Orthogonal ligation: a three piece assembly of a PNA–peptide–PNA conjugate†

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A PNA–peptide–PNA conjugate was assembled from three fragments using a combination of native chemical ligation and an orthogonal, auxiliary-mediated ligation.

We have applied two complementary ligation strategies to assemble three unprotected fragments (peptide and peptide nucleic acids (PNAs)). This novel strategy for regioselective bioconjugate construction will have broad scope in the synthesis of defined three component conjugates, as well as libraries of these bioconjugates.[1]

The target chosen for the development of this synthetic approach was a branched PNA–peptide–PNA conjugate (Fig. 1a). PNAs are oligonucleotide mimics in which the nucleobases are attached to a polyamide backbone composed of *N*-(2-aminoethylglycine) repeats.[2] PNAs are promising as antigene therapeutics. They bind dsDNA with high affinity and specificity but their utility has been limited by their poor solubility and cellular uptake and the slow kinetics of dsDNA strand invasion.[2,3] Conjugating PNAs to cell-penetrating peptides including Penetratin (Pen) has been shown to improve cellular uptake.[4,5] We want to explore a novel dsDNA binding construct for inhibition of gene transcription, a three component PNA–peptide–PNA conjugate. The major groove binding peptide Pen,[6] which corresponds to helix 3 of the Antennapedia homeodomain,[7] was selected as a platform. PNAs were appended *via* amide bonds to the side chains of lysines located on the *N*- and *C*-terminus of Pen, providing a construct as shown in Fig. 1a. This structure is expected to favour binding to the major groove without effecting strand invasion as proposed in Fig. 1b. To improve DNA binding of the construct, libraries of conjugates will have to be screened. We thus needed a rapid construction method for accessing these libraries. This necessitated the development of a regioselective convergent ligation approach for installing the PNAs onto the peptide scaffold.

In addition to enabling rapid library construction, a convergent ligation approach provides numerous advantages for bioconjugate construction. The advantages of ligation over stepwise synthesis on a solid phase or protected fragment coupling are well known for protein synthesis. These include greater ease of handling, purification and characterization of the fragments and producing a homogeneous final material in a single step that does not require further modification. Additionally, purification of the final product is simplified since impurities differ from the product by the deletion of a whole fragment rather than a single residue. Lastly, no protecting group manipulations are involved, simplifying synthesis and removing the possibility of deprotection artifacts.[8] The PNA–peptide–PNA conjugate was a challenging target for a ligation assembly as it required a strategy for the regiocontrolled assembly of the three fragments. The principal problem was how to steer each of the PNAs to exclusively one of the two nucleophilic ligation sites without reintroducing protecting groups.

A three piece ligation strategy based on the consecutive use of two amide bond ligations, native chemical ligation (NCL)[8] and an auxiliary-mediated ligation (Scheme 1), was envisioned to regioselectively attach the PNA sequences to the Pen peptide. NCL is best known for its application to the synthesis of proteins but it has also been applied to biopolymers including oligonucleotides[9] and PNAs.[10] Earlier we disclosed an auxiliary-mediated peptide ligation effective for the synthesis of proteins termed extended ligation (EL).[11] This ligation uses the 4,5,6-trimethoxy-2-mercaptobenzyl (Tmb) auxiliary, which functions analogously to cysteine in NCL, combining thioester exchange with efficient intramolecular acyl transfer to the N^{α}-terminus of the peptide. The Tmb group and closely related auxiliaries have been successfully applied to the synthesis of peptides and glycopeptides.[11–13] We previously reported that cysteine (NCL) and Tmb-mediated ligation (EL) of two peptides require different buffer conditions to proceed efficiently. In particular, EL is

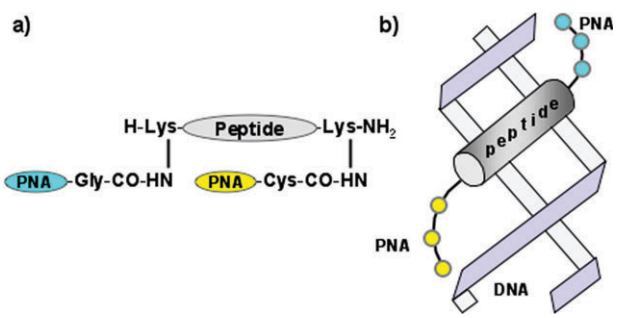

Fig. 1 (a) Structure of the conjugate. (b) Targeted binding to DNA.

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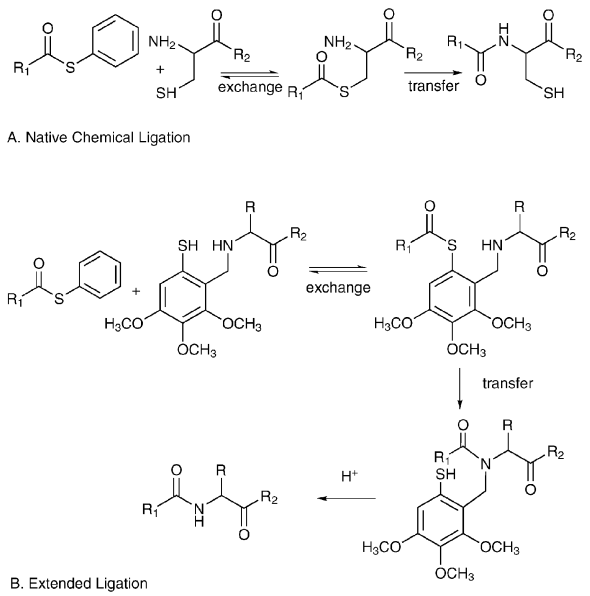


Scheme 1 Proposed mechanism of NCL and EL.

suppressed by addition of alkylthiols.[11,14] This initial observation suggested that conditions for the regio-controlled ligation of a PNA thioester to the Cys of a peptide also containing Tmb would be possible in the presence of alkylthiols, as the nucleophilicity of the Tmb piece would be masked.

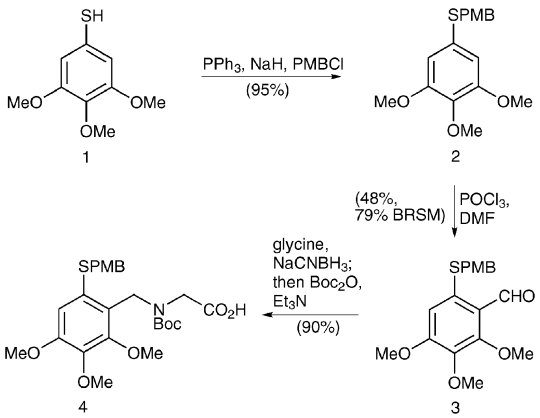
To investigate conditions for NCL and EL of PNAs to the Pen peptide, three Pen derivatives (Pen peptide is Penetratin without modifications) and two model PNA thioesters were synthesized (Table 1). The Pen peptides were constructed using solid phase stepwise synthesis and the Tmb group was installed by coupling a Boc derivatized Tmb-Gly monomer to the lysine side chain. The synthesis of the monomer is shown in Scheme 2. The PNA thioesters were synthesized using Boc protected PNA monomers on solid phase with *in situ* neutralization on a mercaptopropionic acid leucine (*MPAL*) resin[15] and cleaved with HF to give the *MPAL* thioester derivatives. All components were characterized by electrospray or MALDI-TOF mass spectrometry (see ESI†).

Ligation of T_4 PNA to PenCys was efficient under standard conditions for NCL (*i.e.* 1 mM of both fragments in phosphate buffer pH 7 containing 6 M guanidine hydrochloride (Gd·HCl), 2% thiophenol and 2% benzylmercaptan).[8] The half-life for the reaction was estimated at 3 h, with no starting material observed after overnight reaction (see ESI†). The conjugate was isolated with 58% yield after HPLC purification (C4 column). When the PNA thioester was mixed with the TmbPen peptide under identical conditions, no conjugate was detected, even overnight. This observation confirms that EL can be completely suppressed under standard NCL conditions.

Table 1 Peptide and PNA sequences

Name	Sequence
PenCys	KRQIKIWFQNRRMKWKK[N^{ε}-C][a]
TmbPen	K[N^{ε}-TmbG]RQIKIWFQNRRMKWKK[a]
TmbPenCys	K[N^{ε}-TmbG]RQIKIWFQNRRMKWKK[N^{ε}-C][a]
T_4	$(aeg.T)_4$-MPAL[b]
C T_4	aeg.C$(aeg.T)_4$-MPAL[b]

[a] Cys and/or Tmb-Gly are linked *via* an isopeptide bond to the side-chain of the lysine residue. [b] *N*-(2-aminoethyl)-glycine PNA unit functionalized by thymine (aeg.T) or cytosine (aeg.C).



Scheme 2

The conditions for coupling of PNA thioester to Tmb-containing peptides were then investigated. No peptide–PNA conjugate could be detected under conditions known to give fast and complete ligation of peptide–*MPAL* thioesters with peptides containing a *N*-terminal Tmb (*i.e.* phosphate buffer pH 7 containing 6 M Gd·HCl and tris(2-carboxyethyl)phosphine (TCEP)).[11] Ligation only occurred when the pH was raised to 7.5 and thiophenol was added to form the more reactive PNA thiophenol thioester species *in situ*.[16] Using these reaction conditions and with peptide and PNA concentrations of 7.5 mM, the reaction was completed within 36 h, giving the expected PNA–peptide conjugate (see ESI†). The product was isolated with 45% yield after HPLC purification. To better define the parameters that control the Tmb-mediated ligation of PNA thioester, we repeated the experiment in presence of benzylmercaptan. This resulted in a decrease in the ligation rate. This result is in contrast to peptide–peptide extended ligations where benzylmercaptan alone completely suppressed the ligation reaction.[11] Thus, the combination of both alkylthiol addition and lower pH is necessary to suppress the ligation of PNA thioesters to *N*-terminal Tmb-containing peptides.

With orthogonal conditions identified for the attachment of PNA thioesters to either a Cys or Tmb, the construction of the target PNA–peptide–PNA conjugate was attempted (Fig. 2). The first PNA fragment (T_4) was directed to the Cys ligation site of the TmbPenCys peptide by performing the reaction at pH 7 in presence of benzylmercaptan (2%). After 12 h, more than 90% of the peptide had been converted to the expected PNA–peptide conjugate (Fig. 2b, compound **6**). No trace of double conjugation of the PNA on the peptide was detected by HPLC or mass spectrometry. After thiol removal by single pass preparative HPLC (compound **6** isolated with 55% yield), the second PNA thioester (CT_4) was ligated onto the *N*-terminal Tmb-Gly of the conjugate in the absence of benzylmercaptan at pH 7.5. The reaction was completed within 2 days (greater than 90% conversion of compound **6** into product **7***, Fig. 2b). The conjugate was purified by HPLC (41% isolated) and the Tmb auxiliary was quantitatively removed under acidic conditions (see ESI†).[11]

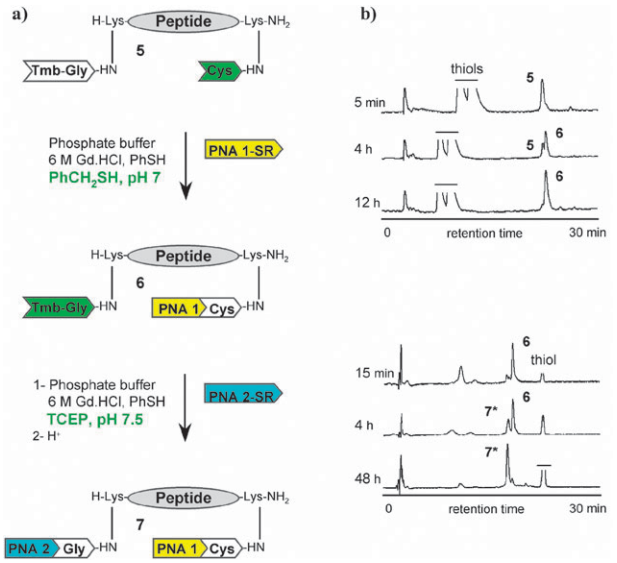


Fig. 2 (a) Assembly of the PNA–peptide–PNA conjugate using orthogonal ligations. (b) Analytical HPLC of the reaction time course. Top: Cys-mediated ligation (RP-C4 column). Bottom: Tmb-mediated ligation (RP-C18 column), **7*** corresponds to product **7** before Tmb removal.

In the present study we have documented the unexpectedly low reactivity of PNA thioesters toward Cys and Tmb-mediated ligation reactions. The rate limiting step of ligation is thioester exchange.[8] The reaction rate is slowed considerably as the *C*-terminal thioester becomes more sterically hindered.[11–13,15] While the PNA thioester possesses the sterically accessible *C*-terminal glycine residue, its reactivity is closer to sterically hindered *C*-terminal β-branched residue thioesters. The slow rate of PNA thioester exchange may be due to the constraints induced by the presence of a tertiary amide bond in the PNA backbone.

The central tenet of chemical ligation, which gives it such great practical value, is the omission of protecting groups. Their absence increases peptide solubility in aqueous solution, eliminates deprotection steps, the requirement of the target to be stable to deprotection and simplifies characterisation. However, when multiple fragment couplings by NCL are required the reintroduction of protecting groups has proved necessary.[17] Combining NCL with an orthogonal EL obviates cysteine protection and confers greater synthetic flexibility.

This study demonstrates that by minor alterations to the conditions (pH, thiol composition and fragment concentration) a thioester can be steered selectively towards a cysteine in the presence of Tmb. This was used to realize the sequential assembly of three unprotected fragments through amide bonds, obviating the need for ligation compatible protecting groups. Products showed excellent purity by HPLC and mass spectrometry. The three piece ligation strategy reported here appears to be particularly well adapted to the constitution of a small library of PNA–peptide–PNA conjugates; different *C*- or *N*-terminal PNA thioesters can simply be introduced at the position of choice. This will now be used to define the best orientation of the PNAs within the conjugate for binding to DNA. Modified residues can also be introduced in the peptide and PNA domains to improve binding. We are currently studying extension of the three piece ligation strategy to the synthesis of proteins and glycoproteins.

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Notes and references

1. D. W. Romanini and M. B. Francis, *Bioconjugate Chem.*, 2008, **19**, 153.
2. A. Ray and B. Nordén, *FASEB J.*, 2000, **14**, 1041; G. Wang and X. S. Xu, *Cell Res.*, 2004, **14**, 111.
3. B. A. Armitage, *Drug Discovery Today*, 2003, **8**, 222.
4. J. J. Díaz-Mochón, L. Bialy, J. Watson, R. M. Sánchez-Martín and M. Bradley, *Chem. Commun.*, 2005, 3316.
5. A. Joliot and A. Prochiantz, *Adv. Drug Delivery Rev.*, 2008, **60**, 608.
6. The affinity of Pen for a DNA duplex containing the target TAATNN sequence is about 2 orders of magnitude lower than that of the Antennapedia homeodomain (Y. H. Shim, P. B. Arimondo, A. Laigle, A. Garbesi and S. Lavielle, *Org. Biomol. Chem.*, 2004, **2**, 915). Stabilizing the structure of peptides based on helix 3 of Engrailed for the design of miniature homeodomains greatly increased affinity for DNA (J. K. Montclare and A. Shepartz, *J. Am. Chem. Soc.*, 2003, **125**, 3416; L. Guerrero, O. S. Smart, A. Woolley and R. K. Allemann, *J. Am. Chem. Soc.*, 2005, **127**, 15624).
7. E. Fraenkel and C. O. Pabo, *Nat. Struct. Biol.*, 1998, **5**, 692.
8. P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. H. Kent, *Science*, 1994, **266**, 776; P. E. Dawson, M. Churchill, M. R. Ghadiri and S. B. H. Kent, *J. Am. Chem. Soc.*, 1997, **119**, 4325.
9. D. A. Stetsenko and M. J. Gait, *J. Org. Chem.*, 2000, **65**, 4900.
10. M. C. de Koning, D. V. Filippov, N. Meeuwenoord, M. Overhand, G. A. van der Marel and J. H. van Boom, *Synlett*, 2001, **10**, 1516; A. Mattes and O. Steiz, *Chem. Commun.*, 2001, 2050.
11. J. Offer and P. E. Dawson, *Org. Lett.*, 2000, **2**, 23; J. Offer, C. N. C. Boddy and P. E. Dawson, *J. Am. Chem. Soc.*, 2002, **124**, 4642.
12. D. Macmillan and D. W. Anderson, *Org. Lett.*, 2004, **6**, 4659; B. Wu, J. Chen, J. D. Warren, G. Chen, Z. Hua and S. J. Danishefsky, *Angew. Chem., Int. Ed.*, 2006, **45**, 4116; J. Chen, G. Chen, B. Wu, Q. Wan, Z. Tan, Z. Hua and S. J. Danishefsky, *Tetrahedron Lett.*, 2006, **47**, 8013; T. Kawakami, K. Akaji and S. Aimoto, *Org. Lett.*, 2001, **9**, 1403; C. Haase and O. Seitz, *Angew. Chem., Int. Ed.*, 2008, **47**, 1553.
13. S. Tchertchian, O. Hartley and P. Botti, *J. Org. Chem.*, 2004, **69**, 9208; V. M. Cardona, O. Hartley and P. Botti, *J. Pept. Res.*, 2003, **61**, 152; D. W. Low, M. G. Hill, M. R. Carrasco, S. B. Kent and P. Botti, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 6554.
14. NCL is also suppressed by alkyl thiols but a much higher concentration is required to abolish ligation.
15. T. M. Hackeng, J. H. Griffin and P. E. Dawson, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 10068.
16. Above pH 7.5 the hydrolysis of thiophenol thioesters becomes a serious competing reaction. Alkyl thioesters are moderately stable up to pH 8.5 over the lifetime of a ligation reaction (24 h). However ligations are usually performed at pH 7.0 to prevent complications such as epimerization of the *C*-terminal residue.
17. D. Bang and S. B. Kent, *Angew. Chem., Int. Ed.*, 2004, **43**, 2534; D. Bang, B. L. Pentelute and S. B. Kent, *Angew. Chem., Int. Ed.*, 2006, **45**, 3985.