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Review Article

Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications

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1. Introduction

The antisense and antigene strategy for treatment of diseases at the level of gene expression has attracted wide attention in medicinal chemistry. As indicated in Figure 1, interference with gene expression can be accomplished by the binding of an oligonucleotide (or oligonucleotide analogue) to DNA or RNA. The binding is governed by standard base pairing rules, and therefore the design of antisense agents should be

Key words: Peptide nucleic acids, PNA, antisense, DNA analogue.

straightforward. Several excellent reviews[1-6] describe the general principles and results obtained in this field, and the reader is referred to these for more details.

Natural oligonucleotides have been shown to exhibit both antisense and antigene properties in vitro.[1,2,6] However, both DNA and RNA are rapidly degraded by nucleases in vivo and in attempts to overcome this serious obstacle an impressive number of oligonucleotide analogues have been synthesized during the last two decades (Fig. 2). Chemical modification generally improves the biological stability of oligonucleotides. However, it is of equal importance to retain the DNA and RNA binding affinity and specificity and to obtain adequate pharmacokinetic behaviour of the analogues. Most of the analogues being investigated so far are closely related to natural oligonucleotides and only few attempts to radically modify the backbone of DNA have been successful.

Peptide nucleic acid (PNA) is a DNA mimic in which the deoxyribose phosphate backbone has been replaced by a pseudo peptide backbone and only the four natural nucleobases are retained. This review deals with the synthesis, hybridization and biological properties as well as the potential applications of this DNA mimic. In addition, structural features of PNA–oligonucleotide complexes will be discussed.

2. Peptide Nucleic Acids (PNA)

Peptide nucleic acids were first described by Nielsen et al. in 1991.[12] It was shown that replacement of the natural deoxyribose phosphate backbone of DNA by repeating *N*-(2-aminoethyl)glycine units with the nucleobases attached through methylenecarbonyl linkers (Fig. 3) afforded a DNA mimic with remarkable properties.

PNA was designed by computer assisted model building using as scaffold a normal TAT triplex.[13] The deoxyribose phosphate backbone of the Hoogsteen strand was removed and a pseudo peptide backbone was built in its place. The use of peptide chemistry provides several benefits. Firstly, a neutral DNA mimic can be obtained (the charges at the C- and N-terminals are easily modified to give neutral groups) which is

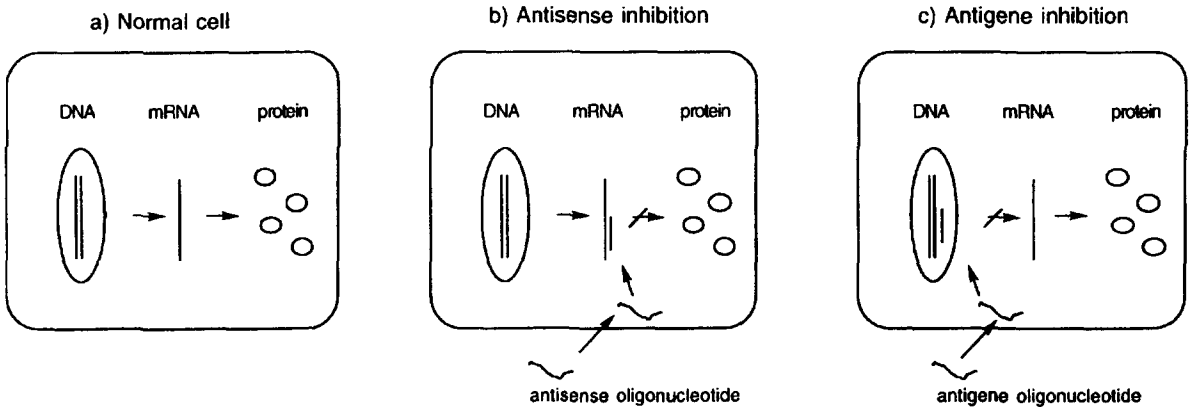


Figure 1. Schematic illustration of (a) Normal gene expression. DNA is transcribed into mRNA followed by translation to give multiple copies of the protein gene product; (b) Antisense inhibition. An antisense oligonucleotide binds specifically to mRNA via Watson–Crick hydrogen bonding whereby it inhibits translation of mRNA into protein; (c) Antigene inhibition. Transcription is inhibited by the binding of an antigene oligonucleotide to DNA. The binding may be mediated through triplex formation or strand displacement. Several different mechanisms have been suggested to operate in (b) and (c) to give the desired suppression of gene expression. See references in text.

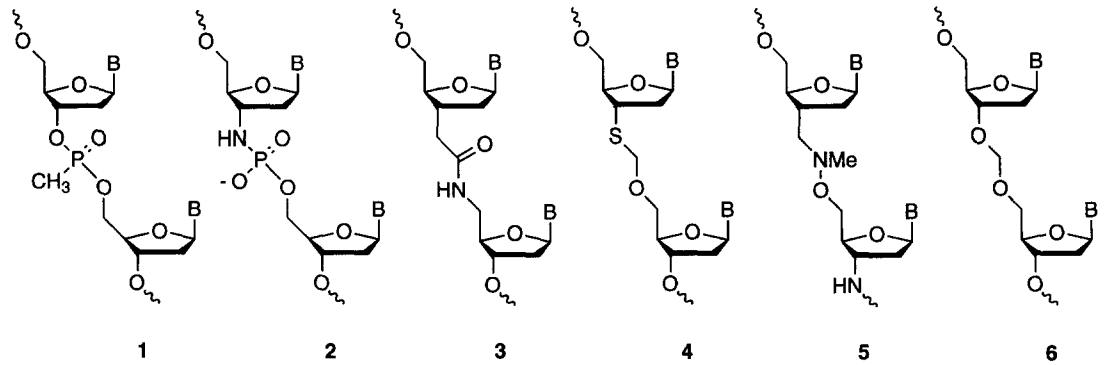


Figure 2. Examples of modified oligonucleotides. References: **1**,[7] **2**,[8] **3**,[9] **4**,[10] **5**,[11] **6**.[10]

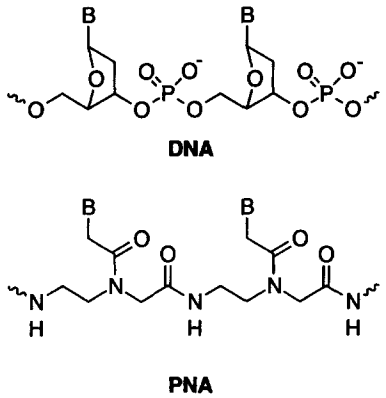


Figure 3. The structure of PNA compared to DNA (B = nucleobase).

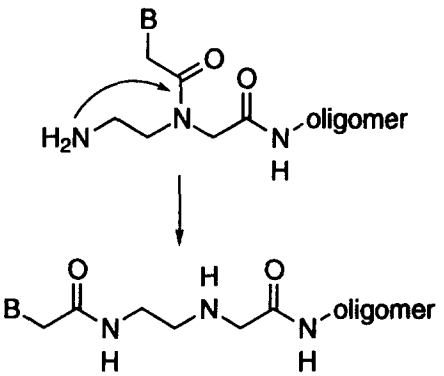


Figure 4. The *N*-acyl transfer reaction which may occur under neutral and alkaline conditions.

advantageous, since electrostatic repulsion between this mimic and negatively charged DNA and RNA is avoided. Secondly, oligomers composed of modified amino acid building blocks (PNA monomers) can be assembled by well established solid phase peptide synthesis protocols supplying oligomers in milligram to gram quantities. Reporter groups, intercalators, metal binders, etc. can be attached through either the N-terminal amino group or the C-terminal carboxylic acid. Another important feature of PNA is the high biostability. PNA oligomers are neither degraded by nucleases nor proteases.[14] Finally, PNA is achiral, thereby avoiding problems of enantiomeric purity.

2.1 Synthesis of PNA oligomers

As mentioned above, the preparation of PNA oligomers is based on standard solid phase peptide synthesis protocols. Two different protection schemes have been employed, both providing oligomers in high yields and purity. In the strategy first reported, the *tert*-butyloxycarbonyl (Boc) group was chosen as the protective group for the primary amino group of the backbone and the benzyloxycarbonyl (Cbz) group was used to protect exocyclic amino groups of the nucleobases.[15] Acid labile protective groups seemed to be most appropriate since alkaline conditions were found to induce a rearrangement of the N-terminal nucleobase to the primary amino group of the backbone (Fig. 4).[16] The acyl migration is, however, a slow process, and Thomson et al. did not observe any migration during piperidine treatment of growing PNA chains.[17] Piperidine was employed to remove the 9-fluorenylmethoxycarbonyl (Fmoc) protective group used as an alternative to the Boc group.

The initial step in the synthesis of PNA monomers involves alkylation of the (protected) nucleobases with bromoacetic acid (Scheme 1). The thymine monomer was the first to be reported[18] since this nucleobase requires no protective groups and can be easily alkylated with methyl bromoacetate. Following hydrolysis of the resultant methyl ester the desired thymin-1-ylacetic acid is obtained.

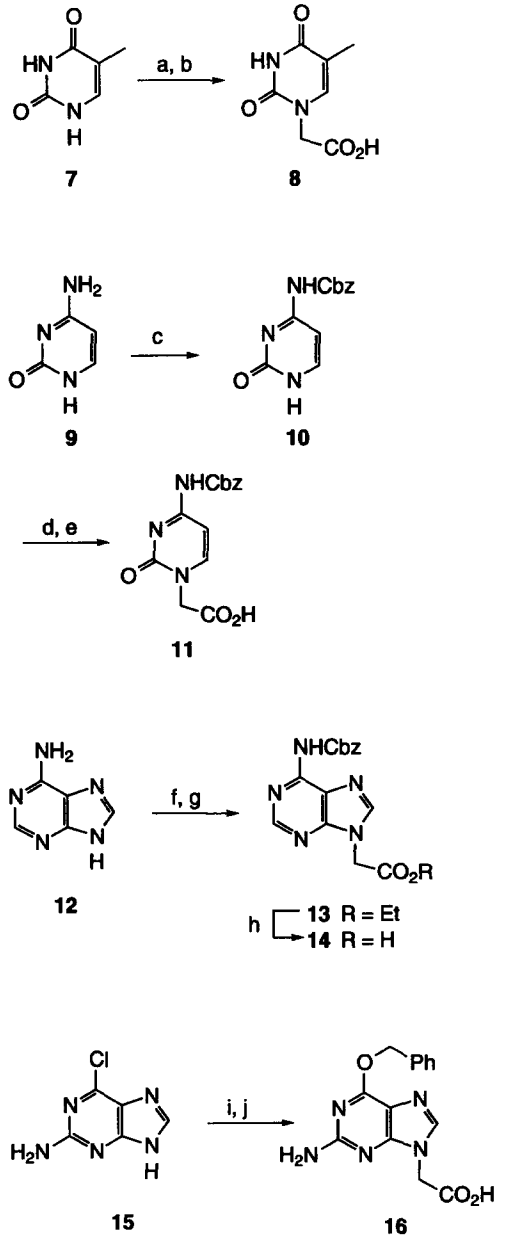
The exocyclic amino group of cytosine requires protection in order to prevent chain extension from this position or acetylation during the capping procedure employed in the oligomerization.[15,17,19] Introduction of the Cbz group prior to alkylation was found to be most appropriate (Scheme 1), in part because this lipophilic group improves the solubility of cytosine in organic solvents considerably.

Adenine was alkylated by ethyl bromoacetate in DMF using either potassium carbonate[20] or sodium hydride[15] as base (Scheme 1). In both cases only alkylation of the 9-position was observed as verified by X-ray crystallography.[21] The Cbz group was subsequently introduced on the 6-amino group using *N*-(benzyloxycarbonyl)-*N'*-ethylimidazoliumtetrafluoroborate,"Rapoport's reagent", as the acylating agent. This provided a much cleaner reaction than observed with benzyloxycarbonyl chloride.[15] The reverse order involving Cbz-protection of adenine prior to alkylation with *tert*-butyl bromoacetate has also been reported.[17] However, the yields were somewhat lower for these steps, and both the N-7 and N-9 alkylated products were obtained. These could be separated by recrystallization and the *tert*-butyl ester was removed with trifluoroacetic acid.

Since guanine itself cannot be alkylated to give exclusively the 9-isomer, 2-amino-6-chloropurine has been used as the starting material for preparation of the G-monomer.[15,20] The reaction between 2-amino-6-chloropurine and bromoacetic acid also produces a mixture of the 9- and 7-alkylated compounds. However, the desired 9-isomer is the major product, and it can easily be separated from the 7-isomer (Scheme 1).[15] Due to the poor solubility of the resulting alkylated purine, the chloro group was exchanged for a benzyloxy group. This group is removed during treatment with TFA in the oligomerization procedure. While attempts to protect the 2-amino group of **16** with the Cbz group were unsuccessful, treatment with acetic anhydride under the conditions used for capping in the oligomerization caused partial acetylation. To prevent this unwanted acetylation, capping is omitted after introduction of the first guanine residue in oligomer synthesis using this

G-monomer. N^2-Cbz protected guanin-9-yl acetic acid has been prepared for application in the Fmoc based synthesis of PNA. 2-Amino-6-chloropurine was alkylated with allyl bromide and following separation of the two regioisomers, the chloro group was hydrolyzed to give the guanine derivative. Subsequently, the N-2 amino group was Cbz protected and ozonolysis of the allyl moiety provided the desired free acid.[17] Recently, the use of the Boc- and N^2-Cbz protected G-monomer has also been reported.[16]

Initially, thymin-1-ylacetic acid (**8**) was activated as the pentafluorophenyl ester and coupled to the Boc-protected backbone *N*-(2-Boc-aminoethyl)glycine.[18]

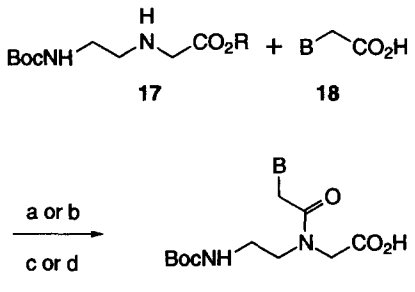


Scheme 1. Alkylation of the nucleobases as reported by Dueholm et al.[18] (a) $BrCH_2CO_2CH_3/K_2CO_3$; (b) Aqueous NaOH; (c) $PhCH_2OCOCl$, pyridine; (d) $BrCH_2CO_2CH_3/K_2CO_3$; (e) Aqueous NaOH; (f) $BrCH_2CO_2Et$, NaH; (g) $PhCH_2OCOIm^+EtBF_4^-$; (h) Aqueous NaOH; (i) $BrCH_2COOH/K_2CO_3$; (j) $PhCH_2ONa$.

Esterification of the backbone, however, improves work up and purification, and usually the methyl or ethyl ester backbone is employed. This concomitantly reduces the nucleophilicity of the secondary amino group of the backbone and therefore more potent activation of the nucleobase-acetic acid is required. In situ activation using 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DhbtOH) and *N,N'*-dicyclohexylcarbodiimide (DCC) in combination with the ethyl ester backbone was found to be advantageous for introduction of thymin-1-yl-, (N^4-Cbz-cytosin-1-yl)-, and (N^6-Cbz-adenin-9-yl)acetic acid (Scheme 2).[15] The monomer ethyl esters were hydrolyzed with LiOH in H_2O/THF to afford the T-, N^4-Cbz C-, and N^6-Cbz A-monomers. (2-Amino-6-(benzyloxy)purin-9-yl)acetic acid (**16**) was attached to the backbone via condensation using bromotris(pyrrolidino)phosphonium hexafluorophosphate (PyBrop) (Scheme 2). This afforded the O^6-Bn G-monomer ester which was hydrolyzed to the free acid in aqueous sodium hydroxide.[15] The Boc-protected backbone esters were prepared by a reductive amination of Boc-aminoacetaldehyde with glycine methyl or ethyl ester.[22]

A two-step synthesis provided the Fmoc-protected backbone as the *tert*-butyl ester.[17] Excess ethylenediamine was alkylated by *tert*-butyl bromoacetate and the Fmoc group was introduced selectively at the primary amine. The purine nucleobase acetic acids were coupled to the backbone using BOP (*O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate) and HOBT (1-hydroxybenzotriazole) as the coupling reagents, whereas EDC (1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide) was employed for the pyrimidine bases. All monomers were converted to the *p*-nitrophenyl esters and used as such in the oligomerization process.

Different protocols for the assembly of Boc-protected PNA monomers to oligomers have been reported.[15,16,18–20] All are based on the standard procedures for solid phase peptide synthesis using a (4-methylbenzhydryl)amine polystyrene resin as the



Scheme 2. Coupling of the nucleobases to the backbone. (a) DCC, DhbtOH (B = T, N^4-Cbz-C, or N^6-Cbz-A; R = Et); (b) PyBrop, DIEA (B = O^6-Bn-G; R = Me); (c) LiOH, THF, H_2O (B = T, N^4-Cbz-C, or N^6-Cbz-A; R = Et); (d) NaOH, EtOH, H_2O (B = O^6-Bn-G; R = Me).

solid support. Homothymine oligomers were synthesized from activated pentafluorophenyl ester monomers in good yields.[18] This activation, however, was inefficient for the incorporation of cytosine residues[15] whereas in situ DCC coupling resulted in close to quantitative coupling yields for both T- and C-monomers.[19] The adenine and guanine residues could be incorporated as well utilizing this coupling scheme[20] but *N,N'*-diisopropylcarbodiimide (DIPCDI) was found to be a more potent coupling reagent for these monomers.[15] In a more recent study, the activation by several uronium salts and a single phosphonium salt has been compared.[16] All coupling reagents performed well with only minor differences in coupling yields but *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) consistently seemed to give the best yields. As previously mentioned, the rearrangement outlined in Figure 4 may take place under neutral conditions,[16] and it is therefore important that the coupling reaction proceeds rapidly and that neutralization of the resin is performed in situ in the presence of the activated monomer. The exact coupling conditions, i.e., concentration of reactants, choice of base for in situ neutralization, coupling reagent, solvent, capping reagent and additives have been optimized to ensure >95% average coupling yields. This allows preparation of oligomers up to at least 20 residues in length incorporating all four natural nucleobases.[16]

Similarly, the Fmoc protected monomers activated as *p*-nitrophenyl esters were oligomerized in high yields (95–99%) as determined by UV monitoring of the dibenzofulvene–piperidine adduct formed upon removal of the Fmoc group.[17] The last Fmoc group was retained on the oligomers to facilitate HPLC purification and was subsequently removed prior to a second round of purification.

The PNA oligomers are cleaved from the solid support either by treatment with anhydrous HF or by the 'high–low' trifluoromethanesulphonic acid procedure[15,17] as in traditional peptide chemistry. The crude products are usually more than 80% pure as analyzed by reversed phase HPLC and the identity of the oligomers can be verified by FAB, MALDI-TOF, or electrospray mass spectrometry. PNA oligomers are readily soluble in water (>1.5 mM for homopyrimidine decamers terminating in a lysine amide[18]), however, increased length and/or increased purine:pyrimidine ratio seem to lower the solubility.

Base sequence analysis of PNA oligomers have been performed by positive-ion fast atom bombardment (FAB) tandem mass spectrometry.[23] The MS/MS product ions revealed a reproducible cleavage pattern along the PNA backbone which allowed identification of each PNA unit in the sequence. The composition of a PNA decamer containing both purines and pyrimidines was verified by this method. Furthermore, due to the high resolution of FABMS, H-T_4-LysOH and H-T_4-LysNH_2, which differ by only one mass unit, could be differentiated.

3. Hybridization Properties of PNA

Although PNA was designed to bind as the Hoogsteen strand to complementary double stranded DNA, $(DNA)_2$–PNA triplexes have not yet been observed. Still, PNA has proven to be a very potent DNA mimic capable of participating in both duplex and triplex formation. Furthermore, it displays interesting properties beyond those of natural DNA.

3.1 PNA–DNA duplexes

The properties of PNA–DNA duplexes have been most intensively investigated utilizing the pentadecamer H-TGTACGTCACAACTA-NH_2.[24,25] This oligomer forms a duplex with complementary antiparallel DNA (antiparallel: amino terminal of the PNA facing the 3'-end of the oligonucleotide) with a T_m of 69.5 °C.[26] The corresponding DNA–DNA complex has a T_m of 53.3 °C. Interestingly, PNA is also able to bind to the parallel DNA target although with lower affinity (T_m = 56.1 °C). The preference for the antiparallel binding orientation seems to be a general feature of mixed sequence PNA–DNA duplexes. Kinetic binding studies employing capillary gel electrophoresis have shown that PNA–DNA duplex formation is very fast (<30 s) for antiparallel hybrids whereas considerably slower kinetics were observed for parallel complexes.[27] The thermal stability of PNA–RNA duplexes is consistently slightly higher than found for PNA–DNA duplexes.[24]

A systematic examination of the effect of base pair mismatches in the middle of a pentadecamer PNA–DNA duplex revealed decreases in T_m of 8–20 °C for single mismatches. The sequence discrimination is generally higher for PNA recognizing DNA than for DNA recognizing DNA.[24]

The increased thermal stability of PNA–DNA duplexes relative to the corresponding DNA–DNA duplexes (ca. 1 °C per base pair) is predominantly ascribed to lack of electrostatic repulsion between the two strands.[24] This contention is supported by experiments showing that PNA–DNA and DNA–DNA duplexes have equal thermal stability at ionic strength above 1 M Na^+. As expected the stability of PNA–DNA hybrids is little affected by changes in ionic strength.

3.2 $(PNA)_2$–DNA triplexes

Homopyrimidine PNA oligomers or PNAs with a high pyrimidine:purine ratio bind to complementary DNA via the formation of $(PNA)_2$–DNA triplexes.[12,18–20,28] Monophasic, well-defined melting curves are obtained for these very stable complexes (T_m > 70 °C for decamer hybrids) where the binding most probably is governed by Watson–Crick and Hoogsteen base pairing. The stability of the complexes depends on the length of the oligomers in a regular manner with an increase in T_m of ca. 10 °C per base pair.[18] Triplex formation involving cytosine in the homopyrimidine

PNA is pH dependent in accordance with the formation of C^+GC triplets (lower pH increases hybrid stability).[19] It is interesting to note that even at pH 9 where cytosine is not expected to be protonated, UV-titrations indicate a $(PNA)_2$–DNA stoichiometry. This could mean that cytosine binds with only a single hydrogen bond in the Hoogsteen strand or that the local pK_a of cytosine is increased considerably to ensure at least partial protonation. Thermal melting of $(PNA)_2$–DNA triplexes exhibits a pronounced hysterisis (e.g., ca. 30 °C for PNA H-TCTCT$_3$-LysNH$_2$ hybridized to its complementary target) indicating a very slow rate of triplex formation under these conditions.[29,30]

3.3 Strand displacement

The exceptionally high stability of $(PNA)_2$–DNA triplexes enables strand displacement to take place upon targeting double stranded DNA.[12,31–33] In this unique binding mode, homopyrimidine PNA oligomers displace the pyrimidine strand of complementary dsDNA targets upon formation of a $(PNA)_2$–DNA triplex with the homopurine strand (Fig. 5). The parallel binding orientation has been shown to be slightly preferred to the antiparallel. Although restricted to salt concentrations below 50 mM for simple PNAs[32,34] this interesting property of PNA is being intensively investigated and so far several applications have been demonstrated.[31,34–36]

Strand displacement has been evidenced by a number of techniques. Footprinting experiments utilizing diazoacridine showed protection of the d(A)$_{10}$ target in a 248-bp fragment when incubated with the PNA acridine-T$_{10}$-LysNH$_2$.[12] The d(T)$_{10}$ region of the same target was cleaved by Staphylococcus nuclease or S1 nuclease in the presence of PNA. Both nucleases

Figure 5. Strand displacement of dsDNA by homopyrimidine PNA (PNA shown in bold).

preferentially cleave single stranded DNA. The displaced d(T)$_{10}$ strand could also be probed by potassium permanganate which oxidizes the C5—C6 double bond in thymines not involved in base stacking. The claim that a $(PNA)_2$–DNA triplex rather than a PNA–DNA duplex is formed with the purine tract of the double stranded target is based on two types of experiments.[33] Dimethyl sulphate probing has shown that N-7 of guanine is protected from methylation. This is taken as strong evidence that guanine participates in Hoogsteen hydrogen bonding. Furthermore, sensitivity to pH with preference for acidic conditions (pH 5.5) is consistent with protonation of cytosine involved in triplex formation.

Strand displacement with PNA (termed P-loop formation) has been visualized directly using electron microscopy.[32,37] A linear dsDNA target containing a d(A)$_{98}$/d(T)$_{98}$ insert was challenged with PNA H-T$_{10}$-LysNH$_2$.[32] The resultant P-loops of 90–100 bases in length were detectable within the resolution of electron microscopy and were found at the expected position of the DNA molecule. The same target was employed in a topoisomerase relaxation assay using two-dimensional gel electrophoresis. Complexing with PNA caused an unwinding of one helical turn per PNA decamer bound. Biotinylation of PNA decamers enabled single PNA binding sites on dsDNA to be detected using streptavidin as an electron microscopy marker.[37] In some experiments two streptavidin molecules could be detected per target supporting a $(PNA)_2$–DNA stoichiometry. Binding was chiefly observed at the fully complementary target. However, increased PNA concentration or prolonged incubation time led to binding to sites with one mismatch.

As mentioned previously, the formation of strand displacement complexes is highly dependent on the ionic strength. Salt concentrations above ca. 50 mM decrease the binding rate of simple PNAs with double stranded DNA dramatically. However, if the P-loop is preformed in a low-salt buffer it withstands salt concentrations up to at least 500 mM NaCl.[32,33,38] Complexes between dsDNA and PNA also have high thermal stability. For instance, directing H-T$_{10}$-LysNH$_2$ towards a homo-adenine target in dsDNA results in a stable hybrid up to at least 70 °C.[13]

The mechanism as well as the kinetics of PNA binding to duplex DNA have been investigated using gel shift and nuclease S1 cleavage assays.[39] The reaction obeys pseudo-first-order kinetics with a pseudo-first-order rate constant, k_{ps}, of 4.2×10^{-4} min^{-1} for the binding of H-T$_{10}$-LysNH$_2$ to its target dsDNA (the concentration of PNA was 10 μM corresponding to a 200-fold excess of PNA). A single mismatch in the PNA reduced k_{ps} by more than a factor 100. For other sequences the discrimination was found to be even larger (>1000-fold). Thus the observed selectivity between matched and mismatched complexes was found to be kinetically controlled, and consequently high PNA concentrations and long incubation times provided complete strand displacement even for singly mismatched decamers. A kinetic model was proposed

(Fig. 6) in which the PNA oligomer binds via Watson–Crick base pairing to the complementary DNA strand in breathing dsDNA. This process is reversible and accompanied by partial hybridization of PNA to target sequences (DP*). Also the sequence discrimination is exerted at this step. The final trapping of the complex DP by the second PNA strand results in a kinetically and thermodynamically very stable product which, according to the model, is not in equilibrium with other complexes. Assuming a constant concentration of PNA and steady state concentrations of all intermediate species, the model accounts for the observed kinetics, however, other possible mechanisms are not ruled out based on these data.[39]

It should be stressed that strand displacement has only been observed with PNAs able to participate in triplex formation and it therefore suggests that the very high stability of $(PNA)_2$–DNA triplexes is essential for strand displacement. An increase in the stability of PNA–DNA duplexes might therefore allow targeting of double stranded DNA at any sequence.

The very slow dissociation of PNA from dsDNA has been monitored by the addition of a 25-fold excess of an oligonucleotide complementary to the PNA strand in a strand displacement complex.[38] At various time intervals samples were withdrawn and transcription with RNA polymerase II was initiated. Since PNA blocks transcription a slow re-emergence of the full length transcript (ca. 50% between 5 and 20 h) was taken as evidence for the dissociation of PNA from the complex.

3.4 Bis-PNAs

As already discussed, the antiparallel configuration for duplex formation between PNA and DNA is preferred whereas the parallel orientation of the Hoogsteen strand seems to result in the most stable $(PNA)_2$–DNA triplexes.[38,40] Increased stability of triplexes has consequently been obtained by connecting two PNAs in a continuous synthesis with one strand antiparallel to the target DNA (designed for Watson–Crick recognition) and the second strand parallel to the DNA target (designed for Hoogsteen hydrogen bonding).[29] The two strands were connected by three units of 8-amino-3,6-dioxaoctanoic acid (eg1). The thermal stability of H-TCTCT$_3$-(eg1)$_3$-T$_3$CTCT-LysNH$_2$ hybridized to 5′-d(CGCAGAGA$_3$CGC) was determined to be 49 °C whereas a combination of the two PNA fragments H-TCTCT$_3$-LysNH$_2$ and H-T$_3$CTCT-LysNH$_2$ with the DNA target resulted in a T_m of 45 °C. The small but significant gain in melting temperature presumably stems from a smaller entropy loss.

The requirement for protonation of cytosine in the Hoogsteen strand results in a strong pH dependence of T_m which, however, can be eliminated by replacing cytosine in the parallel strand by pseudoisocytosine (Fig. 7).[29] This base is able to form two hydrogen bonds with guanine in the Hoogsteen motif (or three hydrogen bonds in the Watson–Crick motif depending on the tautomeric form). If pseudoisocytosine is placed in the antiparallel strand, it exerts no effect on the pH dependence. This demonstrates that the preference for the antiparallel strand to participate in Watson–Crick rather than Hoogsteen hydrogen bonding is stronger than the possibility to avoid protonation of cytosine even at pH 9.

The sequence discrimination of bis-PNAs was found to be very high reflecting the two-fold recognition process. Another important benefit of connecting the two PNA strands is faster triplex formation. As mentioned in Section 3.2 a pronounced hysteresis of the melting behaviour is observed with $(PNA)_2$–DNA triplexes, indicating a very slow rate of triplex formation. This hysteresis is reduced to 2–3 °C for bis-PNAs as a result of the high local concentration of the covalently linked second PNA strand. The ability to form strand displacement complexes with double stranded DNA is also improved for bis-PNAs compared to single PNA, presumably as a result of both the increased thermal stability and the faster kinetics.[29]

Griffith and coworkers also investigated the ability of bis-PNAs to strand invade dsDNA.[30] They found that a

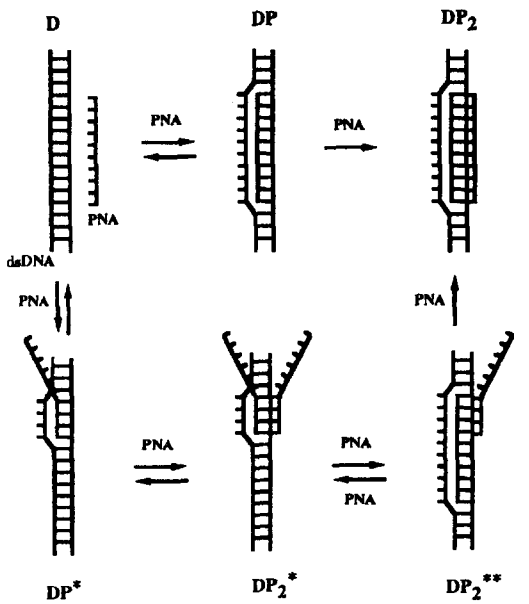


Figure 6. Schematic showing a possible pathway of PNA–dsDNA strand displacement.

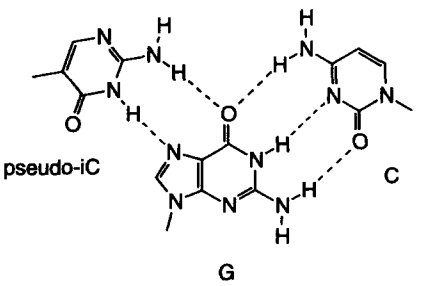


Figure 7. Triplex formation employing pseudoisocytosine in the Hoogsteen strand.

positively charged (lysine–aminohexyl) linker between the two PNA strands lead to a considerably faster and more efficient binding to duplex DNA than observed with a neutral poly(ethylene glycol) based linker. Thus, 50% binding to dsDNA was obtained at a concentration of 50 nM bisPNA with the charged linker whereas 22 μM bisPNA with neutral linker was required to cause the same effect. All experiments were performed at a relatively high salt concentration (100 mM Na^+). The kinetics revealed a much faster on-rate for the charged bis-PNA than for the corresponding neutral compound, and exceedingly slow off-rates were demonstrated for both compounds. The different behaviour of the two bisPNAs is most probably due to electrostatic interactions of the cationic linker with the negative charges of DNA targets.

3.5 PNA–PNA duplexes

Complementary PNA decamers containing all four nucleobases form antiparallel duplexes of very high thermal stability (e.g., PNA–PNA $T_m = 67\,°C$; PNA–DNA $T_m = 51\,°C$; DNA–DNA $T_m = 33.5\,°C$ for identical decamer sequences) with a high sequence specificity indicating Watson–Crick base pairing.[41] As observed for PNA–DNA duplexes, the antiparallel orientation is preferred. However, the parallel complex is still considerably more stable (13.5 °C) than the corresponding DNA–DNA duplex.

A helical structure of PNA–PNA complexes has been inferred from circular dichroism.[41] A preferred handedness of the helix can be induced by a terminal amino acid where the L- and D-forms give rise to opposite chiralities of the helix. The structure of the helix is presumably closely related to natural B-form duplex since the CD spectrum of the PNA duplex resembles that of the corresponding DNA duplex. Hypochromicity studies show an immediate decrease in absorption suggesting very fast hybridization. However, the CD signal appears much slower (within minutes), and a reorganization process of an already base paired racemic mixture of hybrids has been proposed to account for the observed kinetics.

4 The Structure of PNA–DNA Hybrids

The remarkable properties of PNA have obviously resulted in a desire to elucidate the structural details of PNA–oligonucleotide hybrids. Various techniques including NMR spectroscopy, X-ray crystallography, circular and linear dichroism spectroscopy, and molecular mechanics calculations have been applied.

4.1 Dichroism spectroscopy of PNA–DNA hybrids

Circular dichroism (CD) spectroscopy of PNA–DNA and PNA–RNA duplexes has provided information about the overall base-pair geometry in these complexes.[24] The CD spectra of DNA–DNA and antiparallel PNA–DNA and PNA–RNA duplexes are largely similar, suggesting that PNA participates in the formation of right-handed helices with a base-pair geometry not much different from that found in a B- or an A-form DNA helix. In contrast, the spectra of parallel PNA–DNA and PNA–RNA deviates more from the DNA–DNA spectrum indicating a different structure of these duplexes. This is in agreement with the different mobility of parallel and antiparallel PNA–DNA complexes in capillary gel electrophoresis.[27]

Triplex formation between poly(dA) and PNA H-T_8-$LysNH_2$ has been examined using circular and linear dichroism spectroscopy.[28] As expected, titration of poly(dA) with PNA resulted in saturation of both types of signals at a 2 PNA:1 DNA base ratio. The CD spectrum of the complex was compared to the spectra of the poly(dA)–poly(dT) duplex and the poly(dA)–$(poly(dT))_2$ triplex. The resemblance between these three spectra suggests that the PNA–DNA triplex has a right-handed helical structure with base pair stacking similar to that of a DNA triple helix. Poly(dA) alone shows no signal in flow linear dichroism (LD) due to its very high flexibility. Addition of increasing amounts of PNA H-T_8-$LysNH_2$ resulted in a strong negative LD signal indicating that the stiffness of the complex had increased considerably.[28] However, the LD intensity was not proportional to the PNA concentration as was the case for the CD signal. The intensity increased slowly up to ca. 1.7 equivalents of PNA followed by a steep increase during the addition of PNA up to saturation (2 equivalents). The existence of two isobestic points in the CD signal argues that only two different species are present during the titration. This is in accordance with the LD spectrum which suggests that no duplex stretches are present but rather segments of $(PNA)_2$–DNA triplexes are distributed evenly on the poly(dA) separated by flexible single stranded regions. Only close to the saturation point the complex becomes rigid due to triplex formation and base stacking between each PNA triplex segment. The preference for triplex formation seems in part to be due to strong interactions (hydrogen bonding) between the DNA phosphates and the PNA backbone amides according to the crystal structure of a $(PNA)_2$–DNA triplex.[42] Finally, reduced LD indicates that the base tilt in the triplex between poly(dA) and PNA H-T_8-$LysNH_2$ is similar to that of poly(dA)–$(poly(dT))_2$.

4.2 Molecular mechanics calculations

PNA–DNA, PNA–RNA and $(PNA)_2$–DNA duplexes and triplexes have been subjected to molecular modelling, mainly to address the importance of hydrogen bonding within the PNA backbone.[43,44] As shown in Figure 8, a potential hydrogen bond can be formed between the carbonyl oxygen of the tertiary amide in PNA and either the intra- or inter-residue backbone NH group. The calculations emphasized a crucial role played by the inter-residue hydrogen bond both for pre-organizing ssPNA and in hybrids between

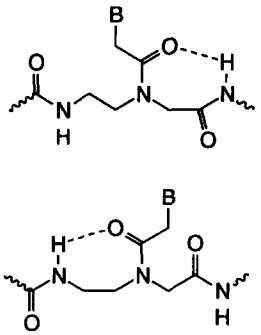


Figure 8. Potential intra- and inter-residue hydrogen bonds within the PNA backbone.

PNA and natural oligonucleotides. The analogue shown in Figure 9 does not possess the ability to form such a hydrogen bond (but it also has drastically altered conformational flexibility) and in accordance with the calculations a 9-mer of this analogue did not show any detectable binding to complementary DNA. These results contrast NMR data[45,46] which rule out any significance of the proposed hydrogen bond. It should, however, be noted that the molecular modelling studies did not take any solvent effects into account and they might therefore overestimate the significance of hydrogen bonding. Calculations on $(PNA)_2$–DNA triplexes predicted, in agreement with experimental results,[29] an antiparallel arrangement of the two PNA strands to be most stable.

Conformational analysis (by means of AMBER 3.0) of both duplexes and triplexes containing PNA showed that the conformational flexibility of these complexes is practically like the flexibility of the corresponding DNA complexes.[47] The amide bonds were found to be almost planar and the *cis* and *trans* configurations were close in energy. The energy gain for PNA–DNA helices was calculated to be 20 kcal/mol/monomer unit relative to duplex DNA whereas it was as high as 40 kcal/mol for $(PNA)_2$–DNA triplexes.

4.3 NMR studies of PNA–DNA and PNA–RNA hybrids

The most detailed information about the structure of PNA–DNA and PNA–RNA duplexes has been obtained by NMR spectroscopy.[45,46,48] Two PNA–DNA duplexes of mixed sequence (8-mer and 10-mer) have been investigated.[45] Although the PNA protons could not be completely assigned due to overlaps among the methylene protons and a low duplex concentration (ca. 1 mM), central features concerning the overall duplex conformation have been established. The imino proton region reveals that the nucleobases are engaged in hydrogen bonding, presumably of the Watson–Crick type. Dispersion of the chemical shifts is larger in the PNA–DNA complex than in the DNA–DNA duplex indicating that the base pair stacking is less regular in the PNA–DNA hybrid. The cross-peak pattern in NOESY spectra, the DQF-COSY spectrum, as well as modelling based on interproton distances suggest a B-like conformation of the DNA strand with the glycosidic torsion angles in the anti conformation and a sugar pucker close to C2′-*endo*.

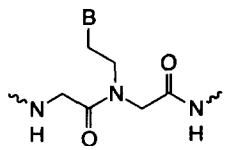


Figure 9. PNA analogue where hydrogen bonding between the linker to the nucleobase and the backbone is not possible.

The ^1H NMR spectrum of a PNA–RNA hexamer duplex has been completely assigned.[46] The thymine residue in PNA was synthesized with >98% enrichment of ^{13}C and ^{15}N in the backbone to facilitate assignment. All bases were found to be involved in standard Watson–Crick hydrogen bonding and the glycosidic torsion angles of RNA were *anti* combined with a typical C3′-*endo* sugar pucker. The overall structure of the RNA strand was close to a standard A-form helical geometry. This implies that the carbonyl group of the tertiary amide in the backbone of PNA is positioned isosteric to an RNA C2′ hydroxyl group, facilitating maximal solvent exposure of the additional backbone carbonyl oxygen. The tertiary amide bonds in PNA are found exclusively in the *cis* conformation and there is no indication for intra- or inter-residue hydrogen bonding.

4.4 Crystal structures of PNA–DNA complexes

The X-ray crystal structure of a $(PNA)_2$–DNA triplex has recently been described.[42] The two PNA strands (9-mers) were designed to be antiparallel and connected through a short peptide linker. At a resolution of 2.5 Å it was shown that the bases were engaged in standard Watson–Crick and Hoogsteen hydrogen bonding as well as in base stacking interactions. The overall structure was distinct from both A- and B-form DNA and was referred to as a P-form helix with 16 base triplets per turn. A large cavity down the helix axis reflected A-form characteristics whereas the bases were nearly perpendicular to the helix axis as in B-form DNA. Interestingly, the phosphates of the DNA backbone formed hydrogen bonds to amide protons in the PNA backbone of the Hoogsteen strand. These contacts combined with extensive van der Waal's interactions between these two strands were suggested to be important for the ability of homopyrimidine PNA oligomers to form very stable triplexes with complementary oligonucleotides. The minor groove was hydrated by a continuous network of water molecules which stabilized the Watson–Crick PNA strand in a conformation that was nearly identical to the Hoogsteen PNA strand.

Preliminary crystallographic data on a PNA–DNA duplex has also been reported. PNA H-GTAGATCACT-NH_2 hybridized to its complementary, antiparallel DNA target has been crystallized.[49] However, the crystals

only diffracted to a resolution of 5 Å and no details about this structure are yet available.

5. PNA as a Potential Antisense and Antigene Drug

The potential use of PNA as an antisense or antigene drug for sequence-specific modulation of gene expression has bright prospects. However, several issues must be addressed before reaching this ultimate goal. In vitro assays examining the effect of PNA on replication, transcription and translation all look very promising.[34,35,50,51] Low concentrations of PNA are sufficient to obtain the desired effects, the sequence specificity is high and, furthermore, the biological stability of PNA appears to be sufficient for the application of PNA as a drug.[14] The drawbacks include poor cellular uptake of PNA[52,53] and possibly the sensitivity of strand displacement complex formation to high salt concentrations. The cellular uptake may improve by attachment of lipophilic or other helper groups to PNA, by formation of PNA–DNA chimeras, or by the use of liposomes or other techniques for drug delivery. Moreover, the pharmacological properties of PNA have not been thoroughly investigated.

5.1 Transcription arrest by PNA

In vitro transcription arrest by PNA has been reported by different laboratories.[35,50,51] In one experiment the phage RNA polymerases T3 and T7 were employed in combination with Bluescript KS^+ plasmids containing the appropriate homopurine targets for PNA binding.[35] Concentration dependent transcription elongation arrest was observed when 10-mer PNA was bound to the template strand whereas virtually no effect was exerted by complexing PNA to the non-template strand. This selectivity should allow independent targeting of either mRNA with the antisense sequence or dsDNA with the sense sequence in the potential application of PNA in vivo. In all experiments the strand displacement complexes with PNAs were preformed in a low-salt buffer and subsequently transferred to the transcription buffer. If four promoter fragments were present simultaneously, covering all combinations of T3 or T7 promoters with an A_{10} target present or absent, only arrest at the expected PNA binding sites were observed at least up to 5 μM PNA (five times the concentration needed to obtain complete arrest at the targets). This emphasizes a pronounced sequence selectivity. High resolution sequencing gels were employed to determine the exact position of transcription arrest. The lengths of the transcripts showed that polymerase T7 transcribed 2–3 nt into the target while T3 was arrested before the binding site for PNA H-T_{10}-LysNH_2. Interestingly, the efficiency was also dependent on the C:T ratio in the PNA oligomer. Higher C-content caused longer transcripts indicating a less efficient blocking of the polymerases. This may be explained by the need for protonation of cytosine in the Hoogsteen motif. Shorter PNAs containing six or eight PNA-units, respectively, arrested transcription as well, however much higher concentrations of PNA were required. The arrest occurred with the PNA bound at the far end of the target, implying that the RNA polymerase 'pushes' the PNA along the DNA target.

Hanvey et al. demonstrated PNA dependent transcription elongation arrest with eukaryote RNA polymerase II.[51] Decamer and pentadecamer PNAs with a high pyrimidine:purine ratio were used and concentrations of 1 μM PNA proved sufficient to completely inhibit transcription. No nonspecific inhibition was observed at this dose and the effect of the PNA persisted for at least 1 h. Targeting of PNA to the non-transcribed strand caused only a minor decrease in transcription. In contrast, DNA triplex formation requires covalent crosslinking of the third strand to dsDNA to achieve efficient termination of transcription elongation.

Incubation of homopyrimidine PNA with RNA containing the appropriate target sequence results in the formation of highly stable $(PNA)_2$–RNA triplexes.[51] Triplex formation between PNA H-T_{10}-LysNH_2 and RNA containing a $r(A)_{10}$ target has been shown to cause inhibition of RNase H^- MMLV reverse transcriptase.[51] The effect was dose and sequence dependent, and when $d(T)_{10}$ was applied instead of PNA, no inhibition was observed. Thus, a PNA, unlike DNA, can inhibit elongation of reverse transcriptase, presumably due to the high stability of the PNA–RNA complex. Furthermore, it should be emphasized that only binding to dsDNA is salt dependent whereas hybridization of PNA to ssDNA or RNA is not affected significantly by the salt concentration.

Strand displacement loops between homopyrimidine PNA and dsDNA targets have been shown to interfere with the binding of several DNA recognizing proteins.[34,35,50,51] For instance, cleavage by the restriction enzymes *Bam*H1, *Sal*I, and *Pst*I was fully inhibited at a PNA:target ratio of 60 when PNA was bound proximal to the cleavage sites.[34] The concomitant cleavage at a second unblocked site with *Pvu*II ruled out the possibility that PNA could inhibit enzymes in general by direct binding to these. Only ca. 10% inhibition of cleavage was observed if a single mismatch was present and PNAs with two mismatches had no effect on the cleavage. In another experiment PNA dose-dependent inhibition of cleavage by Hind III was observed.[51] A molar ratio of 30:1, PNA:DNA completely prevented cleavage by the enzyme. Based on these results, transcription inhibition by promoter targeting should be feasible.

5.2 P-Loops as artificial transcription promoters

Although usually not considered an antigene effect, we find it appropriate to mention here the ability of PNA to activate transcription. Activation of transcription might turn out to be a valuable tool in medicinal chemistry as well.

The resemblance between PNA strand displacement hybrids and transcription initiation complexes became

obvious in an experiment demonstrating that transcription initiation takes place at P-loops.[36] The displaced DNA strand mimics the ca. 12 bp open region formed at DNA promoters by either the polymerase itself or in combination with transcription factors. Strand invasion was performed in a low-salt buffer and subsequently *E. coli* RNA polymerase was added. Transcription took place with the looped-out strand as template and the length of the resulting transcripts corresponded exactly to run-off transcripts initiated at the bound PNA. The efficiency of transcription increased when two decamer PNA targets were present either on the same strand (*cis*) or on opposite strands (*trans*). The *trans* binding mode resulted in two transcripts corresponding to transcription initiation from both P-loops. Reverse transcriptase primer extension experiments on the produced transcripts suggested that transcription was initiated at several positions within the single-stranded DNA loop. If the *lac* UV5 *E. coli* promoter was present on the same dsDNA as the PNA binding site, transcription from both the promoter and the P-loop took place. Again larger loops caused the most efficient transcription initiation and in the case where three PNA targets were present close to each other (two on the template strand, one on the non-template strand) only transcripts initiated at the PNA binding site were observed. This emphasizes that the 'PNA promoter' is very strong and might have the potential to act as a gene activator in vivo provided that strand invasion is accessible inside cells. As mentioned previously, PNA is also able to arrest transcription, however, the two processes might be distinguished by the size of the P-loop (transcription arrest requires only decamer targets[50,51] whereas larger loops are more efficient to promote transcription initiation).

5.3 Inhibition of replication by PNA

In a primer extension experiment, PNA H-T_{10}-LysNH_2 was incubated with a single stranded DNA target and a primer oligonucleotide.[50] Addition of the *Taq* DNA polymerase caused formation of a truncated product corresponding to termination of replication at the PNA binding site. Similar results were obtained using the large fragment (Klenow fragment) of *E. coli* DNA polymerase.

5.4 Translation arrest by PNA

The potential application of PNA as an antisense agent has been evaluated by examining the ability of PNA to inhibit in vitro translation.[51] PNA H-$T_3ACT_2CT_2$-NH_2 was incubated with RNA containing the complementary sequence in the presence of nuclease treated rabbit reticulocyte lysate. Analysis of the translation products revealed a concentration dependent termination of translation elongation at the PNA binding site. Since a PNA–RNA complex was reported not to be a substrate for RNase H, the termination is probably not due to cleavage of the RNA target but rather caused by a steric blockage of the ribosomes. A scrambled PNA control sequence with the same base composition as the target PNA did not cause any significant translation arrest.

Cell culture studies of PNA are complicated by the fact that PNA only very slowly or not at all penetrates cell membranes.[52,53] This obstacle has been circumvented in a study employing microinjection.[51] As in the in vitro translation experiments, PNA was targeted to the SV40 T Ag gene (or corresponding mRNA) in cells from the tsa 8 cell line. After microinjection of PNA, the amount of T Ag protein was determined by indirect immunocytochemistry. To account for both nonspecific PNA effects and for cell viability after injection, a β-galactosidase expression vector was included in all injections. When a 15-mer PNA was used, ca. 40% of the injected β-gal-expressing cells showed significantly reduced T Ag staining at an estimated concentration of 1 μM PNA per injected cell. A 20-mer PNA effected 50% inhibition at the same dose. In contrast a 10-mer PNA was not capable of inhibiting T Ag protein synthesis at concentrations ranging from 1 to 10 μM. PNA Concentrations exceeding 5 μM generally resulted in nonspecific inhibition of β-gal expression. Whether the observed inhibition is caused by an antisense or an antigene effect is not known. However, the high ionic strength in cells most likely prevents binding to dsDNA and therefore argues that PNA binds to mRNA.

5.5 Biological stability of PNA

The biostability of antisense and antigene compounds is of major concern. Since PNA is a pseudopeptide, it appeared relevant to study its stability towards both proteases and peptidases. PNA H-T_{10}-LysNH_2 was found not to be degraded to any detectable degree during 2 h of incubation in human serum, in bacterial cell extracts, or in nuclear mouse ascites tumor cell extract.[14] Furthermore, a PNA pentadecamer containing all four nucleobases and terminating in a carboxamide (rather than a lysine amide) was shown to be stable in human serum. After 2 h in a eukaryotic cytoplasmic extract a decrease in the amount of this PNA of maximally 20% was observed. It has not been determined whether the disappearance of PNA is due to degradation or caused by its aggregation with cellular components. PNA H-T_5-LysNH_2 resisted degradation by isolated fungal proteinase K and peptidase from porcine intestinal mucosa at very high enzyme concentrations. In all experiments PNA was incubated in the presence of a control peptide to ensure adequate proteolytic activity in the media. Thus, PNA appears to have ample biological stability for drug development.

6. Other Applications of PNA

Obviously, PNA has a great potential to be used as a tool in molecular biology, diagnostics and related fields. In addition, PNA has established itself as a

valuable tool in elucidating the mechanism, binding forces etc. involved in DNA processes.[24,28,36,41,54]

6.1 PNA directed PCR clamping

The high stability and sequence selectivity of PNA–DNA complexes have been exploited in the analysis of single base pair mutations in DNA employing PCR amplification.[55] PNA can either be directed towards the primer binding site, the region adjacent to the primer site, or towards the middle of the PCR region. Competition between the primer and PNA for binding to the primer site resulted in decreased amplification since PNA cannot function as a primer for DNA polymerase. When a mixed sequence PNA 15-mer overlapping the PCR primer site was included in the PCR mixture, it completely prevented formation of the expected PCR product at PNA concentrations at or above 0.3 μM. To investigate whether a single mutation in the primer site could be detected, the target plasmid and the corresponding plasmid with a single mutation in the primer region were mixed with the two sets of primers (15-mers). In the absence of PNA two PCR products of the expected sizes were produced. Addition of the complementary 15-mer PNA resulted in suppression of both products, indicating that PNA binds to both the matched and the mismatched targets. However, when the length of the mutant primer was increased (and thereby the stability of mutant primer–DNA complexes) it was able to compete with PNA for the primer binding site and at a primer length of 25 nucleotides only PCR product from the mutant plasmid was observed. The required length of the mutant primer was dependent on the ΔT_m between matched and mismatched PNA–DNA complexes and for three other mismatches (having larger ΔT_m's) a primer length of 15 nucleotides was sufficient.

When the PNA target site is located adjacent to the primer site the observed clamping is likely to reflect that the polymerase cannot access the PCR primer or that primer elongation is prevented. Conversely, PNA binding at a distance from the primer site is expected to cause elongation arrest of the polymerase. Dependent on the length of the PNA and the exact assay, PNA–DNA duplex formation in the middle of the PCR region was shown to block the read-through of the polymerase, however, this type of clamping is less efficient than targeting of the primer site since a 20-mer PNA was required to obtain a sufficient inhibition in the above described assay system.

The most efficient and discriminative clamping was observed with homopyrimidine PNA decamers. Formation of the extraordinary stable $(PNA)_2$–DNA triplexes caused inhibition of the polymerase at any PNA binding site. The high sequence selectivity was demonstrated in an experiment where three plasmids containing the targets for PNAs H-T_{10}-LysNH_2, H-T_5CT_4-LysNH_2, or no target, respectively, were mixed. In all samples the control PCR product from the no-target plasmid was produced whereas the formation of the two other products could be suppressed selectively by addition of the respective complementary PNAs.

6.2 PNA and S1 nuclease as 'artificial restriction enzymes'

PNAs in combination with nuclease S1 have been used as 'artificial restriction enzymes'.[31] Three different approaches were tested as shown in Figure 10. The first model illustrates hybridization of a homopyrimidine PNA decamer to the complementary target on double stranded DNA. Treatment with nuclease S1 caused a significant fraction of DNA to be cut into well-defined fragments. It was suggested that the enzyme first digested the displaced strand, then the gap was to some extent enlarged, thereby allowing the opposite strand to act as a substrate for nuclease S1. When PNAs with a single mismatch were employed, only very weak cleavage was observed. Due to the high concentration of enzyme required in this system, the ends of the double stranded DNA were also digested to some extent. This problem could be eliminated by the use of a dimeric target either in *cis* or *trans* (Figure 10). The binding of PNA to two adjacent targets in *cis* lead to an opening of the entire region including the intervening (six) base pairs and thereby provided an easily accessible substrate for the nuclease. This considerably increased the sensitivity to cleavage, and an even more favorable situation arose if the two PNA targets were on opposite strands. The efficiency was dependent on both the concentration of PNA, the digestion time, and the amount of nuclease S1 used. Higher than optimal concentrations of PNA resulted in unspecific cleavage at multiple sites. This was ascribed to the binding of PNA to lower affinity mismatch sites. Digestion of the displaced single stranded DNA took place over a distribution of cleavage sites and therefore the opposite DNA strand was produced with overhangs of different length corresponding to the PNA binding site.

6.3 Isolation of specific active genes by PNA strand invasion

A biotinylated 21-mer PNA (H-CTGCTGCTGCT-GCGCTGCTG-NH_2) has been used in a procedure to

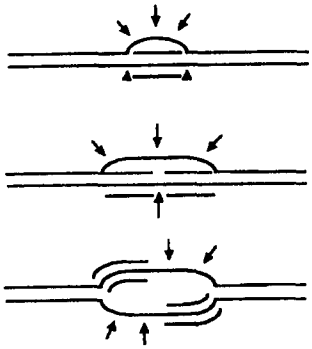


Figure 10. P-Loops with (1) a single PNA target, (2) two PNA targets in *cis* and (3) two PNA targets in *trans*. Arrows indicate cleavage sites for nuclease S1.

isolate transcriptionally active chromatin fragments containing multiple tandem CAG triplets.[56] The chromatin fragments of interest were challenged with excess PNA oligomer at low ionic strength. Subsequently, the PNA–DNA hybrids were separated from free PNA by density gradient centrifugation and isolated using streptavidin coated magnetic particles. The molecular binding mechanism responsible is unclear at present since the PNA employed would not be expected to engage in stable Watson–Crick–Hoogsteen type triplex strand displacement, and no controlled binding studies were performed.

6.4 Interactions of DNA binding ligands with PNA–DNA complexes

The close similarity between PNA–DNA and DNA–DNA duplexes offers the opportunity to investigate the forces responsible for the binding of different ligands to dsDNA (and triplexes).[54] Assuming that binding forces can be divided into electrostatic and hydrophobic contributions, the effect of replacing the negatively charged deoxyribose backbone with a neutral pseudopeptide backbone might help assess the role of the phosphate groups in binding of a given ligand. DAPI, a synthetic trypanosid drug, and distamycin A, a naturally occurring agent, are both positively charged and known to bind preferentially in the minor groove of AT-rich DNA sequences. Circular dichroism (CD) studies have shown that both ligands are able to bind to PNA–DNA duplexes containing appropriate target sites whereas they show no affinity for duplexes lacking an AT stretch.[54] The similarity of the CD spectra with the spectra of the ligands bound to dsDNA strongly suggests that binding takes place in the minor groove of the PNA–DNA duplex. The binding affinity was decreased by a factor of 100 relative to the binding to DNA–DNA. This might be a consequence of the reduced negative charge in the minor groove and is in accordance with the fact that no binding to a charge neutral PNA–PNA duplex was observed. DAPI, but not distamycin A, was also found to bind to a $(PNA)_2$–DNA triplex. This difference between the two ligands might reflect that DAPI carries two positive charges while distamycin A only has a single charged group, and that distamycin A therefore lost the affinity for the triplex (its negative charge being reduced from -3 per base triplet to -1).

The DNA intercalators ethidium bromide (positively charged), 8-methoxypsoralen (neutral), and the Δ and Λ enantiomers of $Ru(phen)_2dppz^{2+}$ were tested with respect to their interaction with a PNA–DNA duplex, a PNA–PNA duplex, and a $(PNA)_2$–DNA triplex, respectively.[54] In no case was intercalation observed. Extension of the PNA backbone by a single methylene group (see Section 7.1) in either the amino ethyl part or in the glycine part did not improve the binding, although such modifications are expected to increase the flexibility of the backbone. Whether the absence of intercalation is due to decreased breathing of PNA complexes or caused by other special features of hybrids containing PNA is not known.

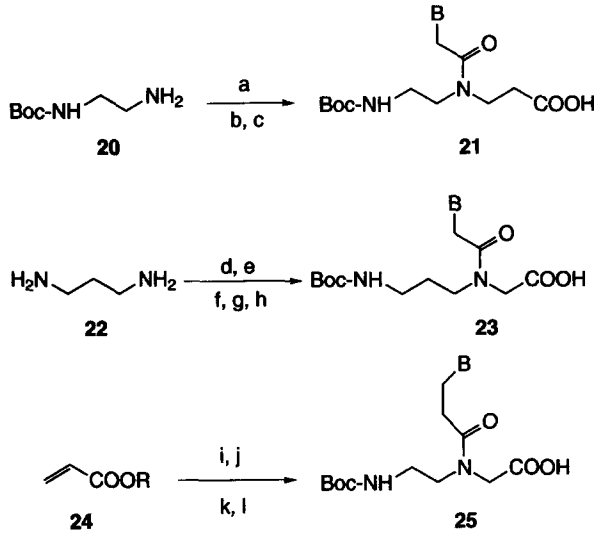
7. Modifications of PNA

The design of successful DNA analogues can be guided by structure–activity studies on already existing analogues. Much effort has been directed towards understanding the essential as well as the undesirable features of these analogues, typically by preparing closely related compounds and comparing their characteristics. Since PNA has demonstrated unique hybridization properties towards complementary oligonucleotides, it has been of utmost interest to understand which structural elements are responsible for this behaviour. Furthermore, it is important to know which kind of modifications can be accommodated while retaining binding to DNA and RNA.

7.1 PNA containing extended units

The number of bonds connecting the nucleobases in PNA and DNA are identical. It is an obvious question to ask whether this is a necessary condition to be fulfilled by DNA analogues and mimics in general to obtain the desired properties. In an attempt to address this, PNA oligomers containing residues extended in either the backbone or in the linker to the nucleobases by a single methylene group have been prepared and their hybridization properties examined.[57,58] The syntheses of the modified PNA pyrimidine monomers are outlined in Scheme 3. These monomers were incorporated into PNA oligomers by standard coupling procedures. The following types of modified oligomers were prepared: (1) homopyrimidine PNA decamers containing a single modified unit in the middle, (2) homopyrimidine PNA decamers built up exclusively of modified units, and (3) mixed sequence PNA decamers with one modified unit in the middle of the oligomers.

As could be expected, homopyrimidine PNAs containing a single modified unit formed $(PNA)_2$–DNA triplexes with complementary DNA, presumably mediated through Watson–Crick and Hoogsteen base pairing (indicated by the pH dependence of T_m in complexes containing cytosine and the sequence specificity of the binding). Also the modified mixed sequence PNAs behaved like unmodified PNA in terms of formation of duplexes with complementary DNA or PNA, showing a preference for the antiparallel binding orientation. However, all modified PNA–DNA hybrids were considerably less stable than the corresponding unmodified complexes. For instance a single backbone extended unit in a $(H\text{-}T_4(X)T_5\text{-}LysNH_2)_2/d(A_{10})$ triplex (X corresponding to **21** or **23**) caused a decrease in T_m from 72 °C in the unmodified complex to 59 or 61 °C, respectively, in the modified complexes. Similarly, the melting temperature was lowered by 10–12 °C when a single of these modifications were incorporated in mixed sequence PNA–DNA duplexes. Extension of the linker between the backbone and the nucleobase



Scheme 3. Synthesis of PNA monomers with extended backbone or extended linker to the nucleobase. (a) $CH_2CHCOOCH_3$, CH_3CN; (b) $BCH_2COOPfp$, Et_3N in DMF; (c) Aqueous NaOH; (d) $ClCH_2COOH$; (e) MeOH, HCl; (f) p-NO_2-C_6H_4O-Boc, H_2O/dioxane, pH 10; (g) BCH_2COOH, DhbtOH, DCC in DMF/CH_2Cl_2; (h) NaOH, MeOH; (i) Thymine in MeOH, (R = CH_3), cat. NaOH; or N^4-Cbz-cytosine in DMF (R = C_2H_5), NaH; (j) 2 M NaOH; (k) $BocNHCH_2CH_2NHCH_2COOC_2H_5$ in DMF/CH_2Cl_2, DhbtOH, DCC; (l) 2 M NaOH in MeOH (B = thyminyl); or 1 M LiOH in THF (B = N^4-Cbz-cytosyl). B = thyminyl or N^4-Cbz-cytosyl.

resulted in even lower T_m values for the same sequences. It is, however, noteworthy that all hybrids exhibited a pronounced sequence selectivity with ΔT_m between 6 and 21 °C for a single mismatch opposite the modified unit. This is regarded as strong evidence for base pairing between the modified units and the complementary bases in DNA, and the decreased stability is most likely caused by geometric constraints in the PNA and/or a larger loss in entropy upon complex formation.

PNA decamers composed exclusively of backbone extended units did not show any detectable binding to complementary DNA, whereas H-$(paT)_{10}$-$LysNH_2$ (paT corresponding to **25**) with all linkers between backbone and nucleobases extended, formed a triplex with $d(A_{10})$. The stability was very low (T_m = 22 °C) but again the binding was discriminative for mismatches. These results emphasize the importance of maintaining the correct distance between the nucleobases, and also suggest that the backbone distances might be of greater significance than the distance between the backbone and the nucleobases when all units are modified. PNA oligomers with shorter backbones than in unmodified PNA have not yet been described and it remains to be determined if they bind to DNA.

7.2 'Retro-inverso' PNA

'Retro-inverso' PNA designates modified PNA where a methylene group from the aminoethyl part of the backbone has been 'transferred' to the glycine part to yield an *N*-aminomethyl β-alanine backbone (Fig. 11).[59,60] This results in an interchange of the hydrogen bond donors and acceptors of the amide groups in the backbone while retaining the number of bonds between the nucleobases. The syntheses of the modified Cbz-A- and T-monomers are shown in Scheme 4.[59]

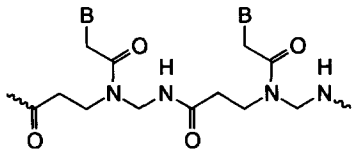
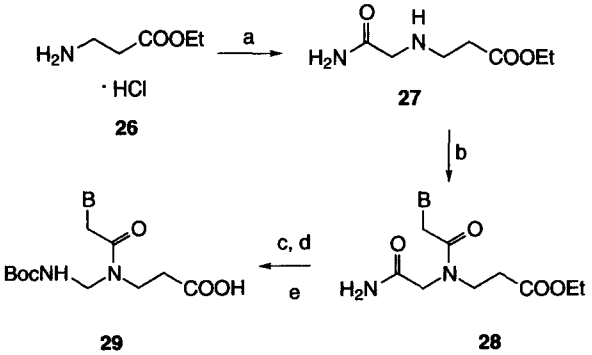


Figure 11. 'Retro-inverso' PNA.

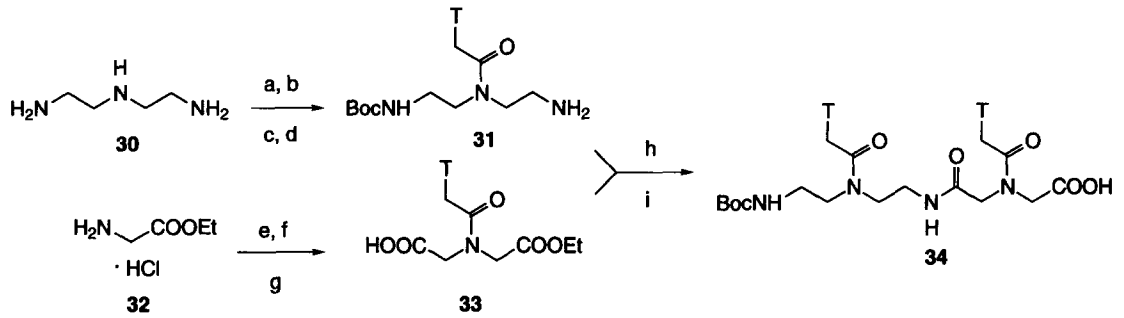
The inherent lability of the geminal diamino groups complicated the oligomerization process. Unprotected monomers exhibited a half life of 1.6 and 0.9 days at pH 7 and 9, respectively, whereas acidic conditions stabilized the N-terminus considerably. Single modifications were incorporated into PNA oligomers by standard oligomerization chemistry, however, equal amounts of the desired product and the decomposed (truncated) oligomer were isolated.[60] An improvement of this procedure was achieved by condensing Boc-deprotected **29** with a PNA monomer containing the normal aminoethyl terminus and using this dimer as a building block in solid phase synthesis. Block synthesis solution chemistry was employed to obtain oligomers modified in all units. Octamers were prepared by this strategy and the Cbz-protective group of adenine was removed by catalytic hydrogenation. The hybridization properties of these oligomers to complementary oligonucleotides have not yet been reported.

7.3 PNA containing a heterodimer

An alternative interchange of groups in the PNA backbone is illustrated in Scheme 5. In this heterodimer (detT—idaT) the amide bond connecting the two units is positioned with the carbonyl group towards the C-terminal rather than the N-terminal.[40] Incorporation of the dimer into a PNA decamer, H-TT(detT-idaT)CCTCTC-$LysNH_2$, was found not to influence the thermal stability of hybrids of this oligomer with complementary DNA. No data were reported to reveal whether more than one heterodimer could be



Scheme 4. Synthesis of 'retro-inverso' PNA monomers. (a) $ClCH_2CONH_2$, K_2CO_3, DMF; (b) BCH_2COOH, DhbtOH, DCC or HBTU, DMF; (c) $PhI(O_2CCF_3)_2$, CH_3CN, H_2O; (d) $(Boc)_2O$, K_2CO_3, dioxane; (e) LiOH, THF, H_2O. B = T or N^6-Cbz-A.



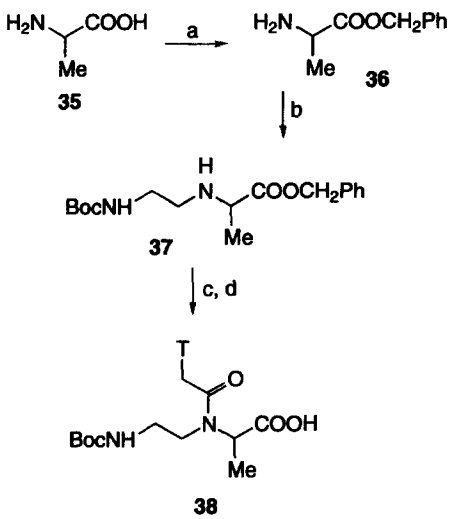
Scheme 5. Synthesis of PNA heterodimer. (a) *p*-NO_2-C_6H_4O-Boc, $CHCl_3$; (b) p-NO_2-C_6H_4O-Cbz, dioxane, H_2O; (c) TCH_2COOH, DCC, DhbtOH; (d) H_2, Pd–C; (e) $BrCH_2COOBn$, Et_3N, EtOH; (f) TCH_2COOH, DCC, DhbtOH; (g) H_2, Pd–C; (h) DCC, DhbtOH; (i) 1 M NaOH.

accommodated or whether the sequence selectivity was retained.

7.4 Chiral PNA

Despite the advantages offered by the achirality of PNA, it has been of interest to replace the glycine moiety of the backbone with chiral amino acids. The side chains of the amino acids were envisaged to be valuable for controlling binding affinity, specificity, hydrophobicity and attachment of ligands to PNA. Finally, chirality might be used to control the binding orientation of PNA to DNA.[61]

The synthesis of the alanine based PNA T-monomer (alaT) is outlined in Scheme 6.[61] Racemization of the chiral center during monomer synthesis was ruled out based on NMR studies of the (*S*)-2-butanol esters of **36**, **37**, and **38**. These esters had been subjected to the same operations as the corresponding benzyl esters. Alkaline hydrolysis of the monomer ester was found to be the only step causing partial racemization and therefore the benzyl ester, which could be removed quantitatively by hydrogenation, was employed.



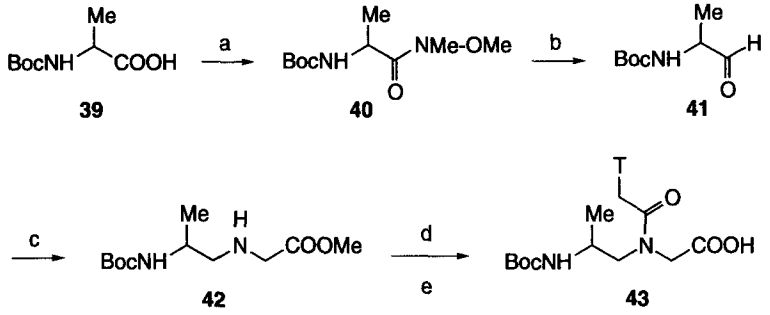
Scheme 6. Synthesis of the PNA alanine monomer. (a) $HOCH_2Ph$, $SOCl_2$; (b) $BocNHCH_2CHO$, $NaBH_3CN$, AcOH; (c) TCH_2COOH, DCC, DhbtOH; (d) H_2, Pd–C.

The PNA oligomer H-G(alaT)AGA(alaT)CAC(alaT)-NH_2 was prepared by standard oligomerization protocols[15] except for couplings of alaT which were performed with TDBTU (*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate) as coupling reagent in the presence of diisopropylethylamine (DIEA). The combination of TDBTU and DIEA was found to suppress racemization during oligomerization. Both the oligomers containing L- and D-form alanine were prepared and their binding to complementary parallel and antiparallel DNA was examined. The melting temperatures indicate that the antiparallel orientation is preferred for both the L- and D-alanine containing oligomers as is the case for glycine-based PNA. The D-configuration gives rise to more stable hybrids than the L-form (T_m = 50 °C vs. 46.5 °C, antiparallel target) and its T_m is very close to that of the corresponding unmodified complex (51 °C).

Chirality has been introduced in the ethylene portion of PNA monomers using a natural amino acid as starting material (Scheme 7).[62] Substituents in this part of the backbone might reduce the flexibility of PNA. Syntheses and binding characteristics of oligomers containing these PNA monomers have yet to be reported.

8. PNA–DNA Chimeras

PNA–DNA chimeras are potentially interesting because they may combine advantageous properties of PNA and DNA. Such chimeras would for instance allow DNA recognizing enzymes like RNase H and DNA polymerases to interact with the DNA part while the PNA part would provide high binding affinity and specificity. A linker to connect the two oligomers in a way that allows stacking between the neighbouring bases in the PNA and the DNA part of the chimera is required. Model building suggested that a PNA terminating in a hydroxyl group instead of the primary amine would constitute an appropriate linker in terms of base stacking, number of bonds between the nucleobases, and orientation (both parts of the chimera could bind in the preferred antiparallel orientation to DNA) (Fig. 12).[63]



Scheme 7. Synthesis of a chiral PNA monomer. (a) 'BuOCOCl, *N*-methylmorpholine, NHMe–OMe; (b) $LiAlH_4$; (c) H_2NCH_2COOMe, $NaBH_3CN$; (d) TCH_2COOH, 'BuOCOCl, *N*-methylmorpholine; (e) Aqueous NaOH.

The synthesis of the linking PNA unit is outlined in Scheme 8. Oligomerization was performed on controlled pore glass (CPG) to allow continuous synthesis of the DNA part on an automated DNA synthesizer. Only thymine containing PNA (5′-d($CTCTC_2$)-3′-T_4-GlyOH) was employed since removal of the Cbz-protective groups of the other nucleobases would require too harsh conditions for the DNA part to survive. Melting temperatures of the chimeras hybridized to complementary DNA showed a dramatic decrease in stability compared to both PNA–DNA and DNA–DNA complexes. The binding did not improve by connecting the PNA to the 5′-end of DNA instead of the 3′-end. If only the PNA or the DNA parts of the chimera were prepared no hybridization to the DNA target was observed. This indicates that both parts of the chimera are involved in binding. Titration revealed a 2:1 ratio of chimera to DNA, however, T_m was almost independent of pH which could argue for duplex rather than triplex formation. Since only the DNA part contains cytosines, a possible explanation for this apparent discrepancy might be that only the PNA part of the chimera participates in triplex formation. A higher stability observed for the chimera–PNA complexes compared to the corresponding chimera–DNA complexes may reflect a greater flexibility of the PNA backbone, allowing it to better accommodate the PNA–DNA junction.

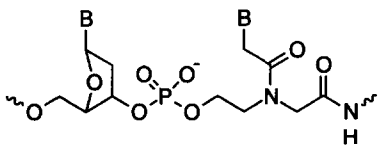


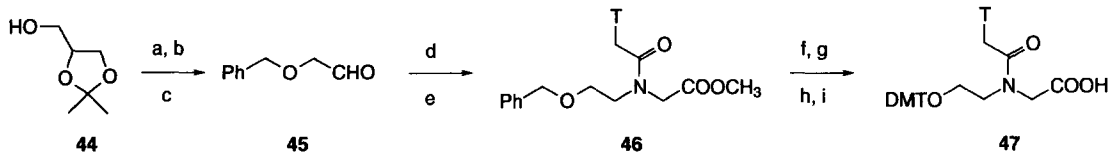
Figure 12. PNA–DNA chimera.

9. Concluding Remarks

The properties of PNA have demonstrated that a sugar phosphate backbone is not a prerequisite for obtaining helical duplex structures governed by Watson–Crick base pairing. This has inspired speculations that PNA-like compounds might have played a role in the prebiotic evolution of life since they have the potential to carry genetic information, and at the same time they are stable compounds which may survive the harsh conditions supposed to have prevailed in the prebiotic soup.[13,64,65] PNA has also been considered in relation to the origin of chirality.[65] As discussed in Section 3.5, a chiral amino acid at the C-terminal of PNA is able to induce a preferred handedness in a PNA–PNA duplex.[41] Such a helical seeding mechanism could have accounted for promotion of chirality in the prebiotic world. This would require reversible binding of the chiral unit and fixation of the preferred handedness before release of the chiral auxiliary group.

The high stability of both PNA–DNA, PNA–RNA, and especially $(PNA)_2$–DNA complexes will surely make PNA useful as a tool in diagnostics, molecular biology, and related fields. In vitro assays will benefit as well from the chemical, biological, and thermal stability of PNA itself. Furthermore, PNA can act as a versatile handle for attachment of various ligands.

The future prospects of PNA as a drug have still to be assessed. The poor cell permeability of PNA may indicate poor bioavailability, and issues like the pharmacological properties of PNA have to be addressed. It has been discussed that the high thermal stability of PNA–nucleic acid complexes could lead to decreased sequence specificity at physiological temperature.[66] While this concern is certainly legitimate, the use of



44 45 46 47

Scheme 8. Synthesis of a PNA–DNA linker. (a) $PhCH_2Cl$, KOH; (b) H^+, H_2O; (c) KIO_4; (d) H_2NCH_2COOMe, $NaBH_3CN$; (e) TCH_2COOH, DCC; (f) H_2, Pd–C; (g) DMTCl; (h) OH^-, H_2O; (i) H^+, H_2O.

shorter PNAs and/or backbone modified PNAs should allow the stability to be controlled.

Finally, unravelling the structure–activity relations of PNA should facilitate the design of new DNA analogues and could contribute to a better understanding of the structural and biological properties of DNA itself.

Acknowledgments

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26. T_m, The melting temperature, is determined as follows: UV absorbance is measured as a function of temperature and the temperature where the 1. derivative of the resultant curve has maximum is recorded as T_m.

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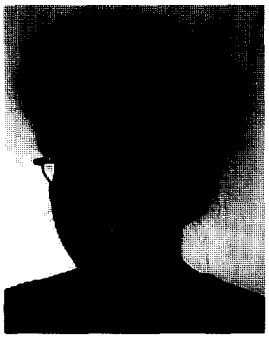

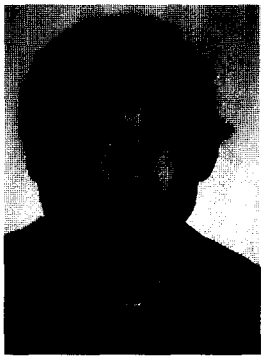

Biographical Sketch

Birgitte Hyrup was born in Ringe, Denmark in 1967. She received her Masters Degree in Chemistry in 1993 from the University of Copenhagen, and is now a Ph.D. student with Professor Peter E. Nielsen, University of Copenhagen. A part of her graduate work has been carried out at the ETH, Zürich, with Professor Steven A. Benner. Her major research interests include DNA–RNA analogues as antisense compounds, and the design and synthesis of artificial enzymes.

Peter E. Nielsen was born in Copenhagen, Denmark in 1951. He received his Ph.D. in Chemistry in 1980 from the University of Copenhagen and has, apart from a postdoctoral stay at U.C. Berkeley, worked at the Department of Biochemistry, The Panum Institute, University of Copenhagen, where he presently is a professor and director of the Center for Biomolecular Recognition. His research interest is molecular recognition in general and DNA recognition in particular. He has developed the uranyl photofootprinting technique and is a co-inventor of PNA.