlighted in bold, **13a**: X = G; **13b**: X = T; **13c**: X = A; **13d**: X = C). The PNA base opposite to the mutation site is also in bold. Concentration of ligation probes **11**, **12**, and target DNA **13a**–**d**: 50 nM.

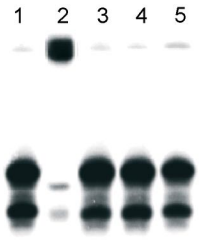


Figure 4. Phosphorimage autoradiogram of denaturing PAGE analysis of the single-nucleotide-specific click ligation of PNA-**11** and DNA-**12** in the presence of DNA templates **13a**–**d** (lane 1: no template; lane 2: **13a**; lane 3: **13b**; lane 4: **13c**; lane 5: **13d**). The ligation reactions were carried out at room temperature for 60 min with 50 nM of PNA and DNA substrate.

Having established the optimum conditions for the PNA–DNA click ligation reaction, the approach was applied to the preparation of extended PNAs by using click–click ligation. The click–click reaction was performed with a 50-mer (ODN-**4**) template, a DNA substrate (ODN-**2**), and four equivalents of PNA-**6**. The template is partly com-

plementary to the 10-mer ODN-**2**. The remaining part contains four repeating oligonucleotide sequences (the italicized sequence in ODN-**4**), which are fully complementary to PNA-**6**, also a 10-mer. To determine the efficiency of the click–click ligation using denaturing PAGE, the 3′-^{32}p-labeled DNA template was used in this experiment. PNA-**6** contains an azide group at the N-terminus and an alkyne at the C-terminus. After hybridization and the addition of the Cu^{I} catalyst/ligand, we observed around 70% of full length ligated product **10** by denaturing PAGE (Figure 5). This indicated highly efficient click–click ligation. By choosing different templates and PNA substrates one can make any length of DNA–PNA or PNA–PNA conjugate. Apart from the main product, a small amount (<5%) of byproducts was observed, which we attribute to the DNA conjugating to different numbers of PNA-**6** (1, 2, 3, and 4 × PNA-**6**).

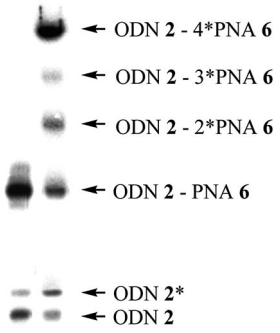


Figure 5. Phosphorimage autoradiogram of denaturing PAGE analysis of the click–click ligation of ODN-**2** and PNA-**6** in the presence of template ODN-**4** [lane 1: ODN-**2**/PNA-**6**/ODN-**17** (1:1:1); lane 2: ODN-**2**/PNA-**6**/ODN-**4** (1:4:1)]. The ligation reactions were carried out at room temperature for 60 min with 50 nM of PNA and DNA substrate.

Conclusions

We have demonstrated an efficient, versatile PNA ligation method using click chemistry, which enables quantitative conjugation of PNA with DNA, PNA, or peptide under mild conditions. The PNA click ligation is template-dependent and is capable of discriminating between templates differing by a single nucleotide. Thus, PNA probes could be used for single nucleotide specific detection of DNA, in hybridization and polymerase chain reaction (PCR) based protocols. This approach could also be useful for the construction of PNA nanomaterials.

Supporting Information (see footnote on the first page of this article): Experimental details, ESI-MS of oligonucleotides, and phosphorimage autoradiograms.

Acknowledgments

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