The peptide nucleic acids: a new way for chromosomal investigation on isolated cells?

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The development of nucleic acid analogues has become an important feature due to the potential use of this new biomolecular tool in genetic diagnostics and investigations. Among all the synthetic oligonucleotides designed, the peptide nucleic acids (PNA) constitute a remarkable class of nucleic acid mimics, with important physico-chemical properties which have been exploited to develop a wide range of powerful biomolecular tools, including molecular probes, biosensors and anti-gene agents. New applications of PNA involve their use as hybridization probes, and consequently the PNA technology is now developing within the field of in situ hybridization techniques. Recent studies have reported the successful use of centromeric PNA probes on human lymphocytes, sperm as well as on isolated oocytes and blastomeres. Muticolour PNA protocols have been described for the specific identification of several human chromosomes. These data show that PNA could become a powerful complement to fluorescence in situ hybridization (FISH) for in situ chromosomal investigation, especially on isolated cells. The present paper gives an overview of the properties of PNA and the assays exploiting PNA technology in molecular genetics and cytogenetics.

Key words: aneuploidy/PNA–DNA/PNA–FISH/PNA–PCR

Introduction

The advent of molecular technologies has brought forth new procedures for increasingly sensitive, fast and reliable diagnostics. For chromosomal investigations, molecular cytogenetic methods have revolutionized the exploration of nucleic acid sequences in individual cells and chromosomes. Thanks to the introduction of various hapten and fluorescent chemicals such as biotin, digoxigenin and fluorescein, two new techniques were introduced, fluorescence in situ hybridization (FISH) and primed in situ labelling (PRINS), which constitute two distinct approaches for in situ chromosomal analysis (Pinkel et al., 1986; Koch et al., 1989). Because of its efficiency, its relative simplicity and the commercial availability of numerous DNA probes, FISH quickly became the assay of choice for in situ localization of specific nucleic acid sequences. The introduction of FISH surpassed previously available techniques to become the foremost assay for chromosomal abnormality detection, as illustrated by its wide spectrum of applications in research and medical diagnostics (Trask, 2002). The adaptation of FISH to single cell analysis has constituted one of the most interesting applications, largely contributing to the development of preimplantation genetic diagnosis (PGD). To date, over half of all PGD carried out worldwide have been performed for chromosomal screening using FISH probes (Harper and Wells, 1999).

Recently, a new approach has been introduced in cytogenetics, based on the use of DNA analogues to recognize and bind to specific nucleic acid sequences in situ. These new types of probes, named peptide nucleic acids (PNA), were developed by Nielsen et al. (1991). Originally conceived and utilized as DNA binding reagents for studying the mechanism of double helix invasion, PNA have quickly evolved from basic research to application in biological assays. The PNA-based protocols benefit from the unique physico-chemical properties of these new synthetic molecules, leading to the development of simple and robust assays. Powerful applications of PNA have thus been developed in microbiology, virology and pharmacology, but have also recently emerged in genetics and cytogenetics. This new family of probes might have significant impact on the exploration of chromosomal and genomic aberrations and lead to marked progress in cytogenetic procedures.

PNA structure and properties

PNA are synthetic DNA analogues in which the phosphodiester backbone is replaced by repetitive units of
DNA or DNA–RNA duplexes (Jensen et al., 1993). Thus, the PNA hybridize virtually independently of the salt concentration. An additional consequence of the polyamide backbone is that the PNA backbone is not charged. Consequently, there is no electrostatic repulsion when PNA hybridizes to its target nucleic acid sequence, giving a higher stability to the PNA–DNA or PNA–RNA duplexes than the natural homo- or heteroduplexes. This greater stability results in higher thermal melting temperature \( T_m \) values than is observed for DNA–DNA or DNA–RNA duplexes (Jensen et al., 1997). An additional consequence of the polyamide backbone is that the PNA backbone is virtually independent of the salt concentration. Thus, the \( T_m \) of PNA–DNA duplex is barely affected by low ionic strength. This significantly facilitates the hybridization with the PNA. The unnatural backbone of PNA also means that PNA are particularly resistant to protease and nuclease degradation (Demidov et al., 1993). Because of this resistance to the enzyme degradation, the lifetime of PNA is extended both \( \text{in vivo} \) and \( \text{in vitro} \). Also, PNA are not recognized by polymerases and therefore cannot be directly used as primers or be copied (Nielsen and Egholm, 1999).

PNA hybridize to complementary DNA or RNA in a sequence-dependent manner, according to the Watson–Crick hydrogen bonding scheme. In contrast to DNA, PNA can bind in either parallel or anti-parallel fashion. These data indicate that the PNA backbone is more flexible than native nucleic acid backbone. PNA probes can bind to either single-stranded DNA or RNA, or to double-stranded DNA (Figure 2).

N-(2-aminoethyl) glycine to which the purine and pyrimidine bases are attached via a methyl carbonyl linker (Figure 1). The unique chemical make-up provides PNA with unique hybridization characteristics. Unlike DNA and RNA, the PNA backbone is not charged. Consequently, there is no electrostatic repulsion when PNA hybridizes to its target nucleic acid sequence, giving a higher stability to the PNA–DNA or PNA–RNA duplexes than the natural homo- or heteroduplexes. This greater stability results in higher thermal melting temperature \( T_m \) values than is observed for DNA–DNA or DNA–RNA duplexes (Jensen et al., 1997). An additional consequence of the polyamide backbone is that the PNA backbone is virtually independent of the salt concentration. Thus, the \( T_m \) of PNA–DNA duplex is barely affected by low ionic strength. This significantly facilitates the hybridization with the PNA. The unnatural backbone of PNA also means that PNA are particularly resistant to protease and nuclease degradation (Demidov et al., 1993). Because of this resistance to the enzyme degradation, the lifetime of PNA is extended both \( \text{in vivo} \) and \( \text{in vitro} \). Also, PNA are not recognized by polymerases and therefore cannot be directly used as primers or be copied (Nielsen and Egholm, 1999).

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**Figure 1.** Chemical structures of peptide nucleic acids (PNA) as compared to DNA and protein. The backbone of PNA displays 2-aminoethyl glycine linkages in place of the regular phosphodiester backbone of DNA, and the nucleotide bases are attached to this backbone at the amino-nitrogens through methylene carbonyl linkages. The amide bond characteristic for both PNA and protein is boxed. By convention, PNA are illustrated like peptides, with the N-terminus at the left (or at the top) position and the C-terminus at the right (or at the bottom) position. PNA hybridize to complementary nucleic acids in both parallel and anti-parallel orientation. However, the anti-parallel orientation illustrated in this figure is preferred. The Watson–Crick hydrogen bonds are indicated by ‘…’.

**Figure 2.** Schema of peptide nucleic acid (PNA) binding modes for targeting double-stranded DNA. PNA oligomers are drawn in bold. (1) Standard duplex invasion complex formed with some homopyrimidine PNA. (2) Double-duplex invasion complex, very stable but only possible with PNA containing modified nucleobases. (3) Conventional triple helical structure (triplex) formed with cytosine-rich homopyrimidine PNA binding to complementary homopurine DNA targets. (4) Stable triplex invasion complex, leading to the displacement of the second DNA strand into a ‘D-loop’.

Homopyrimidine PNA, as well as PNA containing a high proportion of pyrimidine residues, bind to complementary DNA sequences to form highly stable (PNA)_2–DNA triplex helices displaying high \( T_m \). In these triplexes, one PNA strand hybridizes to DNA through standard Watson–Crick base pairing rules, while the other PNA strand binds to DNA through Hoogsteen hydrogen bonds. The resulting structure is called P-loops (Nielsen, 2001). The stability of these triple helices is so high that short homopyrimidine PNA targeted to purine tracts of double-stranded DNA invades the duplex by displacing one of the DNA strands. Also, PNA–DNA hybridization is significantly more affected by base mismatches than DNA–DNA hybridization. A single mismatch in a mixed PNA–DNA 15 mer duplex decreases the \( T_m \) by up to 15°C, whereas in the corresponding DNA–DNA complex, a single mismatch decreases the \( T_m \) by only 11°C (Giesen et al., 1998). This high level of discrimination at single base level has indicated that short PNA probes could offer high specificity and has thus allowed the further development of several efficient PNA-based strategies for molecular investigations and diagnosis.

**Use of PNA in molecular genetics**

Following the introduction of these new synthetic compounds, it quickly became evident that their unique physico-chemical properties could be exploited in a large variety of genetic procedures.

**PNA as antigenic and antisense agents**

PNA molecules were first used in antigenic and antisense assays. Several \textit{in vitro} studies demonstrated the ability of PNA to inhibit both eukaryotic translation and transcription (Hanvey et al., 1992; Vickers et al., 1995; Boffa et al., 1996). PNA-mediated inhibition of gene transcription is mainly due to the formation of strand-invaded complexes or strand displacement in DNA targets. PNA targeted against the promoter region of a gene can form stable PNA–DNA complexes that restrict the DNA access of the polymerase, whereas PNA complexes located far from the promoter can...
block the polymerase progression and lead to the production of truncated RNA transcripts. Nielsen et al. (1994) have demonstrated that even an 8 mer PNA can efficiently block transcriptional elongation by (PNA)\textsubscript{2}–DNA triplex formation. Another way to alter gene transcription is based on the use of PNA as competitors with endogenous cis-element(s) present in the target gene for trans-acting factors. This results in an attenuation of the authentic interactions of trans-factors with their cis-elements (Mischiai et al., 1999).

PNA are able to interact with mRNA independently of the RNA secondary structure. Studies on the mechanisms of antisense activity have demonstrated that PNA inhibits expression differently from anti-sense oligonucleotides, acting through RNase-H-mediated degradation of the mRNA–oligonucleotide hybrid. Since PNA are not substrates for RNase, their anti-sense effect acts through steric interference of either RNA processing, transport into cytoplasm or translation, caused by binding to the mRNA (Knudsen and Nielsen, 1996).

The good stability of PNA oligomers, their strong binding efficiency as well as their lack of toxicity at even relatively high concentrations suggested that PNA could constitute highly efficient compounds for effective antisense/antigene application. However, despite the initial rapid success of PNA-based approaches in vitro, progress in the use of PNA as tools for regulating gene expression was hampered by the slow cellular uptake of ‘naked’ PNA by living cells. Subsequent modifications of PNA have led to significant improvements in the uptake of PNA in eukaryotic cells. The delivery into the cell can be speeded up by coupling PNA to DNA oligomers, to receptor ligands or more efficiently to peptides such as liposomes or cell-penetrating peptides that facilitate the entry of PNA into the cell. This may also be facilitated by the formation of PNA–DNA chimeras, which are recognized by DNA polymerase and thus can be used as mimics of the primer in the PCR reaction. Thus, during the last few years, the bulk of the interest in PNA has focused on their exploitation as probes for chromosomal analysis (Jensen et al., 1997).

PNA–PCR strategies

PNA probes have no direct interaction with DNA polymerase but PNA can terminate the elongation of oligonucleotide primers by binding to the template or competing with the primers. Moreover, PNA–DNA chimeras can be recognized by the DNA polymerase and can thus be used as primers for PCR reactions (Misra et al., 1998). The high-affinity binding of PNA has also been used for detecting single base pair mutations by PCR. This strategy, named PNA-directed PCR clamping, uses PNA to inhibit the amplification of a specific target by direct competition of the PNA targeted against one of the PCR primer sites and the conventional PCR primer. This PNA–DNA complex formed at one of the primer sites effectively blocks the formation of the PCR product. The procedure is so powerful that it can be used to detect single base pair gene variants for mutation screening and gene isolation (Orum et al., 1993).

More recently, novel automated real-time PCR has been developed using PNA. In this method, named Q-PNA PCR, a generic quencher labelled PNA (Q-PNA) is hybridized to the 5’ tag sequence of a fluorescent dye-labelled DNA primer in order to quench the fluorescence of the primer. During PCR, the Q-PNA is displaced by incorporation of the primer into amplicons and the fluorescence of the dye label is liberated (Fiandaca et al., 2001).

Solid-phase hybridization techniques

The neutral backbone of PNA significantly increases the rate of hybridization in assays where either the target or the probe is immobilized. Thus, PNA can be used for sequence-specific capture of single-stranded nucleic acids, taking advantage of the tight complex formation at low ionic strength which destabilizes nucleic acid secondary structure. A system for capture of double-stranded DNA was also experimented using (PNA)\textsubscript{2}–DNA openers, creating a large single-stranded DNA loop to which a biotinylated oligonucleotide could hybridize. This complex allows the capture of the DNA via streptavidin beads (Bukanov et al., 1998).

The high-affinity binding of PNA oligomers might lead to faster and easier procedures in most standard hybridization techniques, such as southern and northern blotting (Nielsen and Egholm, 1999). An alternative to southern analysis is the PNA pre-gel hybridization process, which significantly simplifies the procedure of southern hybridization. Labelled PNA are then used as probes, allowing hybridization to a denatured double-stranded DNA sample at low ionic strength prior to loading on the gel. This is different from conventional southern blotting where hybridization occurs after gel electrophoresis and membrane transfer. Here, the mixture is directly subjected to electrophoresis for separation of bound and unbound PNA probes. Because of their neutral charge, excess unbound PNA probes do not migrate in an electrical field. The PNA–DNA hybrids are then blotted onto a nylon membrane and detected using standard chemiluminescent techniques. The method is sensitive enough to detect a single mismatch in a DNA sample (Perry-O’Keefe et al., 1996).

Likewise hybridization PNA-based biosensor procedures have been developed in which a single-stranded PNA probe is immobilized onto optical or mass-sensitive transducers to detect the complementary strand or corresponding mismatch in a DNA sample solution. The hybridization events are converted into electric signals by the transducers (Jensen et al., 1997).

PNA as probes for chromosomal analysis

PNA can be used in many of the same hybridization applications as natural or synthetic DNA probes but with the added advantages of tighter binding and higher specificity. Thus, during the last few years, the bulk of the interest in PNA has focused on their exploitation as probes for in situ hybridization assays. Thanks to its high binding specificity, a single 15 mer PNA probe can substitute for a set of longer DNA probes. Also, the neutral backbone of PNA allows them to bind to DNA or RNA under low ionic strength conditions, which discourage renaturation of complementary genomic strands. This is particularly advantageous for in situ targeting of repeat sequences for which both the length and the repetitive nature can favour renaturation over hybridization with probes. Additional benefits of using PNA are
lower background signals and unlimited stability of the probe mixture (Williams et al., 2002). PNA are compatible with a wide range of reporter molecules and fluorochromes including fluorescein, rhodamine as well as cyanine and Alex dyes available in a wide variety of colours. Because the introduction of PNA in cytogenetics is recent, the commercial availability of PNA probes is still limited to consensus telomeric and human-specific satellite probes, and their prices remain 20% more expensive than the FISH probes. One can hope that the success of the first generation of PNA probes will stimulate the future production of an extended variety of PNA probes and the decrease of their cost.

The PNA–FISH technique was first used for quantitative telomere analysis. The study of telomere behaviour has become a sensitive subject because of telomere involvement in the processes of cancer evolution and cellular senescence. The FISH technique has been successfully utilized for the in situ detection of telomeric repeat sequences in chromosomes of various species, using synthetic oligonucleotide probes, but the efficiency of these probes has not been sufficient to extend this procedure beyond qualitative analysis of repeat telomeric sequences. To monitor telomere length quantitatively, Lansdorp et al. (1996) utilized fluorescein-labelled PNA probes. By comparing fluorescein-labelled DNA, RNA and PNA probes for the detection of telomeric repeat sequences on human metaphase chromosomes, they first showed that PNA probes yielded superior staining of telomeres. The PNA–FISH approach allowed the distinction of fluorescence of individual sister chromatid ends and the accurate estimate of individual and global telomere length in metaphase chromosomes of various cultured human haematopoietic cells. Subsequently, telomere PNA probes were used in several in situ studies of cancer and ageing (Zijlman et al., 1997; Boei et al., 2000; Mathioudakis et al., 2000). Since PRINS provided better efficiency than FISH for the identification of repeat telomeric sequences (Krejci and Koch, 1998), the performance of PNA method for in situ detection and sizing of telomeric repetitive sequences was compared to the PRINS technique. The two techniques were compared on mouse, hamster and human cell lines and the results were identical in terms of labelling efficiency and sensitivity (Serakinci and Koch, 1999).

Further developments of PNA technology were focused on the improvement of the specificity of PNA probes and the in situ detection of numerical chromosome abnormalities. Chen et al. (1999) reported that PNA probes could discriminate between two centromeric DNA repeat sequences that differ by only a single base pair. Identical results were obtained with PRINS primers (Pellestor et al., 1995) and oligonucleotide probes (O’Keefe et al., 1996), but never with standard DNA probes. The identification of chromosomal variation and the analysis of polymorphisms could greatly benefit from the discrimination power of PNA. The procedure of PNA synthesis allows us to consider the further production of allele-specific probes. This will constitute an evident improvement over the current labelling techniques.

Several chromosome-specific PNA probes were designed and tested. Chen et al. (2000) defined short (15–18 mer) and specific PNA probes for alpha-satellite domains of nine chromosomes (chromosomes 1, 2, 7, 9, 11, 17, 18, X and Y) and successfully used them on metaphases and interphase nuclei. To demonstrate the potential utility of PNA probes in clinical application, cultured and uncultured amniocyte preparations were also analysed, giving rates of hybridization efficiency of 90–97%. Taneja et al. (2001) tested other PNA probes for chromosomes 1, X and Y, 18–22 mer in size and directly labelled with fluorochromes, on normal human lymphocytes and fibroblasts with abnormal chromosome contents. A fast and simple multicolour PNA protocol was utilized, demonstrating the easy use of PNA probes for in situ labelling assays.

Recently, Pellestor et al. (2003) experimented with PNA technology on human sperm. The adaptation of PNA technology to human sperm constituted an interesting challenge because of the particularities of the sperm nucleus in terms of genomic compaction and accessibility of DNA sequences. To estimate and validate the efficiency of PNA labelling on human sperm, comparative estimates of disomy X, Y and 1 were performed on sperm preparations from healthy subjects using multicolour FISH, PRINS and PNA procedures in parallel. An equivalent quality of in situ nuclear labelling and similar disomy rates were obtained with the three methods. However, the hybridization timing of PNA probes (i.e. 45 min) was considerably shortened in comparison with FISH reaction, which requires an overnight hybridization in order to be efficiently completed on sperm preparations. The fast hybridization kinetics of PNA on sperm was similar to the kinetics of PRINS reaction (20–30 min). This similarity might be due to the small size of both PNA and PRINS primers. These data highlighted the importance of the probe size for in situ sperm labelling, and consequently the great potential of PNA probes for chromosomal screening on difficult biological material.

Lastly, the PNA strategy has been experimented on isolated human oocytes, polar bodies and blastomeres in order to assess the possibility of using PNA probes for preimplantation genetic diagnosis (PGD; Paulasova et al., 2004). Using directly labelled satellite PNA probes for chromosomes 1, 4, 9, 16, 18, X and Y, we have tested simple and sequential multicolour PNA labelling procedures on 34 in vitro unfertilized oocytes and 23 isolated blastomeres. The combined use of PNA and FISH was also investigated and, in a few cases, FISH labelling was utilized as control in parallel with the PNA reactions. Both rates and types of chromosomal abnormalities scored were in good agreement with results of previous FISH studies. This first use of PNA probes on isolated cells confirms the efficiency of PNA technology for in situ chromosomal analysis and demonstrates the feasibility of using PNA on unique cells. This procedure could become an efficient complement to FISH for PGD because of its simplicity, its fast kinetics of hybridization (45–60 min) and the high affinity of PNA probes.

All these studies indicated that PNA probes have multiple advantages for the in situ analysis of nucleic acid sequences. Consequently, the PNA hybridization method might develop...
quickly within the field of in situ labelling methodology and become a powerful complement to FISH and PRINS for in situ chromosomal investigations.

**Future developments**

PNA are already being implemented in research protocols and medical assays of increasing diagnostic value, adding both the sensitivity and the specificity of PNA probes to the standard procedures. New chemical modifications of the original PNA backbone might contribute to increase the efficiency of PNA molecules and to develop novel applications.

Interesting new contributions of PNA could come from the development of new applications that cannot be performed using DNA probes. Because of their remarkable sequence discrimination, PNA oligomers have great potential in the growing area of whole genome analysis. It is now evident that future genetic and chromosomal investigations will feature increasingly higher-order multiplexing, as indicated by the rapid development of DNA microarrays. The ability to simultaneously assay many molecular signatures in cells constitutes an important challenge for our understanding of functional cell states, single cell versus tissue-level gene expression, and also for the future of diagnostic medicine. Technical platforms for arraying probes, hybridization and data analysis are already in place, and DNA microarrays using the CGH technology are already commercially available for the determination of the copy number of all human chromosomes and individual telomeres. Microarray technology appears to be a very powerful and realistic new diagnostic procedure that may be applied to isolated cells. The preliminary results reported by Bermudez et al. (2004) show movement in this direction. The remarkable hybridization properties of PNA (stability and mismatch discrimination) suggest that PNA oligomers may be efficiently incorporated into microarrays and could improve the timing of the whole procedure, especially when applied on single cells (Weiler et al., 1997). In association with different fluorochromes, short PNA sequences could constitute a new class of genomic biomarkers for microarray platforms and contribute to the next challenge of extending microarray technology to the single cell level and preimplantation diagnosis.

Another promising feature of PNA might be linked to the development of in situ fluorescence imaging. The ability to introduce fluorescent probes into living cells will allow deeper study of live gene expression and mRNA transfer (Tyagi and Kramer, 1996). This innovative approach will be more easily applied than non-hybridization-based green fluorescent protein (GFP)-fusion protein systems, and the new multiphoton microscopy will provide efficient tools to visualize multiple gene expression patterns in single living cells (König, 2000). Due to their high in vivo stability and resistance to enzymes and the flexibility of their synthesis procedure, PNA oligomers, conjugated to cell-permeable peptides or liposomes, have a great potential for the future of non-invasive medical imaging.

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**References**


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