Optimizing the synthesis of Peptide Nucleic Acids using PurePep® Chorus

Application Note 0028 D0043505/A

Introduction

Peptide Nucleic Acids (PNA) are synthetic analogues of DNA and/or RNA that possess several unique physicochemical properties, as the entire phosphodiester backbone is replaced by a pseudopeptide backbone to which the nucleobases are attached via a methyl carbonyl linker (Figure 1). [1]

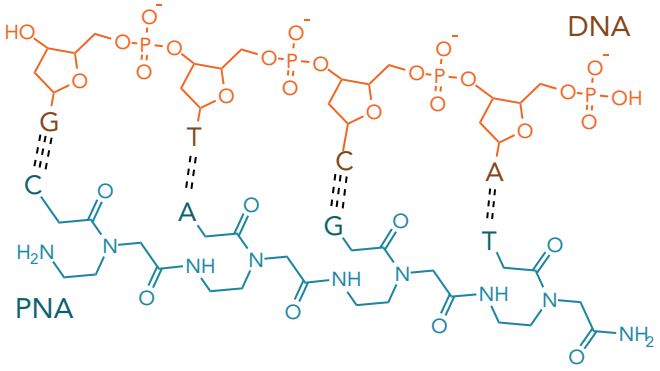

Figure 1. Structure of a peptide nucleic acid, and its binding to complementary DNA.

PNAs are non-ionic nucleic acid analogues whose neutral polyamide backbone was designed to minimize non-specific electrostatic effects often observed within DNA oligonucleotides. Hence, sequences as short as 12 bases allow for strong, specific hybridization, with improved sequence mismatch discrimination, being more responsive to single-point mutations than natural oligonucleotides. Moreover, PNAs are more resistant to both nucleases and proteases than native peptides or nucleic acids. [2]

PNAs are capable of inhibiting gene expression by binding to complementary RNAs and also, under certain conditions, can promote gene expression by binding to a DNA duplex sequence by a strand-invasion mechanism, which promotes the formation of a D-loop and consequent expression of that gene. Their high affinity and specificity make them valuable tools not only as therapeutic agents but also for their application in diagnostics. [3,4]

The synthesis of PNAs can often be regarded as a challenge, as they are prone to aggregation, and deprotection steps are often accompanied by side reactions. [5] One of the strategies to overcome the first issue consists of using large excesses of reagents in the coupling steps; however, the N-protected PNA monomers required for the synthesis are relatively expensive, making this approach less favorable.

In this application note, we report the optimization of PNA synthesis using PurePep® Chorus. PNA1 was used as a reference sequence to assess different synthesis conditions: the number of couplings, excess of PNA monomers, reaction time, and temperature. The best conditions, yielding PNA1 with the highest crude purity, were then used to synthesize five other sequences (Table 1), which differed in length and purine (adenine and guanine) content. The latter constitutes a relevant factor for the syntheses, as their coupling is often associated with poor yields due to the difficulty of coupling hindered monomers.

Table 1. Sequences of the PNA synthesized during this work.

PNA	Sequence(a)	Number of residues	Purine Content [%]	Molecular weight [Da]	Source
PNA1	acagtgtRR-NH_2	9	44	2246.22	[6]
PNA2	cagtccagttG-NH_2	11	27	2759.63	[7]
PNA3	ctcatactctG-NH_2	11	18	2694.59	[8]
PNA4	agacgacccaG-NH_2	11	55	2762.65	[9]
PNA5	acgcacactgtcG-NH_2	13	38	3271.13	[9]
PNA6	ggccgggacacaG-NH_2	13	62	3361.18	[9]

(a) Sequences written in one-letter code, where adenine (a), cytosine (c), guanine (g) and thymine (t) are written in lowercase letters, and natural amino acids (arginine – R; and glycine – G) are written in capital letters.

Results and Discussion

PNA1 was synthesized using PurePep Chorus (Gyros Protein Technologies) at a 25 µmol scale, using a low-loading Rink Amide MBHA resin (0.27 mmol.g-1), and following a Fmoc/tBu orthogonal protection scheme. Deprotection of the resin was carried out with a solution of 20% Piperidine in DMF for 2 x 5 min at room temperature. Coupling steps were conducted in the presence of HCTU and NMM, and conditions varied significantly throughout all the syntheses (Table 2). Coupling steps were followed by a capping step, with a solution of 10% acetic anhydride in DMF, for 5 min at room temperature. After the final deprotection, the resin was cleaved with a solution of trifluoroacetic acid/triisopropylsilane(TIS)/water (95:2.5:2.5) for 4 hours at room temperature.

Table 2. Coupling strategies studied in this work.

Method	PNA monomer (50 mM)	HCTU (100 mM)	NMM (200 mM)	Time [min]	Temp. [°C]	Crude Purity
A	10 eq.	10 eq.	20 eq.	60	RT	81%
B	4 eq.	4 eq.	8 eq.	60	RT	72%
C	4 eq.	4 eq.	8 eq.	5	45	61%
D	4 eq.	4 eq.	8 eq.	10	45	82%
E	2 eq. (4 eq. in total)	2 eq. (4 eq. in total)	4 eq. (8 eq. in total)	2 x 5	45	87%

Analyzing Table 2, we can observe that using 10 eq. of PNA monomer results in a very good crude purity (81%); however, this is economically prohibitive since Fmoc-protected PNA monomers are expensive. By reducing the excess of PNA to 4 eq., the crude purity decreased to 72%. By keeping a low excess of PNA monomer but increasing the temperature to 45°C and reducing the reaction time to 5 min, the crude purity was further reduced (61%). Hence, as 5 min might be too short for the coupling to be completed, the same conditions were applied but the reaction time was increased to 10 min (experiment **D**), yielding similar crude purity (82%) as for experiment **A**, which used more than twice the equivalents of PNA monomers. **Method E** was employed as a final attempt to further optimize this reaction, where double coupling was carried out with 2 eq. at a time. Using the same number of equivalents in total and the same amount of time, the crude purity further improved by 5%.

Once identified the coupling protocol that produced the best crude purity of PNA1, the remaining sequences were synthesized using the same strategy (**Method E**). Results are shown in **Table 3**.

Table 3. Crude purities obtained for PNAs synthesized with Method E.

PNA	Crude Purity
PNA2	66%
PNA3	68%
PNA4	83%
PNA5	80%
PNA6	91%

The crude purity of the synthesized PNA sequences ranges from 66-91%, which can be considered excellent crude yields, especially regarding peptide nucleic acids. Interestingly, the PNA sequences with higher purine content were produced with a better crude purity.

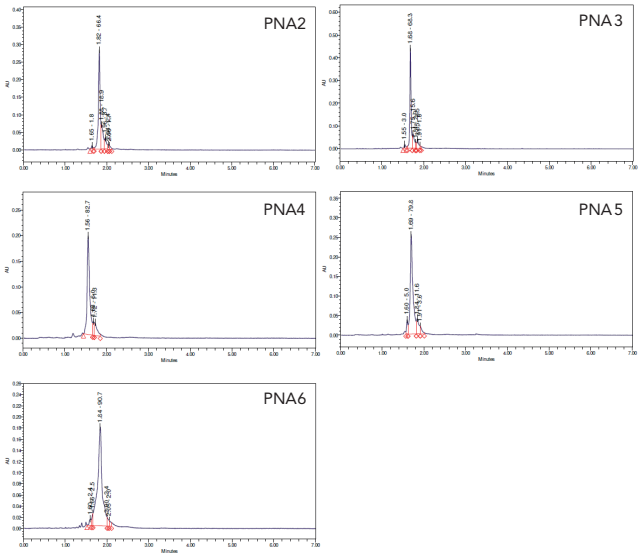

Figure 2. Chromatograms obtained for crude PNAs. UPLC-UV chromatograms (210 nm) were recorded with an analytical Acquity H-Class UPLC/ESI-MS system from Waters on a C-18 column (1.7 µm, 2.1 x 500 mm). In the mobile phase, mixtures of water (A) and MeCN (B) with 0.1% TFA were used.

Conclusions

In this application note, we describe the optimization of a coupling strategy for PNA synthesis using PurePep Chorus. We started by employing 10 eq. of PNA monomer in a coupling reaction of 1 hour at room temperature – a strategy that produces a very good crude PNA (81%). We expected to reduce the number of equivalents and minimize the loss of crude purity to make this reaction more financially sustainable and "green". Ultimately, we developed a coupling strategy that not only reduced the number of equivalents and the reaction time but also produced a better crude PNA than the original strategy. By doing double couplings, each with 2 eq., for 5 min, at 45°C, we were able to increase the crude purity of PNA1 from 81% to 87%. Finally, this coupling strategy was employed in the synthesis of the remaining sequences. This protocol produced very good crude peptide nucleic acids with varying lengths and purine content. PurePep Chorus proved to be a crucial tool for this work, as synthesis programs can be easily adjusted: from the number of equivalents and repetitions to temperature and time.

References

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