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γ-Hydroxymethyl PNAs: Synthesis, interaction with DNA and inhibition of protein/DNA interactions

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ABSTRACT

The ability of PNA to interact with DNA double stranded has been recently investigated. In a decoy approach these interactions are of great importance as may lead to inhibition of interactions of DNA sequences to specific transcription factors and may be employed as a strategy for the inhibition of gene transcription alternative to the antisense strategy (targeting transcription factors mRNAs) and the transcription factor decoy approach (targeting transcription factors). We explored the ability of PNA and PNAs with modified monomers to bind to DNA and to interfere in the formation of DNA/transcription factor complex. We report a procedure for the synthesis of Fmoc–γ-hydroxymetyl PNA, the synthesis and CD analysis of PNA oligomers containing the modified monomer in different positions and EMSA assays to test the: (a) binding to double stranded DNA and (b) inhibition of DNA–protein interactions.

1. Introduction

PNAs (aeg PNA) are oligonucleotide mimics with an uncharged backbone, composed by amino-ethyl glycine units [1]. Due to their chemical and enzymatic stability and to their high affinity towards complementary DNA and RNA, they are widely used for regulating gene expression with basically two mechanisms of action, the first being antisense against target mRNAs, the second being antigene for the targeting of DNA stretches [2]. Other applications of PNAs include the identification of single nucleotide polymorphism, and isolation and purification of nucleic acids [3,4]. The research on PNAs focused on improving those properties which are considered the Achilles heel of PNAs, as solubility and ability to cross cell membranes. Conjugation of PNAs to peptides, oligonucleotides and small molecules strongly improved the PNA cellular uptake [5]. On the other side PNAs with modified backbone have been obtained to improve cellular uptake and selectivity towards DNA and or RNA. Chiral PNAs bearing amino acid side chain in position alpha and gamma of the backbone are widely investigated [6]. Appending guanidine groups in the gamma position yields PNAs with an increased ability to be taken up by cells, as compared to aeg PNAs. The presence of chiral centers contributes to increase the selectivity in the recognition of complementary DNA. Studies on gamma modified PNAs revealed that the introduction of a side chain in this position does not hamper formation of duplexes with DNA and RNA; furthermore, insertion of such modified PNAs into a standard aegPNA oligomer induces structural organization and allows for an improvement in the binding affinity [7,8].

The binding of PNAs to DNA duplexes has been investigated, paying attention to homopyrimidine sequences of single and bis-PNA able to strand invade DNA and work as antigene. Binding of homopyrimidine PNA to duplex DNA results in the formation of triple helices [9]. Strand invasion has also been reported by PNA targeting regions of duplex DNA subject to breathing [10]. In this case the resulting complex is a duplex PNA/DNA instead of a triplex. Examples of invasion by mixed sequence PNA and PNA-peptide conjugates have been described by Lundin and Corey respectively on supercoiled DNA and chromosomal DNA [11,12]. Recently it was found that mixed-sequence 15–20 mer PNAs composed of only gamma-methyl modified monomers can invade double-helical B-DNA [13]. This last activity is of some interest, since binding of PNAs to double stranded DNAs may lead to inhibition of interactions of DNA sequences to specific transcription factors (TFs) and may be employed as a strategy for the inhibition of gene transcription alternative to the antisense strategy (targeting transcription factors mRNAs) and the transcription factor decoy approach (targeting transcription factors). In this context antisense strategy against TFs is expected to be unefficient, due to the fact that usual regulation of gene transcription is dependent from several TFs, belonging to the same superfamily, but recognizing the same target gene sequence; on the other hand PNA-based decoy molecules targeting TFs have been

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reported to exhibit (with the exception of PNA–DNA chimeras) low activity, as compared to ODN decoys.

In this work we explored the ability of gamma-hydroxymethyl PNA to interfere in the binding of the transcription factor Sp1 to its target DNA.

We describe a procedure for the synthesis of PNA monomers with the backbone Fmoc–(O-benzyloxymethyl) amino-ethyl glycine, and oligomers containing modified monomers. In order to investigate the biological activity of the modified oligomers, we tested the ability of oligomers containing γ-hydroxymethyl PNA in: (a) binding to double stranded DNA and (b) inhibiting DNA–protein interactions. In both cases electrophoretic mobility shift assays have been performed.

2. Experimental

2.1. Synthesis of the PNA monomer and oligomers

Reagents for the synthesis of the PNA t^{OH} monomer, Silica gel 60 (0.04–0.063 mm) for flash chromatography were purchased at Fluka. TLC plates (Silica gel 60 F254, 5 × 20, 0.25 cm) are from Merk. Fmoc (Bhoc) PNA monomers (Link Technologies), PAL–PEG (0.19 mmol/g) (Applied Biosystem) were employed for the synthesis of the PNA oligomers. ^{1}He ^{13}C NMR were recorded on a Varian Innova 400 MHz spectrometer at 25 °C. LC–MS were carried out on a Surveyor HPLC equipped with a mass spectrometer Thermo Finnigan (electrospray source MSQ). Phenomenex Jupiter 5 μ C18 300 Å and Phenomenex Jupiter 10 μ Proteo 90 Å columns were employed respectively for analytical and semipreparative runs. Circular dichroism spectra were recorded on a Jasco J-810 spectropolarimeter equipped with a Peltier thermal controller unity using a 1 cm quartz cell at a temperature of 25 °C.

The modified monomer Fmoc–(BzlOCH$_2$)–T–OH was synthesised starting from the Boc (Bzl–OCH$_2$)–T–OMe derivative obtained following procedures reported in the literature [8].

2.1.1. Boc–(Bzl–OCH$_2$)–T–OH (1)

56.0 mg of Boc (Bzl–OCH$_2$)–T–OMe (0.11 mmol) are dissolved in 1.5 mL of dioxane and treated with 0.27 mL of NaOH 2 M solution. The mixture is stirred 20 min, HCl 1 M is added until the pH of the solution is 2. The crude is washed with ethyl acetate. The organic layer is washed with H_2O (2×) and dried with Na_2SO_4. The solvent is evaporated and the resulting oil is dried under reduced pressure, to give 55 mg of Boc–(Bzl–OCH$_2$)–T–OH. (Yield 100%).

Rf: 0.48 (chloroform/methanol/water 14/6/1 v/v/v).

Mass analysis: *m*/*z* (ESI), [M+H]+ expected 504.3; found 504.2.

^{1}H NMR ($CDCl_3$) (2 rotamers): 1.64 (9H, s, t-butyl–CH$_3$–), 2.05 & 2.06 (3H, s, CH$_3$ thymine), 3.51–3.68 (2H, m, –CH–CH$_2$–O–), 3.74 (1H, m, –NHCH–CH$_2$–), 3.85 (2H, s, –CO–CH$_2$–thymine), 4.21–4.50 (2H, m, –CH–CH$_2$–N–), 4.70–4.78 (2H, m, –O–CH$_2$–Ph), 4.95 (2H, dd, –N–CH$_2$–CO), 5.10 (1H, br s, BocNH), 7.41 & 7.44 (1H, s, H(6)–thymine), 7.46–7.56 (5H, m, –Ph–H), 8.10 (1H, s, NH–thymine). ^{13}C NMR (CD_3OD) 12.55 (CH$_3$–thymine), 29.02 (t-butyl–CH$_3$), 48.66 (N–CH$_2$–CO), 49.65 (CH–CH$_2$–N–), 49.79 (NH–CH–CH$_2$–), 49.94 (–CO–CH$_2$–thymine), 68.38 (–CH–CH$_2$–O–), 74.52 (–O–CH$_2$–Ph), 80.82 (t-butyl–C), 111.15 (C(5)–thymine), 128.98, 129.14, 129.66, 129.76, 139.74 (–Ph), 143.90 (C(6)–thymine), 153.20 (C(2)–thymine), 158.10 (t-butyl–O–CO), 167.23 (C(4)–thymine), 170.50 (–CO–CH$_2$–thymine), 172.53 (COOH).

2.1.2. NH$_2$-(Bzl-OCH$_2$)-T-OH (2)

46.6 mg of Boc-(Bzl-OCH$_2$)-T-OH are dissolved in 1 mL of TFA/CH_2Cl_2/TIS 47/50/3 v/v/v and stirred 10 min. TFA is evaporated and the product is precipitated in cold ether. The precipitate is suspended in H_2O, purified by preparative HPLC and lyophilized. (Yield 100%.)

Rf: 0.48 (chloroform/methanol/water 14/6/1 v/v/v).

Mass analysis: *m*/*z* (ESI), [M+H]+ expected 404.3; found 404.2.

^{1}H NMR (DMSO) (2 rotamers): 1.85 & 1.86 (3H, s, CH$_3$ thymine), 4.10 (2H, s, –CO–CH$_2$–thymine), 4.34 (2H, s, –N–CH$_2$–COOH), 4.63 (2H, s, –OCH$_2$–Ph), 7.37 & 7.41 (1H, s, H(6)-timina), 7.47 (5H, m, -Ph-H), 11.41 & 11.45 (1H, s, COOH). Peaks corresponding to –NH–CH–CH$_2$–, –CH–CH$_2$–N–e–CH–CH$_2$–O– are covered by the signal of water contained in DMSO in the range 3.2–3.6. ^{13}C NMR (DMSO) 15.93 (CH$_3$–thymine), 50.41 (N–CH$_2$–COOH), 51.78 (CH–CH$_2$–N–), 52.6 (NH–CH–CH$_2$–), 52.91 (–CO–CH$_2$–thymine), 71.61 (–CH–CH$_2$–O–), 76.50 (–O–CH$_2$–Ph), 112.18 (C(5)–thymine), 131.70–132.33 (–Ph), 141.52 (C(6)–thymine), 155.00 (C(2)–thymine), 168.31 (C(4)–thymine), 172.97 (–CO–CH$_2$–thymine), 174.66 (COOH).

2.1.3. Fmoc–NH (Bzl–OCH$_2$)–T–OH (3)

46.4 mg of NH$_2$–(Bzl–OCH$_2$)–T–OH (0.11 mmol) are dissolved in THF, 0.072 mL of a K_2CO_{3aq} 20% solution are added. The mixture is stirred at 0 °C. 36.3 mg of Fmoc-OSu (0.11 mmol) are added and the reaction is stirred at room temperature 1 h. The crude is extracted with ethyl acetate. The organic layer is washed with water (2×) and dried over anhydrous Na_2SO_4. The solvent is evaporated and the product is purified by silica gel chromatography. (Yield 84%.)

Rf: 0.58 (ethyl acetate/methanol/formic acid 95/3/2 v/v/v).

Mass analysis: *m*/*z* (ESI), [M+H]+ expected 627.1; found 627.2.

^{1}H NMR ($CDCl_3$) (2 rotamers): 1.76 (3H, s, CH$_3$ thymine), 3.46–3.70 (2H, m, –CH–CH$_2$–O–), 3.99–4.05 (1H, m, –NH–CH–CH$_2$–), 4.17–4.11 (2H, m, –CO–CH$_2$–thymine), 4.25–4.27 (2H, m, –CH–CH$_2$–N–), 4.36 (2H br s, –O–CH$_2$–Ph), 4.44 (1H, br s, CH–Fmoc), 4.51 (2H, s, –N–CH$_2$–COOH), 4.60 (2H, br s, CH$_2$–Fmoc), 6.80 & 6.98 (1H, s, H(6)–thymine), 7.19–7.34 (5H, m, OCH$_2$Ph),7.46–7.92 (8H, m, fluorenyl), 10.19 (1H, br s, COOH). ^{13}C NMR ($CDCl_3$) 11.98 (CH$_3$–thymine), 47.04 (CH–Fmoc), 48.71 (–CH–CH$_2$–N–), 48.91 (CH–CH$_2$–N–), 49.60 ((N–CH$_2$–COOH), 49.89 (–CO–CH$_2$–thymine), 67.04 (CH$_2$–Fmoc), 69.99 (–CH–CH$_2$–O–), 73.26 (–O–CH$_2$–Ph), 111.04 (C(5)–thymine), 125.01, 127.09, 127.76, 127.97, 128.08, 128.41, 128.58, 137.62 (Ph), 137.30 (C(6)–thymine), 141.22, 143.67 (Cq fluorenyl), 151.65 (C(2)–thymine), 156.48 (CO–Fmoc), 165.02 (C(4)–thymine), 168.68 (–CO–CH$_2$–thymine), 173.06 (COOH).

2.2. Synthesis of the oligomers

The PNAs were synthesised by Fmoc chemistry on a PAL–PEG resin (2 μmol scale) on a Expedite 8909 synthesiser. The modified monomer Fmoc–(BzlOCH$_2$)–T–OH was coupled following procedures reported in the literature [8]. Cleavage and deprotection were performed by treatment of the resin with a TFA/TFMSA/m-cresol/thioanisole mixture (6:2:1:1); the oligomers were precipitated with ethyl ether. The oligomers were purified by reverse-phase HPLC using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA), from 5% to 40% in 30 min. Purified molecules were characterized by electrospray mass analysis.

Sequences obtained:

PNA: tgaggcgtggcct
PNA-C: tgaggcgtggcct^{OH}
PNA-M: tgaggcgt^{OH}ggcct
PNA-C+M: tgaggcgt ^{OH}ggcct^{OH}
PNA-N: ^{OH}tgaggcgtggcct

Mass analysis:

PNA: tgaggcgtggcct expected: 3590.4; 1197.79 $[M+3H]^{3+}$; 898.59 $[M+4H]^{4+}$; found: 1198.10 $[M+3H]^{3+}$; 898.35 $[M+4H]^{4+}$
PNA-C: tgaggcgtggcct^{OH}, **PNA-M**: tgaggcgt^{OH}ggcct and **PNA-N**: ^{OH}tgaggcgtggcct: expected: 3622.08; 1208.36 $[M+3H]^{3+}$; 906.52 $[M+4H]^{4+}$: found: 1208.95 $[M+3H]^{3+}$; 906.20 $[M+4H]^{4+}$
PNA-C+M: tgaggcgt ^{OH}ggcct^{OH}: expected 3652.08; 1218.36 $[M+3H]^{3+}$; 914.02 $[M+4H]^{4+}$: found: 1217.75 $[M+3H]^{3+}$; 914.7 $[M+4H]^{4+}$

2.3. Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) [14,15] was performed by using double stranded ^{32}P-labeled oligonucleotides as target DNA. Binding reactions were set up as described elsewhere in binding buffer (10% glycerol, 0.05% NP-40, 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 0.5 mM DTT, 10 mM $MgCl_2$), in the presence of poly(dI:dC)·poly(dI:dC) (1 μg/reaction) (Pharmacia, Uppsala, Sweden), 5.75 μg/reaction of crude nuclear extracts from K562 cells or 45 ng of purified nuclear factor human Sp1 (Promega, Milano) and labeled oligonucleotide, in a total volume of 20 μl. For analysis of molecular interactions between PNA and double stranded DNA (dsDNA), binding reactions were carried on for 40 min at room temperature. In both cases, the samples were electrophoresed at constant voltage (200 V for 1 h) through a low ionic strength (0.25× TBE buffer) (1× TBE = 0.089 M Tris–borate, 0.002 M EDTA) on 6% polyacrylamide gels until tracking dye (bromophenol blue) reached the end of a 16 cm slab. Gels were dried and exposed for autoradiography with intensifying screens at −80 °C. In studies on the inhibition of protein/DNA interactions, addition of the reagents was as follows: (a) poly(dI:dC).poly(dI:dC); (b) labeled oligonucleotides (c) competitor molecules (50 ng, 100 ng, 400 ng/reaction); (d) binding buffer; (e) nuclear factors or purified Sp1, for Fig. 4A and C, while for Fig. 4B and D nuclear extracts were added before additions of PNAs. The nucleotide sequences of double stranded target DNA utilized in these experiments were: 5′-TGA GGC GTG GCC T-3′ (sense strand) and the complementary 5′-AGG CCA CGC CTC A-3′.

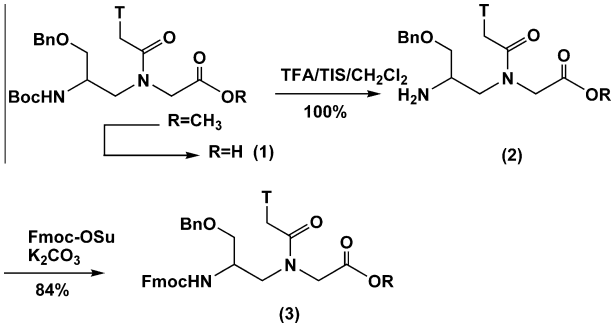
2.4. CD analysis

CD spectra were recorded using concentration of PNA single strand and PNA/DNA duplexes 2 μM in 10 mM Phosphate buffer pH 7. The spectra are an average of 5 consecutive scans from 320 to 220 nm, recorded with a bandwidth of 0.5 nm, a time constant of 4 s and a scan rate of 20 nm/min. Concentration of the PNAs and DNA single strands was calculated measuring the absorbance at 260 nm and using a molar extinction coefficient of 129.5 mL μmol^{-1} cm^{-1} for PNA C, PNA M, PNA C+M, PNA N and 124.4 mL μmol^{-1} cm^{-1} for the complementary DNA strand (AGGCCACGCCTCA).

3. Results and discussion

3.1. Synthesis of the Fmoc protected monomer and the oligomer

We developed a procedure for the synthesis of the Fmoc–(Bzl–OCH_2)–T–OH monomer, to use on an automated PNA synthesiser (Scheme 1). Yields in PNA synthesis increase when reactions are performed under argon, as on a PNA synthesiser. The protocol requires first the synthesis of the Boc–(Bzl–OCH_2)–T–OH analog and then the successive conversion of protecting groups. The Boc–(Bzl–OCH_2)–T–OCH_3 monomer was obtained following procedures reported in the literature [8]. The methyl ester was removed by treatment with a sodium hydroxide solution, followed by a reaction with trifluoroacetic acid, to free the amino terminus. The Fmoc group was installed and the product purified (Scheme 1). Oligomers were assembled on a low loading PAL–PEG resin using standard Fmoc–(Bhoc)–PNA monomers and the modified Fmoc–(Bzl–OCH_2)–T–OH. Fmoc (Bhoc) monomers were coupled following standard procedures, while the modified monomer was coupled using the mixture N-methyldicyclohexylamine/pyridine as a base and HBTU as coupling reagent, as described in the literature [8]. Treatment of resin bound oligomer with TFA/TFMSA/thioanisole/m-cresol afforded the crude product, which was purified by HPLC and analyzed by mass spectrometry. The sequence of PNA oligomers is the DNA sequence recognized by Sp1, a transcription factor involved in the regulation of the expression of several genes, as those encoding for the vascular endothelial growth factor, plasminogen activator inhibitor type 1, urokinase plasminogen activator and its receptor [16].



Scheme 1. Synthesis of the Fmoc (Bzl–OCH_2)–T–OH PNA monomer.

Sequences obtained are reported in Table 1. The chemical structure of the modified monomer as it is in the deprotected oligomers is illustrated in Fig. 1 and is indicated as t^{OH}.

3.2. CD analysis

Single strand PNAs and their hybrids with the complementary DNA were analyzed by Circular Dichorism, in Phosphate buffer pH 7 (Fig. 2). PNA bearing hydroxymethyl in position gamma are reported to display optical activity, due to the presence of chiral centers [8]. The PNA obtained by us, bearing one or two chiral centers exhibit very low optical activity; the PNA single strand with the modification at the C-terminus (PNA C), in the middle (PNA M) and at the C-terminus and in the middle (PNA C+M) have superimposable CD spectra, while the PNA modified at the N-terminus hardly shows optical activity. The low optical activity may be due

Table 1
PNA sequences.

Name	Sequence
PNA	tgaggcgtggcct
PNA C	HO tgaggcgtggcct
PNA M	HO tgaggcgtggcct
PNA C+M	HO HO tgaggcgtggcct
PNA N	HO tgaggcgtggcct

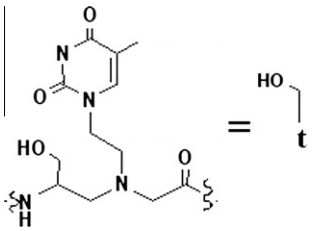


Fig. 1. Chemical structure of the γ-hydroxymethyl PNA monomer employed in these studies.

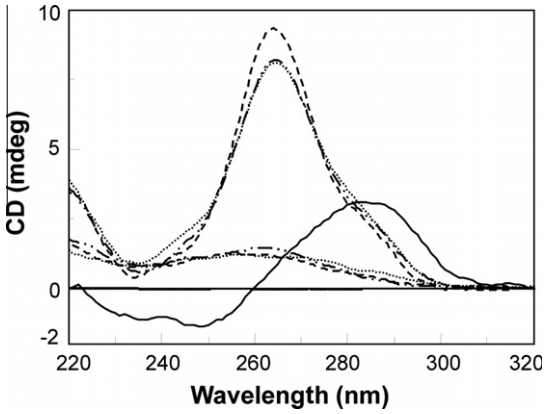


Fig. 2. CD spectra of: duplexes PNA N/DNA (—); PNA M/DNA (· · ·); PNA C/DNA (- - -); PNA C+M/DNA (- · · -) and single strands PNA M (· · ·); PNA C (- - -); PNA C+M (- · · -) in Phosphate buffer 10 mM pH 7.

to the scarce incidence of one or two chiral monomer on a sequence of 13 bases, a phenomenon particularly manifest for PNA N. With the exception of the duplex formed by PNA N, all duplexes obtained after annealing to complementary PNAs are very similar in secondary structure. The PNA N/DNA duplex shows one positive band around 280 and one negative band, of comparable intensity around 245; this spectrum resembles to the spectrum of an antiparallel PNA/DNA duplex. Hybrids formed by PNA C, PNA M and PNA C+M, instead show a strong positive band around 265 and a minimum around 240; the structure of these duplexes looks very similar to that of PNA/RNA duplexes, suggesting that the presence of the hydroxymethyl chain influences the structure of the duplex [17]. Overall CD data indicate that the position in which the chiral monomer is inserted and the length of the oligomer dictates the secondary structure of the single strand and of the hybrids with DNA.

3.3. Binding studies: PNA analogs bind to target DNA and inhibit DNA–protein interactions

In a first set of experiments the interactions of the modified PNA and double stranded Sp1 target DNA were analyzed and compared to that of standard control PNA (Fig. 3). As evident from the Fig. 3, a sharp decrease of free ds-DNA (panel A) and the appearance of low-migrating dsDNA/PNA complexes (panel B) are clearly evident with addition of 50 and 100 ng/reaction of the PNA molecules. In these experimental conditions PNA molecules, when used at 50 ng/reaction, are about 10-fold in respect to target ^{32}P-labeled double stranded Sp1 DNA. In using DNA-based oligonucleotides, specific competition is usually obtained in the range of 15–50 ng/reaction using experimental conditions similar to those followed in the present paper. Among the four PNAs analyzed, PNA-C and the standard reference PNA appear to be slightly more efficient. This is evident with two complementary observations: (a) the absence of free probe only in the presence of PNA-C and the standard reference PNA and (b) the high amount of blocked band evident when 50 ng of PNA are added. The retarded bands may be attributed to a complex formed after binding of the PNA on the DNA duplex, likely after strand invasion. This phenomenon was reported for homopurine PNAs [18] and in our case it may be attributed to the use of a PNA oligomer with a high content of purines. The effect observed may be further driven by the use of large excesses of PNA. The ability to sequester the DNA is lowered when the modified monomer is inserted at in the center or when two such monomers are present. PNA-N demonstrated an activity similar to standard PNA.

In the second set of experiments, EMSA was performed using two alternative approaches. In the first one (panels A and C of Fig. 4), standard PNAs and PNA analogs were pre-incubated with ^{32}P-labeled double stranded Sp1 DNA and then allowed to interact with purified Sp1 nuclear factor (Fig. 4A) or nuclear extracts purified from K562 cells (Fig. 4C). In the second approach (panels B and D of Fig. 4), purified Sp1 nuclear factor (Fig. 4B) or nuclear extracts (Fig. 4D) were allowed to interact with target ^{32}P-labeled double stranded Sp1 DNA and then PNA molecules added.

Fig. 4A and C shows the data obtained performing the first approach, demonstrating that the synthesised PNA analogs inhibit Sp1/DNA interactions when pre-incubated with the target double stranded DNA. The efficiency of inhibition of the Sp1/DNA interaction exhibited by the PNA analogs approached that of the standard PNA. Again, PNA-C and the standard reference PNA appear to be slightly more efficient.

Fig. 4B and D, on the other hand, suggests that PNA-mediated inhibition does not take place on pre-formed Sp1/DNA complexes; even when (in the case of the use of nuclear extracts) PNA analogs are added at 400 ng/reaction the inhibition does not take place.

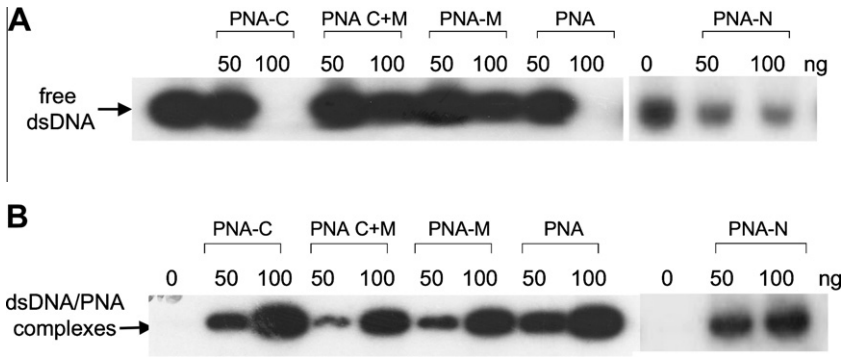


Fig. 3. Efficiency of addition of PNA, PNA-C, PNA C+M, PNA M on the conversion of free-dsDNA (A) to dsDNA/PNA complexes. Free ds-DNA (panel A) and PNA complexed dsDNA (panel B) were identified by polyacrylamide gel electrophoresis. Free ds-DNA migrated as a fast band, while dsDNA/PNA complexes exhibited a very low migration efficiency.

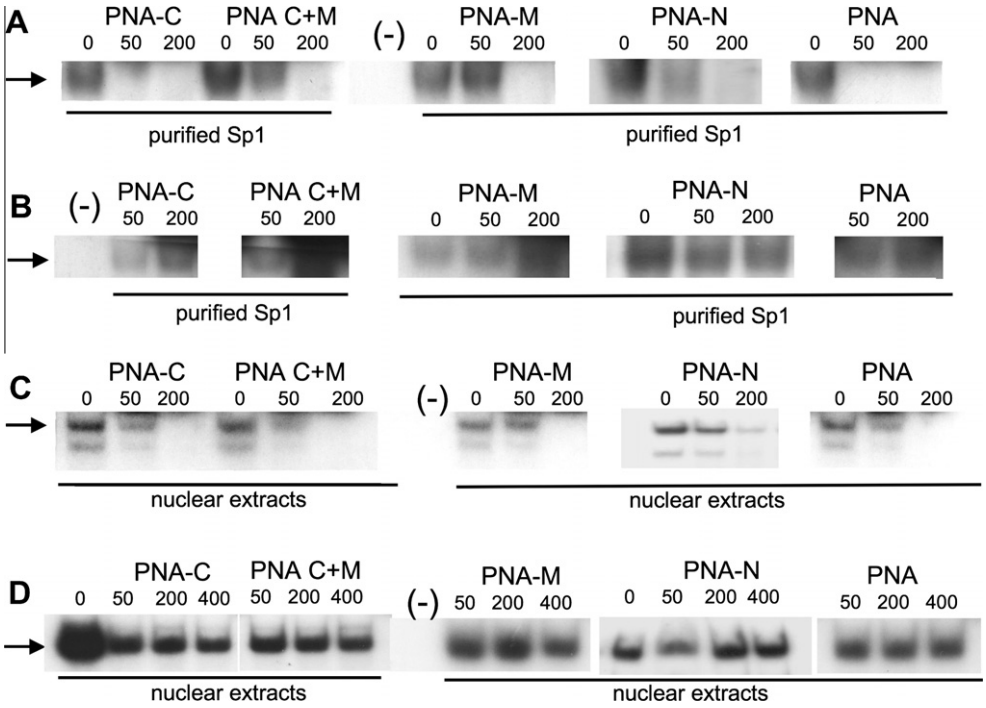


Fig. 4. Effects of PNA molecules on Sp1/DNA interactions. The effects of the indicated concentrations of standard PNA PNA-C, PNA C+M, PNA-M, and PNA-N were determined on the *de novo* formation of Sp1/DNA complexes (A and C) and on pre-formed Sp1/DNA complexes (B and D). In the experiments described in panels A and C PNA molecules were pre-incubated with ^{32}P-labeled double stranded Sp1 DNA and the purified Sp1 nuclear factor (A) or K562 nuclear extracts (B) were added. In the experiment described in panels B and D, purified Sp1 factor (B) or nuclear factors (D) were pre-incubated with ^{32}P-labeled double stranded Sp1 DNA and then PNA molecules added. (–) = no addition of purified Sp1 or nuclear extracts.

The complex formed by the transcription factor Sp1 and its target DNA is thus very strong and, probably, the DNA is not easily accessed by external molecules, therefore the PNA oligomer cannot reach its target and move the equilibrium

$$\text{DNA} + \text{protein} \rightleftarrows \text{complex DNA/protein}$$

backwords, sequestering the DNA.

Overall, these data suggest that: (1) mixed sequences aegPNAs form complexes with DNA double strand, but the introduction of a modification in the middle of a sequence or at its C-terminus prevents complex formation. (2) C-terminal modified PNAs keep the ability to sequester DNA duplexes; (3) the complex PNA/DNA does not interact with the transcription factor; (4) the capability of PNA oligomers to sequester the Sp1 DNA duplex is strongly reduced when the DNA is in complex with the transcription factor, probably due either to the fact that the DNA duplex cannot be approached by the PNA oligomer for steric reasons and to the lack of interactions of the modified PNA with the transcription factor.

4. Conclusions

In conclusion this work sustains the concept that mixed sequence PNAs can form complexes with duplex DNA. This feature might be used in the future for approaches aimed at interfering with transcription of targeted genes. This strategy for interfering in the DNA transcription mediated by transcription factors was proposed by several groups, based on the use of molecules able to inhibit the recognition of specific DNA sequences by transcription factors. In this context, the ability of PNA oligomers to bind to DNA duplexes is a required pre-requisite for proposing these molecules for further studies in this field. The introduction of one or more modified PNA monomers in the oligomer influences the ability to complex DNA. Among the novel molecules designed by us and here reported, the PNA analogs carrying the modified monomer at the C-terminus appears to be the most promising (see Fig. 3). On the other hand, our results suggest that the addition of these PNA analogs has inhibitory effects on the generation of Sp1/DNA complexes. In addition to the generation of PNA/DNA/DNA complexes unable to interact with Sp1, our data are also compatible with the generation of PNA/DNA duplexes which, as published by Borgatti et al. [19] are not able to interact efficiency with Sp1. In any case, these (as well as the employed standard PNA) are unable to disassemble pre-formed Sp1/DNA complexes. In our opinion, this is very important, since we can hypothesize these molecules as reagents able to interact and affect only "open" chromatin structure interacting with transcription factors. Whatever being the mechanism of action, our data sustain the concept that this approach might interfere to the recruitment of transcription factors to the promoter site, thereby inhibiting transcription, allowing to sustain strategies alternative to the use of transcription factor decoy oligonucleotides to control gene expression.

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