Low-Scale Automated Synthesis of a PNA-Peptide Conjugate on the Prelude®

Introduction

Peptide nucleic acid (PNA) is a DNA mimic in which the deoxyribose phosphate backbone is replaced with a neutral pseudopeptide backbone composed of 2-aminoethyl glycine linkages. The four natural nucleobases (adenine, guanine, cytosine and thymine) are retained as side chains (**Figure 1**).
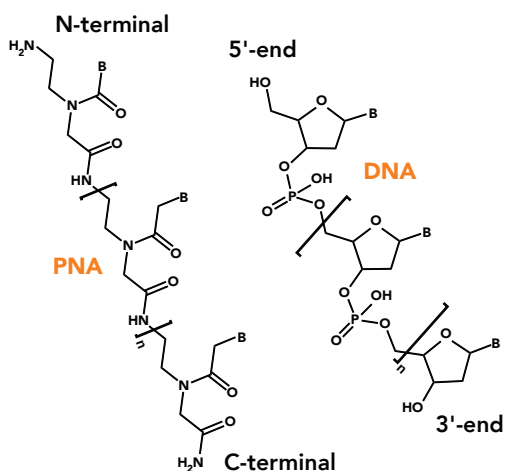

Figure 1: Comparative structures of PNA and DNA.

PNA probes can bind more strongly than DNA probes to DNA and RNA strands because there is no charge repulsion between backbones. This means PNA probes have greater specificity. If a target is already in a DNA duplex, the PNA probe can displace the complementary strand by helix invasion. Its neutral backbone means PNA binding strength is independent of salt concentration, and PNA-containing duplexes are also stable over a wide pH range. Because their structure is not entirely protein and not entirely oligonucleotide, they are resistant to enzyme degradation by both proteases and nucleases. Due to its useful properties, PNA is used in many areas of chemistry, biology, and medicine including molecular recognition, genetic diagnostics, and drug discovery.

Because of its structure, PNA has low water solubility and poor cell membrane permeability. Peptides can be linked to PNA probes in order to overcome these disadvantages [1]. Most automated PNA synthesis methods today are performed on DNA synthesizers. If a PNA-peptide conjugate is desired, the PNA is first synthesized on a DNA synthesizer, and then switched to a peptide synthesizer for the peptide portion. In this application note, a PNA-peptide conjugate is synthesized at the 10 μmol scale in a single synthesis on the **Prelude®** peptide synthesizer. The **Prelude** is ideal for working with expensive special reagents and monomers. With its Single-ShotTM delivery feature, the **Prelude** can deliver the entire contents of one of its 27 amino acid positions to any reaction vessel without priming or waste! It is also great for labeling or other modification reactions. The **Prelude** can deliver volumes as low as 150 μL, so you can be as frugal as you like with solvents and reagents.

There are several guidelines to follow when designing a PNA sequence. PNA is notorious for being insoluble. The higher the purine content, the lower the solubility in aqueous solution. Adding a lysine to one or both termini increases the solubility. In addition, it is recommended not to have more than a 60% purine content and no more than 3 G's in a row. It is also important that the sequence not form hairpin structures with itself or be self-complementary. The synthesis will be fine, but analysis and later handling will be difficult due to the resulting aggregation. A model PNA sequence was designed to have no self-complementary sequences and less than 60% purine content. Initially, it was synthesized alone with just a lysine on the C-terminus for solubility (**Sequence 1**). This synthesis was to verify the synthesis chemistry for PNA. It was then synthesized with the peptide G-LHRH and a lysine added on the C-terminus (**Sequence 2**) to demonstrate how a PNA-peptide conjugate could be synthesized on the **Prelude** in a single synthesis with minimal solvent usage and waste.

Sequence 1: CAGTCCAGTT-Lys-OH

Sequence 2: CAGTCCAGTT-Gly-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Lys-OH

Method

Materials: PNA monomers, Fmoc-A(Bhoc)-aeg-OH, Fmoc-C(Bhoc)-aeg-OH, Fmoc-G(Bhoc)-aeg-OH, and Fmoc-T-aeg.OH, were purchased from Panagene (Daejeon, Korea). Fmoc-Lys(Boc) Wang resin was purchased from Novabiochem. HATU was purchased

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from GenScript Corp. Amino acids, solvents and reagents were provided by Protein Technologies, Inc. or purchased from Sigma-Aldrich.

Synthesis: Sequence 1 (CAGTCCAGTT-Lys-OH) was synthesized on Fmoc-Lys(Boc)-Wang PS resin (0.24 mmol/g) using a Protein Technologies, Inc. **Prelude** peptide synthesizer (Tucson, AZ), and a 10-fold excess of PNA monomer with respect to the resin. Deprotection was performed for 2x5 min using 20% Piperidine in DMF. Coupling was performed for 1 hour using a 1:1:2 monomer/HATU/NMM in DMF (0.5 mL of 200 mM monomer, 0.3 mL of 333 mM HATU, and 0.3 mL of 667 mM NMM all in DMF). Cytosine was dissolved in DMF at 80°C using sonication. Washing with 2:1 DMF/NMP, methanol, DCM, and 2:1 DMF/NMP was performed twice between coupling and deprotection steps and once between deprotection steps. The product was cleaved from the resin by treatment with 85/10/2.5/2.5 TFA/m-cresol/water/TIS for 5 hours.

Sequence 2 (CAGTCCAGTT-Gly-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Lys-OH) was synthesized on Fmoc-Lys(Boc)-Wang PS resin (0.24 mmol/g) using a Protein Technologies, Inc. **Prelude** peptide synthesizer (Tucson, AZ), and a 5-fold excess of amino acid and 10-fold excess of PNA monomer with respect to the resin. Deprotection was performed using 20% Piperidine in DMF. Coupling was performed using a 1:1:2 monomer/HATU/NMM in DMF (0.5 mL of 200 mM monomer, 0.3 mL of 333 mM HATU, and 0.3 mL of 667 mM NMM all in DMF except cytosine, which was dissolved in DMSO). Washing with DMF was performed between deprotection and coupling steps. The peptide Gly-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Lys-OH was first synthesized at the 20 mol scale using deprotection times of 2x30 sec, and coupling times of 5 minutes. Half the resin was cleaved by treatment with 95/2/2/1 TFA/water/anisole/EDT for 2 hours to verify the peptide was successfully synthesized. The remaining resin was used to complete the synthesis of the PNA-peptide conjugate at the 10 mol scale using deprotection times of 2x5 min, and coupling times of 1 hour. The PNA-peptide conjugate was then cleaved from the resin by treatment with 85/10/2.5/2.5 TFA/m-cresol/water/TIS for 5 hours.

Analysis: Crude products were precipitated with anhydrous ether, dissolved in water and analyzed using a Varian ProStar HPLC.

Gly-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Lys-OH was analyzed on a C18, 300 Å, 5 µm, 50 x 4.6 mm column (Varian Microsorb-MV), over 7 minutes with a flow of 2 mL/min and using a gradient of 5-95% B where Buffer A was 0.1% TFA in water and Buffer B was 0.1% TFA in acetonitrile. Detection was at 214 nm.

PNA products were analyzed on a C18, 300 Å, 5 µm, 250 x 4.6 mm column (Varian Microsorb-MV), over 60 minutes with a flow of 1 mL/min and using a gradient of 5-95% B where Buffer A was 0.1% TFA in water and Buffer B was 0.1% TFA in acetonitrile. Detection was at 260 nm.

Product masses were verified on a MALDI-TOF mass spectrometer (Voyager-DETM Biospectrometry Workstation from PerSeptive Biosystems) or at the University of Arizona mass spectrometry facility.

Results

Sequence 1 was synthesized in order to verify the chemistry protocol for the synthesis of PNA. Sequence 1 was successfully synthesized and easily purified by HPLC (**Figure 2a**). Mass analysis confirmed the identity of the product (2831.281 is the product, 2698.044 is a fragment formed during the analysis) (**Figure 2b**). Sequence 2 was then synthesized to demonstrate
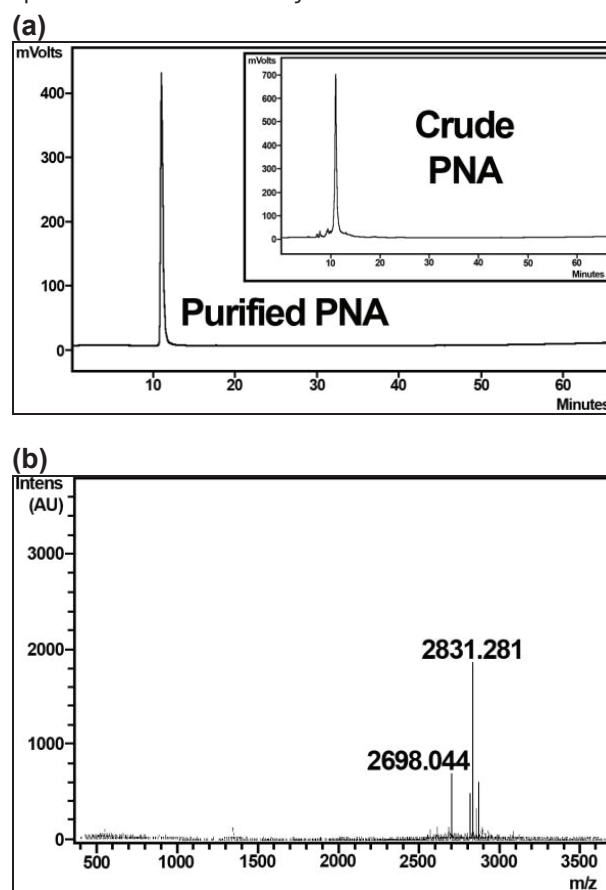

Figure 2: Sequence 1 (CAGTCCAGTT-Lys-OH) results. (a) HPLC analysis. Crude and purified HPLCs are shown. (b) Mass spectrometry analysis. 2831.281 is the product, 2698.044 is a fragment formed during the analysis.

the synthesis of a PNA-peptide conjugate in a single synthesis on the **Prelude** peptide synthesizer. Washing steps were simplified, and consumption values were recorded to demonstrate the frugality of the **Prelude** (**Table 1**).

Item	Consumption
DMF [mL]	1480
DCM [mL]	75
20% piperidine [mL]	60
333 mM HATU [mL]	7.6
667 mM DIPEA [mL]	7.9
TFA cocktail [mL]	4.7
Nitrogen [psi]	90

The consumptions of expensive PNA monomers and HATU activator were minimized. 0.5 mL of a 200 mM solution of monomer was used for each coupling. Dissolved monomers were delivered using the **Prelude**'s Single-ShotTM delivery feature which delivered the entire contents of the amino acid vial without priming or waste. The HATU solution was also placed in a 10 mL Single-ShotTM vial, but delivered without the Single-ShotTM function. Only 7.6 mL of 333 mM HATU solution were used for the 20 couplings of Sequence 2 (**Table 1**). Analysis of the peptide portion of Sequence 2 showed that the crude peptide was successfully synthesized with no fragments observed by HPLC or mass spectrometry (**Figure 3**).
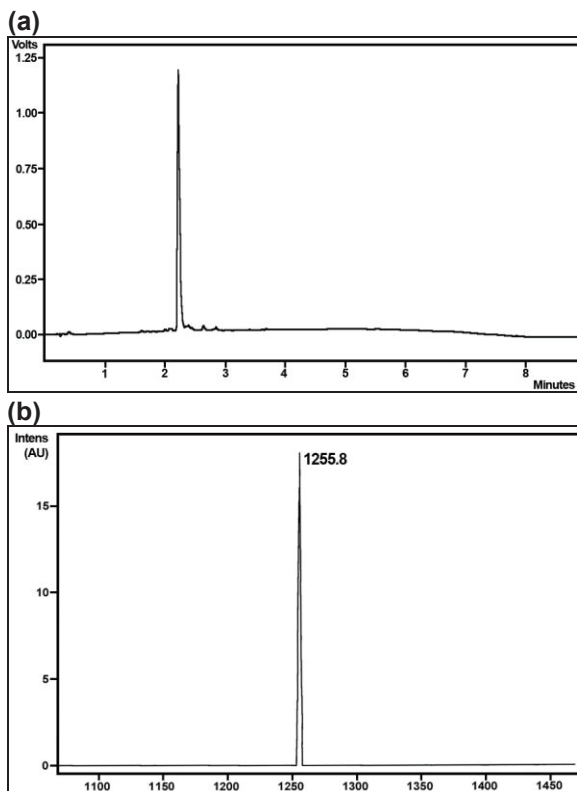

Figure 3: Gly-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Lys-OH results. (a) HPLC analysis of crude peptide. (b) Mass spectrometry analysis. 1255.8 is the product.

The PNA portion was then added, and the complete Sequence 2 was purified. Mass analysis verified the identity of the product, plus a few deletion fragments that together totaled less than 10% of the total sample (3941.617 is the product, 2384.298 = -CAGTCC + 27, 2635.423 = -CAGTC + 27, 2885.456 = -CAGT +26, 3152.536 = -CAG + 26, 4191.650 = -CAGTCCA dimer, and 7883.544 = product dimer) (**Figure 4**).
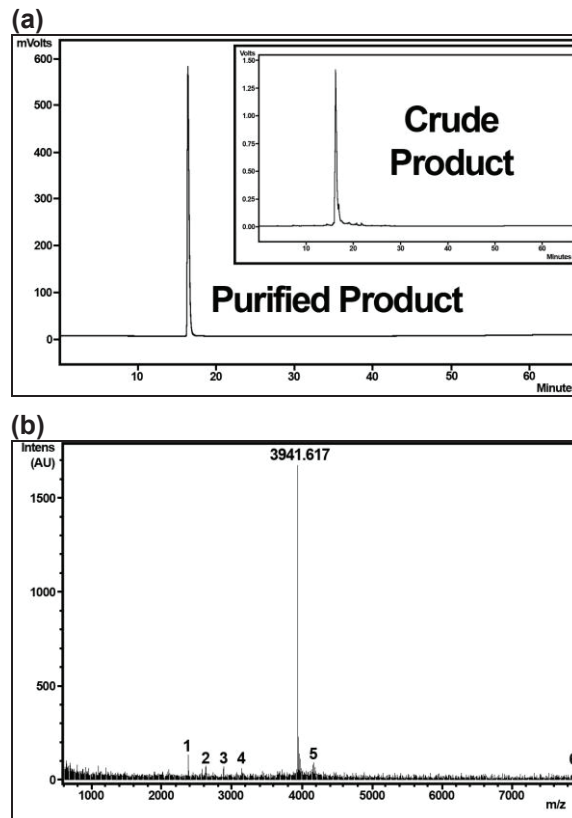

Figure 4: Sequence 2 (CAGTCCAGTT-Gly-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Lys-OH) results. (a) HPLC analysis. Crude and purified HPLCs are shown. (b) Mass spectrometry analysis. 3941.617 is the product, 1 = 2384.298 = -CAGTCC + 27, 2 = 2635.423 = -CAGTC + 27, 3 = 2885.456 = -CAGT +26, 4 = 3152.536 = -CAG + 26, 5 = 4191.650 = -CAGTCCA dimer, and 6 = 7883.544 = product dimer

Conclusion

A model PNA sequence was successfully synthesized alone, and as part of a PNA-peptide conjugate on the **Prelude** peptide synthesizer in a single synthesis at the 10 µmol scale. The **Prelude**'s Single-ShotTM delivery feature and vials were used to minimize the use of expensive PNA monomers and HATU activator. Delivery volumes ranging from 300 µL to 1 mL were used to minimize overall consumptions.

References

[1] Martijn C. de Koning, Gijs A. van der Marel and Mark Overhand, Current Opinion in Chemical Biology 7, 734-740 (2003).

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