

Review

Recent advances in peptide nucleic acid for cancer bionanotechnology

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Peptide nucleic acid (PNA) is an oligomer, in which the phosphate backbone has been replaced by a pseudopeptide backbone that is meant to mimic DNA. Peptide nucleic acids are of the utmost importance in the biomedical field because of their ability to hybridize with neutral nucleic acids and their special chemical and biological properties. In recent years, PNAs have emerged in nanobiotechnology for cancer diagnosis and therapy due to their high affinity and sequence selectivity toward corresponding DNA and RNA. In this review, we summarize the recent progresses that have been made in cancer detection and therapy with PNA biotechnology. In addition, we emphasize nanoparticle PNA-based strategies for the efficient delivery of drugs in anticancer therapies.

Keywords: peptide nucleic acids; bionanotechnology; cancer diagnostics and therapy

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Introduction

Peptide nucleic acid (PNA) was originally invented in 1991 by Peter E NIELSEN (University of Copenhagen), Michael EGHOLM (University of Copenhagen), Rolf H BERG (Risø National Laboratory), and Ole BUCHARDT (University of Copenhagen) to act as a sequence-specific DNA binding reagent^[1]. PNAs play an important role as therapeutic agents, anticancer agents, molecular tools and biosensor probes, as well as in nanotechnology. PNA bionanotechnology, which includes nanoparticles, nanodevices, and nanoscale phenomena within the discipline of nanotechnology, affords a new approach for scientists to create new functional materials for cancer detection and therapy. PNAs have a distinct N-(2-aminoethyl)glycine backbone. Purines and pyrimidines are then connected to the backbone by methylene carbonyl linkages^[2]. It is easy to hybridize DNA/PNA sequences because the structure of PNA is similar to natural nucleic acid (Figure 1). Specific PNA oligomers have been used as anticancer therapeutics to detect tumor cells or deliver small molecule drugs targeting nucleic acids^[3,4]. However, all reported methodologies require labeling or conjugating steps, which are time-consuming and inefficient. Nanotechnology provides direct, simple, rapid

and label-free methods for PNA application. This technical approach provides an extremely attractive solution for cancer diagnosis and therapy.

Nanoparticles usually act as a bridge between bulky materials and molecular structures. Incorporating PNAs with nanoparticles enables the complexes to possess the properties of both nanoparticles and PNAs. For example, nanoparticles often possess optical properties. Surface-coating fluorescent nanoparticles could be used to image tumor tissues or RNA molecules in living cells^[5]. Some nanoparticle-PNA complexes have high surface-to-volume ratios, which greatly enhance the detection limit of biosensors. As PNA has an uncharged backbone, PNA/DNA duplexes are more stable than the DNA/DNA equivalents. Therefore, single-base mismatches of PNA/DNA duplexes have a better destabilizing effect. The neutral amide backbone of PNAs has a significant advantage in that it can hybridize with DNA under low salt conditions. This indicates that there is no need to counteract the inter-strand repulsion between the two negatively charged nucleic acids with positive ions. PNA oligomers can not only effectively bind to complementary DNA/RNA but also have extensive storage temperature and pH ranges^[6]. The unnatural backbone of PNAs makes them resistant to both nucleases and proteases^[7]. Based on the excellent chemical and biological stability of PNAs, there is a growing interest in PNAs as gene therapy drugs, diagnostic reagents and molecular beacons^[8–10]. In this review, we summarize the current research on PNA

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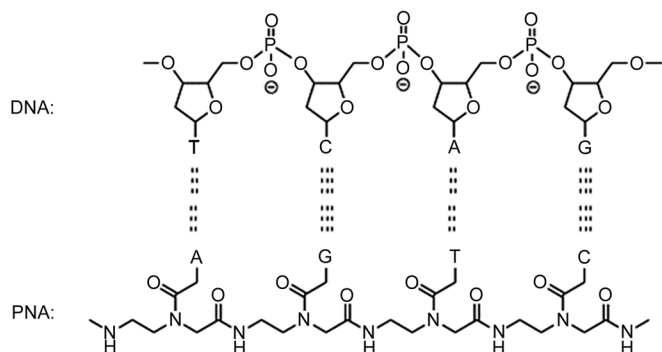


Figure 1. PNAs hybridize to complementary DNA by hydrogen bonding. A=adenine, G=guanine, C=cytosine and T=thymine.

bionanotechnology in cancer treatment and its limitations. We focus on the use of nanoparticle-based delivery systems, PNA anticancer drugs for tumor therapy, and PNA probes for cancer diagnosis.

Synthesis of PNA

In recent years, PNA oligomers have been used as antibacterial and antisense drugs, as well as for nucleic acid diagnosis and molecular biology. With the excellent binding ability of PNA, it is unnecessary to design long PNA oligomers for these applications. However, specificity is the main concern in designing long PNA oligomers. Unlike the phosphodiester backbone of nucleic acids, a PNA has an achiral backbone of N-(2-aminoethyl)glycine, which connects to the nucleotide bases. This unique achiral structure enables PNAs to be easily synthesized without considering stereoselectivity.

The synthesis of PNA oligomers can be carried out easily by

a standard, solid-phase protocol using Fmoc-protected PNA monomers^[11-13]. Typically, the PNA oligomers are deprotected and cleaved off of the resin using 4-trifluoromethylsalicylic acid (TFMSA)/trifluoroacetic acid (TFA) and then purified by reverse-phase high-performance liquid chromatography (HPLC). PNA oligomers can be characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. PNA-peptide conjugates can be synthesized by continuous synthesis using standard peptide conjugation techniques, such as maleimide cysteine coupling or thioester condensation. Inspired by the synthesis of PNAs, a large number of PNA analogs^[14], such as a variety of non-natural nucleobases, have been synthesized^[15-18] (Figure 2). The amino groups of the PNA monomers are usually protected by Fmoc (9-fluorenylmethoxycarbonyl group) or Bhoc (benzhydryloxycarbonyl group)^[12]. Originally, PNA monomers were connected on a Merrifield resin (methylbenzhydrylamine) in a fritted vial. After capping the unreacted groups in resin, the Fmoc group was deprotected by adding piperidine solution (20% piperidine in dimethylformamide). The following monomers of PNA were connected to the resin one after another by reacting with the corresponding coupling reagents. At the end of the synthesis, the Fmoc group was removed by a piperidine solution. The Bhoc group, which protects the A, C, G, T monomers, was removed by trifluoroacetic acid (Figure 3).

Advantages of PNA

Thermal stability of PNA and its hybrid complexes

PNA oligomers have several important advantages for biological applications. As compared with DNA/DNA and DNA/RNA duplexes, PNA/RNA and PNA/DNA duplexes have excellent thermal stability^[19]. The neutral PNA backbone

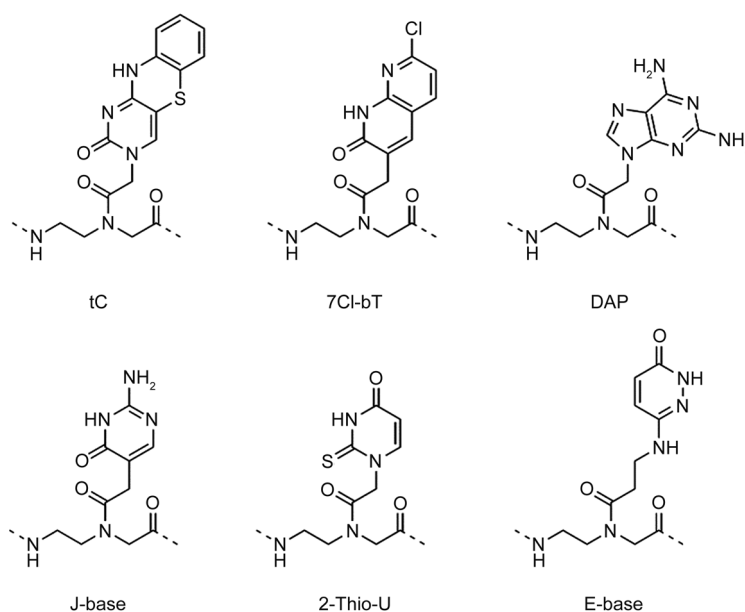


Figure 2. Fmoc/Bhoc-protected PNA monomers.

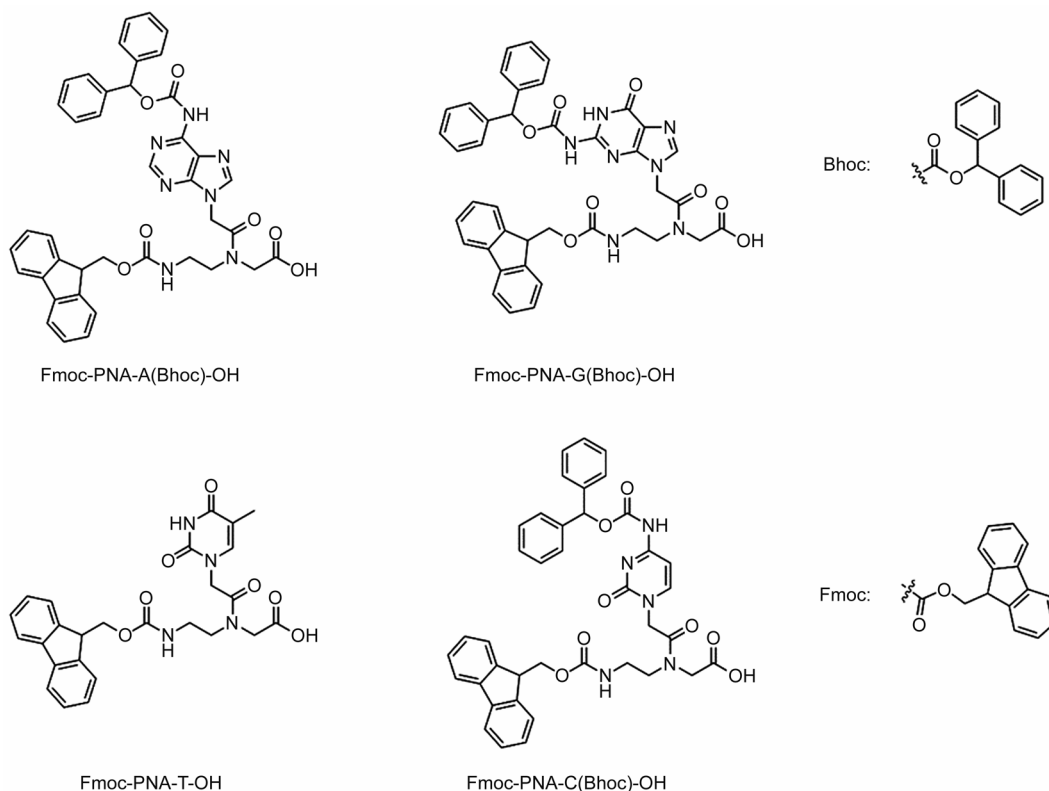


Figure 3. Chemical structures of the unnatural nucleobases used in PNA oligomers.

lacks a repulsion charge when binding with DNA/RNA, making the complexes more stable. As reported by Shakeel *et al*, a 15-mer PNA/DNA or PNA/RNA duplex has a 70°C melting temperature, whereas the corresponding DNA/DNA or DNA/RNA duplexes are approximately 55°C. Generally, in 100 mmol/L NaCl solution, the melting temperature of PNA/DNA duplexes is 1°C higher (per base pair), as compared with the melting temperature of the corresponding DNA/RNA duplexes. For a 15-mer PNA/DNA or PNA/RNA duplex, the melting temperature values are approximately 20°C higher than the natural nucleic acid duplexes^[7].

Stronger binding ability in high ionic strength

The neutral backbone enables PNAs to form stable PNA/DNA duplexes at high salt concentrations. The melting temperatures of the duplexes in high salt concentration are higher than the DNA/DNA duplexes^[20]. At low ionic strength, at certain temperatures, PNAs have a stronger ability to bind complementary DNA/RNA sequences than DNA hybridization. In contrast to Mg²⁺ inhibition of DNA/DNA duplex formation, hybridization of a PNA can be carried out in the absence of Mg²⁺. Therefore, the appropriate ionic strength can eliminate the influence of DNA/RNA in the sample.

Greater interaction specificity

Another advantage is the greater specificity of PNA when interacting with complementary PNA/RNA^[21]. A PNA/DNA or PNA/RNA base pair mismatch usually is much more

destabilizing than a mismatch in a neutral nucleic acid duplex. On average, a single mismatch in PNA/DNA or PNA/RNA 15-mers decreases the melting temperature by 15°C. Additionally, a single mismatch of the DNA/DNA duplexes lowers the melting temperature by 11°C.

Strong strand invasion ability

PNA oligomers have a strong strand invasion ability. PNA oligomers that contain cytosine and thymine usually bind with nucleic acids in a 2/1 (PNA/DNA) manner and form PNA/DNA/PNA triplexes^[22, 23]. The triplex is formed by first assembling a PNA/DNA duplex. Then, the second PNA oligomer is inserted into the major groove of the PNA/DNA double helix. Because the triplexes are very stable (melting temperature >70°C for a 10-mer complex), PNAs can even invade the strands of neutral nucleic acid duplexes by displacing one of the DNA strands and forming a D loop in the double-stranded DNA. This characteristic has been applied in gene expression analysis.

Resistance to nucleases and proteases

The different skeletons of PNAs (as comparing with DNA/RNA) determine their peptide backbone and are not easily degraded by nucleases or proteases^[6].

PNA bionanotechnology for cancer therapy

PNA as anticancer drug carriers

PNAs designed as cancer drugs are valuable for cell-specific

nucleic acid delivery because they can strongly bind to specific nucleic acids (*eg*, microRNA). In some diseases, microRNAs, for example miR-21, are key regulators of oncogenic processes and are usually overexpressed^[24-26]. MicroRNAs are endogenous short RNAs that have important cellular functions^[27-30] and they are ideal diagnostic and therapeutic targets in cancer therapy^[31, 32].

Lee *et al* developed a miRNA-responsive theranostic platform in the treatment of cancer^[33]. The microRNA-responsive drug is composed of a photosensitizer, chlorin e6 (Ce6), which is connected to the PNA sequence and the dextran-coated reduced graphene oxide nanocolloid (Dex-RGON). The synthesized PNA has a complementary sequence to cancer-specific miR-21, which can selectively hybridize to cancer cells. Upon binding to cancer cells, Ce6-PNA is released from Dex-RGON, and the fluorescence of Ce6 is restored. Through near-infrared irradiation, Ce6 acts as a photosensitizer and can kill cancer cells.

To increase the selectivity and transfection efficiency of therapeutic nucleic acids in tumor cells, Zhang *et al* developed a dual receptor-targeted quantum dots (QDs) gene carrier^[34]. The carrier is composed of four segments: CdSe/ZnS QDs, biotinylated anti-survivin PNAs (anti-survivin antisense oligonucleotides: 5'-(biotin-SS)-CCCAGCCTTCCAGCTCCTTG-3'), streptavidin and two targeting peptides, GE11 and c(RGDfK). The target PNA sequence can selectively bind to epidermal growth factor receptors (EGFRs) and integrin $\alpha v\beta 3$ receptors, which increase the cellular uptake of QD carriers. The gene carrier can effectively increase the efficiency of gene silencing and shows the potential for great application in cancer therapy. Furthermore, they investigated a quantum dot probe for detecting the down-regulation of folate receptor (hFR) in breast cancer cells. The probe is composed of a biotinylated antisense oligonucleotide (AS-ODN, a 21-mer single-strand DNA molecule), a biotinylated p160 peptide (VPWMEPAY-QRFL) and a streptavidin-coated core-shell CdSe/ZnS QD. The probe could specifically target MCF-7 human breast cancer cells and real-time track AS-ODN cellular delivery. AS-ODNs can bind to hFR and inhibit its expression. Thus, with the binding of hFR, the probe can be used to track gene function in anticancer therapy^[35].

PNA as cancer drugs

The development of chemical approaches for controlling gene expression will facilitate investigations of new gene therapy drugs for cancer therapy^[36]. PNAs are perfect candidates in the design of gene therapeutic drugs due to their strong strand invasion ability and both chemical and biological stabilities^[37-39]. Two strategies have been involved in using PNAs as therapeutic drugs, namely, antisense and anti-gene therapies.

Antisense oligonucleotides have been widely used to block the expression of genes or alter splicing^[40]. The antisense effect of PNA is normally due to the steric blocking of RNA transport and translation into the cytoplasm. PNAs have exceptionally high affinity to complementary sequences and possess an uncharged backbone; thus, it is naturally difficult

for them to interact with many cellular proteins that bind anionic macromolecules^[41]. *In vitro* translation experiments with rabbit reticulocyte lysates display that PNAs are capable of inhibiting translation by overlapping the AUG start codon at the targets and forming duplex and triplex complexes. In the mRNA coding region, PNAs are able to bind the translation machinery by forming triplexes. Unlike duplex-forming PNAs, the formation of the triplex requires a target containing 10 to 15 homopurine bases. Triplex-forming PNAs have the ability to inhibit translation at initiation codon targets.

PNAs have been used to image the expression of an endogenous gene in brain cancer and to act as a targeted PNA radiopharmaceutical^[42]. Suzuki *et al* designed two PNAs, which were antisense to either rat caveolin-1- (CAV) mRNA or rat glial fibrillary acidic protein (GFAP) mRNA. These two PNAs contain an amino-ended diethylenetriaminepentaacetic acid and a carboxyl-terminal-N-biotinyl lysine moiety. The amino-terminal diethylenetriaminepentaacetic acid was designed to chelate radioactive ¹¹¹In, and the carboxyl-terminal-biotinyl lysine residue was used to connect with the delivery system (Figure 4). The carboxyl-terminal-biotinyl lysine residue is composed of streptavidin (SA) and a murine OX26 monoclonal antibody, which is connected by a thiol-ether linker. The control group PNAs were designed without SA-OX26. By attaching the PNA antisense agent, the antisense radiopharmaceutical is able to penetrate the membrane barriers and access the target mRNA. The PNA-modified radiopharmaceutical can then be used to image endogenous gene expression. Another strategy is the use of PNAs as anti-gene therapy drugs. The unmatched ability of PNAs to invade duplex DNA suggests that they can bind to chromosomal targets and act as anti-gene agents. For this purpose, PNAs were synthesized to recognize or hybridize to specific sequences in a particular gene. By forming a triplex or a strand invasion complex with

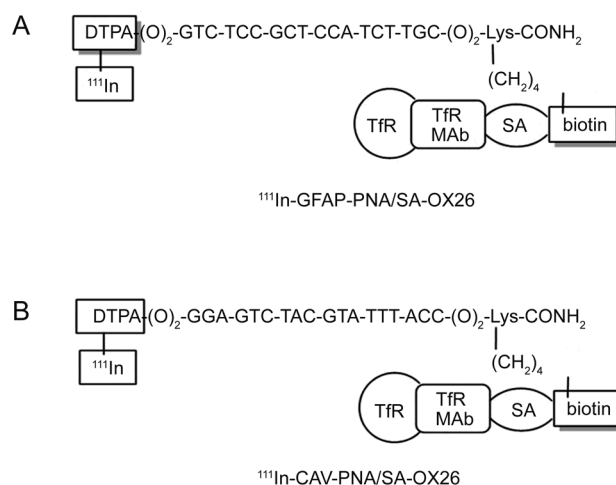


Figure 4. (A) The structure of an 18-mer PNA which is antisense to rat GFAP mRNA. (B) The structure of an 18-mer PNA, which is antisense to rat CAV mRNA. Tfr represents rat transferrin receptor; SA represents streptavidin.

DNA, PNAs are capable of arresting transcriptional processes. These complexes can work as anti-gene agents by blocking the stable functioning of RNA polymerase through the formation of a structural hindrance. *In vitro* studies show that the transcription processes of both prokaryotic and eukaryotic RNA polymerases can be affected via these complexes. Moreover, PNAs can also affect polymerase progression and transcription elongation by targeting the promoter regions or further downstream from the promoter region.

PNAs are further used in gene editing technology. McNeer *et al* found that PNA/DNA-containing nanoparticles are more effective than DNA-containing nanoparticles to produce *in vivo* gene editing in human hematopoietic cells^[8]. Instead of gene knock-in, this non-toxic, virus-free poly(lactic-co-glycolic acid) (PLGA) nanoparticle can be easily reengineered to target various genes, cells and mediate site-specific modification in human hematopoietic stem and progenitor cells (HSPCs) *in vitro*. This high efficiency and low toxicity gene editing technology can be applied in the treatment of cancer, which involves multiple organs and target cells *in vivo*.

PNAs as antisense and anti-gene therapy drugs open the door for anticancer drug design. However, unlike nucleic acid oligonucleotides, it is difficult for the N-(2-aminoethyl) glycine backbone of PNAs to enter cells by endocytosis^[43]. Many novel strategies have been explored to increase the ability of PNAs to penetrate the cell. One strategy is to graft the positively charged residues to PNA molecules. For example, lysine and arginine have been introduced into PNA sequences to make them more accessible from the cell membrane. Short peptides or other ligands, such as antibodies and steroids, are involved in the construction of PNAs to increase the efficiency of delivery^[44, 45].

PNA bionanotechnology for cancer detection

PNAs were originally designed for the recognition of double-stranded DNA. However, their extraordinary thermal stability and synthesis technology enable their potential use as excellent probes for cancer detection.

PNA-silicon nanowire biosensor for detection of miRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules found in animals and plants. MicroRNAs play an important role in gene regulation. Because many miRNAs are overexpressed in cancer diseases^[46], the detection of miRNAs is crucial in cancer diagnosis. PNA has a neutral backbone, which increases its hybridization ability with miRNA. Using PNA-functionalized silicon nanowires (SiNWs), single base differences in miRNA can be discriminated. Thus, specific miRNAs can be identified in the total RNA extracted from cancer cells. Furthermore, SiNWs have a high surface-to-volume ratio, which enhances the sensitivity of the detection.

Zhang *et al* reported a label-free PNA-SiNWs biosensor for the ultrasensitive detection of miRNA. The biosensor is composed of an SiNW array and a PNA molecule. The PNA is covalently immobilized on the electrically addressable SiNW surface and serves as a receptor to recognize miRNAs. The

biosensor can sensitively bind to miRNAs without labeling. The excellent sensitivity of the biosensor is suitable for the early detection of miRNAs in cancer diagnostics^[47].

PNA as a detector for nucleic acid single base detection

The detection of specific single bases in nucleic acid sequences is of great importance in the diagnosis of inherited diseases and genetic disorder-induced cancers. Short PNA oligomers have excellent thermal stabilities upon binding to nucleic acids. Single base mismatches will affect the thermal stability of the effectively formed duplex. Thus, PNA oligomers have potential applications in the detection of single, specific nucleic acid bases. Ficht *et al* reported that several labeled PNA probes can be used to detect specific, single DNA nucleotides. Using native chemical ligation, two adjacent PNA probes were hybridized together, which then served as an indicator for the target DNA^[48-51].

PNA as a biosensor for the detection of nucleic acids

PNAs have attracted increasing interest as biosensors for the detection of nucleic acids. PNA biosensors display real-time spatial and temporal resolution in the detection of nucleic acids. In living cells, they can distinguish bound DNA/RNA from unbound with a high signal-to-background ratio. Currently, as compared with traditional detection methods, PNA probes exhibit significant advantages in nucleic acid detection for cancer diagnosis^[52]. Ostromohov *et al* developed a novel assay that can rapidly detect nucleic acids with high sensitivity. The detection assay is composed of isotachopheresis (ITP) and PNA probes. PNA probes have low electrophoretic mobility, which cannot be identified under ITP unless bound to their complementary sequence. In this way, they can easily identify nucleic acid sequences via ITP focusing^[53].

PNA oligonucleotides with unnatural backbones have been used as molecular beacons (MBs) for the detection of nucleic acids due to their excellent sensitivity and selectivity. PNA MBs are composed of a fluorescent dye and a quencher molecule at each terminus. They have stable stem-loop structures, which are composed of a fluorescent reporter and a quencher. By transferring energy to a proximate quencher, the fluorescence of the reporter dye can be effectively quenched in solution^[54]. The mechanism is achieved first by hybridizing with the nucleic acid sequence. Then, the stem-loop structure of the PNA is destroyed by hybridizing with DNA/RNA, which separates the fluorescent dye and the quencher. PNA beacons have unique, thermodynamically stable structure, efficient intrinsic signal switching, diverse fluorophore-quencher pairs, and good sensitivity and selectivity, making them excellent candidates for the detection of nucleic acids^[55-59].

PNA MBs usually bind to double-stranded DNA in the major groove via four different hybridizing modes (Figure 5). Among these four binding modes, double duplex invasion requires a DNA sequence with at least 50% adenine-thymine content, whereas the other three modes require a homopyrimidine or homopurine DNA sequence^[60]. A well-designed PNA MB can be used as a powerful tool for cancer cell imaging.

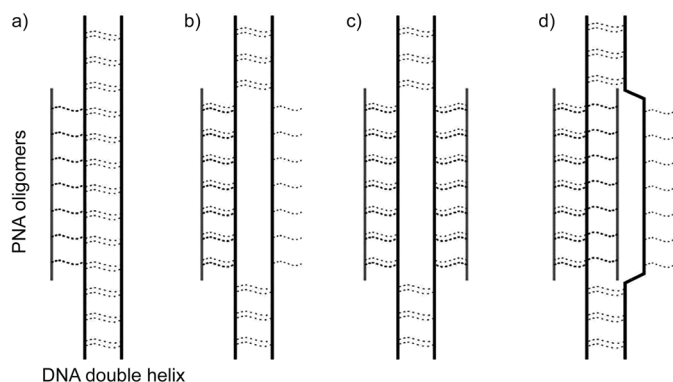


Figure 5. The scheme shows that PNA has 4 different ways to hybridize duplex DNA. a) Conventionally-formed triplex; b) Triplex invasion complexes; c) Double duplex invasion complexes; and d) Duplex invasion complexes.

Kam *et al* reported a PNA molecular beacon that served as a diagnostic probe for *in vitro* and *ex vivo* detection of colorectal cancer (CRC). The molecular beacon was composed of a colon cancer-associated transcript 1 (CCAT1) specific PNA, a thiazole orange (TO) fluorescent dye and a cell-penetrating peptide. They observed the *in situ* hybridization of a PNA beacon containing CCAT1 with human CRC specimens. This PNA MB is a powerful diagnostic tool for the detection of CRC^[61].

Because PNA oligomers have good stability and specific hybridization with nucleic acids, PNA MBs are ideal for nucleic acid detection^[62, 63]. Ortiz *et al* reported a PNA-DNA MB that was composed of the fluorescent dye 7-amino-4-methylcoumarin-3-acetic acid and the fluorescent quencher p-(dimethylaminophenylazo) benzoic acid (Figure 6). Upon binding to a complementary DNA sequence, the probe underwent fluorescence enhancement.

PNA MBs can also be used to detect quadruplex DNA. Datta *et al* reported a PNA MB that could invade quadruplex structures of the thrombin binding aptamer (TBA) and form stable complexes^[64].

PNA probe for microarray

PNAs have been used in microarrays^[65]. Unlike DNA microarrays, PNA microarrays exhibit excellent sensitivity, selectivity and stability under various conditions. Due to their unique properties, PNA chips have better accuracy, good reproducibility and lengthier storage time. PNA microarrays have been used for the study of pathogen identification and functional genomics. The capability of PNA microarrays largely depends on the properties for attaching PNA molecules to the surface.

Brandt *et al* used PNA microarrays to hybridize unlabeled DNA samples. PNAs were first complexed with gold electrodes on the chip surface. DNAs were then added to the microarray. After hybridization with PNAs, the complexes were further bound by adding the intercalating dye. These hybridization events were monitored by electrochemical methods (Figure 7)^[66, 67].

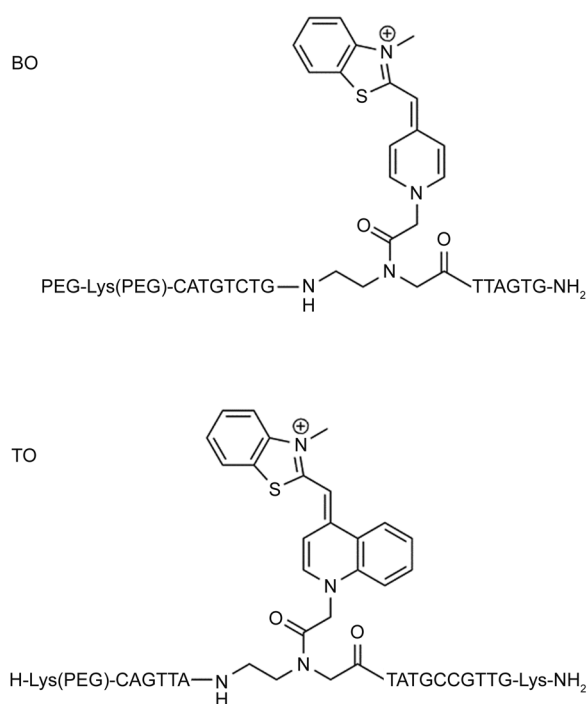


Figure 6. Chemical structures of the PNA intercalating dyes (BO, TO).

Other PNA nanotechnologies

Using PNA in southern hybridization

Using fragments of defined length derived from restriction enzyme digestion, southern hybridization is usually used to analyze genetic loci. Southern hybridization is very useful because it can provide precise size and sequence information of nucleic acids for cancer diagnosis. Pre-gel hybridization of PNA reduces the cumbersome post separation, probing and washing steps in the process of southern hybridization. Perry-O'Keefe *et al* prepared denatured DNA to hybridize with fluorescently labeled PNA oligomers at low ionic strength. By hybridizing with PNA, single-stranded DNA fragments exhibited higher electrophoretic mobility. After separation, the PNA/DNA hybrids were detected using standard chemiluminescent techniques. PNA is ideal for the detection and separation of specific DNA sequences because it has the ability to bind with DNA at low ionic strength. This technology enables cancer diagnosis to be faster and more accurate than conventional southern hybridization techniques^[68].

PNA for nucleic acid purification

PNA can hybridize and purify nucleic acids in low-salt concentrations that destabilize native nucleic acid structures. PNAs have high binding affinities, which can be used to purify specific nucleic acids. This technology has potential application in cancer research. Orum *et al* designed an oligo-histidine PNA with six histidine residues to purify a target nucleic acid. The designed 6×His-PNA could strongly bind to Ni(II) ions. By using a Ni(II)-NTA (nitrilotriacetic acid) resin, the 6×His-PNA/DNA complex could be purified by metal ion affinity

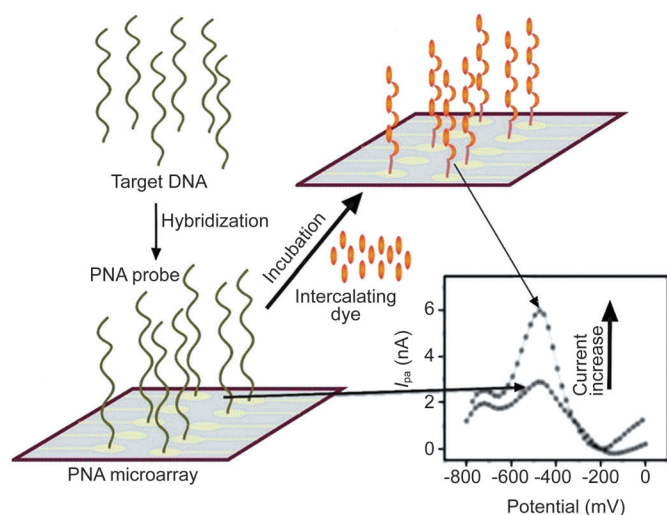


Figure 7. PNA microarray for the detection of target DNA on gold electrodes.

chromatography. They further demonstrated that large RNAs (2224 nucleotides) can be captured with high efficiency using multiple 6×His-PNA probes^[69].

Conclusions

Peptide nucleic acids are of the utmost importance in the biomedical field because of their ability to hybridize with neutral nucleic acids and their special chemical and biological properties, which ensure that PNA oligomers are ideal candidates for application in the fields of cancer diagnosis and therapy. With future research efforts, PNA bionanotechnology will be able to develop an even wider field of application.

Acknowledgements

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