Parallel Synthesis of PNA-Peptide Conjugate Libraries

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Abstract: An optimized semi-automatic protocol for parallel synthesis of up to 96 peptide nucleic acids (PNA) or PNA-peptide conjugates using Boc-protection strategy has been developed using a robotic system. The approach is illustrated by synthesizing PNA and PNA-peptide libraries varying between 15 and 27 "amino acid" units. The peptides (NLS (nuclear localization signal) or Tat-peptide) were attached to N-terminus of the PNA. The method was found to be far superior to that based on the SPOT/Fmoc protocol by which PNA oligomers are synthesized on a modified cellulose membrane. On a 0.5 micromole scale the method typically yielded 2 mg product of 90% purity by HPLC/MALDI-TOF analysis. This approach is suitable for screening of a large number of PNA and/or peptide sequences for biochemical and biological studies.

Key words: Peptide nucleic acid, PNA libraries, Parallel PNA synthesis, PNA peptide conjugates

INTRODUCTION

The pseudo peptide DNA mimic PNA (peptide nucleic acid) (Figure 1) is finding widespread applications within genetic diagnostics[1-7], drug development and molecular biology apart from its purely scientific interest as a polyamide with DNA like structural properties [16-21]. PNA oligomers are routinely synthesized by conventional solid phase peptide chemistry using either the Boc or the Fmoc protection strategy [22-24]. For many applications in diagnostics (*e.g.* for hybridization arrays) or drug development (*e.g.* for mRNA gene walks in antisense studies) it is highly advantageous to be able to synthesize large series of oligomers in parallel. Furthermore, it would be of interest to extend such libraries to include PNA peptide conjugates, which have shown great promise for delivery of PNA to both mammalian and bacterial cells [11,25-27].

Using a simple X-Y robotic dispenser, the production of PNA arrays by direct synthesis on cellulose membranes was recently described as an extension of peptide arrays synthesized by this method [28,29]. For drug development applications, one is primarily interested in obtaining soluble libraries of PNAs and PNA-peptide conjugates. We have therefore developed this technique further, and now report a method in which such a robot is used to synthesize 96 PNAs in parallel at 0.5 μmole scale (typically yielding 1-2 mg of PNA) by Boc chemistry, or a combination of Boc and Fmoc chemistry for PNA-peptide conjugates. The method may be extended to yield 384 PNAs in parallel.

RESULTS AND DISCUSSION

Initially, a PNA library gene walk against the *E. coli* β-lactamase gene was synthesized on a cellulose membrane using the Fmoc chemistry as reported earlier [28]. Briefly, prior to the actual PNA synthesis, the trypsin sensitive tetra peptide spacer Lys(Boc)-Glu-(OtBu)-(6-aminohexanoic acid)$_2$ was manually attached to the entire amino functionalized membrane via the acid cleavable Rink-amide linker using Fmoc chemistry. PNA monomers and HATU were each dissolved in NMP at a concentration of 0.3 M, and DIEA at 0.4 M. Fmoc group was removed by 20% piperidine followed by vigorous washing of the membrane with DMF and subsequently with ethanol, and the membrane was dried in air prior to the next coupling. The PNA monomers were activated by mixing with the HATU and DIEA solutions in the ratio of 1:1:1 for 1 min. and spotted with 0.3 μl aliquots. Except for spotting, all other steps including capping, washing and deprotection were carried out manually. Although, all coupling steps went well as judged by the bromophenol blue color reaction, mass spectrometric analysis indicated that the amount of the correct PNAs were far exceeded by truncated byproducts (Figure 2). Moreover, the yields were very low (< 100 μg per spot) regardless of whether acid cleavage or trypsin digest was used.

We therefore chose to explore a multi-well approach for PNA and PNA-peptide parallel library synthesis using a 96-well manifold. Because of its robustness and our desire to exploit various non-standard PNA monomers developed by us, we chose the Boc-protection strategy rather than the Fmoc. The PNA synthesis was optimized on 5 mg MBHA resin (down-loaded to 0.1 mmol/g, 0.5 μmol). The Boc group was removed by TFA followed by washing of the resin with DMF/DCM, DMF and neutralizing with pyridine. Subsequently, the next PNA monomer was activated by mixing with HBTU and DIEA (1:1:1) for one min. and coupled to the growing PNA chain for 30 min. Each coupling was repeated to achieve 100% coupling in each cycle. After each coupling, non-reacted PNA oligomer was capped, and the resin was washed with piperidine and the terminal amine was deprotected for the next cycle. No Kaiser test was performed in any stage of the synthesis. The detailed synthesis protocol for a single PNA cycle is presented in Table 1. At the end of the synthesis, the PNA

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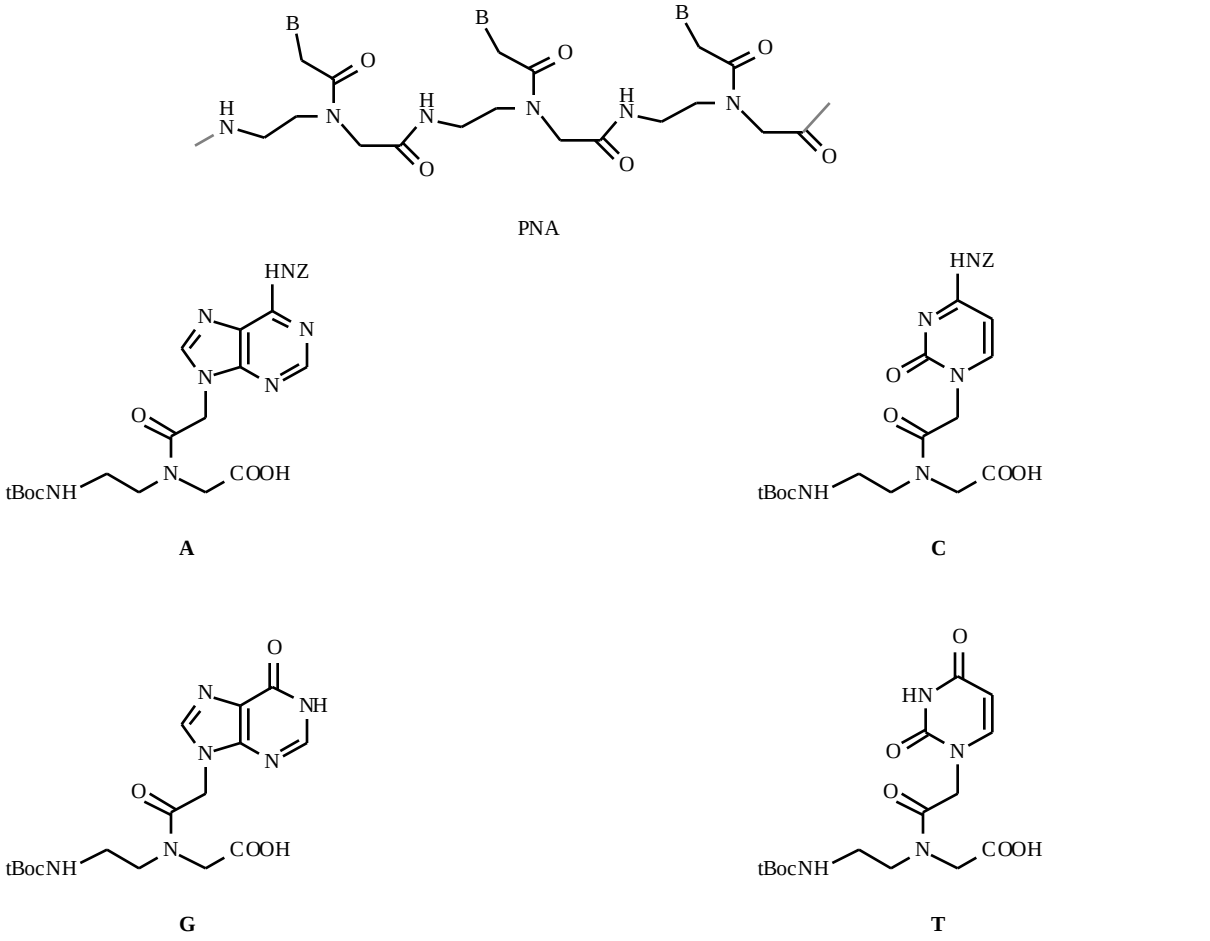


Fig. (1). Chemical structure of PNA oligomer and of Boc-protected PNA monomers of the adenine (A), guanine (G), cytosine (C) and thymine (T) nucleobases.

oligomers were cleaved from resin by the high-low TFA-TFMSA method in glass vials [22]. All PNAs or PNA-peptide conjugates were obtained with a free amino group and a carboxamide group at the N and C-terminus, respectively. The crude PNAs were precipitated in cold dry ether and washed thoroughly to completely remove scavengers. HPLC analysis of the crude PNA and PNA-peptide conjugates showed ≥ 90% purity as indicated by mass spectrometry (Figure 3). The PNAs synthesized by this method could be used for antisense experiments without further purification (manuscript in preparation). An example of a gene walk synthesis is presented in Table 2.

Table 1. Protocol for Parallel PNA Synthesis Using Boc Chemistry

Resin	MBHA, 5 mg, loading 0.1 mol/gm (0.5 µmol scale)
Total volume per well	30 µL
Resin swelled in DMF	~3-4 h
PNA stock solution in NMP or DMF	0.24 M (G monomer in DMSO)
HBTU stock solution in DMF	0.23 M
DIEA stock solution in DMF	0.46 M
Capping reagent	(DMF/acetic anhydride/ collidine : 8:1:1)
Piperidine washing	(5% piperidine in DMF)
Step involves in a single PNA synthesis cycle	
1. Boc deprotection	50 µL TFA (3 x 4 min for the first time, subsequently 2 x 4 min)
2. DMF/DCM (1:1; vol/vol) wash	3 x 100 µL
3. DMF wash	1 x 100 µL
4. Pyridine wash	2 x 100 µL
5. Coupling	30 min
Total volume 30 µL (10 µL PNA +10 µL HBTU +10 µL DIEA from stock solution), preactivation time = 1 min	
6. Wash resin with DMF	2 x 100 µL
7. Repeat coupling	30 min (as step 5)
8. DCM / DMF wash	3 x 100 µL
9. Capping reagent	50 µL (2 min)
10. DCM/ DMF wash	3 x 100 µL
11. Piperidine wash	100 µL (4 min)
12. DMF wash	3 x 100 µL
13. DMF/DCM wash	2 x 100 µL

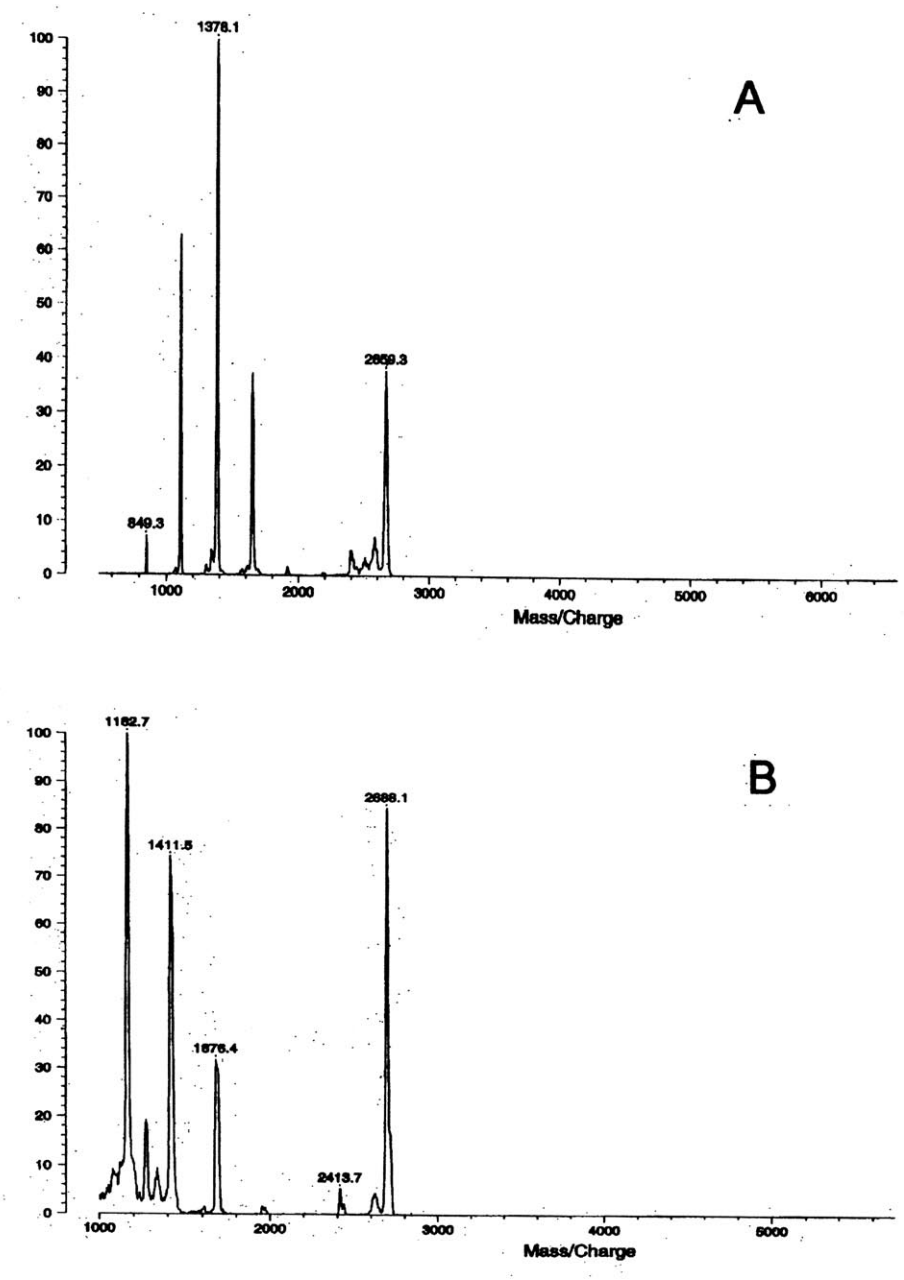


Fig. (2). Positive ion Maldi-TOF spectra of PNA oligomers H-ACCATCTGGC-NH_2 (calc. mass: 2686) and H-CTTTTACTTT-NH_2 (calc. mass: 2657) synthesized on cellulose membranes by Fmoc chemistry.

In order to illustrate the scope of parallel PNA synthesis in a multi-well Boc-format, PNAs varying from 10 to 15 nucleobases in length were synthesized (Table 3). Furthermore, two different peptides (nuclear localization signal, and Tat peptide) were selected for continuous synthesis conjugation as they are commonly used for cellular delivery of DNA, PNA or proteins *in vivo* and *in vitro* [30]. In particular the NLS peptide has been used for cellular delivery of PNA [27]. The PNA oligomers were synthesized using Boc chemistry, while the peptide moiety was subsequently synthesized by standard Fmoc chemistry. The 8-amino-2,6-dioxaoctanoyl linker (eg1) was used as linker between PNA and peptide to avoid any steric hindrance caused by the peptide upon hybridization to the target site on the mRNA. In addition, the adamantyl group, which has been shown to aid liposome mediated cellular uptake of PNA (sequence SP 13-15 and 19-21) was attached to the N-terminal of the PNA. Furthermore [31], non-natural PNA nucleobases (DAP or tC) (Figure 4) were successfully incorporated into PNA oligomers without any change in the synthesis protocol. Finally, an extensive gene walk series targeted to the *E. coli* β-lactamase gene was synthesized. These PNAs were all conjugated at the N-terminal by continous synthesis to the peptide, $(KFF)_3K$, which has been found to dramatically improve the bacterial uptake when conjugated to a PNA oligomer [11].

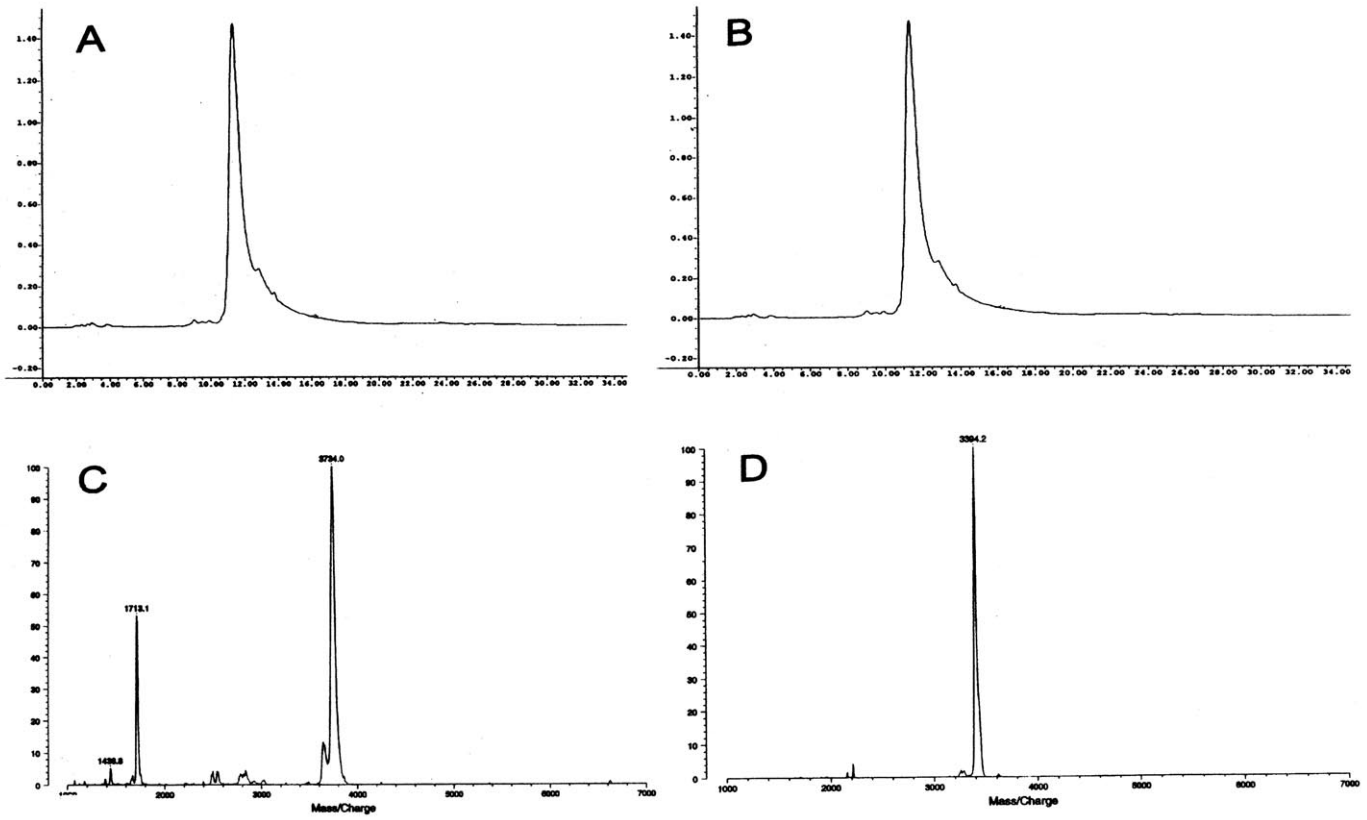


Fig. (3). HPLC analysis of crude A) PNA-peptide (H-PKKKRKV-eg1-TGTACGTCACAACTA-NH_2) and B) PNA (H-CCCACCAGCACCA--NH_2) as well as and MALDI-TOF mass spectra C) and D) of these two PNAs synthesized semi automatically on the multi-well ABIMED robot by Boc chemistry. The found (and calculated) masses for these two PNAs are 3734 (3736) and 3394 (3393), respectively.

Table 2. **Example of a Gene Walk Library of the *E. coli* β-Lactamase Gene (bla) Prepared by Parallel PNA* Synthesis**

AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT

TCGTTCATCC ATAGTTGCCT GACTCCCCGT CGTGTAGATA ACTACGATAC

GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA

CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG CCGGAAGGGC

CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA

ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC

AACGTTGTTG CCATTGCTGC AGGCATCGTG GTGTCACGCT CG TCGTTTGG TATGGCTTCA TTCAGCTCCG

GTTCCCAACG ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC

TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA TGGCAGCACT GCATAATTCT

CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT

AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAC ACGGGATAAT ACCGCGCCAC ATAGCAGAAC

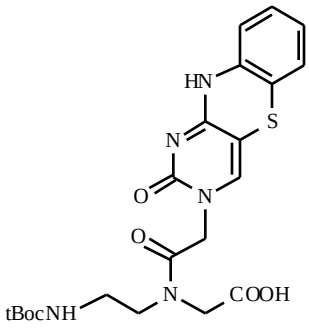
TTTAAAAGTG CTCATCATTG GAAAACGTTC TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA

TCCAGTTCGA TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA

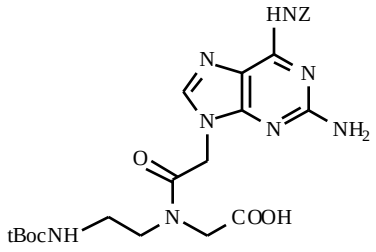
AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC

AGAAGTGGTC TTGTTGCCAT CTTTTACTTT AACGTTCTTC ATGCTTTTCT

*The PNA sequences are written from their amino to carboxy termini, which correspond to the 5' end of a conventional oligonucleotide. The peptide KFFKFFKFFK-eg1 is attached to the amino termini of all PNA sequences.



tC



DAP

Fig. (4). Chemical structures of the non-standard nucleobase tBoc monomeres of 2,6-diaminopurine (DAP) and tC.

Table 3. Examples of Various PNAs and PNA Conjugates Synthesized by the Parallel Manifold Approach

Sequence	target site
H-GRKKRRQRRRPPQ-eg1-ATCTACTGGCTCC AT-NH_2	HIV-1
H-GRKKRRQRRRPPQ-eg1-CAAGCTTTATTGAGG-NH_2	HIV-1
H-AAGCCCTCCCCG-NH_2	ras oncogene
H-AAGCCCTCCCC-NH_2	ras oncogene
H-AAGCCCTCCC-NH_2	ras oncogene
Ada-AAGCCCTCCCCG-NH_2	ras oncogene
Ada-AAGCCCTCCCC-NH_2	ras oncogene
Ada-AAGCCCTCCC-NH_2	ras oncogene
H-CCACCAGCACCAT-NH_2	ras oncogene
H-CCCACCAGCACCA-NH_2	ras oncogene
H-CCCCACCAGCACC-NH_2	ras oncogene
Ada-CCACCAGCACCAT-NH_2	ras oncogene
Ada-CCCACCAGCACCA-NH_2	ras oncogene
Ada-CCCCACCAGCACC-NH_2	ras oncogene
H-PKKKRKV-eg1-GGCCGCCAGCTCCAT-NH_2	her2 gene
H- PKKKRKV-eg1-GTCTTTATTTCATCTT-NH_2	her2 gene
H-KFFKFFKFFK-eg1-CTCATACTCT-NH_2	*acp*P (*E. coli*)
H-KFFKFFKFFK-eg1-CTCAT(DAP)CTCT-NH_2	*acp*P (*E. coli*)
H-KFFKFFKFFK-eg1-CTCATA(tC)CCT-NH_2	*acp*P (*E. coli*)

Abbreviations:
Ada: adamantylacetyl [31]
DAP: 2,6-diaminopurine [32]
tC: 2-hydroxy-10*H*-pyrimido[5,4-b] [1,4]benzothiazine [33]
eg1: 8-amino-2,6-dioxaoctanoyl [34]

Whereas the the synthesis of naked PNA oligomers and PNAs conjugated to the "KFF" or the NLS peptides gave products of high purity (Figure 3B,D), the synthesis of the PNA-Tat conjugates gave less pure products (Figure 3A,C). We also unsuccessfully attempted to synthesize PNA-penetratin conjugates (penetratin = RQIKIWFQNRRMKWKK) by this method. Therefore, the method at present is limited to rather short peptide conjugates that do not contain "synthetically challenging" peptide motifs.

CONCLUSION

We have developed a simple, efficient and convenient method for parallel synthesis of 96 PNA-peptide conjugates at the micromole scale. This produces enough material (1-2 mg) for most *in vitro* and *ex vivo* cell culture experiments and allows the screening of a small library of PNAs. The method as described can easily be expanded to yield 384 PNAs at 2 μmole scale, and therefore should be very useful, *e.g*. in drug development projects, or in the search for optimal hybridization probes.

MATERIALS AND METHODS

The Boc and Fmoc PNA monomers were obtained from Perseptive Biosystems (USA). In the Boc monomer, exocyclic amino groups (A, C, G) were protected by the benzyloxycarbonyl group (Z) while in Fmoc monomers, exocyclic amino groups were protected by the benzhydroloxycarbonyl (Bhoc) group. Amino functionalized cellulose membrane was purchased from ABIMED (Germany) with a support capacity of 400 nmol/cm^2 as reported by the manufacturer. A 96-well (100 μl per well) filter manifold was provided by ABIMED. All Fmoc and Boc amino acids, HBTU and MBHA resin were purchased from Novabiochem (Switzerland). TFA, diisopropylethylamine (DIEA), anisol, m-cresol, NMP, trifluoromethanesulphonic acid (TFMSA), collidine, were purchased from Aldrich (Milwaukee, WI). DMF, DCM, acetonitrile obtained from Lab Scan, UK and were HPLC grade. Bromophenol blue solution was prepared in DMF. Acetic anhydride was purchased from Riedel-de Haen (Germany).

All PNA and PNA-peptide conjugates from the multi-well synthesis were cleaved in TFMSA: TFA: m-cresol:

anisol (100 uL, 2:6:1:1, 2 x1 h), precipitated by adding cold dry ether, and finally washed thoroughly with ether to remove scavengers. PNAs were redissolved in water and stored at -20 ^{o}C. All compounds were characterized by MALDI-TOF mass spectrometry. Some of the PNA and PNA-peptide oligomers were also analyzed and purified by HPLC. The HPLC analysis was performed on a Waters (Milford, MA) Delta Pak C_{18} column (5μm, 3.9 x 150mm) at ambient temperature with a flow rate of 1mL/min and dual wavelength absorbance detection (260 and 230nm). The purification was done on a C_{18} (Delta Pak), 15μm, 19 x 300 mm column at a flow rate of 8 ml/mm a gradient from water to acetonitrile containing 0.1% TFA. Bromophenol blue (0.01%) in DMF solution was used for visualization of the spot on the membrane. The PNA oligomers from the cellulose membrane synthesis were cleaved in a mixture of 90% TFA, 5% water and 5% triethylsilane for 1 h.

Positive ion Maldi mass spectra were obtained using a Kratos MALDI-II time-of-flight mass spectrometer. PNA and PNA-peptide conjugates were analysed by using the 3,5-dimethoxy-4-hydroxycinnamic acid matrix. The PNA oligomer and the matrix were mixed in the ratio of 1:4, and an aliquot of 0.8 μl was deposited on the sample plate and allowed to evaporate at ambient temperature.

ACKNOWLEDGEMENTS

This work was supported by the Danish Cancer Society and The Association for International Cancer Research (AICR). We thank ABIMED, Germany, for providing the 96-well filter manifold.

ABBREVIATIONS

DCM = Dichloromethane

DIEA = Diisopropylethyl amine

DMF = Dimethylformamide

DMSO = Dimethylsulfoxide

HATU = O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HBTU = 2-(1-H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

MBHA = Methylbenzhydrylamine

NMP = N-Methylpyrrolidone

TFA = Trifluoroacetic acid

TFMSA= Trifluoromethanesulphonic acid

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