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Synthesis of γ-Substituted Peptide Nucleic Acids: A New Place to Attach Fluorophores without Affecting DNA Binding

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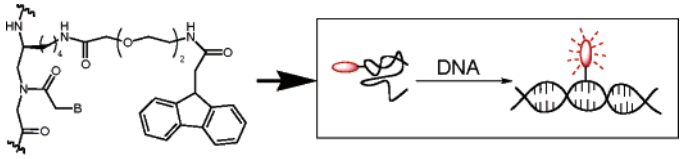
ABSTRACT



γ-Lysine PNA

Molecular beacon strategies using PNA are currently restricted to fluorophore attachment to the ends of the PNA. We report the synthesis of PNA oligomers wherein fluorophores can be attached to the PNA backbone from novel γ-lysine PNA monomers. Oligomers incorporating the modified PNA showed comparable thermal stability to the corresponding *aeg*PNA oligomer with DNA. When the modified PNA oligomer was annealed with complementary DNA, the fluorescence intensity increased 4-fold over the unbound PNA.

Sequence-specific nucleic acid detection is critical for many medicinal and diagnostic applications. In this area, molecular beacons (MBs) are popular tools for *in vivo* nucleic acid detection.[1] In these systems, a nucleic acid probe exhibits a fluorescent signal only in the presence of the target oligonucleotide. MBs usually consist of a fluorophore and a fluorescence-quencher attached at the termini of a nucleic acid oligomer.[2] When the termini are close to one another (due to a stem-loop structure or hydrophobic interactions), the fluorescence is quenched. Upon binding to the target oligonucleotide, separation of the termini is accompanied by an increase in fluorescence.

Recently, quencher-free MBs have been synthesized from DNA that utilize fluorophores quenched by nucleobases.[3] This strategy allows the inclusion of fluorophores at various points in the oligonucleotide sequence, not just at the termini, and allows incorporation of multiple fluorophores in principle. Currently, such MBs rely on covalent attachment of the fluorophore to a nucleobase.[4]

With the inception and continued study of peptide nucleic acid (PNA),[5] molecular beacon strategies incorporating this nonnatural pseudopeptide have become increasingly popular.[6] The use of "stemless" PNA molecular beacons (Figure 1) is especially attractive due to its high binding affinity to natural nucleic acids, high selectivity, and resistance to degradation.[6b] MB strategies using PNA are currently restricted to fluorophore attachment to the ends of the PNA[6] or to the

(1) (a) Paroo, Z.; Corey, D. R. *J. Cell Biochem.* **2003**, *90*, 437. (b) Kaihatsu, K.; Janowski, B. A.; Corey, D. R. *Chem. Biol.* **2004**, *11*, 749. (c) Tan, W.; Wang, K.; Drake, T. J. *Curr. Opin. Chem. Biol.* **2004**, *8*, 547.

(2) Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303.

(3) Hwang, G. T.; Seo, Y. J.; Kim, B. H. *J. Am. Chem. Soc.* **2004**, *126*, 6528−6529.

(4) (a) Sando, S.; Abe, H.; Kool, E. T. *J. Am. Chem. Soc.* **2004**, *126*, 1081. (b) Fujimoto, K.; Shimizu, H.; Inouye, M. *J. Org. Chem.* **2004**, *69*, 3271.

(5) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497.

(6) (a) Seitz, O.; Bergmann, F.; Heindl, D. *Angew. Chem., Int. Ed.* **1999**, *38*, 2203. (b) Svanvik, N.; Nygren, J.; Westman, G.; Kubista, M. *J. Am. Chem. Soc.* **2001**, *123*, 803. (c) 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. (d) Peterson, K.; Vogel, U.; Rockenbauer, E.; Nielsen, K. V.; Kolvraa, S.; Bolund, L.; Nexo, B. *Mol. Cell. Probes* **2004**, *18*, 117.

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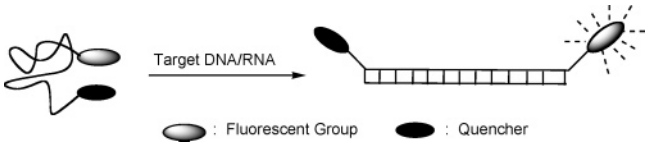

Figure 1. General principle of stemless PNA molecular beacons. Fluorescence is observed upon duplex formation.

nucleobases. Therefore, successful development of PNA-based MBs requires that the termini of the PNA are in close proximity to each other or to nucleobases in the absence of target oligonucleotide (to ensure fluorescence quenching) or that attachment of fluorophores directly to nucleobases does not negatively impact oligonucleotide binding. Such requirements can make the design of PNA-based MBs challenging.

In this communication, we report a new strategy for attaching side chains to the PNA backbone such that fluorophores can be covalently attached. Our strategy should facilitate the design of PNA-based MBs. While many PNA modifications have deleterious effects on the binding,[7] our side chain does not compromise binding to DNA. As an initial test of our strategy, we developed a rudimentary PNA-based biosensor where fluorescent intensity increases upon binding to fully complementary DNA (Figure 2).

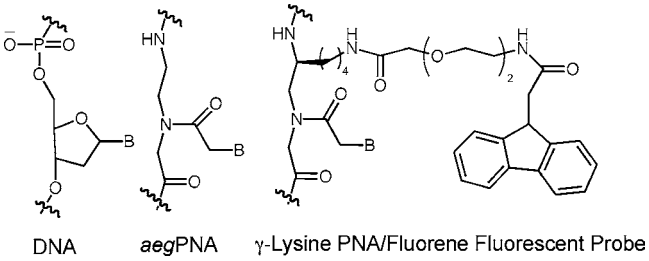

Figure 2. General structure of DNA, *aeg*PNA, and γ-substituted PNA with a linker and fluorophore attached to it.

We designed a PNA monomer with an Fmoc-protected lysine-derived side chain at the γ-carbon (Figure 3, Boc-γ-Lys(Fmoc)-T-OH) to serve as the attachment point for a fluorophore. On the basis of the NMR structure of PNA/DNA duplexes, we deduced that the *S* stereochemistry at the γ carbon would be ideal for side chain accommodation.[8] Furthermore, inspection of the NMR structure indicated that a side chain at the γ-position would be able to tolerate a large flourophore without affecting duplex stability. While PNA side chains are often attached to the α-carbon, we felt that attachment of a large fluorophore at this position would destabilize the PNA/DNA duplex.[9] The side chain-bearing

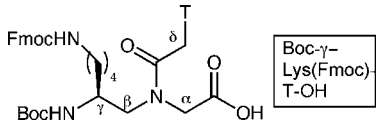

Figure 3. *aeg*PNA derivative synthesized from orthogonally protected (L)-Lysine.[11]

PNA monomers were synthesized from commercially available, orthogonally protected (L)-lysine. Fluorene was chosen as the fluorophore in our initial examination of this system because the fluorophore emission is, in general, effectively quenched by thymine in numerous examples[10] and in recent MB studies.[3] When unbound, the fluorene in this molecule will presumably interact with the thymine in the PNA oligomer due to compaction or hydrophobic interactions[6c] so that the fluorescence is effectivly quenched. Once bound to a complementary nucleic acid in a stable double helix, the fluorene should emit increased fluorescence due to the descreased interaction with the thymine residues. After coupling Boc-γ-Lys(Fmoc)-T-OH to the PNA oligomer on the solid support, the Fmoc was removed, and a MiniPEG linker and 9-fluoreneacetic acid were coupled to the side chain.[11] PNA synthesis was then completed without incident.

Hybridization properties of the side chain-bearing PNA oligomers with DNA (Figure 4) were examined using

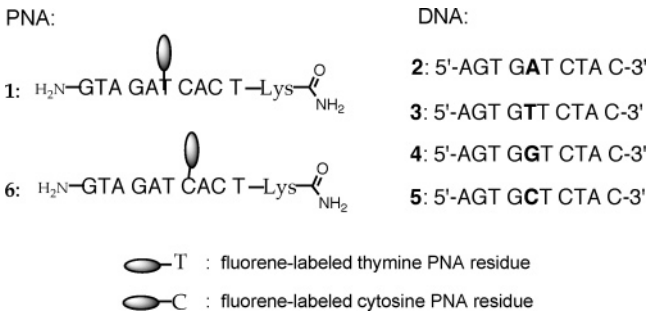

Figure 4. Synthesized PNA oligomers and DNA used in fluorescent and melting temperature (T_m) studies.

variable-temperature UV and compared to unmodified *aeg*PNA (Table 1). The thermal denaturation studies showed that oligomers incorporating the modified PNA residue melted at a slightly higher temperature (T_m) than the corresponding *aeg*PNA oligomer with fully complementary

(7) Hyrup, B.; Egholm, M.; Nielsen, P. E.; Wittung, P.; Norden, B.; Buchardt, O. *J. Am. Chem. Soc.* **1994**, *116*, 7964.

(8) Eriksson, M.; Nielsen, P. E. *Nat. Struct. Biol*. **1996**, *3*, 410.

(9) (a) Dueholm, K. L.; Petersen, K. H.; Jensen, D. K.; Egholm, M.; Nielsen, P. E.; Buchardt, O. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1077. (b) Haaima, G.; Lohse, A.; Buchardt, O.; Nielsen, P. E. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1939. (c) Zhou, P.; Wang, M.; Du, L.; Fisher, G. W.; Waggoner, A.; Ly, D. H. *J. Am. Chem. Soc.* **2003**, *125*, 6878.

(10) (a) Telser, J.; Cruickshank, K. A.; Morrison, L. E.; Nextel, T. L. *J. Am. Chem. Soc.* **1989**, *111*, 6966. (b) Mann, J. S.; Shibata, Y.; Meehan, T. *Bioconjugate Chem.* **1992**, *3*, 554.

(11) See Supporting Information for details.

Table 1. Thermal Melting Temperature (T_m) of Modified PNA with Complementary and Mismatched Antiparallel DNA[a]

	T_m (°C)			
	DNA **2**	DNA **3**[b]	DNA **4**[b]	DNA **5**[b]
*aeg*PNA	48.9	36.0 (12.9)	34.0 (14.9)	32.0 (16.9)
PNA **1**	50.5	30.0 (20.5)	29.9 (20.6)	34.9 (15.6)
PNA **6**	50.3			

[a] Solutions of 1:1 oligonucleotide/PNA were prepared in pH 7.0 buffer consisting of 10 mM sodium phosphate, 0.1 mM EDTA, and 150 mM NaCl. Strand concentrations were 5 μM in each component. Estimated error is ±0.5 °C. [b] ΔT_m (°C) in parentheses represents the difference in T_m between fully complementary duplex and duplex with a single mismatch.

DNA (DNA **2**). Discrimination of single-base mismatches (DNAs **3**−**5**) was also similar to, or higher than, that of the corresponding *aeg*PNA.

We investigated the fluorescent intensity changes of our PNAs when bound to fully complementary and singly mismatched single-stranded DNA. PNA **1** was weakly fluorescent (λ_{max} = 425) in the absence of DNA. When PNA **1** was annealed with complementary DNA (**2**), the fluorescence intensity increased 4-fold over that of the unbound PNA (Figure 5). When combined with DNA **4** or **5**, containing a single-base mismatch opposite the modified PNA segment, the fluorescence remained near baseline levels. Annealing with DNA **3** (TT mismatch) gave a 2.5-fold increase in fluorescence intensity, which is still considerably less than that exhibited by the fully complementary DNA (**2**).

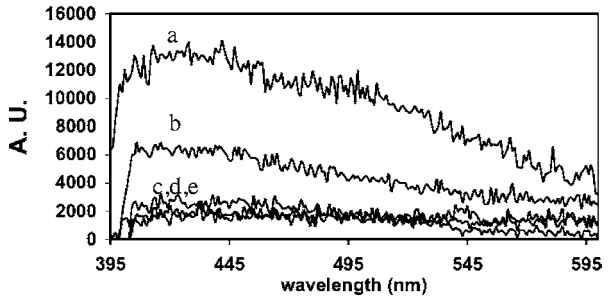

Figure 5. (a) Emission spectra (in arbitrary units) recorded at 23 °C of PNA **1** bound to DNA **2** (5.0 μM). Fluorescent spectra were recorded using an excitation wavelength of 340 nm. Buffer conditions are the same as those described in Table 1. (b) PNA **1** bound to DNA **3**. (c) PNA **1** bound to DNA **4**. (d) PNA **1** bound to DNA **5**. (e) PNA **1** alone.

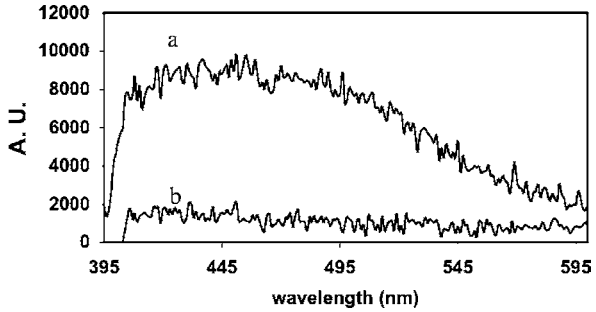

Figure 6. (a) Emission spectra (in arbitrary units) of PNA **6** complexed with DNA **2** with conditions matching those used in Figure 5. (b) PNA **6** alone.

On the basis of our results, we sought to determine if the modification was general or site specific, both in binding characteristics and fluorescent properties. We predicted that the fluorene would remain quenched even if not directly incorporated on a thymine PNA residue. Fluorescent studies of PNA **6** (Figure 6) showed a similar increase (~4-fold) in fluorescent intensity when bound to the complementary DNA (**2**).

In conclusion, we have developed a method to modify the PNA backbone with side chains that can support a fluorescent group. Our modification shows no deleterious effects on DNA binding. Although the fluorene was very efficiently quenched when the oligomers were unbound, the modest increase in fluorescent intensity suggests that the fluorene still interacts with the thymine residues. This could be due to intercalation of the fluorene residue into the duplex or aggregation effects. While the fluorescence increase is not sufficient for most sensor applications, because of the versatility of our strategy we expect to be able to install a variety of different fluorophores at multiple positions to optimize for highly efficient biosensors.

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Supporting Information Available: Synthesis of the γ-Lysine(Fmoc) PNA monomer, along with the solid-phase synthesis of PNAs **1** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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