Optimizing Automated Synthesis of Peptide Nucleic Acids

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Introduction

Peptide nucleic acids (PNAs) continue to gain traction in diagnostic and potentially therapeutic applications where oligonucleotides are the current modality of interest. Despite containing an unnatural backbone, PNA-based mimetics retain the well-established, high fidelity hybridization properties associated with naturally occurring oligonucleotides. As oligonucleotides are approved as therapeutics, PNAs also increase in attractiveness given their resistance to nucleases and proteases, a chemically neutral backbone and synthetic tractability.

Despite these perceived advantages, PNAs have received little traction given their synthetic difficulty and cost. Although synthetically similar strategies are employed when compared to solid phase peptide synthesis, alternative strategies are often employed to overcome significant steric effects that compromise synthetic success. Herein we discuss several strategies toward optimizing automated synthesis of difficult PNA compounds including resin type and substitution level, reaction time and others all while minimizing monomer consumption.

Experimental Protocol

Peptide Synthesis and Analysis

PNA compounds under investigation were synthesized automatically with a Biotage® Initiator+ Alstra™ using default deprotection conditions, DIC and Oxyma as coupling reagents, Fmoc-protected PNA monomers and coupling protocols described herein. Acetylation was performed after each coupling reaction using a solution of 89:5:6 DMF: acetic anhydride : diisopropylethylamine reacted for 1 min, twice unless otherwise mentioned.

PNA cleavage occurred in a cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O for 2 hours at room temperature. The cleavage cocktail was evaporated using the Biotage® V-10 Touch evaporation system and the resulting crude samples were analyzed for purity with an Agilent 1260 Infinity series HPLC and compound identity confirmed using Biotage® Dalton 2000 single quad mass spectrometer.

Results and Discussion

Impact of Coupling Reaction Time

Reported coupling reaction times vary dramatically within the PNA literature, even when filtered for a single automated synthesizer, but are typically longer than standard amino acid coupling reactions. To test the necessity of this extended coupling reaction time, I first compared synthesis results for a PNA synthesized using 5 monomer equivalents and either a 5 or 7 minute coupling reaction time at 75°C, keeping all other synthetic parameters consistent, Figure 1.

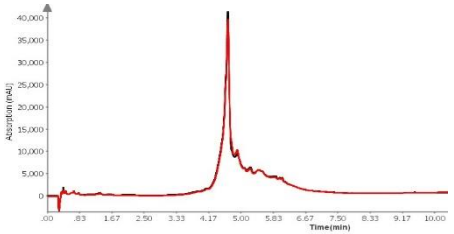

Figure 1. *Crude analytical chromatograms for PNA synthesized using 7 min coupling times (red) or 5 min coupling reactions (black).*

When using elevated reaction temperatures, small reaction time differences can yield wildly different results. For this PNA compound though, the "default" amino acid coupling time of 5 minutes produced a compound with slightly greater crude purity (57% vs. 55% pure). Despite the lower crude purity, the impurity profile remains consistent for the PNA synthesize with longer reaction times. This data suggests that nominal reaction time increases, even at elevated temperatures, could be used to maintain crude purity when monomer equivalents are decreased.

Impact of Resin Choice

Resins historically used in PNA synthesis have significantly lower substitution levels than typically used in peptide synthesis, often requiring additional manipulation of the manufactured resin prior to initiating synthesis. Extremely low resin substitution levels demand significant amounts of resin for any synthesis scale. During the synthesis then, the resin could be suspended in miniscule reagent volumes, compromising mixing efficiency, or more dilute reagents to improve the mixing properties. Either of these scenarios can result in poor crude purity for a compound prepared with relatively expensive reagents. In an effort to improve purity while decreasing resin consumption, the PNA was synthesized using ChemMatrix® resin (0.42 mmol/g) or TentaGel® S-RAM (0.23 mmol/g) using 5 monomer equivalents, reacted for 7 minutes at 75°C, Figure 2.

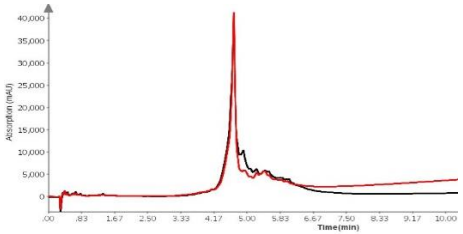

Figure 2. *Crude analytical HPLC chromatograms for PNA synthesized using ChemMatrix® (black) or TentaGel® S-RAM (red) resins.*

The resins selected for this evaluation both contain some amount of polyethylene glycol (Peg) polymeric units. Peg-based resins have been demonstrated to improve the crude purity of particularly difficult peptide sequences given they hydrophilic nature of the bead surface, serving to repel the elongating chain back into the solvent. These data suggest that the hydrophilic nature of the resin surface also yields PNA with higher crude purity despite high substitution levels.

The PNA synthesized using TentaGel® resin did have higher crude purity (about 66% pure) when compared to the PNA synthesized using ChemMatrix® resin (about 56% pure). The difference in purity was surprisingly small, especially given the resin substitution level was nearly doubled. Importantly though, the chromatographic profiles are extremely similar, highlighting the synthetic benefits of resins with surface Peg polymeric units. Despite using approximately *half* the TentaGel® mass for synthesis, the ChemMatrix® resin occupied the same physical space in the reactor vial upon synthesis completion which should also be considered for future syntheses.

Incorporating a Capping Step

Acetylation of any residual free amines at the N-terminus of the growing chain is performed intermittently during solid phase peptide synthesis. This additional reaction adds significant time to the synthesis and increases consumption of wash DMF significantly. Eliminating this step, as is commonly done for peptide synthesis, while still maintaining high crude purity is an attractive update to the standard procedures. To characterize the influence of the capping step during PNA synthesis, PNA was synthesized using 5 monomer equivalents with 7 minute coupling reactions at 75°C in the presence or absence of a subsequent acetylation reaction, Figure 3.

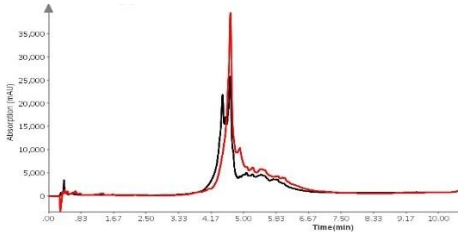

Figure 3. *Crude analytical HPLC chromatograms for PNA synthesized with (red) or without (black) an acetylation reaction performed after the coupling reaction.*

The absence of a capping step, even a very short reaction, proved to have disastrous consequences, despite reducing the synthesis time by approximately 7 hours and wash DMF consumption by more than 50%. Nearly 50% of the crude sample is composed of an early eluting deletion sequence that will be extremely difficult to remove by HPLC. This impurity is nearly undetectable when the capping step is included in the synthesis protocol, clearly demonstrating that the additional synthesis time is a worthwhile investment.

Implementing Preactivation Chemistry

Preactivation - the physical separation of the carboxylic acid activation reaction and the amide condensation reaction – is often performed in PNA synthesis in an effort to improve the crude purity. Heating the coupling reaction has been shown to improve the activation and condensation reaction kinetics such that preactivation chemistry is generally no longer employed in peptide synthesis. In a two-part experiment, the need for preactivation chemistry was evaluated specifically for PNAs using low amounts of monomer equivalents, Figure 4.

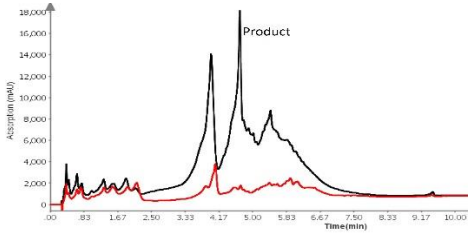

Figure 4. *Crude analytical HPLC chromatograms for PNAs synthesized using 3 monomer equivalents either with (red) or without (black) a 30 second preactivation reaction before transfer to the reactor vial containing resin.*

The PNA were synthesized using only 3 monomer equivalents with a single coupling reaction in this comparison in an attempt to reduce overall synthetic cost. Hypothetically, separating the carboxylic acid activation and condensation reactions should yield a sample with higher crude purity than a sample prepared with *in situ* activation. To further improve activation efficiency, the monomer and coupling reagent solutions were prepared at 0.5 M concentration which was then diluted with DMF to a final 3 mL volume for the heated condensation reaction. These efforts proved futile though. While not the best results observed in this series of experiments, the sample prepared with *in situ* activation and coupling yielded a significant amount of the desired product with readily separable impurities. These data suggest that the carboxylic activation step is less likely to be responsible with an incomplete coupling is observed. Most importantly though, the additional time and DMF wash solvent required to incorporate a preactivation reaction were certainly not worth the expense in this case.

Conclusion

Despite the apparent similarities between peptides and peptide nucleic acids, the synthesis strategies developed diverged. Alternative methods have been developed to accommodate the steric effects caused by the nucleobase "side chain" that reduce synthetic efficiency. Work presented herein explored further methods and strategies that can be more broadly implemented across a wider diversity of PNA sequences, enabling synthetic efficiency approaching that observed for traditional peptides. Future work will include expand these observations to more difficult sequences to ensure methodological fidelity.

This poster was first presented at the Fall National ACS Meeting, Chicago, 2022
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