Pergamon

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Tetrahedron Letters 44 (2003) 8701–8704

TETRAHEDRON LETTERS

New synthesis of a spin-labeled peptide nucleic acid and its interactions with nucleic acids

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Received 10 July 2003; revised 12 September 2003; accepted 17 September 2003

Abstract—A new combined solid-liquid phase synthesis method for a spin labeled peptide nucleic acid (PNA) is developed. The methodology involved initial preparation of a protected PNA on solid phase, followed by efficient solution phase coupling to a spin label containing a reactive carboxylic group. This strategy allows to maintain the integrity of the nitroxide moiety during the various steps of chemical synthesis assuring in the same time the fidelity of the hybridization assay. This compound can be used as a reporter molecule to investigate the binding of peptide nucleic acids to oligonucleotide sequences (DNA or RNA) by EPR spectroscopy.

Peptide nucleic acids (PNAs) are oligonucleotide mimics in which the sugar–phosphate backbone has been replaced by a pseudopeptide chain of *N*-aminoethylglycine monomers.[1,2] PNAs were shown to bind to complementary DNA or RNA sequences with high affinity and specificity.[3,4] In fact, these molecules can hybridize to complementary chains forming very stable duplexes and triple helixes. Their application as sensor molecules and molecular probes leads to an increase in specificity and sensitivity of the analysis, because of their ability to better discriminate the differences among the single base pairs in respect to oligodeoxynucleotide (ODN) probes.[5]

Unlike oligonucleotide probes, even short PNAs are expected to be efficient in hybridization techniques to target DNA.[2] An alternative method of detection is based on electron paramagnetic resonance (EPR) using nitroxide spin labels. EPR studies to elucidate binding of DNA and biostructures by means of the spin labelling procedure have already proven to be a powerful method of analysis.[6–11] It has also been used to study the annealing of labeled oligonucleotides to the complementary DNA sequence.[12]

This study describes a new synthesis of spin-labeled PNA to investigate the binding of peptide nucleic acids to oligonucleotide sequences (DNA or RNA). In combination with EPR-spectroscopy such labeled PNA can be used to detect hybridization processes.

Because of the instability of the nitroxide moiety which undergoes degradation during the repeated deprotection steps, or during the final cleavage from the solid support, it is not possible to insert spin labels linked to residues in internal positions during the solid phase synthesis of peptide nucleic acids. A solid phase method with Boc strategy for the synthesis of the spin labeled conjugate of PNA has been described.[13] Applying this strategy, the final single-step cleavage reaction could create nitroxide protonation followed by disproportion and decomposition of the nitroxide group due to the strong acidic environment (trifluoroacetic acid (TFA)/trifluoromethanesulfonic acid (TFMSA)).[14] To overcome this problem a Fmoc-based strategy, which never requires strong acidic conditions, appeared to be the most appropriate. Moreover, we successfully explored a post-synthetic approach in which the previously assembled PNA was linked to the spin-label moiety in liquid phase to facilitate the chemical synthesis and avoid the possible inactivation of the above mentioned compounds due to the strong final cleavage conditions commonly used in the Fmoc strategy (TFA).

Keywords: spin label; peptide nucleic acid; EPR; oligonucleotides.
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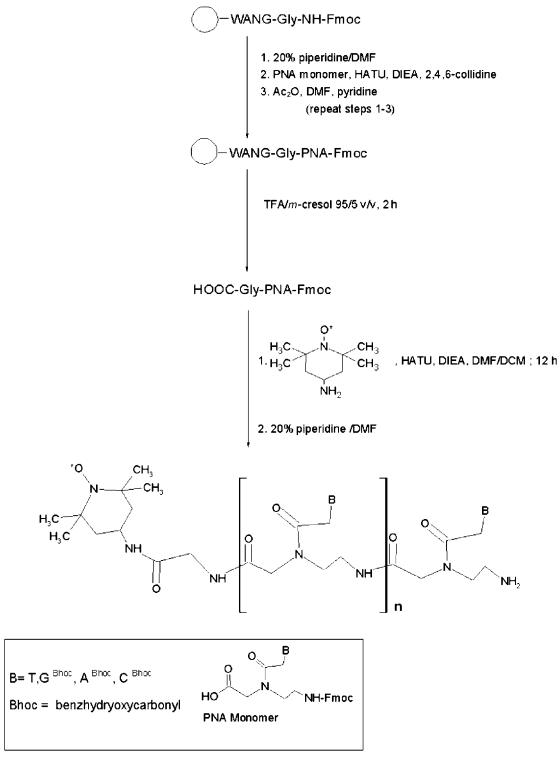
0040-4039/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2003.09.150

The methodology involved initial preparation of PNA bearing a *N*-terminal Fmoc group on solid phase, followed by cleavage and efficient solution phase coupling to a spin label containing a reactive carboxylic group as shown in Scheme 1.

The PNA was prepared by manual, solid phase synthesis using Fmoc-*N*(2-aminoethyl)glycine PNA monomers according to the chemistry and protocols used with Fmoc amino acids.[15] The PNA was assembled on *p*-benzyloxybenzylalcohol polystyrene (Wang) resin (NovaBiochem AG, Luafelfingen, Switzerland) which, after cleavage, produces a free carboxylic group.



Scheme 1.

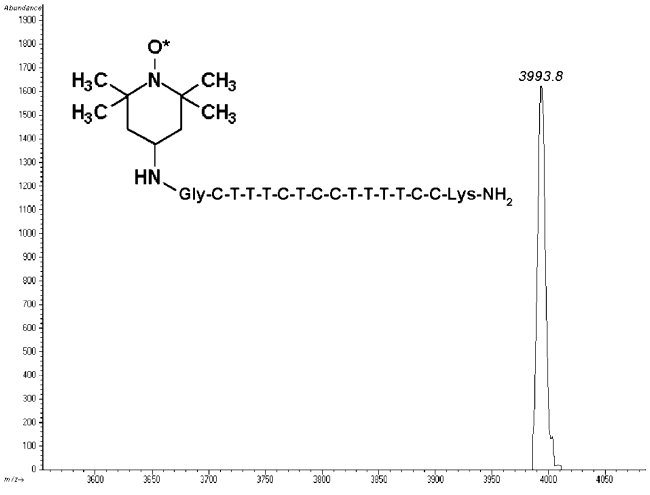


Figure 1. Mass spectrum and molecular structure of the PNA molecule (PNA-T) synthesized.

Deprotection of the Fmoc group was obtained using 20% piperidine in *N*,*N*-dimethyl formamide (DMF) for 20 min (min). Coupling was carried out for 40 min with 5 equiv. of PNA monomers, 4.5 equiv. of activatory *O*-(7-azabenzotriazol-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate (HATU), 5 equiv. of *N*,*N*-diisopropylethylamine (DIEA) and 7.5 equiv. of 2,4,6-collidine in NMP at 40°C. After the assembly of PNA monomers, amino functions that did not react were capped using a mixture of 5% acetic anhydride in DMF.

After synthesis, the last *N*-terminal protecting group was preserved and PNA was released from the resin by treatment with a mixture of TFA/*m*-cresol (95:5) (v/v) for 2 h at room temperature followed by ice-cold diethyl ether precipitation and was purified by reverse phase high pressure chromatography (RP-HPLC) with a Waters C18 μm Bondapack column.[16]

The molecular weight of the PNA was confirmed by electrospray mass spectrometry.

Indeed the spin label was joined by peptidic bond in solution phase to the pre-activated carboxylic group of PNA as follows: 5 equiv. of 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl (4-amino TEMPO) was added to a solution of dichloromethane (DCM)/DMF 1:1 containing 1 equiv. of Fmoc-protected PNA in the presence of 1 equiv. of HATU and 2 equiv. of DIEA (final concentration was 0.1 M in DCM/DMF) and the mixture stirred in the dark for at least 12 h. At that time, the mixture was evaporated to dryness and the Fmoc group removed by piperidine/DMF treatment as already described.

Following this, the spin labeled PNA (PNA-T) was totally precipitated in ice-cold diethyl ether (1:5) and purified by reverse phase HPLC using the same gradient as the previous purification. Fractions containing the PNA-T were collected, vacuum dried and finally lyophilized. The mass spectrum of the purified PNA-T showed a molecular ion at 3993.8 u.m.a. consistent with the neutrally charged molecular ion of the expected PNA molecule (3994.2 u.m.a.) (Fig. 1).The final yield of the total synthesis was over 78%. A control PNA sequence, synthesized using the same chemical methodology, was obtained by scrambling the same bases used for PNA-T (scrambled PNA-T).

The successful labeling was also confirmed by EPR analysis: Figure 2(a) shows the EPR spectrum obtained for PNA-T in water solution at 298 K. The spectrum is constituted by the three hyperfine lines due to the coupling between the unpaired electron spin and the nitrogen nuclear spin ($I=1$). The decreased mobility of the nitroxide group linked to PNA (correlation time for the rotational motion, evaluated from computation of the EPR spectra $=3.6\times10^{-11}$ s) [17] with respect to free unlinked radicals (correlation time for rotational motion, also from computation of the spectral line shape $=1.5\times10^{-11}$ s) is proof of the labeling of PNA at the end of the molecule.

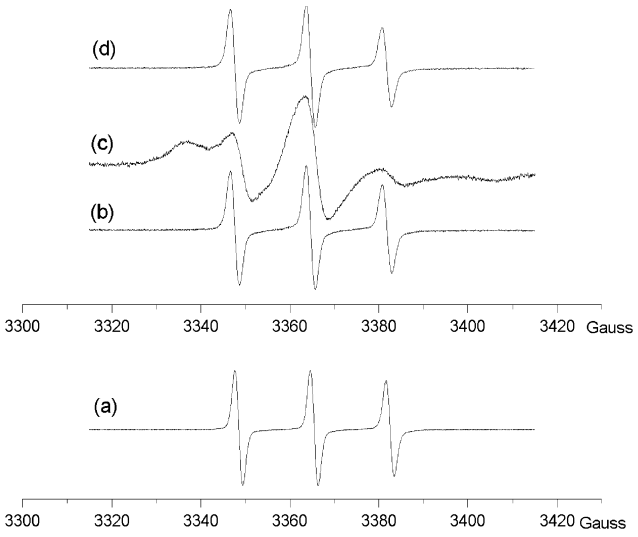


Figure 2. EPR spectra: (a) EPR spectrum of PNA-T in water solution (50 μM) at 298 K; EPR spectra of PNA-T in water solution (50 μM) at 253 K in the absence (b) and in the presence (c) of ODN (PNA-T/ODN = 4); (d) EPR spectrum of scrambled PNA-T (50 μM) at 253 K in the presence of ODN (scrambled PNA-T/ODN = 4).

The ability of PNA-T to report on the binding of PNA to ODN was analyzed by EPR at 253 K. This temperature was selected to better differentiate the rotational mobility of the label in the absence and in the presence of ODN: the samples were directly inserted in the EPR cavity at 253 K to preserve the system from an eventual progressive modification in the structure of the adduct as a function of temperature. Figures 2(b) and (c) show the EPR signals obtained at 253 K for PNA-T in the absence and in the presence of ODN (4:1 molar ratio between PNA-T and ODN). The spectrum in the presence of ODN shows a strong decrease in mobility of the label (correlation time for motion from 1.2×10^{-10} s to 2×10^{-8} s, for the slow motion component), thus monitoring the binding and the formation of a stable adduct. Interestingly, a parallel experiment carried out with scrambled PNA-T did not produce a variation in the spectral line shape on ODN in solution. Figure 2(d) shows the EPR spectrum of scrambled PNA-T in the presence of ODN (4:1 molar ratio between PNA-T and ODN); this spectrum is almost equivalent to the spectrum in Figure 2(b).

The binding of PNA-T with ODN was also analyzed by polyacrylamide gel electrophoresis. For this purpose, complementary ODN (Tib MolBiol, Genoa, Italy) was 5′-end labeled with ^{32}P using T4 polynucleotide kinase (T4 PNK, EC 2.7.1.78) as previously described.[18] The filtrate containing the purified, labeled ODN was then annealed with complementary PNA or PNA-T at a ratio of 1:4. Finally the hybrids were analyzed by non-denaturing polyacrylamide gel electrophoresis (20% AA) in TBE buffer followed by radiography of the gel.

No relevant differences are detectable in the annealing of ^{32}P-labeled ODN and PNA or PNA-T, demonstrating that PNA-T is still able to bind a complementary ODN as shown in Figure 3. The percentage of annealing was of 63% with PNA-T (lane 2) and 48% with PNA (lane 3) respect to the ODN standard (lane 1), meanwhile, with scrambled PNA-T did not anneal to ODN (data not shown).

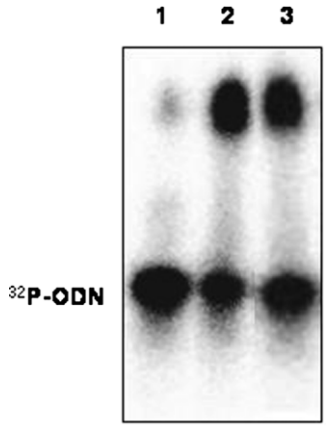


Figure 3. Annealing of ^{32}P-labeled ODN and PNA or PNA-T by non-denaturation polyacrylamide gel electrophoresis. Detection and quantification of ODN-PNA-T or ODN-PNA hybrid formations were performed on a GS-250 Molecular Imager.

In conclusion, we have developed a reasonably simple efficacious method for the synthesis of a spin-labeled PNA using a combined solid-liquid strategy. The labelling of PNA allowed us to follow the fate of this molecule, rendering it visible by means of the EPR technique. Indeed, EPR analysis showed that the labelling does not perturb the PNA ability to bind complementary ODN. Similar conclusions were obtained also by polyacrylamide gel electrophoresis.

Acknowledgements

We are grateful for the help and the technical assistance of Dr. Andrea Armirotti. This work was partially supported by funds from 'Consiglio Nazionale delle Ricerche' Target Project 'Biotechnologies', from 'Consorzio Interuniversitario per le Biotecnologie' Project 2002 and from Strategic Project 'Drug Delivery'. M.F.O. thanks PRIN2002 and MURST for the financial support.

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