CHAPTER II

LITERATURE REVIEWS

Peptide nucleic acid (PNA)

Peptide nucleic acids (PNAs) were first described by Nielsen, et al. [1] (Figure 5) that are remarkable DNA/RNA mimics in which the sugar-phosphate spine of the natural nucleic acid of DNA is by a homomorphous, achiral, uncharged and relatively flexible backbone composed of *N*-(2-aminoethyl)-glycine (*aeg*) units with the pyrimidines and purines attached to the backbone by methylenecarbonyl linkages. Unlike DNA or DNA analogs, PNAs do not contain any (pentose) sugar moieties or phosphate groups.

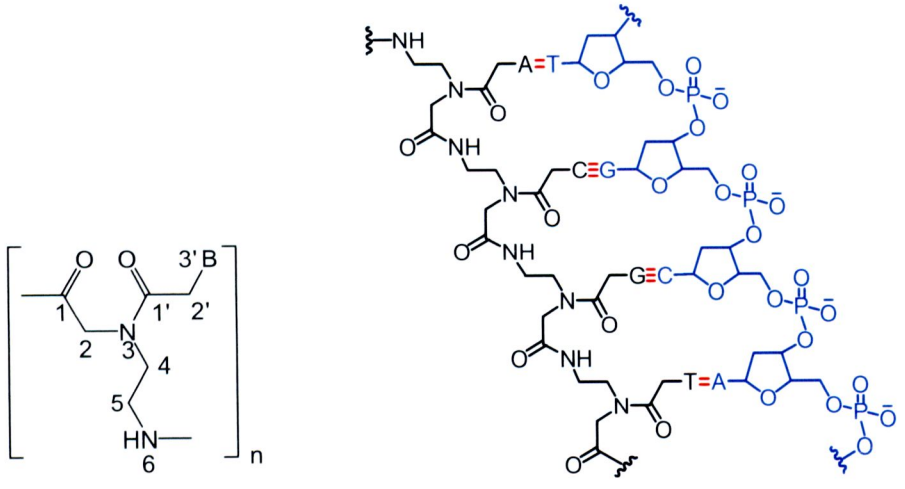


Figure 5 Chemical structures of an *aeg*PNA molecule and the hybridization of *aeg*PNAs with DNAs

PNAs sequences are written like peptides, with PNA's end terminal designated by N- and C-terminal. The orientation in which the N-terminus binds to the 5'-end of DNA designated as parallel, while the N-terminus binds to the 3'-end of DNA designated as antiparallel. Surprisingly, PNA oligonucleotides can hybridize with complementary DNA strands to form complexes according to the Watson-Crick

rules of hydrogen bond mediated base pair formation [7]. The hybrid duplexes formed by PNA with DNA generally have higher thermal stabilities than their duplex DNA counterparts and show unique ionic strength effects because the PNA strand does not bear negatively charged phosphate groups. Furthermore, PNA is resistant to biological degradation and can bind complementary RNA or DNA sequences with extraordinary high affinity and specificity that unlike other DNA-binding peptides or proteins.

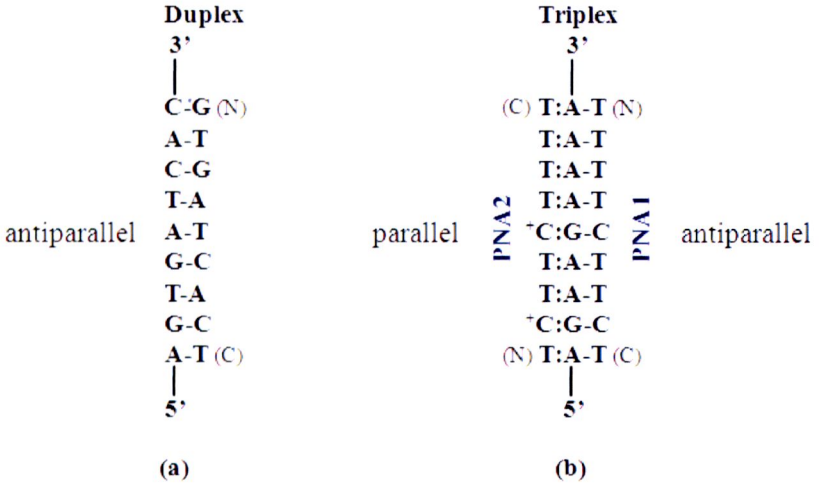


Figure 6 Schematic representations: (a) PNA-DNA duplex in the antiparallel mode (3'-end of the DNA facing the amino-terminal of the PNA); (b) 2PNA/DNA triplex in the preferred binding mode with antiparallel Watson-Crick strand and parallel Hoogsteen strand [8]

The *aeg*PNA can bind to its complementary nucleic acid in both parallel and antiparallel formation (Figure 6) [8]. However, antiparallel PNA·DNA hybrids are considerably more stable than the corresponding DNA·DNA hybrids. Another remarkable property of PNAs is their ability to recognize sequences with in duplex DNA to form triple helices either by triple helix formation or a unique strand invasion mechanism which results in displacement of one of the DNA strand to from the D-loop or P-loop structures as shown in Figure 7 [9].

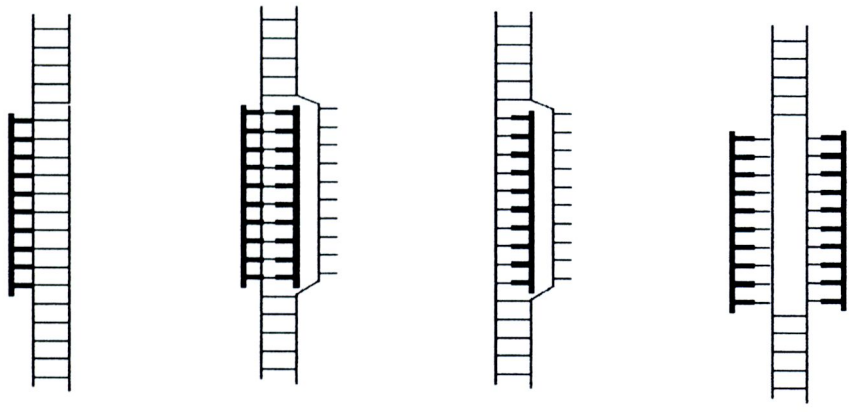


Figure 7 Schematics of *aeg*PNA binding mode for double stranded DNA [9]

In the triplex invasion complex of PNA_2·DNA, the first PNA strand bind with DNA in antiparallel orientation resulting from Watson-Crick base pairing, the second PNA strand binds the PNA·DNA duplex *via* Hoogsteen hydrogen bonding in parallel direction to the DNA strand (Figure 8). It also shows specific hydrogen bonding between amide N-H of the PNA backbone and the phosphate oxygen of the DNA backbone thereby further contributing to the high stability. The hybrids between PNA with DNA or RNA are more stable than the corresponding DNA·DNA or DNA·RNA complexes. The thermal stability follows the order PNA·PNA > PNA·RNA > PNA·DNA (> RNA·DNA > DNA·DNA) [10].

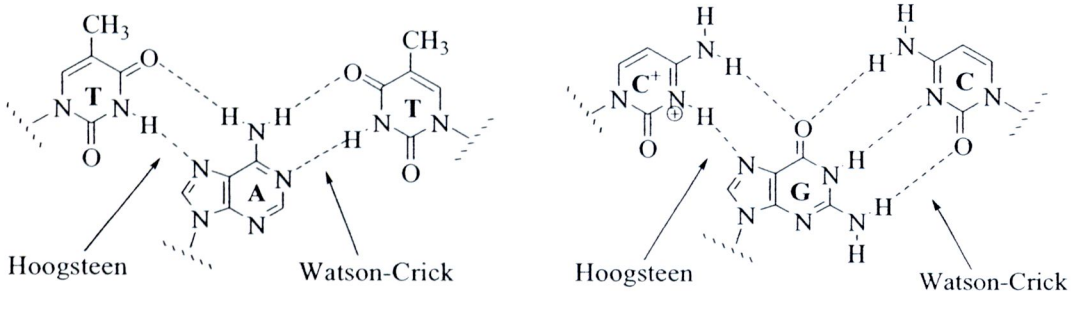


Figure 8 Hydrogen bonding *via* Watson-Crick and Hoogsteen base pairing

The three dimensional structure of four PNA complexes have been determined the structure and physical properties of PNA and its hybrids with nucleic acids by physical techniques, including high-resolution NMR and X-ray crystallography. Three-dimensional structures of duplex structures of PNA·RNA [11] and PNA·DNA [12] duplex were solved by NMR, and PNA_2·RNA triplex [13] and PNA·PNA duplex [14] were also solved by X-ray crystallography as shown in Figure 9. These studies showed that the PNA is flexible enough to adapt itself to form with oligonucleotides partner since the conformation of the RNA strand in the PNA·RNA duplex is essential the A-from; while that of the DNA strand in the PNA·DNA duplex is closer to the B-from [15].

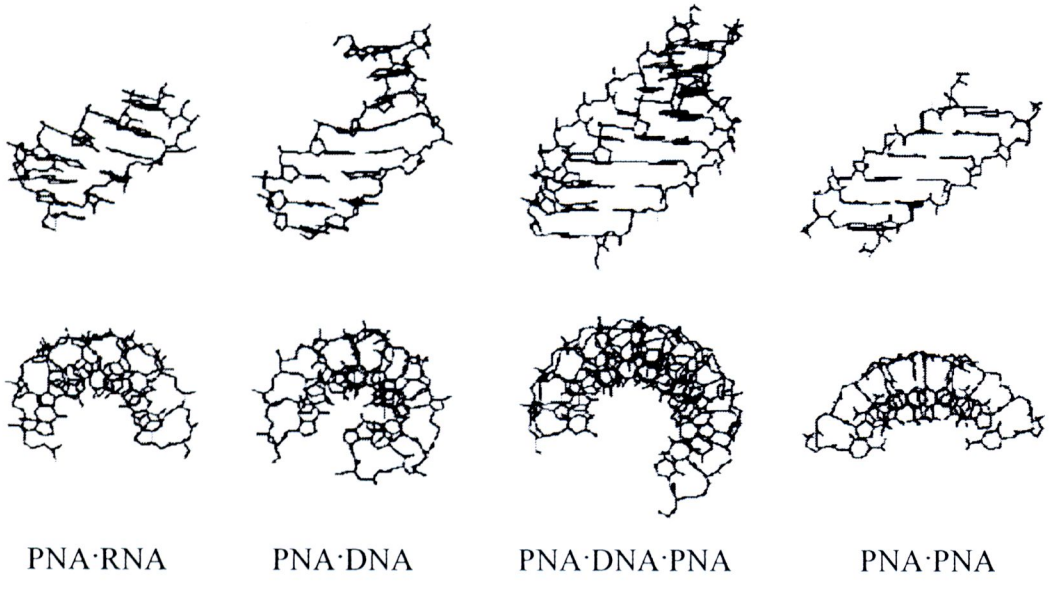


Figure 9 Structure of various PNA complexes shown in side view and top view of PNA·RNA, PNA·DNA, PNA·DNA·PNA and PNA·PNA [16]

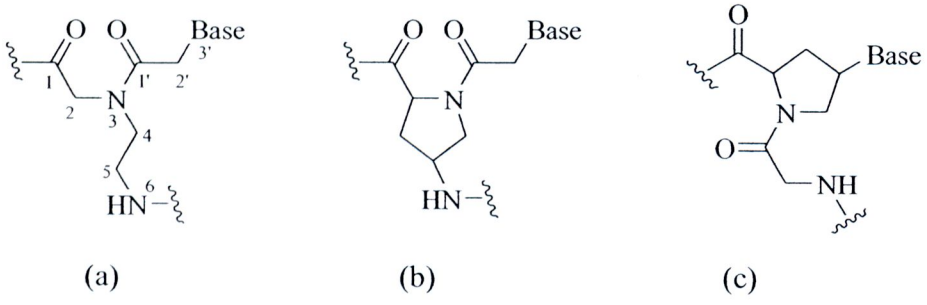
Modification of peptide nucleic acid (PNA)

This original PNA reported by Nielsen [1] shows many important features such as the ability to hybridize with DNA target with high affinity and specificity. Furthermore, PNA are not substrates for common enzymes such as nuclease and protease. These special features of PNA make it potentially useful in antisense and antigene therapeutics. However the main drawbacks of PNA are the rather poor water solubility and poor cellular uptake [2, 3] in addition to the poor discrimination between parallel and antiparallel binding direction. Therefore, extensive works have been focused on structure modification of Nielsen's PNA to overcome these drawbacks [5].

According to thermodynamic consideration, the hybridization of PNA to complementary oligonucleotide caused the enthalpy gain and significant entropy loss due to the conformation restriction of both PNA and DNA as a result of complexation. It can be envisioned that the free energy gain of hybrid formation may be decreased by reducing this energy loss by the formation of a highly ordered and rigid duplex structure from two oligonucleotide strands. The insertion of a rigid structure especially cyclic moiety in the PNA strand has been made to enhance the rigidity of PNA structure. In addition, the presence of chiral residue can have beneficial effect to orientation selectivity in the complementary DNA/RNA binding [10, 17]. For this reason, many research groups have focused on structural preorganization of PNA that introducing a rigid backbone to form a conformationally constrained PNA by inserted a cyclic structure such as morpholine ring, cyclopentane ring, cyclohexane ring and pyrrolidine ring to the structure.

As an example for designing of PNA analogue is based on a methylene group to link between the aminoethylglycyl backbone and the methylene carbonyl side chain of *aeg*PNA (Figure 10a) to form a various five- and six- membered nitrogen heterocyclic analogues [10, 18]. For example, a methylene bridge between the C-5 atom of aminoethyl part and the C-2 atom of the glycine part of the *aeg*PNA to give a rigid pyrrolidine ring with two stereogenic centers resulted in 4-aminopropyl PNAs (Figure 10b) that more than one stereoisomer and each isomer showed unique specific pairing affinity [10, 19]. Homothymine hexamers of three diastereomers (*trans*-D/L, *cis*-L) failed to form any complexes with dA_6. Conversely, the chiral PNAs containing

monosubstitution with prolyl units either at the N-terminus or within the strand of the original PNA are capable to form complexes with complementary DNA in both parallel and antiparallel orientation with higher T_m compared to the original PNA [20]. In another example, a methylene bridge inserted between the C-2 atom of the glycine unit and the C-2' atom of the nucleobase linker of the *aeg*PNA resulted in a novel chiral prolylgylcyl PNA (Figure 10c) [21, 22, 23]. Another interesting proline system of *cis*-L and *trans*-L isomer of both 4-aminoproline and pyrrolidine-2-carboxylic acid derivative carrying nucleobase at C-4 position were studied which could not form the stable complexes [24]. However, these modified PNAs are charge-neutral compounds and hence have poor water solubility compared to DNA that making biological studies difficult [25].



(a) (b) (c)

Figure 10 Structure of modified PNA backbone; (a) aminoethylglycine (*aeg*) PNA, (b) 4-aminoprolyl PNA, (c) prolylglycyl PNA

One of the most interesting design is the so-called aminoethylpropyl (*aep*) PNA developed by Kumar [26] and Vilaivan [27]. In a recent report, a synthesis and binding study of a pyrrolidinyl PNA with an aminoethyl linker has been described. The glycine carbonyl group in the prolylglycyl PNA was replaced with a methylene group to from aminoethylpropyl (*aep*) PNA. This PNA system should make a rather conformationally flexible backbone while the conformation of the side chain is still restricted. Though the increased entropy loss upon hybridization can result from increasing conformational flexibility of the backbone or the decreasing of the binding affinity, this flexibility should allow the *aep*PNA to adopt a wider range of conformations than the prolylglycyl PNA. Moreover, the presence of the positive

charge and the tertiary nitrogen of pyrrolidine ring should attract the negatively charged phosphate group of DNA, providing further stabilization of the hybrid formed with natural DNA. The resulting homothymine oligomer of *aep*PNA showed a strong and specific binding property toward target DNA/RNA sequence [25]. In many other related work, the pyrrolidine ring had been widely used as a rigid component in the PNA structure as shown in Figure 11 [26].

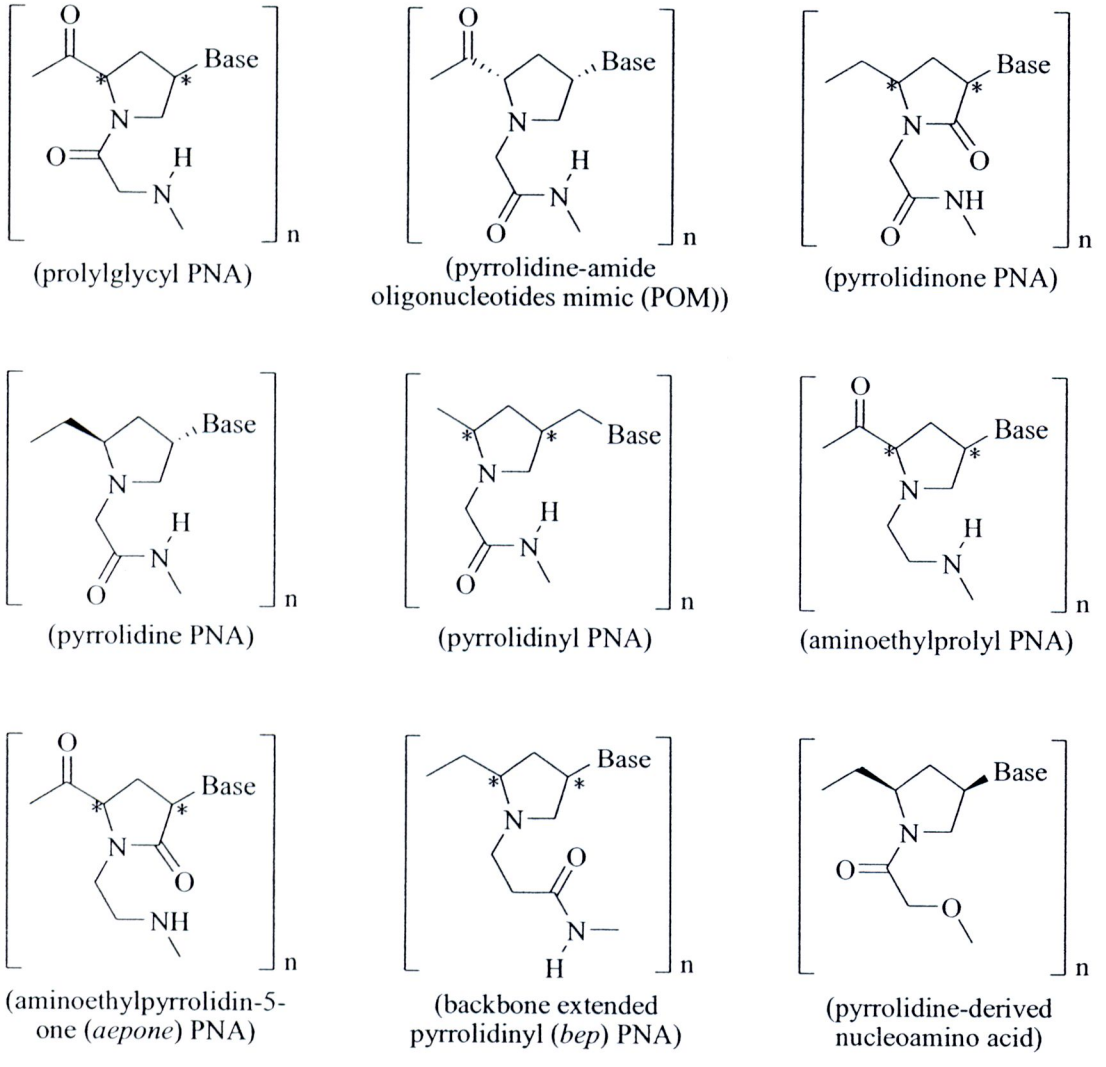
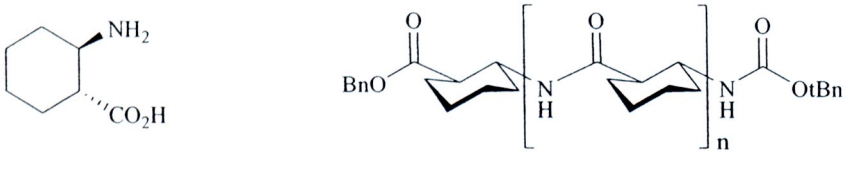


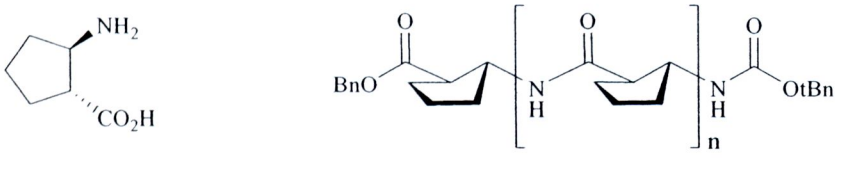
Figure 11 Structure of modified pyrrolidine PNA

In most of the work reported by Kumar group and others, usually only some modified pyrrolidinyl subunits were inserted into the core structure of *aeg*PNA at various positions to form chimeric backbones. The hybridization results of these chimeric PNA with DNA/RNA are not easily interpreted in these cases since the effect on T_m is not only dependent on the structure modification but also to the number and position of the modified residues in the original PNA strand. Consequently comparison and interpretation of these hetero-oligomeric PNA should be done with great care.

During the last decade, Gellman [28, 29] introduced the term of foldamer to describe oligomers with a strong tendency to adopt a specific secondary or tertiary structure particularly of β-amino acids. In a recent report, the conformationally rigid β-peptides such as *trans*-2-aminocyclohexane carboxylic acid (*trans*-ACHC) and *trans*-2-aminocyclopentane carboxylic acid (*trans*-ACPC) (Figure 12) can form stable helical structure in solid state. Their study showed that *trans*-ACHC can form 14-helix and *trans*-ACPC can form 12-helix *via* intramolecular H-bonding between backbone nitrogen and carbonyl groups of amide bond. These features suggested that β-amino acid can be potentially useful to pre-organize PNA oligomer.



(a)



(b)

Figure 12 Structure of monomeric and oligomeric (a) *trans*-2-aminocyclohexane carboxylic acid (*trans*-ACHC) and (b) *trans*-2-aminocyclopentane carboxylic acid (*trans*-ACPC)

Vilaivan and co-worker have reported the synthesis and binding studies of a series of novel modified pyrrolidinyl PNAs bearing various *β*-amino acid spacers. To study the effect of the structure and positive charge on the *β*-amino acid spacer, to designed the new pyrrolidinyl PNA carrying various acyclic and cyclic *β*-amino acid in the backbone and evaluated for their nucleic acid binding properties [30, 31, 32]. For example, a new pyrrolidinyl PNA carrying *N*-amino-*N*-methylglycine spacer (Figure 13a) failed to show binding properties with DNA and RNA [30]. This PNA system reduced the binding affinity with DNA target can be explained by the higher entropy loss upon binding. To minimize such entropy loss, they have developed the novel pyrrolidinyl PNA bearing cyclic *β*-amino acid spacers [31]. The selected spacers include L-aminopyrrolidine-2-carboxylic acid (L-Apc), D-aminopyrrolidine-2-carboxylic acid (D-Apc), (1*R*, 2*S*)-2-aminocyclopentane carboxylic acid (L-Acpc) and *β*-alanine (*β*-ala) as shown in Figure 13. These revealed that the stereochemistry of the spacer of PNA is important for successful hybridization with complementary oligonucleotide. Only the pyrrolidinyl PNA bearing D-aminopyrrolidine-2-carboxylic acid (D-Apc) (*dapc*PNA) but not L-aminopyrrolidine-2-carboxylic acid (L-Apc) spacer showed formed a stable hybrid with its complementary oligonucleotide in antiparallel orientation. The stoichiochemistry studied by UV and CD spectroscopy revealed that only 1:1 PNA·DNA hybrid was formed. Furthermore, the *dapc*PNA system appeared to form more stable hybrid to DNA over RNA [30, 32].

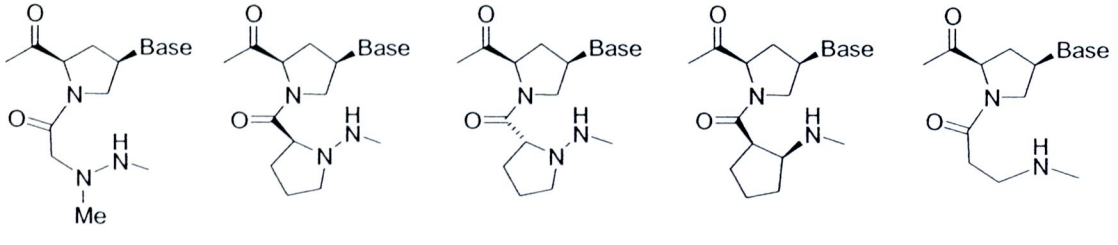


Figure 13 Structure of modified PNA carrying various *β*-amino acid spacers; (a) *N*-amino-*N*-methylglycine, (b) L-aminopyrrolidine-2-carboxylic acid (L-Apc), (c) D-aminopyrrolidine-2-carboxylic acid (D-Apc), (d) (1*R*, 2*S*)-2-aminocyclopentane carboxylic acid (L-Acpc) (e) *β*-alanine (*β*-ala)

Later in 2005 Vilaivan, et al. [27] has developed the pyrrolidinyl PNA bearing a (1*S*, 2*S*)-2-aminocyclopentane carboxylic acid (ACPC) backbone. Study to the DNA hybridization ability of the *acpc*PNA, assessed by melting temperature (T_m) and circular dichroism (CD) spectroscopy analysis, suggested that this PNA system showed a strong binding affinity and high sequence specificity towards its complementary DNA target and only form 1:1 hybrid similar to *dapc*PNA. The melting temperature (T_m) value of a hybrid between the PNA homothymine decamer and its complementary DNA is extremely high (>85 °C). This indicates that the hybrid is far more stable than natural DNA·DNA and other PNA·DNA hybrids including the original PNA and *dapc*PNA [5, 33, 34]. Furthermore, the *acpc*PNA showed a much stronger preference for the antiparallel binding mode. The high binding affinity to complementary DNA and the powerful discrimination for single mismatched DNA, together with the high directional specificity, render the new *acpc*PNA system a potential candidate for the development of a highly effective DNA sensor.

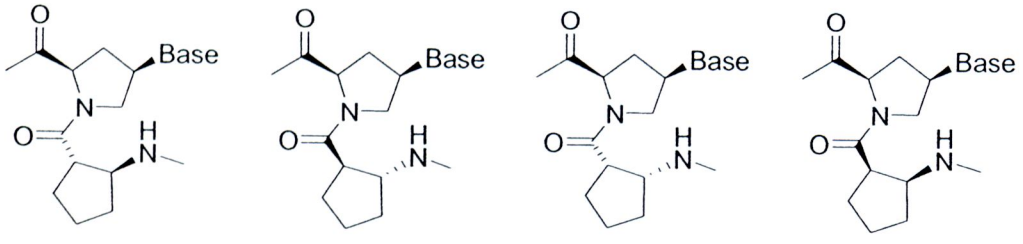
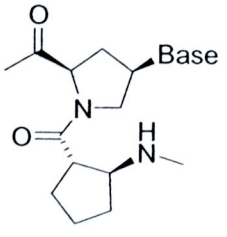


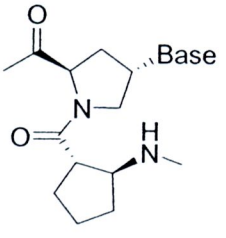
Figure 14 Structure of pyrrolidinyl PNA carrying 2-aminocyclopentane carboxylic acid (ACPC) spacers

Recently, Vilaivan and his collaborators have reported a new pyrrolidinyl peptide nucleic acid (PNA) comprising of a sequence of the nucleobase-modified proline at the 4' position with (2'*R*,4'*S*) configuration and a (1*S*,2*S*)-2-aminocyclopentanecarboxylic acid ((2'*R*,4'*S*)-*acpc*PNA) backbone as shown in Figure 15 [35]. In the presence of complementary DNA, both PNAs form very stable PNA·DNA heteroduplexes with T_m values of 77.0 °C (2'*R*,4'*R*) and 78.6 °C (2'*R*,4'*S*), respectively. This work suggests that the (2'*R*,4'*S*)-*acpc*PNA could substitute (2'*R*,4'*R*)-

*acpc*PNA as probes for nucleic acid detection due to their similar DNA- and RNA-binding properties. The surprise to modulate the self-pairing behaviour of the new (2'*R*,4'*S*)-acpcPNA forms a more stable antiparallel self-hybrid than (2'*R*,4'*R*)-*acpc*PNA. These features suggested should further expand the potential scope of applications of these pyrrolidinyl PNAs. Although the inability to form a self-pairing hybrid of the original (2'*R*,4'*R*)-*acpc*PNA system is highly desirable for certain applications such as DNA targeting.



(2'*R*,4'*R*)-*acpc*PNA



(2'*R*,4'*S*)-acpcPNA

Figure 15 Structure of two configurations of pyrrolidinyl PNA carrying 2-aminocyclopentane carboxylic acid (ACPC) spacers

Application of peptide nucleic acid (PNA) as probes in nucleic acid sequence determination

Sequence-specific nucleic acid detection is critical for many medicinal and diagnostic applications. In the past, DNA probe was primarily use as nucleic biosensors for DNA sequence analysis. Recently, PNA probes are interest tools for nucleic acid detection that PNAs offer some advantages over DNA which can bind with natural nucleic acid through Watson-Crick base pairing with high affinity, specificity and accuracy in the detection of complementary target sequence in a complex nucleic acid mixture. Especially, PNA oligomers are not enzymatically degraded and do not require high salt concentration for binding. A large number of PNA research as a probe to detect DNA base sequence has been demonstrated most of which rely on the use of Nielsen's PNA in conjunction with optical or different characterizing techniques such as optical (mainly on fluorescence label analysis), electrochemical or mass-sensitive transducer. PNA can also labeled and used as a tag,

encoding the structure of the attached molecule, by a defined base sequence. For instance, Zare, et al. [36] presented a colorimetric method in conjunction with PNA to screen for genetic mutation by employing a cyanine dye. This method based on the color change of dye from blue to purple upon binding to PNA·DNA duplexes. The color changing is used to visualize formation/dissociation of hybrid duplex [36].

In 2003, Komiyama and co-worker has presented the remarkable decreasing in stability in the presence of single-base mismatch in PNA·DNA duplexes makes PNA a very useful tool for single nucleotide polymorphisms (SNP) detection. The combination of PNA and S1 nuclease can differentiate single base alteration in DNA target with remarkable specificity. The mixture after the enzymatic digestion was stained by cationic dye namely 3,3'-diethythiadicardocyanine which showed different color upon binding to single stranded PNA and its hybrid with DNA. The mismatched PNA·DNA duplexes are hydrolyzed by S1 while the perfectly-matching hybrids remain intact. When the cationic dye was added, the solution show a purple color for the DNA sample which is complementary with PNA probe, whereas the presence of single base mismatch DNA remains blue color as shown in Figure 16 [37, 38].

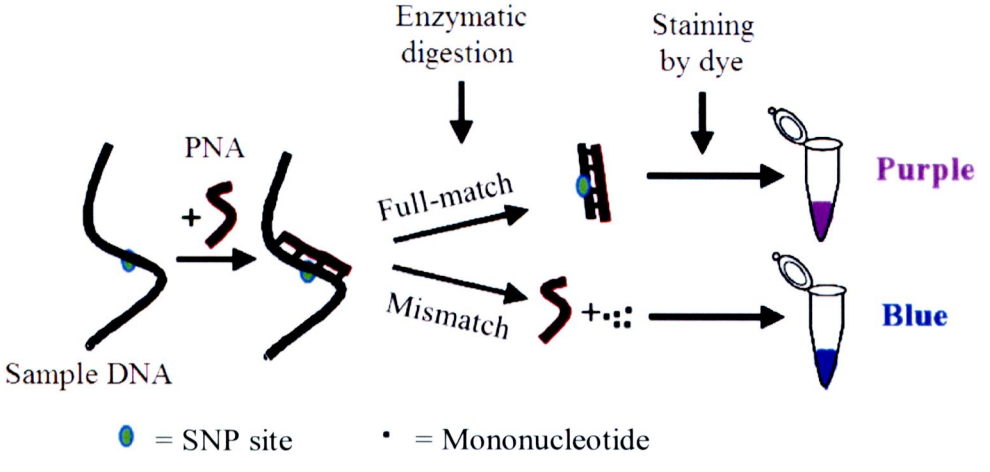


Figure 16 Strategy for SNP detection by the PNA/Nuclease/Dye system

Recently, Svanvik and co-worker [39] reported PNA oligomers incorporating the cyanine dyes thiazole orange (TO) as a probe for DNA sequence determination. This technique focused on the fluorescence enchancement of TO upon binding to complementary DNA. Mixed sequence probes are expected to form PNA–DNA duplexes while homopyrimidine probes should form PNA_2–DNA triplexes. Further, PNA–DNA duplexes can form both in parallel and in antiparallel orientation, and PNA_2–DNA triplexes can form with the two PNA strands either parallel or antiparallel to the DNA. When the PNA hybridizes to DNA target, a strong fluorescent is generated whereas the unhybridized probe showed a low fluorescent signal as illustrated in Figure 17.

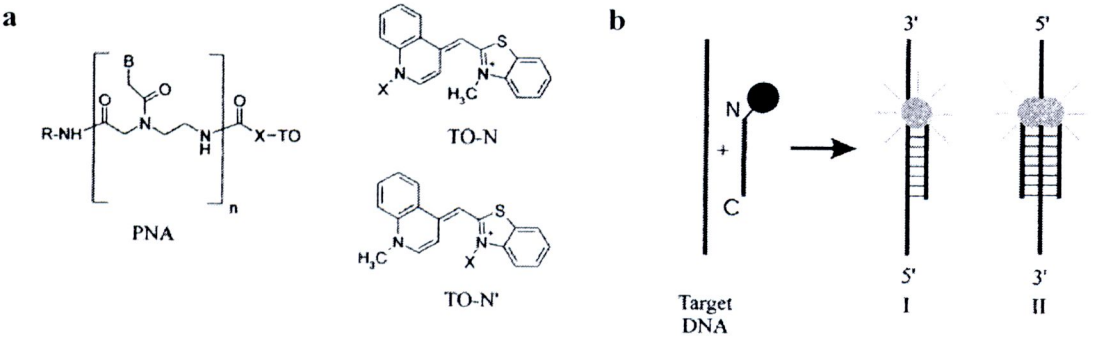
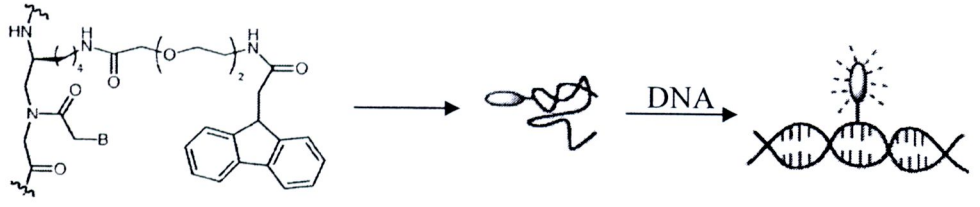


Figure 17 Strategy for nucleic acid detection *via* light-up probe (a) chemical structure of light-up probes (b) schematic showing how mixed sequence (I) and homopyrimidine (II) light-up probes may interact with single-stranded target nucleic acid.

Another example of fluorescent labeled PNAs for nucleic sequence analysis was reported by Appella, et al. [4, 40]. The single-fluorophore-labelled PNA probes (Figure 18) was synthesized base on separation of the fluorophore and the nucleobase which acted as quencher and to cross a restricted to fluorophore attachment to the ends of the PNA. The probe exhibited a fluorescent signal only in the presence of the target oligonucleotide. The modified PNA probe showed comparable thermal stability to the corresponding *aeg*PNA oligomer with DNA.



γ-Lysine PNA/Fluorene Fluorescent Probe

Figure 18 Structure and fluorescence of the single-fluorophore-labelled PNA is observed upon duplex formation

On the other hand, Boontha and co-worker [41] have reported the pyrrolidinyl PNA carrying (1*S*,2*S*)-2-aminocyclopentanecarboxylic acid (*acpc*PNA) was used for DNA sequence analysis by a novel mass spectrometric technique (Figure 19). The sequence of the DNA sample was determine by the selective adsorption of a negatively charged PNA·DNA hybrid by an anion exchanger. Since PNA is a neutral molecule, it cannot be absorbed by the anion exchanger unless it is hybridized with a negatively-charged complementary DNA. The adsorbed hybrid could be isolated from the remaining unhybridized PNA by simple washing and analyzed for the signal of PNA using MALDI-TOF mass spectrometry technique.

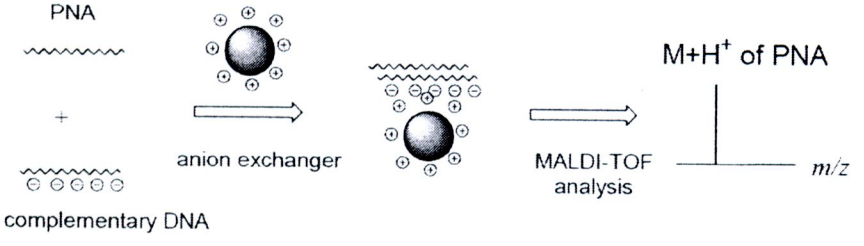
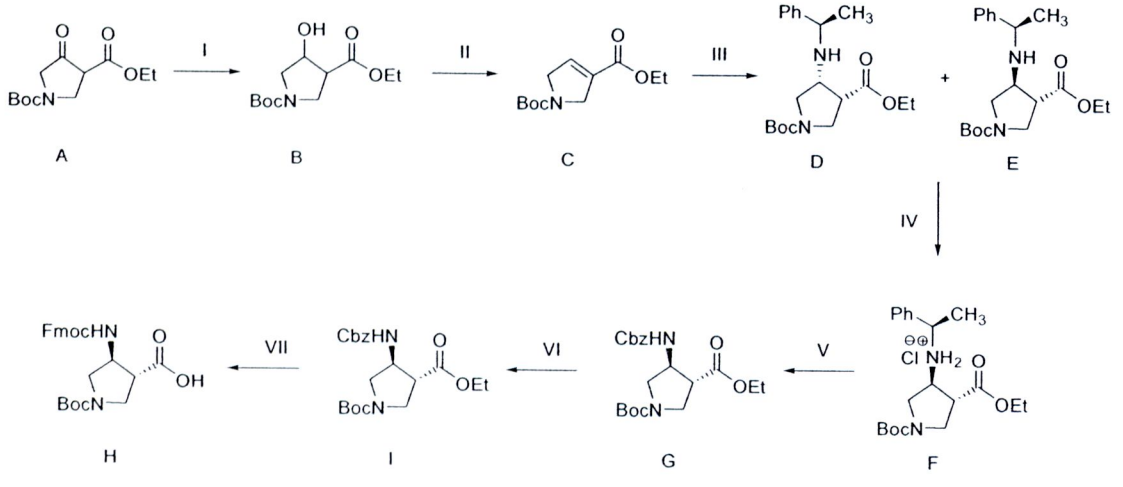


Figure 19 The *acpc*PNA was used for DNA sequence analysis by a new ion-exchange capture technique

Synthesis of *trans*-3-aminopyrrolidine-4-carboxylic acid; alternative spacer for probe application

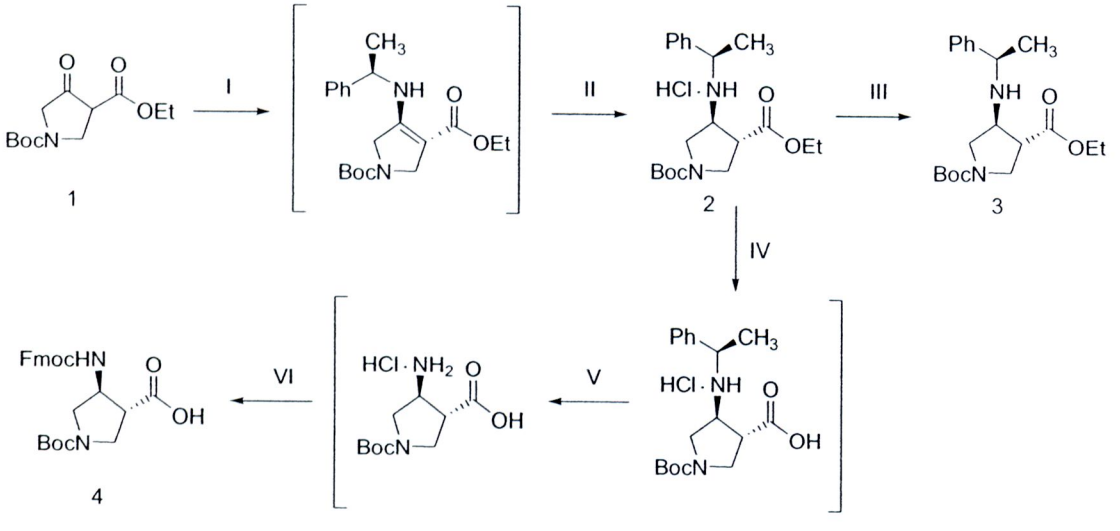
The synthesis of enantiomerically *trans*-3-aminopyrrolidine-4-carboxylic acid (APC) in a protected form reported by Gellman and co-worker in 2000 [42]. The synthesis route started from the known β-ketoester [43]. The key step is reduction followed by elimination produced the α,β-unsaturated ester and Michael addition of enantiomerically pure α-methylbenzylamine then yielded a mixture of the four diastereomeric β-aminoesters as show in Figure 20. The desired isomer was isolated in 13% yield after tedious column chromatography.



Reagent and Condition: (I) $NaBH_3CN$, HCl/MeOH (II) Ph_3P/DEAD, toluene, N_2, r.t., 12 h (III) (*R*)-(+)-methylbenzylamine, water, 55 °C, 67 h (IV) 1) silica gel chromatography 2) HCl/Dioxane (1.5 eq.), EtOAc (V) 1) 10% Pd/C, H_2, 95% EtOH 2) Cbz-OSu, $NaHCO_3$, acetone/H_2O (VI) $LiOH \cdot H_2O$, MeOH/H_2O, 0°C, 15 h (VII) 1) 5% Pd/C, H_2, MeOH 2) Fmoc-OSu, $NaHCO_3$, acetone/H_2O

Figure 20 The synthetic scheme of a protected version of 3-aminopyrrolidine-4-carboxylic acid

The new route of synthesis of 3-aminopyrrolidine-4-carboxylic acid was developed by Gellman and co-worker in 2001 (Figure 21) [44]. The synthetic has been streamlined by reducing the number of chemical operations and by eliminating the need for chromatographic separations. The synthesis route started from β-ketoester is reacted with (*R*)-α-methylbenzylamine in the presence of acetic acid and the resulting enamine is reduced with $NaBH_3CN$. This reduction produces a mixture of four diastereomeric β-aminoesters in which compound 3 is the major product follow by recrystallization protocal allows isolation of hydrochloride salt in diastereomerically pure form. The desired *trans*-(3*S*,4*R*)-configuration was afforded and did the protecting group exchange to get the protected spacers (3*S*,4*R*)-*trans*-3-aminopyrrolidine-4-carboxylic acid. The enantiomer of (3*S*,4*R*)-*trans*-3-amino-pyrrolidine-4-carboxylic acid was prepared by starting with (*S*)-(-)-α-methylbenzylamine.



Reagent and Condition: (I) (*R*)-(+)-methylbenzylamine, AcOH, EtOH, rt (II) $NaBH_3CN$, 75°C and 4 N HCl in EtOAc 0°C (III) saturatated Na_2CO_3, EtOAc (IV) $LiOH \cdot H_2O$, $THF/CH_3OH/H_2O$, 0°C (V) H_2, 10% Pd/C, 95% EtOH (VI) 1) Fmoc-OSu, $NaHCO_3$, $acetone/H_2O$ 0°C to rt

Figure 21 The new route of synthesis of 3-aminopyrrolidine-4-carboxylic acid