Parallel automated solid phase synthesis: efficient high-throughput optimization for therapeutic discovery and development of peptide nucleic acids

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Introduction

Peptide and oligonucleotide therapeutics are increasingly at the forefront of drug development programs, for personalized medicine, cancer therapeutics, and genetic diseases among others. This has driven the search for faster and more efficient solid phase synthesis (SPS) protocols, making method development crucial in the discovery process towards scale-up. Automated synthesizers are part of the SPS toolbox that allows simultaneous optimization and high-throughput synthesis via parallel synthesis.

Peptide Nucleic Acids (PNA) are nucleic acid analogues that contain a neutral, flexible, polyamide backbone of repeating N-(2- aminoethyl)glycine units with nucleobases attached via methylene carbonyl linkers resulting in higher stability analogs with high oligonucleotide binding affinity[1,2]. PNAs are synthesized using solid phase peptide synthesis (SPPS) protocols and adding Lys residues at both N- and C-terminus is a common practice in the synthesis of PNAs to aid with PNA solubility after synthesis.

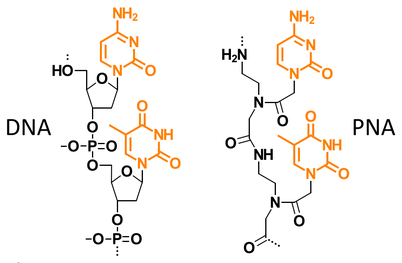

Figure 1. *DNA vs PNA structures.*

Here we show the synthesis of the 3'-end 8mer PNA analogs of the universal primer bluescript SK primer (5'-CGCTCTAGAACTAGTGGATC-3', Fig.2) and an analog of the antisense anticancer drug, Genasense (5'-TCTCCCAGCGTGCGCCAT-3', Fig.3) under several conditions in parallel for synthesis optimization. Genasense® is an oligonucleotide therapeutic that targets the bcl-2 protein leading to its degradation and decreasing protein translation for the treatment of B-cell lymphoma, breast cancer, and others[3].

H-Lys-AGTGGATC-Lys-NH_2

Figure 2. *SK-PNA analog.*

H-Lys-CGCGCCAT-Lys-NH_2

Figure 3. *Genasense-PNA analog.*

Method & Analysis

The SK and Genasense 8-mer PNA analogs were synthesized by Fmoc-chemistry SPPS on a Symphony® X peptide synthesizer at a 40 µmol scale. Using the parallel synthesis capability on the Symphony X, three resins and two coupling reagents were tested in parallel. The resins tested were:

- R Ram Tentagel® Resin (0.19 mmol/g)
- Rink Amide ChemMatrix® resin (0.55 mmol/g)
- Low-loaded Rink Amide MBHA resin (0.22 mmol/g)

Deprotections were done using 20% piperidine in DMF at 25°C for 20 min or at 90°C for 1:30 min. The coupling reactions were done at 25°C for 30 min or 90°C for 2:30 min with a 7.5 excess with initial concentrations of 300 mM AA and 300 mM HATU or COMU® with 500 mM DIPEA/750 mM 2,6-Lutidine. Coupling reaction reagents were dissolved in anhydrous NMP.

Analysis

The cleavage was done using TFA/EDT/H2O/TIS/m-cresol (92.5:2.5:2.5:2.5:2.5) for 2 h at 25°C on the Symphony X followed by precipitation in diethyl ether. The resulting PNA was dissolved in water and analyzed on a Thermo Scientific Ultimate 3000 HPLC using a C18, 100 Å, 2.6 um, 50 X 2.1 mm Kinetex Evo column (Phenomenex), over 4 min with a flow rate of 1 mL/min and a gradient of 0-50% B, where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile. Detection was done at 214 nm. Mass analysis was done on a Shimadzu LCMS-2020 Single-Quad mass spectrometer, equipped with a C18, 100 Å, 2.6 um, 50 x 2.1 mm Kinetex column (Phenomenex), over 7 min with a flow rate of 1 mL/min and a gradient of 0-20% B where A is 0.1% formic acid in water and B is 0.1% formic acid in acetonitrile.

Symphony X

- 24 parallel independent reaction vessels to run different scales, sequences and protocols on multiple RVs all at the same time or on-demand while running other projects
- 12 vessels with pre-activation chemistry
- Real-time UV monitoring of the reaction solution during mixing to control deprotection times and minimize excess waste for better purity and yields
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- Single Shot™ additions with almost no dead volume

Results

Tentagel and Rink Amide MBHA resins produced similar purities in the synthesis of SK-8mer analog using HATU at room temperature whereas ChemMatrix resin produced significantly lower purities (Table 1). Changing the coupling reagent to COMU resulted in increased purity when using Tentagel and ChemMatrix resins. Increasing the coupling reaction temperature to 90°C using a fast protocol was detrimental to the high purity synthesis of SK-8mer PNA analog, with only 15% crude purity using HATU and Tentagel resin.

Using COMU in combination with the Rink ChemMatrix resin resulted in the highest crude purity for Genasense-8mer analog (81%, Table 2, Fig.4B). Comparing all other conditions used for the synthesis of Genasense-8mer analog, similar crude purities were observed, with all resulting purities greater than 60%. This shows that a wider set of conditions could be used for the successful synthesis of high-purity Genasense PNA analogs.

Table 1. *Crude purity and yield of **SK-8mer** PNA analog.*

Resins	% Purity		% Yield	
	HATU	COMU	HATU	COMU
R Ram Tentagel	64.9	73.1	59.8	68.7
Rink ChemMatrix	46.3	66.3	52.2	53.2
Rink MBHA Low Loaded	66.5	63.7	104.4	102.7

Table 2. *Crude purity and yield of **Genasense-8mer** PNA analog.*

Resins	% Purity		% Yield	
	HATU	COMU	HATU	COMU
R Ram Tentagel	75.1	75.8	59.1	59.4
Rink ChemMatrix	72.2	80.5	57.5	60.5
Rink MBHA Low Loaded	62.8	63.3	112.5	119.1

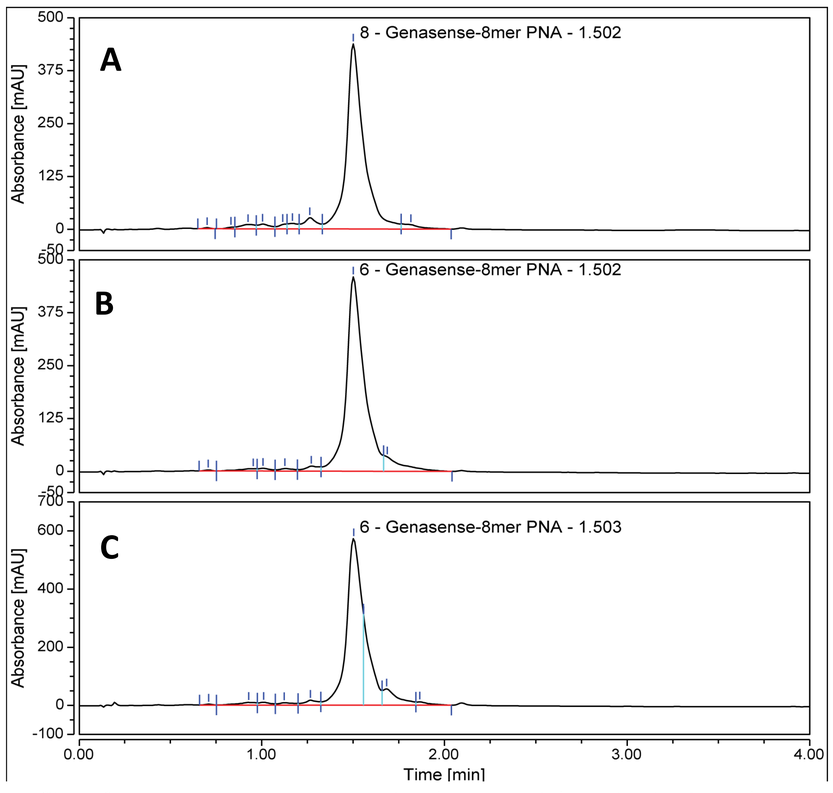

Figure 4. *Crude purity profiles of Genasense-8mer PNA analog (RT: 1.5 min) synthesized on A) R Ram TentaGel , B) Rink Amide ChemMatrix, and C) Rink Amide MBHA resins using COMU/DIPEA:2,6-Lutidine at 25°C.*

Conclusion

- Multi-variable conditions were successfully tested in parallel for the high-throughput optimization of PNA analogs
- The synthesis of SK-8mer PNA analog resulted in optimal crude purity using R Ram Tentagel resin and COMU as the coupling reagent and 30 min couplings
- Synthesis of Genasense-8mer PNA analog resulted in high crude purities with multiple conditions with the Rink ChemMatrix and COMU combination providing crude purities above 80%
- Next steps include overall synthesis time optimization of 8-mer PNA analogs, looking at different reaction times

References

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