Review

Sequence-Specific Recognition of Double-Stranded DNA by Peptide Nucleic Acid Forming Double-Duplex Invasion Complex

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Abstract: Peptide nucleic acid (PNA) is an analog of natural nucleic acids, where the sugar-phosphate backbone of DNA is replaced by an electrostatically neutral N-(2-aminoethyl)glycine backbone. This unique peptide-based backbone enables PNAs to form a very stable duplex with the complementary nucleic acids via Watson–Crick base pairing since there is no electrostatic repulsion between PNA and DNA-RNA. With this high nucleic acid affinity, PNAs have been used in a wide range of fields, from biological applications such as gene targeting, to engineering applications such as probe and sensor developments. In addition to single-stranded DNA, PNA can also recognize double-stranded DNA (dsDNA) through the formation of a double-duplex invasion complex. This double-duplex invasion is hard to achieve with other artificial nucleic acids and is expected to be a promising method to recognize dsDNA in cellula or in vivo since the invasion does not require the prior denaturation of dsDNA. In this paper, we provide basic knowledge of PNA and mainly focus on the research of PNA invasion.

Keywords: PNA; DNA; artificial nucleic acids; invasion

1. Introduction

The “central dogma” is the continuous flow of genetic information from DNA to proteins via mRNA [1]. Since DNA is in its most upstream, the regulation of the DNA function can lead to the specific control of subsequent gene expression. From this perspective, genome editing technology, which replaces genomic DNA with the desired sequence, has been intensively investigated. With the CRISPR-Cas9 system awarded the Nobel Prize in Chemistry in 2020, the understanding of gene function is accelerating [2–4]. In addition to research into DNA-binding proteins, including ZF [5–9], TALE [10,11], as well as CRISPR-Cas9 [2–4], various small molecules which target DNA have also been investigated. For the selective recognition of DNA, minor groove binders (Py-lm polyamides) [12–15] and various artificial nucleic acids have been developed [16–29]. With their high selectivity, artificial nucleic acids, in which a part of the nucleic acid is chemically modified, have been attracting attention as nucleic acid medicines in recent years. Unlike conventional small-molecule drugs that target proteins, nucleic acid drugs are expected to offer completely different selectivity and potency by targeting nucleic acids. However, artificial nucleic acids other than triplex-forming oligonucleotides (TFOs) [30–32] can only target nucleic acids in single-stranded form and have difficulty directly recognizing double-stranded DNA (dsDNA). This is because the recognition of artificial nucleic acids is based on complementarity via Watson–Crick base pairing, and the process of base–pair formation is essential for their recognition. Therefore, artificial nucleic acids are usually utilized to target single-stranded DNA denatured by heat or alkaline conditions from dsDNA. While single-strand recognition is sufficient for the antisense methodology targeting mRNA, artificial nucleic acids recognizing dsDNA are essential for the direct recognition of the genomic
DNA. Human genomic DNA consists of about 3 billion base pairs, and a variety of genes have been identified, including those related to phenotypes and diseases. Developing a technology that can freely recognize such a huge genomic DNA is expected to have a very wide range of applications in the field of biology as well as medicine.

In this review, we focus on the peptide nucleic acid (PNA) that enables the direct recognition of dsDNA, which is difficult to achieve with conventional artificial nucleic acids, and describe its characteristic dsDNA recognition mode (“invasion”), from basic information to applications.

2. Double-Stranded DNA Recognition by Peptide Nucleic Acid (PNA)

2.1. Peptide Nucleic Acid (PNA)

In 1991, Prof. Nielsen and co-workers developed peptide nucleic acid (PNA; Figure 1) [16,33–41]. PNA not only forms stable duplexes with single-stranded nucleic acids via Watson–Crick base pairing but also can directly recognize sequences in dsDNA through a unique DNA recognition called “Invasion” [42] (Figure 2). PNA is a synthetic DNA analog, and its backbone, N-(2-aminoethyl)glycine, consists of six atoms in the monomer unit like DNA. In PNA, the negatively charged sugar-phosphate backbone of DNA was substituted with an electrostatically neutral pseudo-peptide backbone. Consequently, the electrostatic repulsion between PNA and DNA is absent, and PNA/DNA duplexes are much more stable than those between negatively charged DNA strands [43]. In addition, the binding strength of PNAs to mismatched sequences is much lower than that to complementary strands, and PNAs have a higher sequence discrimination ability than DNA [44]. Furthermore, PNA is highly resistant to nucleases and proteases since PNA consists of an unnatural backbone different from DNA and peptide [45]. These characteristics make PNA promising for in-cell and in vivo applications targeting genomic DNA. Although there have been many reports of PNA analogs with modified backbones [40,46–51] (Figure 3), this review focuses on N-(2-aminoethyl)glycine PNA (aegPNA), with an overview of its dsDNA recognition mode, invasion.

Figure 1. Structures of DNA and PNA.

two strands of PNA

Figure 2. The dsDNA recognition by PNA via double-duplex invasion.
2.2. Invasion Complex Formation by PNA

The invasion of PNA is achieved by its high DNA-binding affinity mentioned above. In other words, the energy loss of dissociating the DNA duplex is compensated by more stable PNA/DNA duplex formation. Consequently, by using PNAs with complementary sequences, the sequences in dsDNA can be directly and selectively recognized without a denaturing treatment, which is generally not possible with other artificial nucleic acids. The invasion complex shown in Figure 2 is more correctly called double-duplex invasion complex [42], and the following other invasion complexes have also been reported (Figure 4).

(a) Triplex invasion

(b) Duplex invasion

(c) Double-duplex invasion

Figure 3. Structures of PNA analogs with modified backbone.

Figure 4. Various recognition modes of target dsDNA by PNA: (a) triplex invasion; (b) duplex invasion; and (c) double-duplex invasion.
In the triplex invasion complex [52] (Figure 4a), two PNAs are bound to one strand of a dsDNA by both Watson-Crick and Hoogsteen base pairing, forming a triplex. The DNA recognized by this triplex invasion must be a homopyrimidine/homopurine sequence, which limits the target sequence. Homopyrimidine PNAs are commonly used for triplex invasion, which requires protonation of the PNA nucleobases to form Hoogsteen base pairs. Therefore, the complex is stabilized under acidic conditions. To stabilize the triplex invasion complex, bis-PNA, in which two PNAs are linked by a linker, has been developed [53,54]. Recently, tail-cramp PNAs (tcPNAs), asymmetric bis-PNAs in which the PNA strand for the Watson–Crick side is designed to recognize mix sequences, have been developed and reported for intracellular applications [38,55–58].

The duplex invasion complex (Figure 4b) is formed when one complementary PNA forms a PNA/DNA duplex in a dsDNA by Watson–Crick base pairing [59]. The formation of this invasion complex was first reported for specific sequences with homopyrimidine-homopurine sequences, but it differs from the triplex invasion in that there is no third strand to form the Hoogsteen base pair. The recognition sequences of duplex invasion and triplex invasion are generally restricted to homopyrimidine-homopurine. In 2007, it was revealed that duplex invasion of PNA occurs even in mixed sequences, and the invasion complex was first confirmed by electrophoresis [60]. Subsequently, reports of duplex invasion in mixed sequences have continued with the methodology of enhancing the binding strength of PNA [61,62].

Double-duplex invasion [42] (Figure 4c), mainly discussed in this paper, is a complex formation in which two PNAs with complementary sequences form a Watson–Crick base pair with the target sequences in dsDNA. This complex does not require homopyrimidine/homopurine sequences like triplex and duplex invasion complexes, making it more suitable for various applications. This invasion is unique because PNAs directly recognize (invade into) dsDNA, which is not seen in other artificial nucleic acids. Moreover, another attractive feature is the ability to induce local structural changes in the target DNA during the formation of the invasion complex.

2.3. Double-Duplex Invasion by Pseudo-Complementary PNAs (pcPNAs)

Double-duplex invasion requires a pair of complementary PNAs. Since PNA/PNA is more stable than PNA/DNA, duplex formation between these complementary PNAs preferentially proceeded over invasion complex formation, resulting in lower invasion efficiency. To suppress the formation of PNA self-duplexes, pseudo-complementary PNA (pcPNA) has been used [33,42,63] (Figure 5). In pcPNA, conventional adenine (A) and thymine (T) nucleobases are replaced by the artificial pseudo-complementary nucleobases, 2,6-diaminopurine (D) and 2-thiouracil (Uₜ), respectively (Figure 5a). Though stable base pairs between D-Uₜ and the natural nucleobases T-A are retained (Figure 5b), pcPNA/pcPNA duplexes are greatly destabilized through the steric repulsion between the amino group of D and the thione group of Uₜ (Figure 5c). Consequently, efficient double-duplex invasion complex formation is achieved by using pcPNAs, as pcPNA/pcPNA duplex formation is suppressed and PNA/DNA duplex formation is promoted [42]. Thus, the strategy of pcPNA is very effective for invasion. Although a certain amount of A·T needs to be included for efficient invasion, pcPNA does not have significant sequence restrictions like the homopurine/homopyrimidine sequences of triplex or duplex invasion described above. Therefore, double-duplex invasion is effective for various DNA recognition applications, especially intracellular and in vivo applications, due to its ability to recognize dsDNA directly. In this review, we focus on double-duplex invasion, which has high sequence flexibility among invasion complexes, and the related studies are summarized below (in the following, unless otherwise stated, invasion refers to double-duplex invasion).
3. Promotion of Double-Duplex Invasion by Modified PNAs

Various in vivo applications based on dsDNA recognition are expected to be developed by employing invasion with pcPNAs. However, to make such applications successful, there is a challenge to improve the efficiency of invasion complex formation at high salt concentrations, like those in the intracellular environment. Under high-salt conditions, the stability of the DNA/DNA duplex is enhanced, whereas the DNA binding strength of PNA is slightly reduced, resulting in an overall decrease in the stability of the invasion complex. Therefore, several methodologies such as the chemical modification of PNAs and conjugation with functional molecules have been reported to increase invasion efficiency.

3.1. Chiral PNAs

Unlike DNA and other conventional artificial nucleic acids, aegPNA, composed of an N-(2-aminoethyl)glycine backbone, has an achiral structure. It has been reported that aegPNA itself has no preference for helicity and its helical structure changes depending on the chirality of the C-terminal amino acid [64]. The duplex of PNA decamers with L-lysine at the C-termini gave the CD spectrum similar to that of DNA duplex in the nucleobase absorption region, and the mirror-image spectrum was obtained when L-lysine was substituted with D-lysine. On the other hand, since natural DNA is a chiral molecule and forms a right-handed helical structure, PNAs with chirality introduced into the backbone have been developed to improve the DNA recognition ability. Looking carefully at the structure of PNA, we can imagine that the chirality can be easily introduced by replacing glycine in its backbone with other amino acids. Thus, lysine-incorporated \( D_{\alpha}\)-PNA derived from \( N\)-(2-aminoethyl)-D-lysine was developed in 1996 [65,66] (Figure 6a). This chiral PNA strongly interacts with DNA, whereas the \( L\)-isomer has the opposite effect, suggesting that \( D\)-chirality is very important for high DNA affinity. In addition, the \( L\)-PNA containing a side chain to the PNA backbone, leading to the addition of a positive charge. The resulting conformational control of PNA and electrostatic attraction of PNA to negatively charged DNA enhance the DNA recognition ability of PNA (Figure 6b). It has also been reported that chiral PNAs containing pseudo-complementary nucleobases show a higher efficiency of invasion complex formation than achiral pcPNA [67]. This result is ex-
plained by the fact that in addition to improving the DNA binding strength of pcPNAs, the electrostatic repulsion also causes more significant destabilization of the pcPNA/pcPNA duplex (Figure 6c). This function of chiral PNA assists the effect of pseudo-complementary nucleobases D and Us, and even if the percentage of D and Us in PNA strands is low, high invasion efficiency was maintained by using chiral pcPNAs. Moreover, these chiral pcPNAs show mismatch discrimination ability, although the modification methods that introduce positive charges are often accompanied by reduced selectivity to the target due to nonspecific interactions with negatively charged DNA.

Figure 6. (a) Structures of PNAs with chiral backbone. (b) Enhancement of DNA affinity and suppression of self-duplex formation through electrostatic interaction by cationic PNAs. (c) The promotion of double-duplex invasion by introducing positive charges to PNA strands.

The backbone-modified PNA introduced so far has a side chain at the α-position (Figure 6a), and it is categorized as α-PNA. Chiral α-PNAs using other amino acids instead of lysine have also been reported [48,65]. Furthermore, in addition to these α-PNAs, PNAs with various functional side chains introduced into the PNA backbone at the γ-position (γ-PNA) were developed (Figure 6a) and have been widely studied for their high DNA-binding ability [61,68]. Although γ-PNAs with lysine side chains (γKα-PNAs) also exhibit high DNA-binding ability like γKα-PNA, interestingly, the optical isomerism of lysine suitable for DNA recognition is reversed from that of the aforementioned α-PNAs. When the γD-lysine framework is used for γ-PNA, its DNA binding ability is reduced. γ-PNAs possessing a methyl group or ethylene glycol have been extensively studied, as they have a significant effect on preorganizing the structure in favor of double-strand formation with DNA. Furthermore, the γ-PNA conjugated with an acridine moiety has been reported to allow duplex invasion (not double-duplex invasion) in a mix sequence, which cannot be achieved with unmodified aegPNA, and the formation of the invasion complex has been confirmed by polyacrylamide gel electrophoresis [61].
3.2. PNA Modified with Nuclear Localization Signal (NLS) Peptide (NLS-PNA)

Although the chiral PNAs have improved invasion efficiency, their effect in high-salt conditions like in vivo has not been fully investigated. On the other hand, some modification methods of pcPNA have been reported to improve invasion efficiency at high salt concentrations, and one of them is PNA modified with a nuclear localization signal (NLS) peptide (Figure 7a). The NLS peptide is a functional peptide involved in the nuclear transport of proteins. Since this peptide is rich in basic amino acids (lysine and arginine), it is positively charged and is expected to interact strongly with DNA under neutral pH conditions. PNA, which has a peptide-based backbone, is synthesized by the standard solid-phase peptide synthesis [33], so the modification of peptides to PNAs is much easier than to other artificial nucleic acids, and the introduction of cell-penetrating peptides (CPPs) has been reported [69,70]. Moreover, compared to other PNA modification methods, NLS-PNA is a very simple strategy as it only requires the introduction of additional peptides into the PNA.

![Figure 7.](image_url)

The paper reported in 2013 suggested that NLS-PNAs showed the formation of duplex invasion (not double-duplex invasion) [71]. These NLS-PNAs have been applied to dsDNA recognition because the PNA modified with an NLS peptide has shown antigenic effects, and fluorescence spectroscopic studies have shown complex formation with NLS-PNAs and plasmid DNA. Given that it is challenging to form duplex invasion with unmodified PNA in the mix sequence, the contribution of NLS to improved DNA binding is unquestionable. Interestingly, this NLS-PNA also achieved dsDNA recognition in a consecutive 14 bp GC sequence, which is difficult to be targeted by unmodified pcPNAs.

Inspired by the work described above, pcPNAs modified with an NLS peptide (NLS-pcPNAs) were developed in 2015, and high invasion efficiency was reported with NLS-pcPNAs, enabling the full recognition of target dsDNA with a lower concentration of PNAs than unmodified pcPNAs [72] (Figure 7b). These NLS-PNAs are effective at higher salt concentrations and retain the high invasion efficiency even at 100 mM NaCl, where the invasion efficiency of unmodified pcPNAs is significantly reduced. Furthermore, NLS-pcPNAs show overwhelmingly high invasion efficiency, even under physiological conditions where the invasion efficiency is further reduced with unmodified pcPNAs. The high mismatch discrimination ability was also retained in NLS-pcPNA, despite the fact that it utilizes electrostatic interactions. More interestingly, the NLS peptide used in this study shows its biological function even when conjugated with PNAs, and the NLS-PNA was confirmed to be present in the nucleus upon introduction into the cell [71].

In the NLS-PNAs mentioned above, NLS was introduced at C-termini, but the design of NLS-PNA was also studied in detail, and the invasion efficiency of N-terminal modified NLS-PNAs was examined [73]. The results showed no drastic difference between C- and
N-terminal modified NLS-PNAs, and NLS was effective for invasion in both modifications. N-terminal modification was shown to be slightly more effective at higher salt concentrations, indicating that the amino acids connecting the NLS to PNA may affect invasion.

3.3. Ruthenium-Complex PNA Conjugate (Ru-PNA)

PNA conjugated with ruthenium (Ru) complex (Ru-PNA) has been reported as a modified PNA that exhibits high invasion efficiency under physiological conditions [74]. Ru-polypyridyl complexes are known to have high DNA affinity due to hydrophobic and electrostatic interactions with DNA. Therefore, the DNA binding ability of PNA has been improved by conjugation with Ru complexes, and an increase in invasion efficiency has been achieved. Similar to NLS-PNAs, this method only requires the introduction of the DNA-binding molecule into the PNA, and the preparation of modified PNA is effortless. The introduction of the Ru complexes into PNA is easily achieved via amide condensation between the carboxyl group derivatives of the Ru complex and amino acids having an amino group on the side chain. The synthesis of Ru-PNAs is accomplished by standard solid-phase peptide synthesis, and the Ru complex was introduced to the PNA via an amino group on the side chain of the N-terminal L-2,3-diaminopropionic acid (Dap; Figure 8a).

Figure 8. (a) Structure of Ru-PNA utilizing L-2,3-diaminopropionic acid for introducing the Ru-complex. (b) Ru-PNAs stabilize double-duplex invasion.

Ru-PNA exhibited high invasion efficiency even at low PNA concentrations and 100 mM NaCl (Figure 8b), where with unmodified pcPNA, it is difficult to form an invasion complex. Furthermore, even under physiological conditions, the invasion efficiency was nearly 90% using six equivalents of Ru-PNA. Ru-PNA also showed high sequence-discrimination ability, and no invasion proceeded with mismatched DNA that differs by only one base pair. This increase in invasion efficiency is attributed to the high DNA-binding ability of Ru-PNA, which was confirmed by melting temperature (Tm) measurements. It is suggested by the Tm measurements that the Ru-complex interacts with DNA outside the target sequence of the PNA. Interestingly, it has also been reported that the invasion efficiency of Ru-PNAs varies greatly depending on the introduction manner of the Ru complex. In addition to PNA conjugated with Ru complexes on the side chain of Dap, the introduction of Ru complexes on the side chain of Lys and the amino group of the PNA main chain were also investigated. However, no increase in invasion efficiency was observed with those Ru-PNAs, suggesting that the Ru complexes could not interact appropriately with DNA. These results indicate that the introduction mode and spatial arrangement of the Ru complexes are very important for the appropriate interaction of the Ru complexes with DNA and the improvement of invasion efficiency.
3.4. PNA Containing Cationic Guanine (G\(^+\)-PNA)

In contrast to modified PNAs with chiral backbones or additional functional molecules, cationic-guanine (G\(^+\)) PNA (G\(^+\)-PNA; Figure 9a), in which a nucleobase is modified with a strategy different from pcPNAs, has been developed and reported to show improved invasion efficiency [75]. G\(^+\) is positively charged by methylation of the N\(^2\)-nitrogen atom in a guanine base, resulting in quaternization. Consequently, G\(^+\)-PNA/DNA duplexes are stabilized by electrostatic interaction, and G\(^+\)-PNA/G\(^+\)-PNA duplexes are destabilized by electrostatic repulsion (Figure 9b). The DNA-binding affinity of G\(^+\)-PNAs was evaluated by \(T_m\) measurements with complementary DNAs, and an increase in \(T_m\) of up to 11.2 °C (\(\Delta T_m = 11.2 \text{ °C}\)) was observed by simply replacing a single guanine nucleobase in the PNA with a G\(^+\) nucleobase. On the other hand, the \(T_m\) values of a duplex between G\(^+\)-PNAs were reduced by −4.8 to −8.3 °C by introducing G\(^+\). These results are equivalent to or better than pcPNA (stabilization of pcPNA/DNA; +5 to +6 °C, destabilization of pcPNA/pcPNA; −4 to −6 °C). The relationship between changes in salt concentration and \(T_m\) value suggests that the electrostatic interaction plays an important role in these G\(^+\)-PNAs.

![Figure 9](image_url)

**Figure 9.** (a) Structure of cationic guanine PNA (G\(^+\)-PNA). (b) Enhancement of DNA affinity and suppression of self-duplex formation through electrostatic interaction by using G\(^+\)-PNAs.

Invasion experiments under physiological conditions showed that the introduction of a single G\(^+\) in the PNAs dramatically increased the invasion efficiency, which was about 20 times higher than that of unmodified pcPNAs. Furthermore, when the number of introduced G\(^+\) was increased to three, the invasion efficiency was further improved, showing 24-fold higher invasion efficiency than that of unmodified pcPNAs. On the other hand, when invasion was performed with the target dsDNA containing the single-base mutation, the invasion complex almost disappeared, indicating the high sequence selectivity of G\(^+\)-PNA. G\(^+\) is expected to be one of the new pcPNA options because it can be introduced into GC base pairs, unlike pseudo-complementary nucleobases D and U\(_R\), which can only apply to AT base pairs. In addition, compared to D and U\(_R\), G\(^+\) has the advantage that its monomer can be easily synthesized from commercially available Fmoc-G(Bhoc)-COOH PNA monomers in a single-step methylation reaction (the yield was 98%). Interestingly, although methylation to nucleobases in DNA promotes depurination and generally reduces the stability of the DNA itself [76], no such destabilization was observed with G\(^+\)-PNA, indicating that this nucleobase modification was allowed by using the PNA backbone.

4. Recent Invasion-Related Research

4.1. Development of New Backbone- or Nucleobase-Modified PNAs for DNA Recognition

In addition to the γPNA mentioned above, there have been significant modifications of the PNA backbone to enable dsDNA recognition. Prof. Vilaivan et al. synthesized various pyrrolidinyl PNAs, which have a cyclic structure in the backbone [77–79]. Among them, a PNA having a (2′R,4′R)-pyrrolidine/(2S)-amino-cyclopentane-(1S)-carboxylic acids backbone (acpcPNA; Figure 10a) formed a very stable duplex with DNA. On the other hand, acpcPNAs composed of a bulky backbone cannot form stable acpcPNA/acpcPNA duplexes. In other words, the acpcPNA backbone is pseudo-complementary, suggesting acpcPNA is
an alternative candidate to pcPNA for achieving double-duplex invasion. The stability of each duplex with DNA and acpcPNA is in the following order; PNA/DNA > PNA/PNA, which is completely opposite to that of aegPNA (PNA/PNA > PNA/DNA). Despite the absence of pseudo-complementary nucleobases, the fluorescence change indicated that fluorophore-labeled acpcPNAs recognized the short synthetic dsDNA (30 bp) through invasion (Figure 10b), and the invasion efficiency was around 15% [80]. In contrast, the fluorescence emission is negligible for the mismatch sequence, showing the sequence selectivity of acpcPNA. Furthermore, in polyacrylamide gel electrophoresis, a new band with slower mobility than dsDNA also appeared, indicating that complementary 10-mer acpcPNAs form an invasion complex with 30-bp dsDNA.

![Figure 10. (a) Chemical structure of acpcPNA. (b) Invasion complex formation by fluorophore-labeled acpcPNAs due to steric hindrance between acpcPNAs.](image)

Invasion-like structures in which a single PNA strand recognizes two DNA strands have also been reported. Prof. Ganesh et al. introduced a second nucleobase into the α-(Cα) or γ-position (Cγ) of PNA in addition to the original nucleobase [81–83] (Figure 11a). This new type of PNA termed “bimodal PNA (bm-PNA)” can recognize two DNA strands simultaneously. Furthermore, by using multiple bimodal PNAs and combining duplex and triplex formation with complementary DNAs, fused duplexes, triplexes, and extended PNA/DNA assemblies were created [84] (Figure 11b). Besides, Prof. Ly et al. employed bifacial nucleobases (namely, Janus nucleobases) and succeeded in recognizing dsDNA with one strand of PNA based on the γPNA backbone (JByPNA) [85]. Bifacial nucleobases can selectively recognize A-T, T-A, G-C, and C-G base pairs by forming new base pairs on each side of bifacial nucleobases (Figure 12). Molecular dynamics (MD) simulations demonstrated that DNA/JByPNA on one side maintained a stable structure in antiparallel orientation, whereas JByPNA/DNA on the other side in parallel orientation collapsed its structure. The weaker parallel JByPNA/DNA interaction is attributed to less favorable binding orientation and fewer hydrogen bonds. The structure of JByPNA/JByPNA was collapsed due to the steric collision of its backbone. These results of MD simulations are consistent with circular dichroism (CD) and UV melting experiments. JByPNA with six bifacial nucleobases can invade into not only 6-bp hairpin DNA but also internal binding sites in a stable 26-bp dsDNA under physiological conditions, and its complex formation was confirmed by gel electrophoresis.
JBγPNA/JBγPNA was collapsed due to the steric collision of its backbone. These results of MD simulations are consistent with circular dichroism (CD) and UV melting experiments. JBγPNA with six bifacial nucleobases can invade into not only 6-bp hairpin DNA but also internal binding sites in a stable 26-bp dsDNA under physiological conditions, and its complex formation was confirmed by gel electrophoresis.

**Figure 11.** (a) Chemical structures of Cα and Cγ-bm-PNAs. (b) bm-PNA recognizes two DNA strands forming a double duplex.

**Figure 12.** (a) Structure of γ-miniPEG PNA. (b) Bifacial PNA (JBγPNA) recognizes two strands of dsDNA simultaneously in both antiparallel and parallel orientations. (c) Bifacial nucleobases form hydrogen bonds with canonical nucleobases on the two sides of bifacial nucleobases.

4.2. Double-Stranded DNA Recognition by Non-PNA-Type Artificial Nucleic Acids

Several artificial nucleic acids targeting dsDNA have been reported in a recent study. Prof. Hrdlicka et al. designed chemically modified oligonucleotides with intercalator-conjugated nucleotides (invader probes; Figure 13a) [86–89]. These invader probes form a stable duplex with DNA owing to stacking interactions between base pairs and intercalators. In contrast, the intercalator plays a role in inducing local perturbation and destabilizing the duplex between one probe strand and a complementary probe strand. The stability difference between target DNA/invader probe and invader probe/invader probe duplex provides the driving force for dsDNA recognition via double-duplex invasion. The structure of the invader probe has been optimized, and 2′-O-(pyrene-1-yl)methyl-RNA is mainly used as the monomer for intercalator introduction. dsDNA-targeting properties of invader probes were evaluated by using hairpin DNA as a model target, resulting in successful DNA recognition. In the latest research, the authors have successfully improved dsDNA recognition of invader probes with unique strategies, including toehold and nicked invader designs [90,91]. The authors have been using this system to work on FISH for targeting chromosomal DNA and have detected complex formation between invader probes and chromosomal DNA by fluorescence microscopy.
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![Figure 13](image_url)

**Figure 13.** Sequence-selective recognition of dsDNA with (a) invader probes and (b) a combination of linear probe and unmodified PNA.

Prof. Asanuma et al. achieved dsDNA recognition and sequence-specific fluorescent labeling by combining unmodified PNA with a linear probe [92] (Figure 13b). The linear probe has multiple fluorophores on a D-threoninol scaffold in an oligonucleotide [93,94]. In the single-stranded state, the linear probe does not emit fluorescence due to the self-quenching of fluorophores, but when the linear probe hybridizes with target single-stranded DNA, fluorophores intercalate between the base pairs, triggering a strong fluorescence. In contrast, hybridization between the linear probe and PNA is suppressed since PNA/DNA duplex is known to be inflexible to accommodate intercalators and the fluorophores in the linear probe exhibit inhibitory effect for hybridization with the complementary PNA. Melting temperature analysis demonstrated that the $T_m$ values followed the order of DNA/PNA > linear probe/DNA >> linear probe/PNA, satisfying the requirements for effective invasion. An electrophoresis mobility shift assay demonstrated that linear probe and PNA invaded and fluorescently labeled the target dsDNA. Heat shock treatment enabled double-duplex invasion in the central region of dsDNA. Even without a heat shock, an invasion complex was observed at the ends of 50-bp dsDNA at 40 °C and 100 mM NaCl.

Cross-linkable artificial nucleic acids have also been developed for the formation of a double-duplex invasion complex. Prof. Fujimoto et al. succeeded in performing photo-induced double-duplex invasion using ultrafast photo-cross-linking [95]. Artificial nucleobase cyanovinylcarbazole ($^{CNK}$K; Figure 14a) was developed as the class of photoreactive moiety for nucleic acids, enabling covalent bonds between $^{CNK}$K in oligonucleotide
and a pyrimidine base at the −1 position in complementary DNA or RNA [96,97]. To expand this system to invasion, uracil analog 5-cyanouracil (\(\text{CNU}\)) was also used to inhibit photo-cross-linking between each invader strand [95,98]. The reactivity of photo-cross-linking between \(\text{CNVK}\) and \(\text{CNU}\) was lower than \(\text{CNVK}\) and T (Figure 14b,c). A pair of invader probes containing \(\text{CNVK}\) and \(\text{CNU}\) was used for double-duplex invasion at 37 °C. The denaturing PAGE showed two photo-cross-linked products of DNA and invader probes, indicating thermally irreversible covalent bonds formed between probes and ds-DNA by photoirradiation. Moreover, the antigene effect of this photo-cross-linked system was evaluated by in vitro T7 RNA polymerase assay. After photoirradiation, the shorter RNA products were obtained, suggesting the inhibition of T7 RNA polymerase activity by the photo-cross-linked double duplex formation.

![Figure 14](image)

**Figure 14.** (a) Structures of \(\text{CNVK}\) and \(\text{CNU}\). (b) Scheme of photo-cross-linking reactions of \(\text{CNVK}\) and T. (c) The lower reactivity between \(\text{CNVK}\) and \(\text{CNU}\) suppresses the photo-cross-linking between probes.

5. Application of PNA Invasion to Biological Research

PNA invasion, which can recognize sequences in dsDNA, has been applied in various biological studies. As mentioned above, the backbone of PNA is different from those of DNA and peptide, making it highly resistant to nucleases and proteases, and this is a great advantage in studies including intracellular applications. One example of biological applications of PNA invasion is the control of enzyme functions working on DNA (Figure 15a). Enzymatic functions of restriction enzymes and methylases were successfully inhibited by double-duplex invasion [99,100]. The control (both inhibition and activation) of RNA transcription activity using invasion has also been reported [42,56,101,102]. As a genetic engineering tool, the construction of DNA cutters has been accomplished based on PNA invasion [103,104] (Figure 15b). In addition, genome editing, which is currently a key technology in molecular biology research, has been reported to be achieved by PNA invasion complex formation [38,57,58,105–107].
5. Application of PNA Invasion to Biological Research

The above applications are based on the sequence-specific recognition of dsDNA via invasion complex formation. More interestingly, invasion is accompanied by a significant change in the local structure of the target dsDNA. This is entirely different from the simple DNA binding event and makes it possible to develop novel biotechnologies such as enzyme recognition control and genome editing.

6. Conclusions

In this paper, PNA, which exhibits one of the highest DNA-binding abilities among various artificial nucleic acids, is reviewed, with a particular spotlight on its characteristic dsDNA recognition mode, double-duplex invasion. This invasion enables direct and sequence-specific recognition of sequences in dsDNA, which is difficult with conventional artificial nucleic acids, and is expected to be applied in a wide variety of applications. More specifically, if sequence-selective recognition of genomic DNA in cells and in vivo becomes possible, gene function can be controlled as desired. As PNA and invasion research continue to expand, it would be also possible to establish new non-protein genome editing technologies based on PNAs. As described in this review, various PNA invasion research, including backbone modification and chemical modification, has been intensively studied in recent years. Further optimization of PNA from a chemical approach is expected to promote the application of PNA to research in the fields of biochemistry, chemical biology, and molecular biology.

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References

1. Crick, F.H. On protein synthesis. *Symp. Soc. Exp. Biol.* 1958, 12, 138–163. [PubMed]
2. Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012, 337, 816–821. [CrossRef] [PubMed]
3. Qi, L.S.; Larson, M.H.; Gilbert, L.A.; Doudna, J.A.; Weissman, J.S.; Arkin, A.P.; Lim, W.A. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 2013, 152, 1173–1183. [CrossRef] [PubMed]
4. Doudna, J.A.; Charpentier, E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 2014, 346, 1258096. [CrossRef]
5. Kim, Y.G.; Cha, J.; Chandrasegaran, S. Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. *Proc. Natl. Acad. Sci. USA* 1996, 93, 1156–1160. [CrossRef] [PubMed]
6. Wolfe, S.A.; Nekludova, L.; Pabo, C.O. DNA recognition by Cys(2)His(2) zinc finger proteins. *Annu. Rev. Biophys. Biomol. Struct.* 2000, 29, 183–212. [CrossRef]
7. Jantz, D.; Amann, B.T.; Gatto, G.J.; Berg, J.M. The Design of Functional DNA-Binding Proteins Based on Zinc Finger Domains. *Chem. Rev.* **2004**, *104*, 789–800. [CrossRef] [PubMed]

8. Dhanasekaran, M.; Negi, S.; Sugita, Y. Designer zinc finger proteins: Tools for creating artificial DNA-binding functional proteins. *Acc. Chem. Res.* **2006**, *39*, 45–52. [CrossRef] [PubMed]

9. Urnov, F.D.; Rebar, E.J.; Holmes, M.C.; Zhang, H.S.; Gregory, P.D. Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.* **2010**, *11*, 636–646. [CrossRef] [PubMed]

10. Boch, J.; Scholze, H.; Schornack, S.; Landgraf, A.; Kay, S.; Nickstadt, A.; Bonas, U. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* **2009**, *326*, 1509–1512. [CrossRef] [PubMed]

11. Christian, M.; Cermak, T.; Doyle, E.L.; Schmidt, C.; Zhang, F.; Hummel, A.; Bogdanove, A.J.; Voytas, D.F. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* **2010**, *186*, 757–761. [CrossRef] [PubMed]

12. Trauger, J.W.; Baird, E.E.; Dervan, P.B. Recognition of DNA by designed ligands at subnanomolar concentrations. *Nature* **1996**, *382*, 559–561. [CrossRef]

13. Dervan, P.B.; Egholm, M.; Berg, R.H.; Buchardt, O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* **1991**, *254*, 1497–1500. [CrossRef] [PubMed]

14. Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.-I.; In, Y.; Ishida, T.; Imanishi, T. Synthesis of 2′,4′-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C3′-endo sugar puckering. *Tetrahedron Lett.* **1997**, *38*, 8735–8738. [CrossRef]

15. Steffens, R.; Leumann, C.J. Tricyclo-DNA: A Phosphodiester-Backbone Based DNA Analog Exhibiting Strong Complementary Base-Pairing Properties. *J. Am. Chem. Soc.* **1997**, *119*, 11548–11549. [CrossRef]

16. Oka, N.; Wada, T.; Saigo, K. An Oxazaphospholidine Approach for the Stereocontrolled Synthesis of Oligonucleoside Phosphorothioates. *J. Am. Chem. Soc.* **2003**, *125*, 8307–8317. [CrossRef]

17. Zhang, L.; Peritz, A.; Meggers, E. A Simple Glycol Nucleic Acid. *J. Am. Chem. Soc.* **2005**, *127*, 4174–4175. [CrossRef] [PubMed]

18. Kumar, T.S.; Madsen, A.S.; Wengel, J.; Hrdlicka, P.J. Synthesis and Hybridization Studies of 2′-Amino-α-L-LNA and Tetracyclic “Locked LNA”. *J. Org. Chem.* **2006**, *71*, 4188–4201. [CrossRef] [PubMed]

19. Campbell, M.A.; Wengel, J. Locked vs. unlocked nucleic acids (LNA vs. UNA): Contrasting structures work towards common therapeutic goals. *Chem. Soc. Rev.* **2011**, *40*, 5680–5689. [CrossRef]

20. Kashida, H.; Murayama, K.; Toda, T.; Asanuma, H. Control of the Chirality and Helicity of Oligomers of Serinol Nucleic Acid (SNA) by Sequence Design. *Angew. Chem. Int. Ed.* **2011**, *50*, 1285–1288. [CrossRef]

21. Oka, N.; Wada, T. Stereocontrolled synthesis of oligonucleotide analogs containing chiral internucleotide phosphorus atoms. *Chem. Soc. Rev.* **2011**, *40*, 5829–5843. [CrossRef] [PubMed]

22. Komiyama, M.; Yoshimoto, K.; Sisido, M.; Ariga, K. Chemistry Can Make Strict and Fuzzy Controls for Bio-Systems: DNA Nanoarchitectonics and Cell-Macromolecular Nanoarchitectonics. *Bull. Chem. Soc. Jpn.* **2017**, *90*, 967–1004. [CrossRef]

23. Takezawa, Y.; Muller, J.; Shionoya, M. Artificial DNA Base Pairing Mediated by Diverse Metal Ions. *Chem. Lett.* **2017**, *46*, 622–633. [CrossRef]

24. Ukale, D.U.; Lönneberg, T. 2,6-Dimercuriphenol as a Bifacial Dinuclear Organometallic Nucleobase. *Angew. Chem. Int. Ed.* **2018**, *57*, 16171–16175. [CrossRef]

25. Kimoto, M.; Hirao, I. Genetic alphabet expansion technology by creating unnatural base pairs. *Chem. Soc. Rev.* **2020**, *49*, 7602–7626. [CrossRef]

26. Asanuma, H.; Kamiya, Y.; Kashida, H.; Murayama, K. Xeno nucleic acids (XNAs) having non-ribose scaffolds with unique supramolecular properties. *Chem. Commun.* **2022**, *58*, 3993–4004. [CrossRef]

27. Thuong, N.T.; Hêlène, C. Sequence-Specific Recognition and Modification of Double-Helical DNA by Oligonucleotides. *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 666–690. [CrossRef]

28. Praseuth, D.; Guieysse, A.L.; Hêlène, C. Triplex helix formation and the antigene strategy for sequence-specific control of gene expression. *Biochim. Biophys. Acta Gene Struct. Expr.* **1999**, *1489*, 181–206. [CrossRef]

29. Fox, K.R. Targeting DNA with triplexes. *Curr. Med. Chem.* **2000**, *7*, 17–37. [CrossRef] [PubMed]

30. Komiyama, M.; Aiba, Y.; Ishizuka, T.; Sumaoka, J. Solid-phase synthesis of pseudo-complementary peptide nucleic acids. *Nat. Protoc.* **2008**, *3*, 646–654. [CrossRef] [PubMed]

31. Demidov, V.V.; Frank-Kamenetski, M.D. Two sides of the coin: Affinity and specificity of nucleic acid interactions. *Trends Biochem. Sci.* **2004**, *29*, 62–71. [CrossRef] [PubMed]

32. Nielsen, P.E. Peptide Nucleic Acids (PNA) in Chemical Biology and Drug Discovery. *Chem. Biodivers.* **2010**, *7*, 786–804. [CrossRef]

33. Manicardi, A.; Rozzi, A.; Korom, S.; Corradini, R. Building on the peptide nucleic acid (PNA) scaffold: A biomolecular engineering approach. *Supramol. Chem.* **2017**, *29*, 784–795. [CrossRef]

34. Sharma, C.; Awasthi, S.K. Versatility of peptide nucleic acids (PNAs): Role in chemical biology, drug discovery, and origins of life. *Chem. Biol. Drug Des.* **2017**, *89*, 16–37. [CrossRef]

35. Economos, N.G.; Oyaghire, S.; Quijano, E.; Ricciardi, A.S.; Saltzman, W.M.; Glazer, P.M. Peptide Nucleic Acids and Gene Editing: Perspectives on Structure and Repair. *Molecules* **2020**, *25*, 735. [CrossRef]
39. Muangkaew, P.; Vilaivan, T. Modulation of DNA and RNA by PNA. *Bioorg. Med. Chem. Lett.* 2020, 30, 127064. [CrossRef] [PubMed]
40. Das, A.; Pradhan, B. Evolution of peptide nucleic acid with modifications of its backbone and application in biotechnology. *Chem. Biol. Drug Des.* 2021, 97, 865-892. [CrossRef]
41. Liang, X.; Liu, M.; Komiyma, M. Recognition of Target Site in Various Forms of DNA and RNA by Peptide Nucleic Acid (PNA): From Fundamentals to Practical Applications. *Bull. Chem. Soc. Jpn.* 2021, 94, 1737–1756. [CrossRef]
42. Lohse, J.; Dahl, O.; Nielsen, P.E. Double duplex invasion by peptide nucleic acid: A general principle for sequence-specific targeting of double-stranded DNA. *Proc. Natl. Acad. Sci. USA* 1999, 96, 11804–11808. [CrossRef] [PubMed]
43. Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S.M.; Driver, D.A.; Berg, R.H.; Kim, S.K.; Norden, B.; Nielsen, P.E. PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature* 1993, 365, 566–568. [CrossRef] [PubMed]
44. Jensen, K.K.; Orum, H.; Nielsen, P.E.; Norden, B. Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with the BIAcore technique. *Biochemistry* 1997, 36, 5072–5077. [CrossRef]
45. Demidov, V.V.; Potaman, V.N.; Frank-Kamenetski, M.D.; Egholm, M.; Buchard, O.; Sonnichsen, S.H.; Nielsen, P.E. Stability of peptide nucleic acids in human serum and cellular extracts. *Biochem. Pharmacol.* 1994, 48, 1310–1313. [CrossRef]
46. Kuwahara, M.; Arimitsu, M.; Sisido, M. Novel peptide nucleic acid that shows high sequence specificity and all-or-none-type hybridization with the complementary DNA. *J. Am. Chem. Soc.* 1999, 121, 256–257. [CrossRef]
47. Wada, T.; Minamimoto, N.; Inaki, Y.; Inoue, Y. Peptide Ribonucleic Acids (PRNA). 2. A Novel Strategy for Active Control of DNA Recognition through Borate Ester Formation. *J. Am. Chem. Soc.* 2000, 122, 6900–6910. [CrossRef]
48. Zhou, P.; Wang, M.; Du, L.; Fisher, G.W.; Waggoner, A.; Ly, D.H. Novel binding and efficient cellular uptake of guanidine-based peptide nucleic acids (GPNA). *J. Am. Chem. Soc.* 2003, 125, 6878–6879. [CrossRef]
49. Myers, M.C.; Witschi, M.A.; Larionova, N.V.; Franck, J.M.; Haynes, R.D.; Hara, T.; Grajkowski, A.; Appella, D.H. A cyclopentane conformational restraint for a peptide nucleic acid: Design, asymmetric synthesis, and improved binding affinity to DNA and RNA. *Org. Lett.* 2003, 5, 2695–2698. [CrossRef]
50. Shiroke, P.S.; Kumar, V.A.; Ganesh, K.N. (2S,5R/2R,5S)-aminoethylpipecolyl aepipi-aegPNA chimera: Synthesis and duplex/triplex stability. *Tetrahedron* 2004, 60, 9485–9491. [CrossRef]
51. Govindaraju, T.; Kumar, V.A.; Ganesh, K.N. Synthesis and evaluation of (1S,2R/1R,2S)-aminocyclohexylglycyl PNAs as conformational restraint for a peptide nucleic acid: Design, asymmetric synthesis, and improved binding affinity to DNA and RNA. *Bull. Chem. Soc. Jpn.* 2003, 76, 3677–3680. [CrossRef] [PubMed]
52. Cherny, D.Y.; Belotserkovskii, B.P.; Frank-Kamenetskii, M.D.; Egholm, M.; Buchardt, O.; Berg, R.H.; Nielsen, P.E. DNA unwinding upon strand-displacement binding of a thymine-substituted polyamide to double-stranded DNA. *Proc. Natl. Acad. Sci. USA* 1993, 90, 1667–1670. [CrossRef] [PubMed]
53. Egholm, M.; Christensen, L.; Dueholm, K.L.; Buchardt, O.; Coull, J.; Nielsen, P.E. Efficient pH-independent sequence-specific DNA binding by pseudoisocytosine-containing bis-PNA. *Nucleic Acids Res.* 1995, 23, 217–222. [CrossRef]
54. Griffith, M.C.; Risen, L.M.; Greig, M.J.; Lesnich, E.A.; Sprankle, K.G.; Grif thief, R.H.; Kiely, J.S.; Freier, S.M. Single and Bis Peptide Nucleic-Acids as Triplexing Agents—Binding and Stoichiometry. *J. Am. Chem. Soc.* 1995, 117, 831–832. [CrossRef]
55. Bentin, T.; Larsen, H.J.; Nielsen, P.E. Combined triplex/duplex invasion of double-stranded DNA by “tail-clamp” peptide nucleic acid. *Biochemistry* 2003, 42, 13987–13995. [CrossRef]
56. Kaitha, K.; Shah, R.H.; Zhao, X.; Corey, D.R. Extending recognition by peptide nucleic acids (PNAs): Binding to duplex DNA and inhibition of transcription by tail-clamp PNA-peptide conjugates. *Biochemistry* 2003, 42, 13996–14003. [CrossRef]
57. Bahal, R.; Ali Meneer, N.; Quijano, E.; Liu, Y.; Sulkowski, P.; Turchick, A.; Lu, Y.-C.; Bhunia, D.C.; Manna, A.; Greiner, D.L.; et al. In vivo correction of anaemia in β-thalassemic mice by γPNA-mediated gene editing with nanoparticle delivery. *Nat. Commun.* 2016, 7, 13304. [CrossRef]
58. Ricciardi, A.S.; Bahal, R.; Farrelly, J.S.; Quijano, E.; Bianchi, A.H.; Luks, V.L.; Putman, R.; López-Giráldez, F.; Coşkun, S.; Song, E.; et al. In utero nanoparticle delivery for site-specific genome editing. *Nat. Commun.* 2018, 9, 2481. [CrossRef]
59. Nielsen, P.E.; Christensen, L. Strand displacement binding of a duplex-forming homopurine PNA to a homopyrimidine duplex DNA target. *J. Am. Chem. Soc.* 1996, 118, 2287–2288. [CrossRef]
60. Aiba, Y.; Yamamoto, Y.; Komiyma, M. Activation of double-stranded DNA by one pcPNA strand for its site-selective scission with Ce-IV/EDTA. *Chem. Lett.* 2007, 36, 780–781. [CrossRef]
61. Rapiredy, S.; He, G.; Roy, S.; Armitage, B.A.; Ly, D.H. Strand invasion of mixed-sequence B-DNA by acridine-linked, gammapentaic acid nucleic (gamma-PNA). *J. Am. Chem. Soc.* 2007, 129, 15596–15600. [CrossRef] [PubMed]
62. Shigi, N.; Mizon, Y.; Kunifuda, H.; Matsumura, K.; Komiyma, M. Promotion of Single-Strand Invasion of PNA to Double-Stranded DNA by Pseudo-Complementary Base Pairing. *Bull. Chem. Soc. Jpn.* 2019, 92, 330–335. [CrossRef]
63. Haaima, G.; Hansen, H.F.; Christensen, L.; Dahl, O.; Nielsen, P.E. Increased DNA binding and sequence discrimination of PNA oligomers containing 2,6-diaminopurine. *Nucleic Acids Res.* 1997, 25, 4639–4643. [CrossRef] [PubMed]
64. Wittung, P.; Nielsen, P.E.; Buchardt, O.; Egholm, M.; Norden, B. DNA-like double helix formed by peptide nucleic acid. *Nature* 1994, 368, 561–563. [CrossRef]
65. Haaima, G.; Lohse, A.; Buchardt, O.; Nielsen, P.E. Peptide nucleic acids (PNAs) containing thymine monomers derived from chiral amino acids: Hybridization and solubility properties of D-lysine PNA. *Angew. Chem. Int. Ed.* 1996, 35, 1939–1942.
66. Sforza, S.; Corradini, R.; Ghirardi, S.; Ghirardi, S.; Dossena, A.; Marchelli, R. DNA binding of a D-lysine-based chiral PNA: Direction control and mismatch recognition. Eur. J. Org. Chem. 2000, 2000, 2905–2913. [CrossRef]

67. Ishizuka, T.; Yosida, J.; Yamamoto, Y.; Sumaoka, T.; Pedeschi, T.; Corradini, R.; Sforza, S.; Komiyama, M. Chiral introduction of positive charges to PNA for double-duplex invasion to versatile sequences. Nucleic Acids Res. 2008, 36, 1464–1471. [CrossRef]

68. Englund, E.A.; Appella, D.H. Gamma-substituted peptide nucleic acids constructed from L-lysine are a versatile scaffold for multifunctional display. Angew. Chem. Int. Ed. 2007, 46, 1414–1418. [CrossRef]

69. Pooga, M.; Soomets, U.; Hallbrink, M.; Valkna, A.; Saar, K.; Kahl, U.; Hao, J.X.; Xu, X.J.; Wiesenfeld-Hallin, Z.; et al. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. Nat. Biotechnol. 1998, 16, 857–861. [CrossRef]

70. Bendifallah, N.; Rasmussen, F.W.; Zachar, V.; Ebbesen, P.; Nielsen, P.E.; Koppelhus, U. Evaluation of cell-penetrating peptides (CPPs) as vehicles for intracellular delivery of antisense peptide nucleic acid (PNA). Bioconjugate Chem. 2006, 17, 750–758. [CrossRef]

71. Aiba, Y.; Hamano, Y.; Kameshima, W.; Araki, Y.; Wada, T.; Accetta, A.; Sforza, S.; Corradini, R.; Marchelli, R.; Komiyama, M. Promotion of double-duplex invasion of peptide nucleic acids through conjugation with nuclear localization signal peptide. Chem. Eur. J. 2015, 21, 4021–4026. [CrossRef] [PubMed]

72. Aiba, Y.; Honda, Y.; Komiyama, M. Peptide Nucleic Acid Conjugated with Ruthenium-Complex Stabilizing Double-Duplex Invasion Complex Even under Physiological Conditions. ChemBioChem 2018, 19, 1601–1604. [CrossRef] [PubMed]

73. Hibino, M.; Aiba, Y.; Watanabe, Y.; Shoji, O. Peptide Nucleic Acid Conjugated with a Cationic Guanine Nucleoside Improves the Efficiency of Self-Duplex Invasion to Versatile Sequences. Nucleic Acids Res. 2020, 48, 2546–2549. [CrossRef] [PubMed]

74. Hendler, S.S.; Fuerer, E.; Srinivasan, P.R. Synthesis and chemical properties of monomers and polymers containing 7-methylguanine and an investigation of their substrate or template properties for bacterial deoxyribonucleic acid or ribonucleic acid polymerases. Biochemistry 1970, 9, 4141–4153. [CrossRef]

75. Vilaivan, T.; Srissuwannaket, C. Hybridization of Pyrrolidinyl Peptide Nucleic Acids and DNA: Selectivity, Base-Pairing Specificity, and Direction of Binding. Org. Lett. 2006, 8, 1897–1900. [CrossRef] [PubMed]

76. Mansawat, W.; Vilaivan, C.; Balthas, À.; Atiken, D.J.; Vilaivan, T. Peptide Nucleic Acid Homologues: Effect of Ring Size on Hybridization Properties. Org. Lett. 2012, 14, 1440–1443. [CrossRef]

77. Vilaivan, T. Pyrrolidinyl PNA with a/beta-Dipeptide Backbone: From Development to Applications. Acc. Chem Res. 2015, 48, 1645–1656. [CrossRef] [PubMed]

78. Yokama, T.; Shimata, M.; Kuroda, K.; Ishida, M.; Yamada, T.; Fujita, M. Effect of ring size on hybridization properties of polyamidoamine dendrimers for DNA binding. Bioconjugate Chem. 2016, 27, 1167–1174. [CrossRef]

79. Vilaivan, T.; Aitken, D.J.; Vilaivan, T. Pyrrolidinyl Peptide Nucleic Acid Homologues: Effect of Ring Size on Hybridization Properties. Org. Lett. 2012, 14, 1440–1443. [CrossRef]

80. Vilaivan, T. Pyrrolidinyl PNA with alpha/beta-Dipeptide Backbone: From Development to Applications. Acc. Chem Res. 2015, 48, 1645–1656. [CrossRef] [PubMed]

81. Sforza, S.; Madhanagopal, B.R.; Datta, D.; Ganesh, K.N. Structural Design and Synthesis of Bimodal PNA That Simultaneously Binds Two Complementary DNAs To Form Fused Double Duplexes. J. Org. Chem. 2020, 86, 414–428. [CrossRef] [PubMed]

82. Gupta, M.K.; Madhanagopal, B.R.; Ganesh, K.N. Peptide Nucleic Acid with Double Face: Homothymine–Homocytosine Bimodal PNA That Simultaneously Binds Two Complementary DNA Strands. J. Org. Chem. 2020, 85, 13680–13693. [CrossRef] [PubMed]

83. Bhangardeve, P.; Madhanagopal, B.R.; Ganesh, K.N. Cy(S/R)-Bimodal Peptide Nucleic Acids (Cy-bm-PNA) Form Coupled Double Duplexes by Synchronous Binding to Two Complementary DNA Strands. J. Org. Chem. 2020, 85, 13680–13693. [CrossRef] [PubMed]

84. Gupta, M.K.; Madhanagopal, B.R.; Ganesh, K.N. Peptide Nucleic Acid with Double Face: Homothymine–Homocytosine Bimodal Cx-PNA (bm-Cx-PNA) Forms a Double Duplex of the bm-PNA2:DNA Triplex. J. Org. Chem. 2021, 86, 414–428. [CrossRef] [PubMed]

85. Bhangardeve, P.; Jain, P.; Ganesh, K.N. Molecular Assembly of Triplex of Duplexes from Homothyminyl-Homocytosinyl Cy(S/R)-Bimodal Peptide Nucleic Acids with dA8/dG6 and the Cell Permeability of Bimodal Peptide Nucleic Acids. ACS Omega 2021, 6, 19757–19770. [CrossRef]

86. Thadke, S.A.; Hrideya, V.M.; Perera, J.D.R.; Gil, R.R.; Mukherjee, A.; Ly, D.H. Shape selective bifacial recognition of double helical DNA. Commun. Chem. 2018, 1, 79. [CrossRef]

87. Guenther, D.C.; Anderson, G.H.; Karmakar, S.; Anderson, B.A.; Didion, B.A.; Guo, W.; Verstegen, J.P.; Hrdlicka, P.J. Invader probes: Harnessing the energy of intercalation to facilitate recognition of chromosomal DNA for diagnostic applications. Chem. Sci. 2015, 6, 5006–5015. [CrossRef]

88. Guenther, D.C.; Karmakar, S.; Hrdlicka, P.J. Bulged Invader probes: Activated duplexes for mixed-sequence dsDNA recognition with improved thermodynamic and kinetic profiles. Chem. Commun. 2015, 51, 15051–15054. [CrossRef]

89. Anderson, B.A.; Hrdlicka, P.J. Merging Two Strategies for Mixed-Sequence Recognition of Double-Stranded DNA: Pseudocomplementary Invader Probes. J. Org. Chem. 2016, 81, 3335–3346. [CrossRef]

90. Adhikari, S.P.; Vukelich, P.; Guenther, D.C.; Karmakar, S.; Hrdlicka, P.J. Recognition of double-stranded DNA using LNA-modified toehold Invader probes. Org. Biomol. Chem. 2021, 19, 9276–9290. [CrossRef]
91. Adhikari, S.P.; Karmakar, S.; Hrdlicka, P.J. Nicked Invader probes: Multistranded and sequence-unrestricted recognition of double-stranded DNA. Org. Biomol. Chem. 2022, 12, 3677–3681. [CrossRef] [PubMed]

92. Asanuma, H.; Niwa, R.; Akahane, M.; Murayama, K.; Kashida, H.; Kamiya, Y. Strand-invading linear probe combined with unmodified PNA. Bioorg. Med. Chem. 2016, 24, 4129–4137. [CrossRef]

93. Asanuma, H.; Akahane, M.; Kondo, N.; Osawa, T.; Kato, T.; Kashida, H. Quencher-free linear probe with multiple fluorophores on an acyclic scaffold. Chem. Sci. 2012, 3, 3165–3169. [CrossRef]

94. Asanuma, H.; Akahane, M.; Niwa, R.; Kashida, H.; Kamiya, Y. Highly sensitive and robust linear probe for detection of mRNA in cells. Angew. Chem. Int. Ed. 2015, 54, 4319–4319. [CrossRef] [PubMed]

95. Nakamura, S.; Kawabata, H.; Fujimoto, K. Double duplex invasion of DNA induced by ultrafast photo-cross-linking using 3-cyanovinylcarbazole for antigene methods. Chem. Commun. 2017, 53, 7616–7619. [CrossRef] [PubMed]

96. Yoshimura, Y.; Fujimoto, K. Ultrafast Reversible Photo-Cross-Linking Reaction: Toward in Situ DNA Manipulation. Org. Lett. 2008, 10, 3227–3230. [CrossRef]

97. Fujimoto, K.; Yamada, A.; Yoshimura, Y.; Tsukaguchi, T.; Sakamoto, T. Details of the Ultrafast DNA Photo-Cross-Linking Reaction of 3-Cyanovinylcarbazole Nucleoside: Cis–Trans Isomeric Effect and the Application for SNP-Based Genotyping. J. Am. Chem. Soc. 2013, 135, 16161–16167. [CrossRef]

98. Fujimoto, K.; Hirano, A.; Watanabe, Y.; Shimabara, A.; Nakamura, S. The Inhibition Effect of Photo-Cross-Linking between Probes in Photo-Induced Double Duplex Invasion DNA. ChemBioChem 2021, 22, 3402–3405. [CrossRef]

99. Izvolsky, K.I.; Demidov, V.V.; Nielsen, P.E.; Frank-Kamenetskii, M.D. Sequence-specific protection of duplex DNA against restriction and methylation enzymes by pseudocomplementary PNAs. Biochemistry 2000, 39, 10908–10913. [CrossRef]

100. Protozanova, E.; Demidov, V.V.; Nielsen, P.E.; Frank-Kamenetskii, M.D. Pseudocomplementary PNAs as selective modifiers of protein activity on duplex DNA: The case of type II restriction enzymes. Nucleic Acids Res. 2003, 31, 3929–3935. [CrossRef] [PubMed]

101. Mollegaard, N.E.; Buchardt, O.; Egholm, M.; Nielsen, P.E. Peptide nucleic acid-DNA strand displacement loops as artificial transcription promoters. Proc. Natl. Acad. Sci. USA 1994, 91, 3892–3895. [CrossRef]

102. Wang, G.; Jing, K.; Balczon, R.; Xu, X. Defining the peptide nucleic acids (PNA) length requirement for PNA binding-induced transcription and gene expression. J. Mol. Biol. 2001, 313, 933–940. [CrossRef] [PubMed]

103. Komiyama, M.; Aiba, Y.; Yamamoto, Y.; Sumaoka, J. Artificial restriction DNA cutter for site-selective scission of double-stranded DNA with tunable scission site and specificity. Nat. Protoc. 2008, 3, 655–662. [CrossRef]

104. Lyu, M.; Kong, L.; Yang, Z.; Wu, Y.; McGhee, C.E.; Lu, Y. PNA-Assisted DNAzymes to Cleave Double-Stranded DNA for Genetic Engineering with High Sequence Fidelity. J. Am. Chem. Soc. 2021, 143, 9724–9728. [CrossRef] [PubMed]

105. Faruqi, A.F.; Egholm, M.; Glazer, P.M. Peptide nucleic acid-targeted mutagenesis of a chromosomal gene in mouse cells. Proc. Natl. Acad. Sci. USA 1998, 95, 1389–1403. [CrossRef] [PubMed]

106. Kim, K.H.; Nielsen, P.E.; Glazer, P.M. Site-directed gene mutation at mixed sequence targets by psoralen-conjugated pseudocomplementary peptide nucleic acids. Nucleic Acids Res. 2007, 35, 7604–7613. [CrossRef] [PubMed]

107. Lonkar, P.; Kim, K.H.; Kuan, J.Y.; Chin, J.Y.; Rogers, F.A.; Knauert, M.P.; Kole, R.; Nielsen, P.E.; Glazer, P.M. Targeted correction of a thalassemia-associated β-globin mutation induced by pseudo-complementary peptide nucleic acids. Nucleic Acids Res. 2009, 37, 3635–3644. [CrossRef] [PubMed]