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Original Research Article

Improvement of peptide nucleic acid (PNA) synthesis, by use of DIC/Oxyma and microwave heating

Merita Murtola[1,2*#], Alice Ghidini[2#], Roger Strömberg[2*]
[1]Department of Chemistry, University of Turku, 20014 Turku, Finland
[2]Department of Biosciences and Nutrition, Karolinska Institutet, Novum, 141 83 Huddinge, Stockholm, Sweden.
# Shared first authors (equal contribution)

***Corresponding author**
Roger Strömberg
Email: Roger.Stromberg@ki.se

Abstract: Peptide nucleic acids (PNAs) are promising in diagnostic applications and of great interest due to their therapeutic potential. PNAs unique chemistry can be readily modified with amino acids or other functional groups by the use of peptide type chemistry. In the present study, we show the 10 µmol scale synthesis of a chimeric PNA oligomer containing an internal diaminopropionic acid (Dap) unit. And we compare the result obtained with two different fully automated peptide synthesizers. The product purity obtained with a Biotage instrument using diisopropylcarbodiimide/Oxyma and microwave heating was clearly superior to the purity obtained with the long standing workhorse Applied Biosystems 433A with efficient coupling reagent HATU.
Keywords: microwave, peptide synthesizer, PNA synthesis, peptide nucleic acid, DNA analogue, solid-phase peptide synthesis (SPPS).

INTRODUCTION

Peptide nucleic acids (PNAs), [1] are non-natural DNA/RNA mimetics and considered most promising in diagnostic applications. PNAs are also of great interest due to their therapeutic potential. Owing to its adaptable and neutral backbone, PNA displays excellent hybridization properties, provides a high chemical stability and high resistance to nucleases and proteases. PNA has therefore become a versatile tool in diagnostics and a variety of molecular biology techniques [2, 3]. We develop rationally designed oligonucleotide based artificial nucleases (OBANs) as a part of our interest in cleavage and recognition of RNA targets.[4-7] In the design of OBANs, peptide nucleic acid (PNA) has proven to be a versatile backbone for artificial nuclease constructs.[8-10] Due to PNAs unique chemistry it can be readily modified with amino acids, different RNA interacting units or other functional groups by the use of peptide type chemistry [2,3,11-13]. These PNA constructs can recognize and bind a target RNA, with creation of centrally placed RNA bulges that are cleaved by these artificial enzymes.

In the present study we compare the performance of two procedures with different automated synthesizers in synthesis of a chimeric PNA oligomer (in 10 µmol scale), containing an internal diaminopropionic acid (Dap) unit. Commercially available Fmoc/Bhoc-PNA monomers are used in the synthesis on the automated synthesizers. Solid-phase synthesis of PNA in standard automated peptide synthesizers is typically more difficult (e.g. due to aggregation and/or steric hindrance) than standard peptide synthesis and more in line with the assembly of difficult peptide sequences. This often requires more active coupling agents such as HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) and still leads to incomplete reactions. Here we demonstrate that synthesis of PNA with a microwave heated Biotage Initiator+ Alstra peptide synthesizer and diispropylcarbodiimide/Oxyma as coupling agent gives substantially higher quality PNA than obtained with our standard conditions for PNA synthesis in a ABI 433A peptide synthesizer.

EXPERIMENTAL SECTION

Materials

All materials were obtained from commercial suppliers. Link technologies (Glasgow, UK): (peptide nucleic acid monomers, Fmoc-PNA-A(Bhoc)-OH, Fmoc-PNA-G(Bhoc)-OH, Fmoc-PNA-C(Bhoc)-OH and Fmoc-PNA-T-OH); Iris Biotech GmbH (Marktredwitz, DE): (2,3-diaminopropionic acid (Fmoc-L-Dap(Mtt)-OH), Fmoc-L-Lys(Boc)-OH, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU),

trifluoroacetic acid (TFA), triisopropylsilane (TIS), dichloromethane (DCM), N-methylpyrrolidone (NMP) and piperidine); Merck-Millipore: (ethyl cyano(hydroxyimino) acetate (Oxyma) and N,N'-diisopropylcarbodiimide (DIC)); Sigma-Aldrich: (N,N-Diisopropylethylamine (DIPEA)); Alfa Aesar: (2,6-lutidine); VWR: (acetonitrile); Applied Biosystems: (acetic anhydride); Biotage: (Rink Amide ChemMatrix resin, 0.47 mmol/g) and PCAS BioMatrix (Rink Amide ChemMatrix resin, 0.54 mmol/g).

PNA synthesis with Biotage Initiator+ Alstra microwave peptide synthesizer

PNA was prepared on a Biotage Initiator+ Alstra microwave peptide synthesizer. The sequence was assembled automatically on Rink Amide ChemMatrix resin (supplier Biotage, 0.47 mmol/g) on a 10 µmol scale in a 5 mL reactor vial using Fmoc chemistry under inert gas (N_2). Resin swelling 20 min at 70 °C. Fmoc deprotection was performed at room temperature (RT) in two stages by treating the resin with piperidine-NMP (1:4) for 3 min followed by piperidine-NMP (1:4) for 10 minutes. The resin was then washed with NMP (x4). PNA couplings were performed using 4 eq. of PNA monomer (or Lys or Dap monomers), 4 eq. Oxyma and 4 eq. DIC in NMP. A coupling time of 6 min at 75°C (microwave) was employed and the resin was washed with NMP (x4). This was followed by a capping step using NMP-lutidine-acetic anhydride (89:6:5) for 2 min and then washing with NMP (x4). After the synthesis cycles were completed, the resin was washed with NMP (x5), DCM (x6) and thoroughly dried.

PNA synthesis with Applied Biosystems 433A peptide synthesizer

PNA was prepared on an Applied Biosystems 433A peptide synthesizer. The first amino acid Fmoc-Lys (Boc) was manually coupled to Rink Amide ChemMatrix resin, 0.54 mmol/g (using the same chemistry as in the subsequent automated assembly of the PNA) which gave an amino acid loading of 0.33 mmol/g. The sequence was assembled automatically on Rink Amide ChemMatrix resin (Fmoc-Lys-ChemMatrix, loading 0.33 mmol/g) on a 10 µmol scale in a 3 mL reaction vessel using Fmoc chemistry. Fmoc deprotection was performed in two stages by treating the resin with 22% piperidine in NMP two times for 7 minutes. The resin was then washed with NMP (x6). PNA couplings were performed using 5 eq. of PNA monomer, 4.75 eq. HATU and 10 eq. DIEA in NMP. A coupling time of 60 min at room temperature was employed, followed by a capping step using NMP/2, 6-lutidine/acetic anhydride (89:6:5) for 1 min and then washing with NMP (x3). After the synthesis cycles were completed, the resin was washed with NMP (x3), DCM (x5) and thoroughly dried.

Purification and analysis

Both PNAs were cleaved from the solid support using a cocktail of TFA-H_2O-TIS (95:2.5:2.5) for 1.5 h at room temperature. The PNA products were evaporated to dryness by a nitrogen flow, diluted with deionized (MilliQ) water and evaporated to dryness under reduced pressure. Products were partitioned between diethyl ether and water (x3), and the water phase was evaporated to dryness and HPLC purified with an Ascentis Express Supelco Peptide ES-C18 column (2.7 µm, 150 × 4.6 mm) at 60 °C using a flow rate of 1 mL/min. The following solvent system was used: solvent A, water containing 0.1% TFA; solvent B, 50% CH_3CN: water containing 0.1% TFA (1:1; v/v). PNAs were purified using a linear gradient of 40% B for 30 min. Collected products were evaporated to dryness and lyophilized from water (x3). Mass spectrometry of the collected products was performed on a Micromass LCT electrospray ionization time-of-flight (ESI-TOF) mass spectrometer in a solution of acetonitrile–water 1:1 (v/v) with 0.1% formic acid. The molecular weights of the PNAs were reconstructed from the m/z values using the mass deconvolution program of the instrument (Mass Lynx software package). PNA $C_{130}H_{169}N_{69}O_{37}$, calc. PNA [$M_{cal}$] 3290.

[M_{obs}] 3287 (PNA synthesis with Biotage Initiator+ Alstra microwave peptide synthesizer)
[M_{obs}] 3286 (PNA synthesis with Applied Biosystems 433A peptide synthesizer)

RESULTS AND DISCUSSION

The application of microwave irradiation has been shown to be advantageous for solid phase PNA synthesis [10, 13, 14], especially to enable the synthesis of long or difficult PNA sequences, and to improve crude product purity in general. In this study we have performed two fully automated PNA syntheses using either a conventional procedure on the ABI 433A synthesizer and the active HATU reagent or new conditions with microwave on a Biotage Initiator+ Alstra synthesizer. The PNA sequence included a centrally placed diaminopropionic acid (Figure 1) and it was assembled on a Rink Amide ChemMatrix resin. For the new microwave conditions of PNA synthesis with the coupling agent diisopropylcarbodiimide (DIC), we chose to use the Oxyma [15] additive that has proven to be highly advantageous in peptide synthesis.

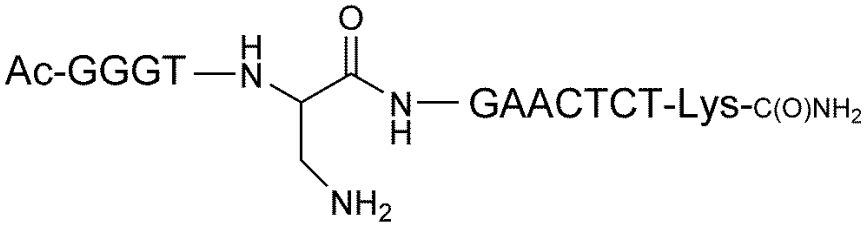

Fig 1: The synthesized PNA containing a centrally placed diaminopropionic acid (PNA1, G, T, A and C designates the PNA residues with the corresponding heterocyclic bases).

A benefit of using peptide/PNA synthesis strategies in construction of modified oligonucleotide conjugates is that PNA has unique properties and the strategy allows for ready conjugation to peptides and other entities. This can with little effort be used to create innovative constructs that can give new desired properties or biological responses of the PNA molecules. **PNA1** (Figure 1) incorporates a diaminopropionic acid (Dap) residue, suitable for post synthetic conjugations of different functional groups, e.g. catalytic units in peptide nucleic acid based artificial enzymes (PNAzymes)[8]. Incorporation of a lysine residue at the C-terminal of PNA oligomer is used to increase solubility.

When synthesizing chimeric oligomers with a fully automated synthesizer the instrument's flexible setup with possibilities for multiple couplings, capping reagents and complete control over all reaction steps becomes important. More optimal solid phase PNA synthesis is obtained by use of a more solvated polymer-matrix that allows efficient reagent accessibility. Here we use a Rink Amide ChemMatrix resin because of its good swelling properties in a variety of solvents used in solid phase peptide synthesis (SPPS).

PNA synthesis with Applied Biosystems 433A peptide synthesizer

PNA1 (Figure 2) was first synthesized using an automated ABI 433A peptide synthesizer. This machine has been considered as the standard workhorse for peptide and PNA synthesis in research laboratories for many years. Synthesis was analogous to standard SPPS at room temperature consisting of cycles of PNA monomer coupling, capping of unreacted amino functions followed by Fmoc deprotection. PNA extension with the ABI 433A was done using 5 equivalents of PNA monomers and an extended coupling time of 60 min at room temperature. For this conventional synthesis we employed the highly reactive coupling reagent (HATU) and the major product was the correct and expected PNA. However, the synthesis resulted in several side products (Figure 2, mainly shorter PNA fragments due to poor coupling efficiency).

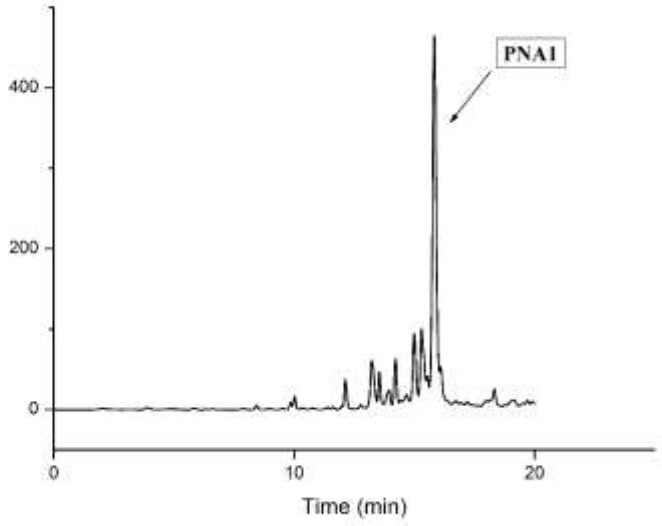

Fig 2: RP-HPLC chromatogram of crude PNA1 synthesized with the ABI 433A peptide synthesizer

RP-HPLC analysis was carried out on a Ascentis Express Supelco Peptide ES-C18 column (2.7 µm, 150 × 4.6 mm) with a linear gradient elution of 0–20% acetonitrile in 0.1% aq. TFA over 30 min at 60 °C, flow rate of 1.0 mL/min and UV detection at 260 nm.

Although the PNA synthesis is acceptable as such, it is in general insufficient when the intentions are to synthesize further modified PNA post-conjugated to different functional entities. It would then be highly advantageous to minimize shorter fragments (many of which also contain the Dap linker) since these typically can cause purification problems at later stages. The outcome shown in Figure 2 is typical of as good results as we got with the ABI 433A, and was achieved with extended reaction time (60 min) and the highly reactive coupling reagent HATU.

PNA synthesis with Biotage Initiator+ Alstra microwave peptide synthesizer

The use of microwave irradiation, combined with oscillating mixing for homogeneous heat distribution, has the potential to increase the coupling efficiency during PNA synthesis and significantly improve the crude purity of the product, especially when synthesizing problematic sequences (e.g. sequences containing consecutive guanosines). In addition, the coupling agent DIC as well as the additive/nucleophilic catalyst Oxyma (that was reported as highly efficient in peptide synthesis [15] and less prone to explosions) seems fully compatible with microwave conditions.

PNA1 was successfully synthesized using DIC/Oxyma on the Biotage Initiator+ Alstra synthesizer with microwave heating and the result was indeed a PNA with high purity (Figure 3). A coupling time of 6 min at 75°C (microwave) was employed. The combination of microwave heating and use of DIC/Oxyma as coupling agent clearly gave a considerably more efficient synthesis of PNA than with the conventional method, even though fewer equivalents of PNA monomers (4 eq) were used. In addition there is a ten-fold time gain in the coupling steps.

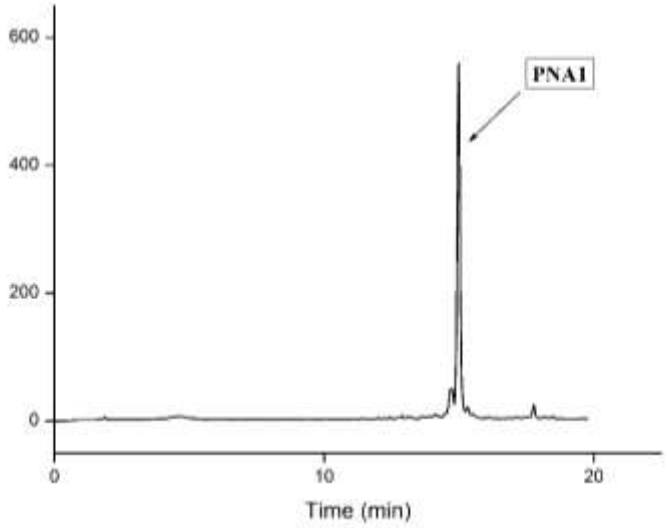

Fig 3: RP-HPLC chromatogram of crude PNA1 synthesized with the Biotage Initiator+ Alstra microwave peptide synthesizer. RP-HPLC analysis was carried out on a Ascentis Express Supelco Peptide ES-C18 column (2.7 µm, 150 × 4.6 mm) with a linear gradient elution of 0–20% acetonitrile in 0.1% aq. TFA over 30 min at 60 °C, flow rate of 1.0 mL/min and UV detection at 260 nm.

CONCLUSIONS

The PNA-amino acid chimera **PNA1** was successfully synthesized with both the ABI 433A and Biotage Initiator+ Alstra automated synthesizer at 10 µmol scale. However the new methodology with DIC/Oxyma and microwave heating is the preferred method as compared to the more conventional HATU/RT methodology. In the ABI method the produced crude PNA contains a substantial amount of truncated sequences while the Microwave/ Biotage/ DIC/Oxyma procedure gives PNA that is virtually free of truncated sequences and with very few impurities altogether. There is clearly a major advantage in the increase of crude product purity, especially when further reactions with the PNA will follow. In addition there is a large reduction in overall synthesis time.

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REFERENCES

1. Nielsen PE, Berg RH. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science. 1991 Dec 6; 254(5037):1497.
2. Lundin KE, Good L, Strömberg R, Gräslund A, Smith CE. Biological activity and biotechnological aspects of peptide nucleic acid. Advances in genetics. 2006 Dec 31; 56:1-51.
3. Peptide Nucleic Acids. *Methods in Molecular Biology* (New York, NY, United States), 2014, 1050.
4. Åström H, Williams NH, Strömberg R. Oligonucleotide based artificial nuclease (OBAN) systems. Bulge size dependence and positioning of catalytic group in cleavage of RNA-bulges. Organic & biomolecular chemistry. 2003; 1(9):1461-5.
5. Åström H, Strömberg R. Synthesis of new OBAN's and further studies on positioning of the catalytic group. Organic & biomolecular chemistry. 2004; 2(13):1901-7.
6. Strömberg MM. 2'-O-Methyloligoribonucleotide based artificial nucleases (2'-O-MeOBANs) cleaving a model of the leukemia related M-BCR/ABL m-RNA. Arkivoc. 2009; 3:84-94.
7. Murtola M, Strömberg R. PNA based artificial nucleases displaying catalysis with turnover in the cleavage of a leukemia related RNA model. Organic & biomolecular chemistry. 2008; 6(20):3837-42.
8. Murtola M, Wenska M, Strömberg R. PNAzymes that are artificial RNA restriction enzymes. Journal of the American Chemical Society. 2010 Jun 14; 132(26):8984-90.
9. Ghidini A, Bergquist H, Murtola M, Punga T, Zain R, Strömberg R. Clamping of RNA with PNA enables targeting of microRNA. Organic & biomolecular chemistry. 2016; 14(23):5210-3.
10. Ghidini A, Murtola M, Strömberg R. Influence of conjugation and other structural changes on the activity of Cu 2+ based PNAzymes. Organic & biomolecular chemistry. 2016; 14(9):2768-73.
11. Sandbrink J, Murtola M, Strömberg R. Solid support post-conjugation of amino acids and a phenanthroline derivative to a central position in peptide nucleic acids. Nucleosides, Nucleotides, and Nucleic Acids. 2007 Nov 26; 26(10-12):1485-9.
12. Ghidini A, Steunenberg P, Murtola M, Strömberg R. Synthesis of PNA Oligoether Conjugates. Molecules. 2014 Mar 13; 19(3):3135-48.
13. Danneberg F, Ghidini A, Dogandzhiyski P, Kalden E, Strömberg R, Göbel MW. Sequence-specific RNA cleavage by PNA conjugates of the metal-free artificial ribonuclease tris (2-aminobenzimidazole). Beilstein journal of organic chemistry. 2015 Apr 16; 11(1):493-8.
14. Fabani MM, Abreu-Goodger C, Williams D, Lyons PA, Torres AG, Smith KG, Enright AJ, Gait MJ, Vigorito E. Efficient inhibition of miR-155 function in vivo by peptide nucleic acids. Nucleic acids research. 2010 Mar 11:gkq160.
15. Subirós-Funosas R, Prohens R, Barbas R, El-Faham A, Albericio F. Oxyma: An Efficient Additive for Peptide Synthesis to Replace the Benzotriazole-Based HOBt and HOAt with a Lower Risk of Explosion. Chemistry–A European Journal. 2009 Sep 21; 15(37):9394-403.