CHAPTER I

INTRODUCTION

1.1 Peptide Nucleic Acids and potential as DNA mimic

Since 1953, Watson and Crick revealed the first proposal of the rules for base pairing of adenine (A) with thymine (T) and cytosine (C) with guanine (G) of two stranded, double-helical model for DNA as known as Watson-Crick base pairing rules [1]. This discovery leads to the revolution of molecular biological and chemical study of the DNA. The key factor of the core structure of this helix is Watson-Crick hydrogen bonding which held single stranded together and further stabilized by π-π interaction and hydrophobic interaction, the so called π-π or base stacking. Nucleobases have to bury themselves into the center of folded structure as to avoid water and negative charge phosphate groups lie on the outside of the double helix. The structure of double helical DNA and Watson-Crick base pairing is shown in **Figure 1.1**.

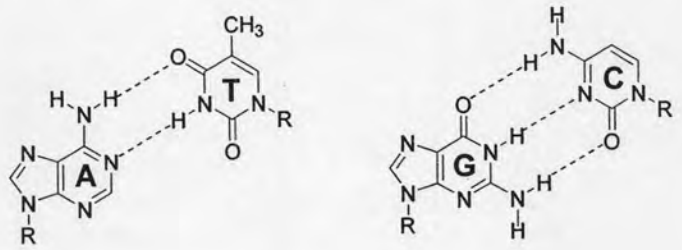


Figure 1.1 Nucleobase pair recognition by Watson-Crick hydrogen bonding and R = ribose or deoxyribose.

The ability of nucleic acid to form double helical hybrid with their complementary strand is the key for biological function such as storage and replication of the genetic information.

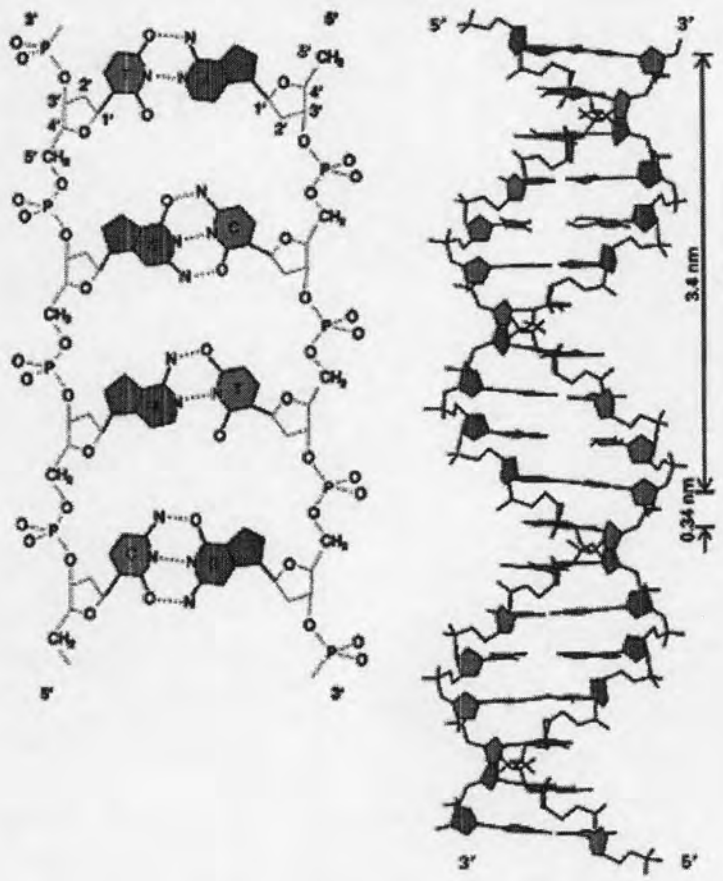


Figure 1.2 Secondary structure of DNA [2].

In the cell, the expression of genetic information consists of 2-stage process. In the first stage, a DNA strand served the template for the synthesis of the complementary strand of ribonucleic acid called transcription. In the second stage, the RNA's sequence bases prescribe the order of amino acid linked with ribosomes known as translation to specify protein structure and function. Since ribosomes cannot translate double-stranded RNA, the translation of a given mRNA can be inhibited by a segment of its complementary sequence, the so-called anti-sense RNA. The requirements for effective antisense oligonucleotide are the stability towards endo and exo-nuclease enzymes. In addition, it must bind to the target mRNA with sufficient affinity and high specificity including the ability to pass the cell membrane. In the similar concept to antisense, antigene oligonucleotide is targeted to the complementary DNA sequence to undertake the origin of problem. The concepts of antisense and antigene are illustrated in **Figure 1.3**.

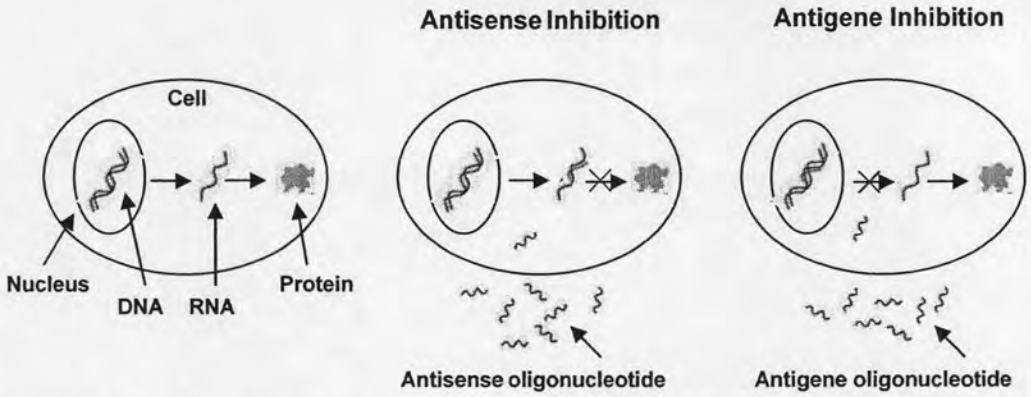


Figure 1.3 Principle of operation of antisense and antigene oligonucleotides [Ref].

The antisense strategy can be potentially used in therapeutics [3-5]. Modification of oligonucleotide for using in antisense applications attracted interests from many research groups. Originally, chemical modification of natural DNA by replacing the phosphodiester linkage with other atoms have been attempted [6-8]. (**Figure 1.4**). Of these, a few of modified structures bind to their complementary DNA/RNA well enough to deserve further interest. However, a majority of them do not form hybrid with DNA/RNA.

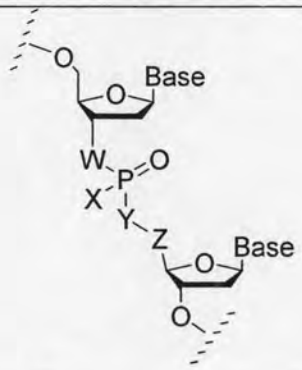
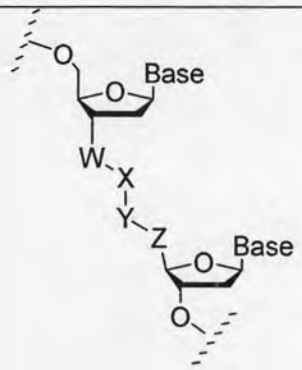
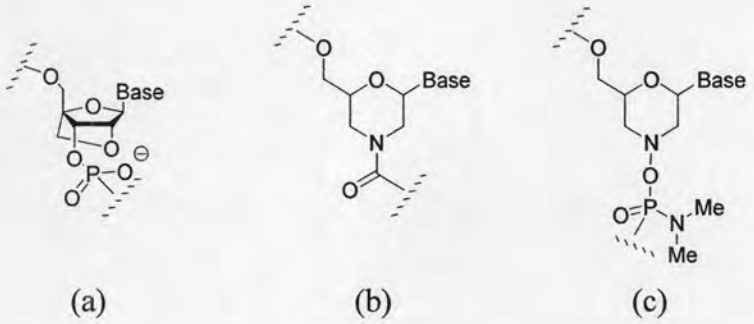
Structure	W	X	Y	Z	Type of modification
	O	O^-	O	CH_2	natural DNA
	O	S^-	O	CH_2	phosphorothioate
	O	CH_3	O	O	methylphosphonate
	NH	O^-	O	O	phosphoroamidate
	CH_2	O^-	O	O	methylene phosphonate
	O	OEt	O	O	Ethyl phosphotriester
	CH_2	CO	NR	CH_2	amide
	S	CH_2	O	CH_2	thioformacetal
	CH_2	NMe	O	CH_2	hydroxylamine
	NH	CH_2	CH_2	CH_2	amine
	NR	CO	NR	CH_2	urea
	O	SO_2	NH	CH_2	sulfamate

Figure 1.4 Examples of modified oligonucleotides.

Recently, a conformationally locked nucleic acids (LNA) introduced by Wengel *et al.* [9-10] which the furanose ring in LNA being part of dioxabicyclo [2.2.1]heptane skeleton is locked was reported (**Figure 1.5a**). The LNA exhibited unprecedented binding affinity towards complementary DNA and RNA. It is also stable to 3′-exonucleolytic degradation and possesses good water solubility. This LNA appears to be currently the most promising candidate for antisense therapy. However, further biochemical studied is required for this structure to evaluate in the antisense/antigene strategy.

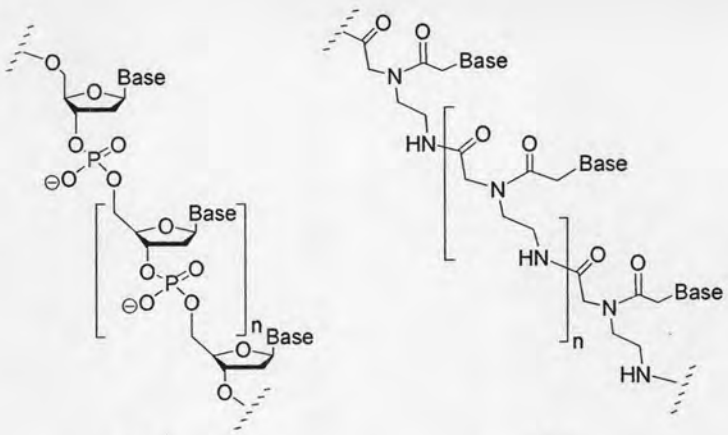


(a) (b) (c)

Figure 1.5 Structure of (a) locked nucleic acid (LNA), (b) and (c) morpholine oligomers.

Recently, the morpholine-modified DNA with carbamate linkages or phosphoramidate linkages (**Figure 1.5b** and **Figure 1.5c**) showed promising antisense activity [7,11].

A new class of DNA analogue, in which the whole sugar phosphate backbone was replaced by a polyamide backbone, was reported by Nielsen in 1999 [12]. The so-called peptide nucleic acid (PNA) consisted of *N*-(2-aminoethyl)glycine (*aeg*) unit which nucleobase are connected to the amino nitrogen *via* a methylene carbonyl linker (**Figure 1.6**). This resulted in an uncharged and achiral mimic of DNA with several remarkable properties.



DNA *aeg*PNA

Figure 1.6 Chemical structures of a DNA molecule and an *aeg*PNA molecule.

PNA forms hybrids with high thermal stability with its complementary DNA/RNA. The hybrids are much more stable than the corresponding DNA·DNA hybrid. The high stability of PNA·DNA/RNA hybrid is attributed to the Watson-Crick base pairing [13], uncharged nature of the PNA backbone and conformational flexibility to allow binding to either DNA or RNA. PNA also binds to double-stranded DNA not by normal triple helix formation, but by a unique strand triplex invasion displacing DNA forming a D-loop or P-loop structure [14] (**Figure 1.7**).

triplex triplex invasion duplex invasion double duplex invasion

Figure 1.7 Schematic of *aeg*PNA binding mode for double stranded DNA [14].

In the triplex invasion complex, the second strand of PNA was bound by a Hoogsteen base-pairing (**Figure 1.8**). It also shows specific interactions (H-bonding) between each amide N-H of the backbone of the Hoogsteen PNA strand and the phosphate oxygen of the DNA backbone thereby also contributing to the high stability. The complexes 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) [15].

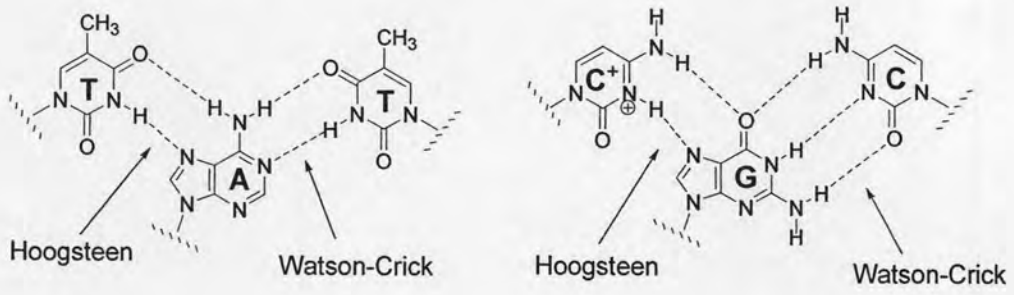


Figure 1.8 Hydrogen bonding *via* Watson-Crick and Hoogsteen base pairing.

A number of research effort focused on the structure and physical properties of PNA and its hybrids with nucleic acids using physical techniques, including high-resolution NMR and X-ray crystallography. Three-dimensional structures of duplex structures of PNA·RNA [16] and PNA·DNA [17] duplex, were solved by NMR, and PNA_2·DNA triplex [18] and PNA·PNA duplex [19] were also solved by X-ray crystallography as shown in **Figure 1.9**.

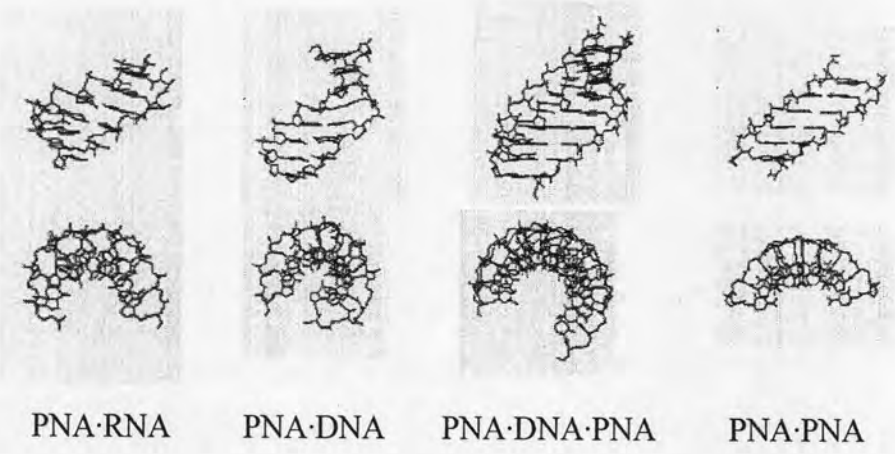


Figure 1.9 Structure of various PNA complexes shown in side view and top view of PNA·RNA, PNA·DNA, PNA·DNA·PNA and PNA·PNA.

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 [20]. As a peptide, PNA's end terminal designated by *N*- and *C*-terminal. The orientation in which the *N*-terminal binds to the 5′-end of DNA designated as parallel, while the *N*-terminal binds to the 3′-end of DNA designated as antiparallel (**Figure 1.10**).

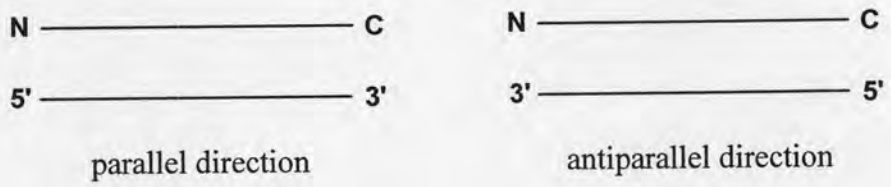


Figure 1.10 The direction of PNA and DNA as parallel and antiparallel.

The *aeg*PNA is known to bind with DNA in both parallel and antiparallel directions [21]. However, the binding in antiparallel orientation has a marginally higher stability. In case of PNA_2·DNA triplexes, the first PNA strand binds with DNA in antiparallel orientation resulting from Watson-Crick base pairing, and the second PNA strand binds the PNA·DNA duplex *via* Hoogsteen hydrogen bonding in parallel direction [22].

1.2 Modified PNA : Variation of structural backbone

In last decades, PNA molecule became a popular topic due to their several unique properties. The ability of PNA to form the stable hybrid with DNA makes it potentially useful in antisense/antigene therapeutics. However the main drawbacks of this molecule are the relatively poor water solubility [23] and poor cellular uptake. [24] Moreover, the mixed purine/pyrimidine PNA oligomers can bind to both parallel and antiparallel DNA target sequences with the antiparallel binding mode only slightly preferred over the parallel one. Consequently, various attempts by many research groups have been made to modify the structure of parent *aeg*PNA to improve the binding, direction selectivity, base specificity, water solubility and cellular uptake. The original PNA was modified in the first stage without touching the basic structure by extending the linker such as aminoethyl was replaced by aminopropyl, glycine unit replaced by β-alanine, acetyl group replaced by propionyl group [25]. The nucleobase

linker was also modified by reducing the amide linker [26] or even reversing the amide bond direction within the backbone as known as retro-inverso PNA. This resulted in interchanging of hydrogen bond donors and acceptors of the amide groups in the backbone, while retaining the number of bonds between the nucleobases as in **Figure 1.11** [27]. Unfortunately, when the modified structure was incorporated into the oligomer PNA sequences, most of these modification destabilize the PNA·DNA hybrids (ΔT_m of -0.7 to -22 °C compared to *aeg*PNA) [15]. The stereochemical issues were investigated by changing the non-functional glycine for other natural amino acids such as lysine, alanine, serine, glutamic acid or aspartic acid [29]. These modifications provided only little change in binding properties with DNA targets (ΔT_m of -3.3 to +1.0 °C compared to *aeg*PNA).

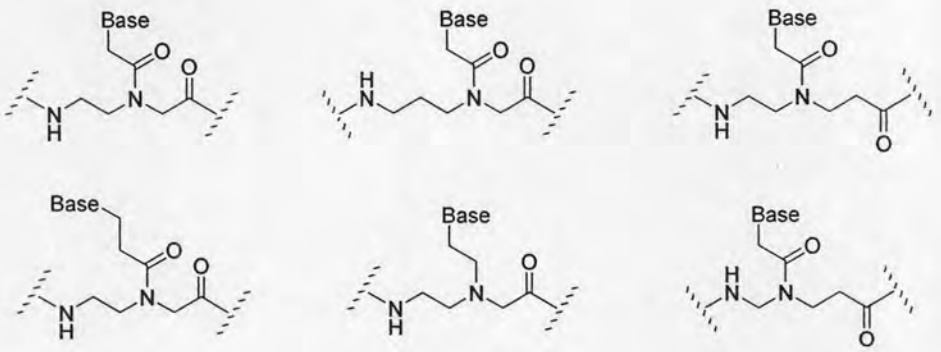


Figure 1.11 Modified PNA at the linker of *aeg*PNA.

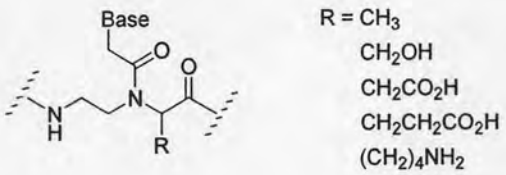


Figure 1.12 Modified PNA by replacing glycine unit with other amino acid.

The next strategy to modify the structure of PNA is considered based on thermodynamic consideration. Hybridization of complementary oligomers is characterized by a large enthalpy and entropy loss. The formation of a highly ordered and rigid duplex structure from two flexible and less ordered strands resulting to the decrease in entropy. In order to reduce this value, the locked conformation and rigid structure were introduced to the core structure of *aeg*PNA. The aminoethyl segment

of the *aeg*PNA was replaced with a 1,2-diaminocyclohexyl moiety in the (*S*,*S*) and (*R*,*R*) form generated the cyclohexyl PNA (*ch*PNA) (**Figure 1.13a**) [30].

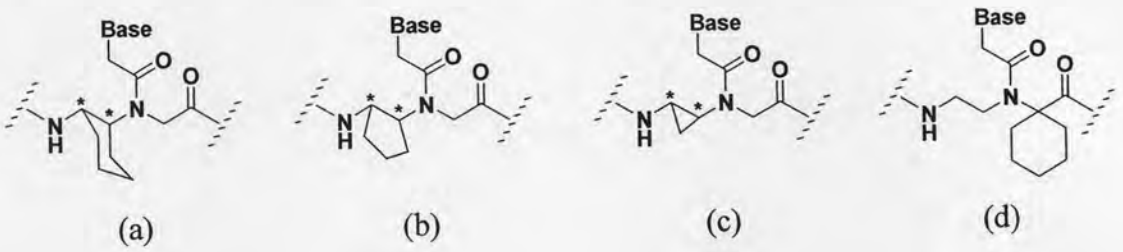


Figure 1.13 The structure of (a) aminocyclohexyl PNA (b) aminocyclopentyl PNA (c) cyclopropane PNA and (d) [(aminoethyl)amino]cyclohexanoic acid PNA.

The conformational flexible ethylene chain was locked in the six-membered cyclic structure and the thermodynamic measurement indeed indicated a decrease in entropy because of conformationally constrained structure. However, the PNA oligomers with (*S*,*S*)-cyclohexyl residues hybridize with DNA or RNA with variable stability depending on their number and the sequence [30]. In 2004, isomers of *ch*PNA (1*S*,2*R*/1*R*,2*S*) was reported by Govindaraju to show an interesting preference for binding to RNA over DNA [31-32]. A related (1*S*,2*R*/1*R*,2*S*) aminocyclopentanyl PNA (*cp*PNA) (**Figure 1.13b**) binds to both RNA and DNA with higher affinity compare to *aeg*PNA and *ch*PNA. The (*S*,*S*)-*trans* cyclopropane PNA (**Figure 1.13c**) [33] was synthesized with a hypothesis that this PNA system should have access to only the range of dihedral angles which are more compatible in Hoogsteen strand of a $(PNA)_2$·DNA triplex than in the Watson-Crick strand. Furthermore, another way to incorporate a cyclohexyl ring in the PNA strand was performed out by replacing the glycyl unit with α-aminocyclohexanoic acid (**Figure 1.13d**) [34]. The synthesis of this PNA monomer was accomplished by an interesting four-component Ugi reaction. However, incorporation of these monomers into oligomeric structures and their DNA/RNA binding properties has not yet been reported.

1.3 *β*-Amino acid : Helical pre-organization

The term "foldamer" was initially introduced by Gellman in 1996, [35] to describe any synthetic oligomers having a powerful tendency to accommodate a specific high-order conformation. The important criteria necessary for creating effective foldamers is capable of backbone to form appropriate structure, and the foldamers should have interesting and chemical useful function and the last, these synthetic molecules should be prepared in an efficient manner. There are a number of unnatural oligomers that adopt well-defined conformation similar to those displayed by proteins and RNA but especially focusing on *β*-peptides [36]. The model of poly(*β*-alanine) studied by Gellman [37] showed six types available of helical form as shown in **Figure 1.14**.

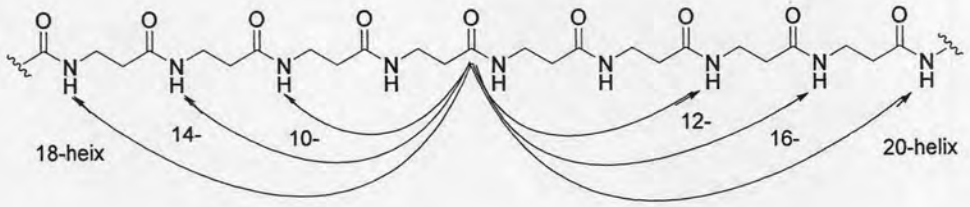
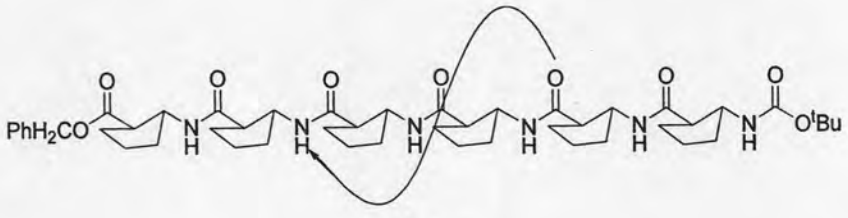


Figure 1.14 Hydrogen bonds defining the six narrowest helices available to a poly-*β*-alanine backbone. The helix designations are based on the number of atoms in the hydrogen-bonded ring.

Appella, *et al* [38] reported that (1*R*,2*R*)-*trans*-2-aminocyclopentanecarboxylic acid (*trans*-ACPC) oligomers adopted the helical secondary structure by a series of interlocking 12-membered ring hydrogen bonds between backbone amide groups confirmed by crystallographic and CD spectra as **Figure 1.15**.



(a)

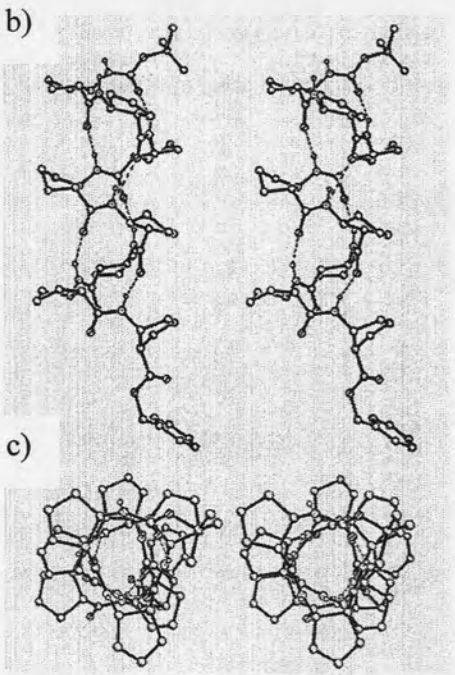
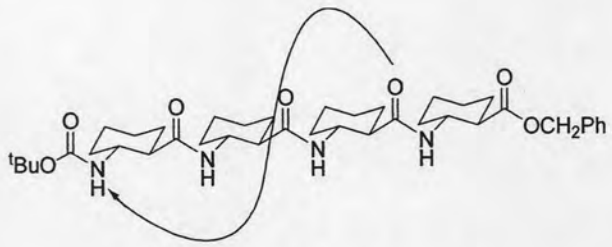
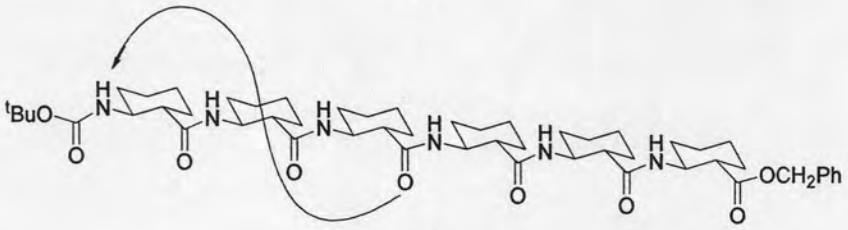


Figure 1.15 (a) Schematic view of the intramolecular 12-helix hydrogen-bonding patterns in *trans*-ACPC hexamer crystals. Solid-state structure of *trans*-ACPC octamer from two perspectives: (b) perpendicular to the helix axis and (c) along the helix axis [38].



(a)



(b)

Figure 1.16 Schematic view of 14-helix intramolecular hydrogen bonding pattern in the crystal of (a) tetramer of (1*R*,2*R*)-ACPC (b) hexamer of (1*S*,2*S*)-ACPC.

The same behavior in oligomers of *trans*-2-aminocyclohexanecarboxylic acid (*trans*-ACHC) was also demonstrated by Gellman and co-workers [39]. They reported that *trans*-ACHC in form of (1*R*,2*R*)-tetramer and (1*S*,2*S*)-hexamer display a helical conformation that involves 14-membered ring hydrogen as shown in **Figure 1.16**. The models of these two oligomers predicted that, unlike α-peptides, the hydrogen bonds pointed in opposite direction between *trans*-ACHC and *trans*-ACPC, relative to the termini of the oligomers.

The structures of these foldamers were also studied in solution using CD spectroscopy which provided similar information previously found in the crystal structures [40]. The conformational behavior of water soluble *β*-peptides has been the subject of intense studies by several groups [41]. Seebach and co-workers [42] have also studied the secondary structure of *β*-peptides of *β*-amino acid analogs and suggested that a large number of cationic functionalities may disorder hydrogen bonding in foldamers. Temperature dependency of *β*-peptides in a 3_{14}-helical conformation was also investigated by NMR and CD [43]. The denaturing process is called cooperative unfolding. A heptapeptide consisted of the *β*-amino acid analogs of alanine, valine and leucine (**Figure 1.17**) was studied. This structure can formed a 3_{14}-helical secondary structure based on the NMR data and molecular dynamics simulations which revealed that at 340 K, the both folded and unfolded peptide were equally populated [44].

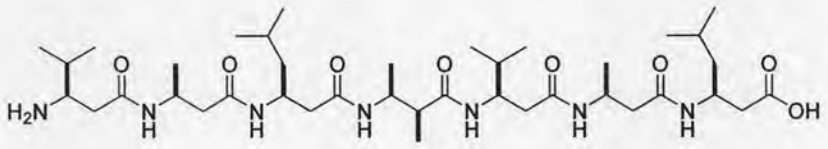


Figure 1.17 A Heptapeptide composed of the *β*-amino acids alanine, valine and leucine.

Insertion of a *trans*-(1*S*,2*S*)-cyclopentanediamine units into the original *aeg*PNA backbone gave rise to a conformationally rigid *tcyp*PNA in **Figure 1.18** [45]. This resulted in increased PNA·DNA stability and sequence specificity. But when the stereochemistry of the cyclopentanediamine is changed to (1*R*,2*R*), no binding to DNA was observed. In addition, the same PNA systems bearing *N*-7 guanine has

improved the stability of PNA·DNA duplex, bis$(PNA)_2$·DNA triplex and PNA_2·DNA_2 quadruplexes [46].

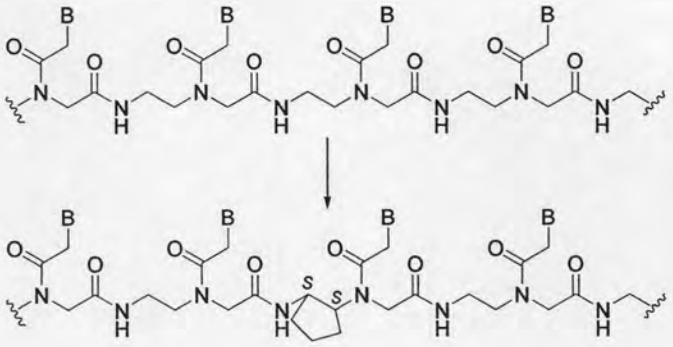


Figure 1.18 General strategy to insert *trans*-(1*S*,2*S*)-cyclopentane into the *aeg*PNA backbone.

In general, PNA core structure is the polyamide usually derived from α-amino acid to form oligopeptides. Replacement of *β*-amino acid residues by α-peptide provided α/*β*-peptides as the heterogeneous backbone, these assumption resulted the unexpected relationship between structure and function [47]. Several research groups have recently reported that short oligomers with a 1:1 alternation of α- and *β*-amino acid residues still can adopt the helical conformations which *β*-amino acid residues are essential for conformational stability such as octamer which contains alternating ACPC and Aib residues adopts an 11-helical conformation in the solid state (**Figure 1.19**) [48-49].

(a)

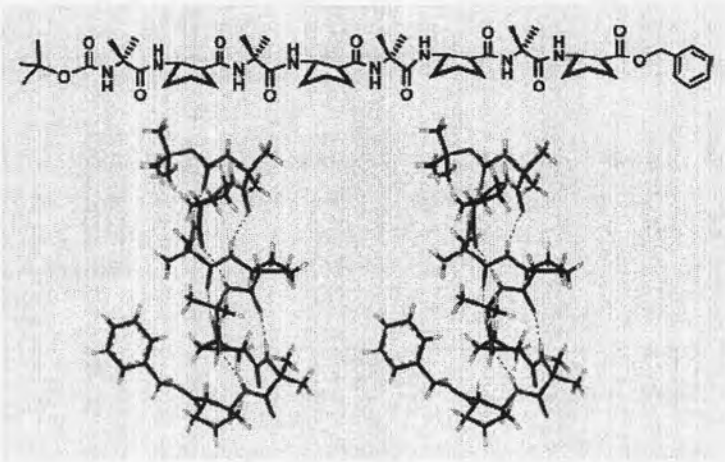


Figure 1.19 Stereoview of X-ray structure of (a); view is perpendicular to the helical axis. Dotted lines indicate hydrogen bonds [48].

1.4 Novel pyrrolidinyl PNA containing *β*-amino acid

In 1991, the first publication of Nielsen's *aeg*PNA [11] was launched. After that, many research groups had attempted to modify the core structure of original PNA by incorporating with chiral residue, cyclic structure, modified nucleobases, additional terminal group etc. These modifications are made for enhancing the stability, specificity and binding affinity with natural oligonucleotides. One of the most popular structural modifications is the insertion of cyclic moiety onto the backbone such as morpholine cyclopentane, cyclohexane or pyrrolidine ring to increase the rigidity to the PNA structure which is believed to decrease the entropy change during the binding. The application of pyrrolidine ring in PNA structure had been widely reported by Vilaivan [51,53,81], Nielsen [67-68], Kumar [63-65], Micklefield [59-62], Ganesh [50,55] and Altmann [56-58] as shown the example of pyrrolidine-based PNA structure in **Figure 1.20** [50].

In 1997, Lowe *et al.* have developed a novel pyrrolidinyl PNA carrying a glycyl group (**1.20c**) as a spacer in backbone [51,81]. The sugar-phosphate backbone of DNA has been replaced with the glycyl-proline residues of both the L- and D-configurations resulting in *cis*- and *trans*-stereochemistry of nucleobase at C-4 position against the carbonyl group of proline. The homothymine decamer of *cis*-D, *cis*-L and *trans*-D isomer were studied the binding properties with both poly(dA) and poly(rA) gave the T_m values of 70 °C and 72 °C respectively and with apparent 1:1 stoichiometry for *cis*-D-isomer and T_m values of 69 °C and 73 °C respectively for *cis*-L-isomer whereas *trans*-D isomer, no melting curve was observed. In the same year, Jordan *et al.* [52] had reported the other *trans*-L-isomer incorporating with *aeg*PNA sequence which showed no hybridization. These results indicated that stereochemistry on the pyrrolidine ring was the important factor to the binding properties in addition to the uncharged nature of the PNA.

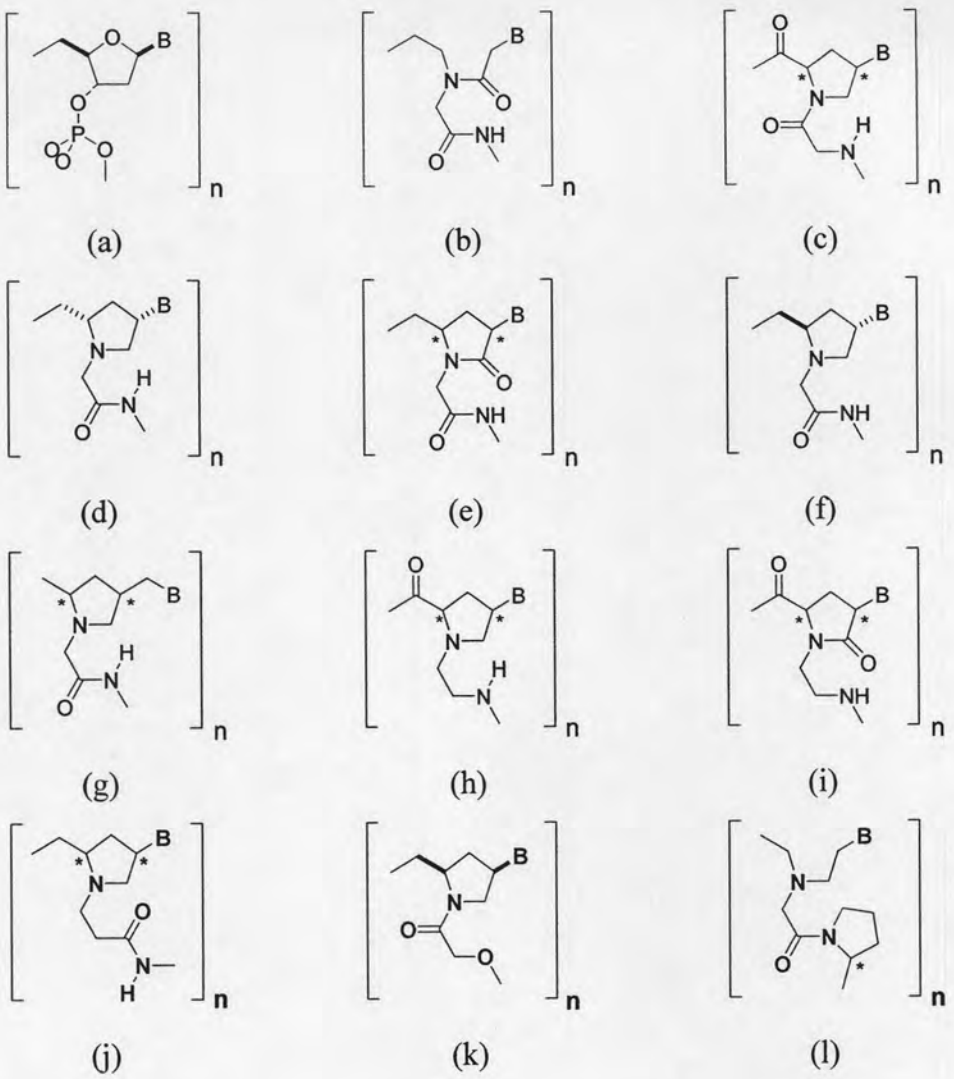
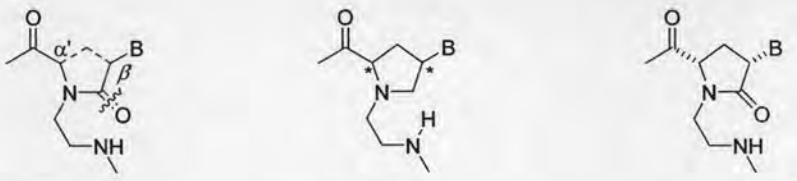


Figure 1.20 Structure of (a) DNA, (b) aminoethylglycine (*aeg*) PNA and modified pyrrolidine PNA (c) prolylglycyl PNA, (d) pyrrolidine-amide oligonucleotides mimic (POM), (e) pyrrolidinone PNA, (f) pyrrolidine PNA (g) pyrrolidinyl PNA, (h) aminoethylprolyl (*aep*) PNA, (i) aminoethylpyrrolidin-5-one (*aepone*) PNA, (j) backbone extended pyrrolidinyl (*bep*) PNA, (k) pyrrolidine-derived nucleo-amino acid (l) *N*-(pyrrolidinyl-2-methyl)glycine-based (*pmg*) PNA.

Ganesh and Kumar [50] had reported the new chiral aminoethylprolyl (*aep*) PNA backbone in 1999, designed by linking the glycyl α′-carbon of the PNA with the acetamido *β*′-carbon *via* a methylene bridge as shown in **Figure 1.21**.

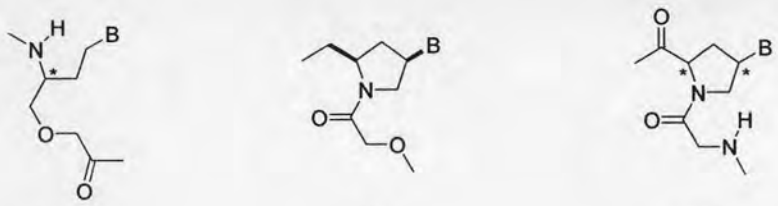


*aeg*PNA *aep*PNA *aepone*PNA

Figure 1.21 Comparison of structure of *aeg*PNA, *aep*PNA and *aepone*PNA.

The balance between rigidity and flexibility of the *aep*PNA backbone together with the presence of positive charge in the strand may contribute to its high affinity to target DNA sequence (T_m > 80 °C for octamer homothymine). A similar PNA system with *N*-aminoethyl-D-proline backbone but in other configuration prepared by Vilaivan *et al.* in 2000 [53] was shown to bind to complementary RNA to form a 2:1 hybrid with high affinity and specificity (T_m 53 °C). Liu *et al.* [54] who studied the same PNA system reported the T_m of homoadenine 12-mers PNA sequence with dT_{10} of 46 °C. As these results from several research groups, the chiral PNA with aminoethylprolyl backbone had the mixed results depending on the stereoisomer of pyrrolidine ring infer that the conformational constraint imposed onto PNA backbone in the form of ring structure may not be uniform for various nucleobases or with sequence context. In 2003, an analogue of *aep*PNA was reported by Ganesh *et al.* [55] derived from aminoethylpyrrolidin-5-one (*aepone*PNA) (**1.20i**) which added the oxo group into the 5-position of pyrrolidine ring. Interestingly, these PNA should give the identical result. On the other hand, *aepone*PNA showed stabilization with DNA and RNA hybrids (T_m were 53 °C and 45 °C for homothymine octamer). The thermal stabilities of the hybrids between these chimeric PNA with DNA are found to be dependent on the type of nucleobases, backbone stereochemistry, and position of modification. Comparison of these hetero-oligomeric PNA must therefore be carefully interpreted.

In 2000, Altmann and co-workers [56] reported a pentadecamer PNA analogues incorporating pyrrolidine-derived nucleo-amino acids (**1.20k**), which a significantly improved binding to complementary RNA over DNA. The PNA derived from the flexible linear δ-amino acid monomer (**1.22a**) showed a contrary behavior as it can bind with both complementary DNA and DNA [57-58].



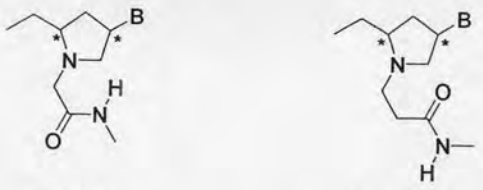
(a) (b) (c)

Figure 1.22 The structure of (a) linear δ-amino acid nucleic acid analogs (b) pyrrolidine-derived nucleoamino acid and (c) prolylglycyl PNA.

The difference between Lowe's system and Altmann's system could conceivably be caused by an increase in conformational flexibility in the latter, which would entropically disfavor hybrid formation. As the results, it should be noted that the stability and selectivity of binding is sensitive upon the configuration and conformation of PNA backbone.

In 2000, Micklefield *et al.* reported another POM PNA (POM is stand for pyrrolidine-amide oligonucleotides mimics) (**1.20d**) [59-61]. The backbone of POM is expected to be protonated and therefore is positively charged at physiological pH. The pentamer of POM-T_5 can form hybrids with both single stranded DNA and RNA. It was also shown that POM binds much faster to RNA than DNA, especially at lower pH and salt concentration. This suggested that electrostatic attraction increases the rate of association. The mixed sequences POM were reported much later in 2007 [62]. The system was capable of hybridizing to both complementary parallel and antiparallel RNA and DNA stranded with some sequence selectivity.

Kumar and Govindarayu had modified the pyrrolidine PNA by extending one carbon conformationally resulting in backbone extended pyrrolidine PNA (*bep*PNA) as shown in **Figure 1.23**. The *bep*PNA backbone was incorporated into *aeg*PNA sequences at predefined positions. The complementary studied with DNA/RNA reveal that these *bep*PNA bring in unprecedented RNA binding selectivity in triplex as well as duplex modes [63-64].



POM *bep*PNA

Figure 1.23 Structure of POM and *bep*PNA.

In 2002, another pyrrolidine PNA was reported by Kumar and Ganesh [65-66] (**1.20g**). The pyrrolidine PNA derived by introducing a methylene bridge between the α-carbon atom of the nucleobase acetamide linker and the *β*-carbon atom of the aminoethyl segment of *aeg*PNA backbone.

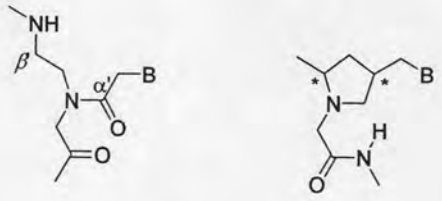


Figure 1.24 Structure of *aeg*PNA and pyrrolidine PNA

All four stereoisomers of this PNA were inserted only one position to the core structure of *aeg*PNA which give the unpredicted discrimination results depend on stereochemistry. This is the drawback of all studies the mixed sequences between *aeg*PNA and any others PNA system. *pmg*PNA is another kind of pyrrolidine-based PNA which introducing pyrrolidine methyl instead of aminoethyl unit; the *pmg*PNA itself showed no sigmoidal transition with DNA and RNA.

In addition, Nielsen and Püschi reported two new classes of pyrrolidine PNA. In 2000, a new conformationally restricted (2*R*,4*S*)-PNA (**1.20f**) [67] was synthesized as adenine decamer which displayed the improvement in binding affinity toward complementary DNA (T_m = 79.0 °C) and RNA (T_m = 60.0 °C) oligonucleotides compared to *aeg*PNA. In 2001, Nielsen revealed another pyrrolidinone PNA, (**1.20e**) [68] in this analogue the aminoethylglycine backbone and the methylenecarbonyl linker are connected, introducing two chiral centers compared to *aeg*PNA. Hybridization properties of all four stereoisomers of this PNA were studied and it was

found that (3*S*,5*R*)-isomer showed to have the highest affinity toward RNA and can recognize RNA and PNA better than DNA.

1.5 Applications of peptide nucleic acid

The favorable DNA/RNA hybridization properties of PNA lead to a number of developments in biomolecular and medical applications. These include hybridization techniques for genetic detection, nucleic acid biosensor, modulation of PCR analysis, array hybridization as well as antisense and antigene technology. Most applications of PNA involve the use of original Nielsen's *aeg*PNA system since it is the only one that is currently commercially available.

1.5.1 PNA as a potential antisense and antigene drug

Transcription and translation arrest by PNA has been reported by many laboratories. PNA are designed to recognize and hybridize to the complementary sequences in a particular gene of interest whereby they should interfere with the transcription of the particular gene (antigene strategy). Alternatively the PNA analogs can be designed to recognize and hybridize to complementary sequences in mRNA and thereby inhibit its translation (antisense strategy) as shown in **Figure 1.3** [69]. The basic mechanism of antisense effects by oligonucleotides is considered to be ribonuclease H (RNase H) mediates cleavage of RNA strand in oligonucleotide-RNA heteroduplex. PNA·RNA duplexes cannot act as substrates for RNase H. Triplex-forming PNA are able to hinder the translation machinery at targets in the codon region of mRNA. However, translation elongation arrest requires a $(PNA)_2$·RNA triplex and therefore needs a homopurine target of 10-15 bases. Triplex-forming PNA can inhibit translation at initiation codon targets and ribosome elongation at codon region targets. Mologni *et al.* [70] reported the effect of different types of antisense PNA on the *in vitro* expression of PML/RARα gene which is complementary to the first AUG (initiation) and starting site for the synthesis of an active protein respectively. The results suggested that the PNA targeting of RNA molecule like PML/RARα and effective translation inhibition can be achieved by combining PNA

directed toward AUG regions. It is also possible by using PNA to inhibit the elongation of DNA primers by DNA polymerase. Efficient inhibition of extra chromosomal mitochondrial DNA, which is single-stranded during replication, has been demonstrated by Taylor *et al.* [71] The PNA can inhibited the replication of mutant human mitochondrial DNA under physiological conditions without affecting the wild-type DNA in mitochondria.

1.5.2 PNA as a probe in nucleic acid biosensor

A single-stranded PNA probe is immobilized onto optical, electrochemical, or mass-sensitive transducers to detect the complementary (or mismatch) DNA strand in a sample solution. PNA can also act as a tag, encoding an attached molecule by a defined base sequence. Winssinger *et al.* [72] reported PNA-tagged small-molecule libraries. These libraries were synthesized on rink resin, using lysine as a branch molecule (**Figure 1.25**). The small molecules were coupled to the α-amino branches of the lysines, whereas the fluorescein-labeled PNA tags were attached at the ε-amino branches. These libraries were incubated with cell lysates. Subsequently, all library molecules that had not interacted with a protein from the cell lysates were removed by size-exclusion filtration. The remaining molecular mixture was applied to a microarray, which contains DNA sequences that were complementary to the PNA tags. The fluorescence signals produced at the various chip positions were indicative of the amount of protein that was present in the cell lysates and interacted with the respective small molecule.

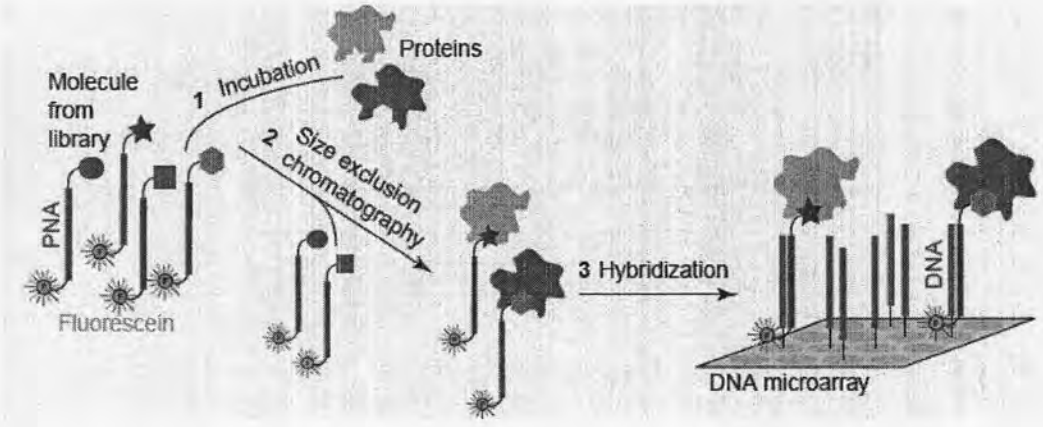


Figure 1.25 Molecule sorting on spatially addressable microarrays [72].

The remarkable decreased in stability in the presence of one-base mismatch in PNA·DNA duplexes was applied to single nucleotide polymorphisms (SNP) detection with high accuracy by Komiyama *et al.* [73] When the target site in DNA sample is completely complementary to the PNA probe, the PNA·DNA hybrid is stable against enzymatic digestion. On the other hand, when the hybrid contains one or more mismatch bases, it will be completely digested as a result of its lower stability compared to the fully matched hybrid. The difference between the (matched) PNA·DNA hybrid and the (mismatched) single stranded PNA plus free nucleotides were detected visually by staining with a 3-3′-diethylthiadicarbocyanine dye which changes its color upon binding to PNA·DNA duplex as shown in **Figure1.26**.

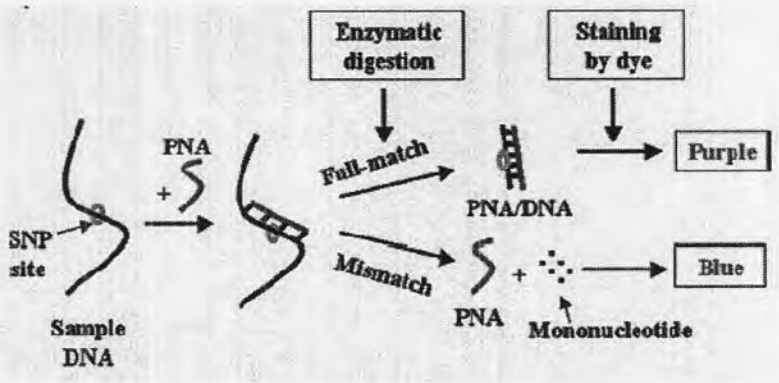


Figure 1.26 Strategy for SNP detection by the PNA/Nuclease/Dye system [73].

1.5.3 PNA as a molecular-biological tool

PNA can terminate the elongation of oligonucleotide primers by hybridization with the DNA template during the polymerase chain reaction (PCR) [74]. PNA has been used for detecting single base pair mutation in a technique called PNA directed PCR clamping [75]. Recently, a quencher labeled PNA has been developed for automated real time PCR. In this strategy, the quencher labeled PNA (Q-PNA) is hybridized to the 5′-tag of fluorescent-labeled DNA primer in order to quench the fluorescence of the primer. During PCR, the Q-PNA is replaced by incorporation of the primer into amplicons and the fluorescence moiety is liberated which can be detected [76].

1.6 Objectives of this research

From the literature reviewed above, there were no specific reports on PNA containing backbones derived from β-amino acid. It was reasoned that the combination of pyrrolidine moiety and β-amino acid in the PNA backbone would introduce rigidity into the structure in an adjustable way. This should improve the binding affinity towards DNA and/or RNA. Consequently, we designed and prepared a new chiral PNA analogue containing pyrrolidine ring and β-amino acid as a linker.

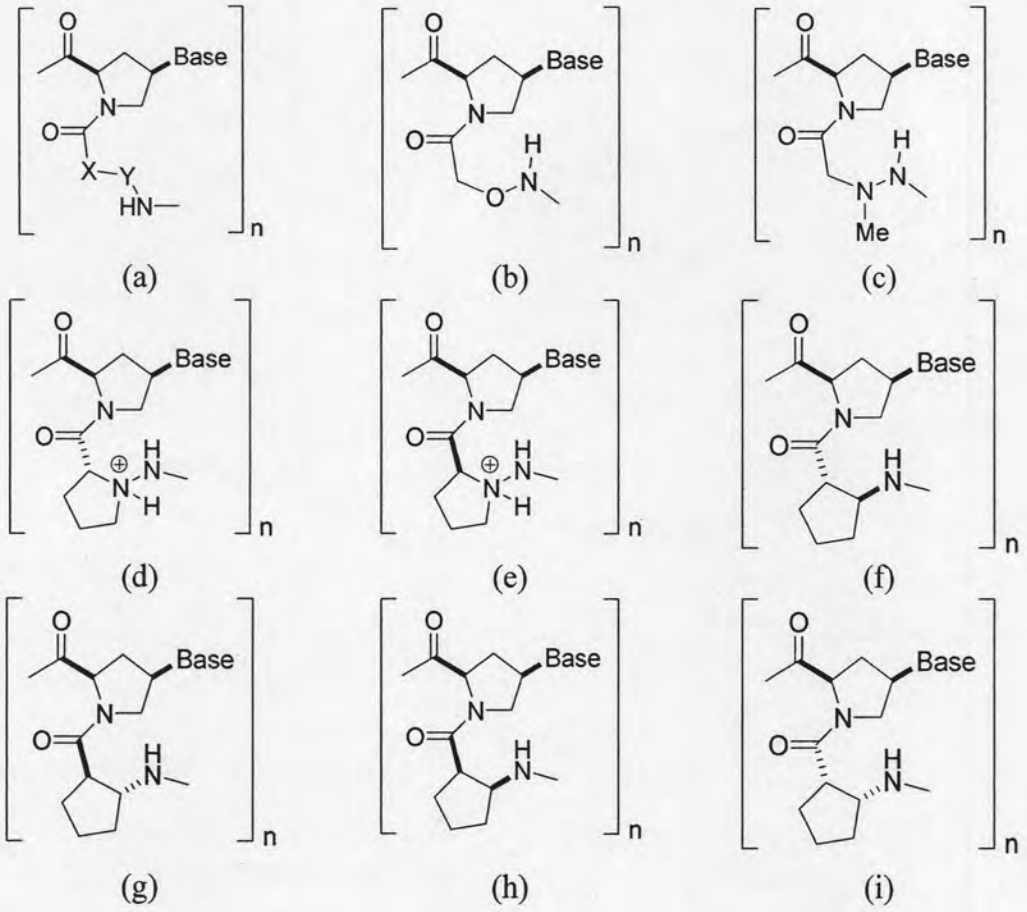


Figure 1.27 Structure of modified PNA bearing various β-amino acid spacer: (a) model β-amino acid, (b) amino-oxy acetic acid, (c) *N*-amino-*N*-methyl glycine, (d) D-aminopyrrolidine-2-carboxylic acid (D-APC), (e) L-amino pyrrolidine-2-carboxylic acid (L-APC) (f) *trans*-(1*S*,2*S*)-2-aminopentane carboxylic acid (1*S*,2*S*)-ACPC, (g) (1*R*,2*R*)-ACPC, (h) (1*S*,2*R*)-ACPC and (i) (1*R*,2*S*)-ACPC.

The fact that oligomers of β-amino acid in a precise conformation can be easily formed lead to a hypothesis that by incorporating β-amino acids in the PNA backbone might help pre-organizing the PNA backbone. The design, synthesis and binding studies of PNA carrying β-amino acid as an alternative spacer are therefore the main objectives of this research.