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Peptide nucleic acids: a review on recent patents and technology transfer

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Introduction: DNA/RNA-based drugs are considered of major interest in molecular diagnosis and nonviral gene therapy. In this field, peptide nucleic acids (PNAs, DNA analogs in which the sugar-phosphate backbone is replaced by *N*-(2-aminoethyl)glycine units or similar building blocks) have been demonstrated to be excellent candidates as diagnostic reagents and biodrugs.
Areas covered: Recent (2002 – 2013) patents based on studies on development of PNA analogs, delivery systems for PNAs, applications of PNAs in molecular diagnosis, and use of PNA for innovative therapeutic protocols.
Expert opinion: PNAs are unique reagents in molecular diagnosis and have been proven to be very active and specific for alteration of gene expression, despite the fact that solubility and uptake by target cells can be limiting factors. Accordingly, patents on PNAs have taken in great consideration delivery strategies. PNAs have been proven stable and effective *in vivo,* despite the fact that possible long-term toxicity should be considered. For possible clinical applications, the use of PNA molecules in combination with drugs already employed in therapy has been suggested. Considering the patents available and the results on *in vivo* testing on animal models, we expect in the near future relevant PNA-based clinical trials.

Keywords: antisense PNAs, delivery, gene therapy, molecular diagnosis, peptide nucleic acids, PNA-DNA chimeras, technology transfer

Expert Opin. Ther. Patents [Early Online]

1. Introduction

The recent progress in biotechnology has been focused, among the several fields of investigation related to technology innovation, on two research areas, i.e., molecular diagnosis and nonviral gene therapy based on the use of DNA mimics [1-3]. In both cases, novel molecules are appealing which, when compared with standard oligonucleotides, exhibit improved characteristics in respect to hybridization efficiency [4], stability in biological fluids [5], and suitability to be delivered to target cells or tissues [6]. **Figure 1** outlines some recently proposed DNA analogs employed in molecular diagnosis and therapeutic interventions, such as 2′-deoxyoligonucleotides [7], 2′-O-methyl (2′-OCH_3)-modified oligoribonucleotides (2′-CH_3) [8], cholesterol moiety-conjugated 2′-OCH_3 [9,10], locked nucleic acid (LNA)-modified oligonucleotides [11], oligonucleotides containing 2′-O-methoxyethyl (2′-MOE) [12], 2′-flouro (2′-F) [12,13], and phosphorothioate backbone modifications [14,15]. Among these DNA mimics, peptide nucleic acids (PNAs) should be considered as very promising reagents in several biomedical applications [16-21]. The objective of this review is to describe recent (2002 – 2013) patents based on studies on the development of PNA analogs, delivery systems for PNAs, applications of PNAs in molecular diagnosis, and use of PNAs for innovative therapeutic protocols.

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Article highlights.

- PNAs, despite a radical structural change with respect to DNA and RNA, are capable of sequence-specific and efficient hybridization with complementary DNA and RNA, forming Watson-Crick double helices.
- PNAs can also generate triple helix with double-stranded DNA and perform strand invasion.
- PNA-based molecules have also been reported to be able to target TFs and behave as TFD agents.
- Accordingly, PNAs have been proposed for antisense and antigene therapy, as well as in a great variety of diagnostic applications.
- Patents are available presenting PNAs as very useful tools for molecular diagnosis.
- Patents are available presenting PNAs as very useful tools to develop therapeutic protocols.
- Studies employing preclinical experimental model systems demonstrate that PNA-based molecules are able to alter gene expression *in vivo*.
- Clinical data demonstrate that PNA-based molecules can be considered for diagnostics as well as prognostic protocols in a variety of human diseases.

This box summarizes key points contained in the article.

2. Peptide nucleic acids (PNAs)

PNAs (**Figure 2**) are DNA analogs in which the sugar-phosphate backbone is replaced by *N*-(2-aminoethyl)glycine units [16-20]. These very interesting molecules have been described for the first time by Nielsen *et al.* [16] and, despite a radical structural change with respect to DNA and RNA, they are capable of sequence-specific and efficient hybridization with complementary DNA and RNA, forming Watson-Crick double helices [16-20]. In addition, they generate triple helix structures with double-stranded DNA and perform strand invasion. Accordingly, they have been proposed for antisense and antigene therapy [18-23]. PNAs, as other DNA analogs, are very promising for RNA recognition, since they exhibit an higher affinity for RNA than for DNA; in addition, they are highly specific and resistant to DNAses and proteases [18]. Moreover, as almost all of the other proposed DNA analogs, PNAs can be modified in order to achieve better performances in terms of cellular permeation, as well as affinity and specificity for the DNA and RNA target sequences [18-23]. With respect to diagnosis, in addition to the already described high hybridization efficiency, PNAs display a remarkable destabilizing effect caused by single-base mismatch, greatly facilitating the use of these molecules in diagnosis protocols finalized to identify point mutations [18,19].

3. Patents and patent applications on peptide nucleic acids: general considerations

The interest on PNAs is demonstrated by the number of patents which can be found consulting suitable Data Banks [24-26]. By searching for "Peptide Nucleic Acids," more than 1500 entries can be retrieved and analyzed. From this analysis, the following considerations should be made: i) PNAs are object of patenting in the field of medicinal chemistry (development of analogs with improved features), pharmaceutical technology (development of delivery systems), molecular diagnosis and therapy (focusing on alteration of gene expression); ii) several patents concern the development of PNA-based molecular biology methods; iii) the possible interest of PNA molecules for biomedical applications and clinical trials is clearly evident; iv) in the case of lack of biological activity of canonical PNAs, PNA analogs were developed to obtain the desired effects (for instance PNA-DNA chimeric molecules are described exhibiting improved biological activity in respect to the reference ODN and PNA molecules); (v) information about companies interested in the technological transfer of these patents are available by looking at the section "applicants." It should be pointed out that in consulting patent databases, a same patent might be identified in different entries (for instance patent US7125994, granted to Panagene, Inc., has been published also as CN1659153A, CN100347161C, DE60330766D1, EP1501812A1, EP150 1812A4, EP1501812B1, EP2174936A1, US6969766, US71 45006, US7179896, US7371859, US7371860, US741 1065, US20030225252, US20050250785, US20050 250786, US20050283005, US20060030709, and WO/ 2003/091231A1) [27]; in this case, we gave arbitrarily priority to codes of issued US patents and US patent applications and World Intellectual Property Organization, avoiding duplications. A second consideration is related to scientific validation and scientific merit. In this respect, within all the considered patents on PNAs, only those based on suitable scientific publications in peer-reviewed journals have been included in the present review. Some examples are shown in **Table 1**, which summarizes key studies reporting the scientific background of PNA-based patents useful for experiments, protocols, and biomedical applications related to alteration of gene expression [28-81].

With respect to key general patents on PNA technology, the major representative US patents that teach preparation of PNAs and possible applications are well recognized [82-84]. Among other interesting patents, one teaches how to identify PNA/DNA and PNA/RNA hybrids by using polyclonal, monoclonal, and recombinant antibodies unable to bind to single-stranded PNAs, double-stranded nucleic acids, and single-stranded nucleic acids [85]. As far as PNA analogs, these are the basis of an interesting patent outlining the possible importance of a chiral backbone to improve the performance of PNAs [86]. As far as applications of PNAs in biotechnology, the use of PNAs has been proposed to enhance the generation of polymerase chain reaction (PCR) products by including a step in which PNA oligomers anneal to sequences containing repeats and possibly interfering with the primer-mediated elongation process [87]. In a more recent patent, DNA-PNA primers have been proposed for highly efficient generation of PCR products [88].

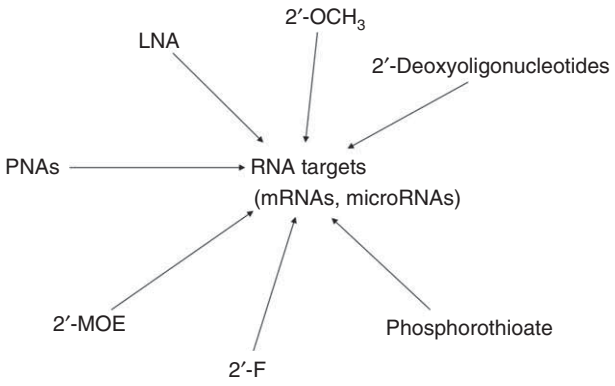

Figure 1. DNA analogs targeting RNA molecules and employed in molecular diagnosis and development of therapeutic protocols. 2′-Deoxyoligonucleotides, 2′-O-methyl (2′-OCH_3)-modified oligoribonucleotides (2′-OCH_3), cholesterol moiety-conjugated 2′-OMe modified, locked nucleic acid (LNA)-modified oligonucleotides, oligonucleotides containing 2′-O-methoxyethyl (2′-MOE), 2′-flouro (2′-F), and phosphorothioate backbone modifications, peptide nucleic acids (PNAs).

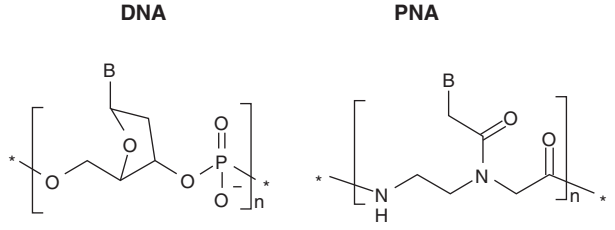

Figure 2. Molecular structures of DNA and PNA.

A final introductory comment is related to the completeness of the list of patents and patent applications present in this review. Despite the fact that the author put a great effort in presenting a balanced picture of the available entries, the possibility of absence of key patents cannot be excluded; in this case the author would like to present his deep apology to the involved inventors and assignees.

4. PNAs and technology transfer

Several biotech companies have included in their major pipelines production and use of PNAs and PNA-based molecules. A partial list is depicted in **Table 2**, reporting examples of biotechnology companies involved in PNA research, production, and development. The issues covered are development of monomers for PNA synthesis, PNA synthesis, PNA modification, and design of PNAs for specific applications, such as PNA clamping and fluorescence *in situ* hybridization (FISH). The major products are PNA-based probes for diagnostics, PNA libraries, PNA arrays, and PNA-based molecules for mRNA/miR (micro-RNA) inhibition. Accordingly, several companies are involved in patenting PNA-based strategies in diagnostics and therapeutic interventions (**Tables 3** and **4** for selected examples) [37,43,52,68,81,89-126]; among the most active in this specific area is Panagene, who is the direct assignee of important patents (see **Tables 3** and **4**). Among approaches for molecular diagnosis, the available patents cover development of highly efficient protocols, PNA clamping, production of PNA arrays for SNP detection, use of PNA-based platforms/methods for transcriptomic studies, including those involving microRNAs (miRs), and use of PNAs for biosensors [37,89-104]. Among patents outlining possible PNA-based therapeutic interventions [43,52,68,81,105-126], the majority are based on the use of antisense PNAs targeting mRNAs or miRs [43,52,81,109-126] for antiviral and anticancer therapy. However, several patents are of interest also in the field of rare diseases, such as a patent application describing PNA molecules capable of targeting a region responsible for exon skipping in muscular dystrophy [43].

5. PNAs in molecular diagnosis

5.1 Scientific background on PNAs in molecular diagnosis

PNAs have been widely proposed in protocols aimed at performing highly sensitive hybridization with nucleic acids [127-137]. In this respect nucleic acid probes have been employed for long time to analyze samples for the presence of nucleic acid from disease-associated bacteria, fungi, virus, or other organisms; in addition, hybridization discriminating between genomic sequences carrying single-nucleotide mismatches can be proposed for examining genetically based disease states or clinical conditions of interest. In this respect, PNA-based molecules have been used in combination with surface-plasmon resonance (SPR) and biosensor technology to discriminate between normal homozygous, affected homozygous, and heterozygous genomes as published in the case of diagnosis of cystic fibrosis W1282X mutation [127]. This PNA-based procedure is rapid and informative and results are efficiently obtained within a few minutes. Other advantages of this methodology are i) that it is a nonradioactive methodology and ii) that gel electrophoresis and/or dot-spot analysis are not required. More importantly, the demonstration that SPR-based BIA could be associated with microarray technology allows us to hypothesize that the method could be used for the development of a protocol employing multispotting on SPR biosensors of many cystic fibrosis PCR (CF-PCR) products and a real-time simultaneous analysis of hybridization to PNA probes [127]. These results are in line with the concept that SPR could be an integral part of a fully automated diagnostic system based on the use of laboratory workstations, biosensors, and arrayed biosensors for DNA isolation, preparation of PCR reactions, and identification of point mutations [127]. The combined employment of SPR-based instruments and PNA probes has been object of several studies and recent

Table 1. Examples of applications of PNAs and PNA-based molecules as gene-expression modifiers.

Molecules	Mechanism of action	Target molecule	Biological effects	Selected references	Representative patents or patent applications
PNA	Antisense	mRNA	Inhibition of translation	Brolin *et al.* [28] Parkash *et al.* [29] Tonelli *et al.* [30] Malchère *et al.* [31] Kilk *et al.* [32] Shiraishi *et al.* [33] Dragulesco-Andrasi *et al.* [34] Pandey *et al.* [35]	US2007020632 (2007): Method for selective inhibition of human n-myc gene in n-myc expressing tumors through antisense and antigéne peptide-nucleic acids (PNA) [36]; US2011123988 (2011): Antisense compounds and methods for diagnostic imaging [37]
PNA	Antisense	Pre-mRNA	Exon skipping	Ivanova *et al.* [38] Saleh *et al.* [39] Yin *et al.* [40] Saleh *et al.* [41] Pankarova *et al.* [42]	WO2009101399 (2009): Treatment of muscular dystrophy using PNA [43]
PNA	Antisense	MicroRNA	Inhibition of regulatory functions of the target miR	Oh *et al.* [44] Wibrand *et al.* [45] Torres *et al.* [46] Torres *et al.* [47] Fabani and Gait [11] Fabbri *et al.* [48] Brognara *et al.* [49] Manicardi *et al.* [50] Fabbri *et al.* [51]	US2010240058 (2010): MicroRNA antisense PNAs, compositions comprising the same, and methods for using and evaluating the same [52]; US2011245481(2011): Method for inhibiting function of microRNA [53]
PNA	Triple-helix formation and strand invasion	Gene promoters	Inhibition of transcription; inhibition of replication	Olsen *et al.* [54] Olsen *et al.* [55] Diviacco *et al.* [56] Betts *et al.* [57] Chin *et al.* [58]	EP0897991 (1999): Small triplex-forming PNA oligos [59]
PNA	Strand invasion	Gene sequences	Inhibition of transcription	Sugiyama *et al.* [60] Yamamoto *et al.* [61] Rapireddy *et al.* [62] Ishizuka *et al.* [63] Aiba and Komiyama [64] Yamazaki *et al.* [65]	WO199635705 (1996): Inhibition of transcription factor-mediated transcriptional activation by oligomer strand invasion [66]
PNA	Artificial promoter	Regulatory promoter sequences	Activation of transcription	Møllegaard *et al.* [67]	WO2002031166 (2003): Artificial transcriptional factors and methods of use [68]; WO/2004/094654 (2004): Transfection kinetics and structural promoters [69]
PNA	Homologous recombination	Genes	Gene correction	Rogers *et al.* [70] Katada and Komiyama [71] Yamamoto *et al.* [72] Miyajima *et al.* [73]	US6936418(2005): Methods and compositions for effecting homologous recombination [74]

Table 1. Examples of applications of PNAs and PNA-based molecules as gene-expression modifiers (continued).

Molecules	Mechanism of action	Target molecule	Biological effects	Selected references	Representative patents or patent applications
PNA-DNA chimeras	Antisense	mRNA	RNase H-mediated degradation	Uhlmann [75]	WO1996040709 (1996): PNA-DNA chimeras and PNA synthons for their preparation [76] US7659258 (2010): Double-stranded synthetic oligonucleotides useful for inducing apoptosis of osteoclasts for the treatment of osteopenic pathologies [80]; US20120095079 (2012): Treatment of transcription factor E3 (TFE3) and insulin receptor substrate 2 (IRS2) related diseases by inhibition of natural antisense transcript to TF3 [81]
PNA-DNA chimeras	TF decoy	Transcription factors	Inhibition of transcription	Finotti *et al.* [77] Gambari *et al.* [78] Borgatti *et al.* [79]	

review articles [138-142]. Unlike nucleic acid hybridization, which is very dependent on ionic strength, the hybridization of a PNA with a nucleic acid is fairly independent of ionic strength and is favored at low ionic strength, conditions which strongly disfavor the hybridization of a nucleic acid to a nucleic acid [93]. The effect of ionic strength on the stability and conformation of PNA complexes has been extensively investigated [143-145]. Sequence discrimination is more efficient for PNA recognizing DNA than for DNA recognizing DNA [19].

5.2 PNAs for PCR-free protocols

PNAs have been demonstrated to be unique reagents for PCR-free molecular diagnosis. For instance, D'Agata *et al.* recently described an ultrasensitive PNA-based analysis of genomic DNA not requiring PCR-mediated target DNA amplification, significantly improving the possibilities in several research and diagnostic applications for which minute cell quantities are available [146]. They have tested (**Figure 3** for a detailed scheme) a nanoparticle-enhanced surface plasmon resonance imaging sensing strategy to detect point mutations in nonamplified genomic DNA using, as model system, genomic DNAs from both healthy individuals and homozygous or heterozygous patients affected by β-thalassemia, demonstrating the specificity and the sensitivity of the described sensing strategy. The assay is ultrasensitive, since attomolar concentrations of target genomic DNA are detected, DNAs from healthy individuals and homozygous or heterozygous patients affected by β-thalassemia are discriminated, and only simple manipulations of the genetic samples are required before the analysis. The proposed ultrasensitive detection of DNA point mutations involved in genomic disorders possibly represents an important advantage in several biomedical applications.

Despite these interesting features, PNAs have been slow to achieve commercial success at least partially due to cost, sequence-specific properties/problems associated with solubility and self-aggregation [147-149], as well as the uncertainty pertaining to nonspecific interactions, which might occur in complex systems such as a cell [150]. However, patents on applications of PNAs to diagnosis are numerous and well suited to technology transfer.

5.3 PNAs in molecular diagnosis: key patents

First of all, we should mention that in the 1990s, the Danish cancer diagnostic company Dako filed a number of patent applications related to PNAs in diagnostics [85,151-154]. As far as other companies involved as assignees, **Table 3** reports that patents and patent applications relevant to diagnosis protocols have been presented by Panagene, Boston probes, Inc., Geneseen LLC, Crosslink Genetics Corp. As far as the object(s) of the patents, it should be underlined that any method, kits, or compositions which could improve the specificity, sensitivity, and reliability of probe-based assays for the detection of diagnosis-relevant DNA or RNA sequences of interest would be a useful advance in the state of the art particularly where the methods

Table 2. Selected top peptide nucleic acid production companies in Pharma Industry.

Company	Activity	Key products	Short description	Biomedical applications
Advanced Peptides	Custom PNA synthesis; PNA modifications	PNA libraries; PNA arrays	An experienced global manufacturer of custom peptides and PNAs. Their scientists have synthesized products for the scientific community for over 25 years and have met the highest standards of quality, service, and technical expertise	This activity is of interest for groups involved in synthesis of PNAs and PNA analogs
ASM Research Chemicals	Monomers for PNA synthesis	PNA monomers	A research and development organization in the field of synthesis of complex organic molecules for various applications	This activity is of interest for groups involved in PNA synthesis
Bio Synthesis	PNA synthesis	PNA FISH probes	This company offers on-demand PNA FISH probes with different fluorophores	Molecular diagnosis; therapeutic applications
Panagene	PNA synthesis; custom PNA oligomers; PNA clamp; PNA FISH probes	K-ras mutation detection kit; PNA miR inhibitors	As a biotechnology platform solution provider, it is a global leading company in molecular diagnostics and novel biomaterials	Molecular diagnosis; alteration of gene expression
PNA Bio	Custom PNA oligos; PNA FISH probes	TALEN & FN; custom PNA oligos; PNA FISH probes; PNA clamp; PNA miR inhibitors	This company offers PNA products of high quality	Molecular diagnosis; imaging; therapy
PolyOrg, Inc.	Peptide nucleic acid monomers	Modified PNA analogs	This company provides a variety of synthesis services for Life Science companies	This activity is of interest for groups involved in PNA synthesis in the fields of pharmaceuticals, biotechnology, and diagnostics

were uniformly applicable to probes of all or substantially all sequence variations. In this respect, patents on PNAs to be used in combination with SPR-based technologies have been reported [155,156]. Of course, several other PNA-based strategies in molecular diagnosis have been objects of recent patents. For instance, PNA probes have been demonstrated useful for rRNA detection in ISH and FISH assays [102,103]. PNA probes have also been used in the analysis of mRNA and viral nucleic acids [157] and the analysis of centromeric sequences in human chromosomes and human telomeres [158]. Similarly, the analysis of trinucleotide repeats in chromosomal DNA using appropriate PNA probes has been suggested [159]. A PNA probe has also been used to detect human X chromosome-specific sequences in a PNA-FISH format [154]. As far as the more recent developments in molecular diagnosis, PNAs have been proposed for detection of microRNAs (miRs). An interesting example is a patent describing the application of PNA probes for developing kits and protocols for expression profiling of miRs [82]. In this patent the proposed length of PNA probes is 13 – 22 bases and includes base sequences complementary to 3 – 10 base sequences in 5′ seed of the target miR. Further examples of patent applications concerning the employment of PNAs for the development of more efficient diagnostic protocols are available [98-101], fully in agreement with previously granted key patents [82-84,160]. The major objective of these patents is to examine tumors quickly and accurately in the early stages to enable effective treatment through early diagnosis. This is achieved by the PNA-based detection of mutations affecting the BRAF, epidermal growth factor receptor (EGFR), K-RAS, and BCR-ABL, respectively, employing PCR clamping.

6. PNAs in imaging

PNA-mediated imaging is possible at cellular level, allowing mRNA identification within cells [161-173]. In this specific field, despite the fact that probes for monitoring mRNA expression *in vivo* are of great interest for the study of biological and biomedical issues [131,161-166], advancements have been hampered by poor signal to noise and effective means for delivering the probes into live cells. An example is that reported by Wang *et al.*, who described a PNA·DNA strand displacement-activated fluorescent probe that can image the expression of inducible-nitric-oxide-synthase mRNA, a marker of inflammation [161]. The probe consists of a fluorescein-labeled antisense PNA annealed to a shorter DABCYL(plus)-labeled DNA which quenches the fluorescence, but when the quencher strand is displaced by the target mRNA the fluorescence is restored. Similar approaches have

Table 3. Partial list of enterprises involved in recent patents or patent applications on PNAs relevant to molecular diagnosis.

Patent or patent application	Title	Original assignee or co-assignee	Short description (claims)	Validity, significance, and biomedical applications
US20100248980 [89]	Method for selective labeling and detection of target nucleic acids using immobilized peptide nucleic acid probes	Panagene	A method for selective labeling of target nucleic acids on an array having PNA probes immobilized on a support	This invention relates to a method for increasing diagnostic efficiency and flexibility
US20110014715 [90]	Synthesis of peptide nucleic acids conjugated with amino acids and their application	Panagene	This invention describes PNA oligomers conjugated with one or more linear-type amino acids containing a plurality of alkylene glycols. In addition, this invention proposes a linear amino acid spacer in a device for detection of a target gene using PNA oligomers fixed on a surface of a functionalized solid support	The patent relates to PNA oligomers useful for the development of PNA arrays, PNA chips, and kits for genetic diagnosis with improved sensitivity and specificity, manufactured using the PNA conjugated with amino acid spacers
US20060147949 [91]	PNA chip using plastic substrate coated with epoxy group-containing polymer, method of manufacturing the PNA chip, and method of detecting single nucleotide polymorphism using the PNA chip	Panagene	Provided is a PNA chip in which a PNA probe containing a desired DNA sequence is immobilized on a plastic substrate coated with an epoxy group-containing polymer	The patent relates to a method based on the immobilization of single-stranded PNAs on a transparent plastic substrate and fluorescence signal detection based on PNA/DNA hybridization, enabling identification of SNP (single-nucleotide polymorphism)
US20090061451 [92]	Biosensors and related methods	Panagene	Provided herein are biosensors that comprise a biological signal source linked to a substrate by a PNA spacer and methods of use of the biosensor	In one embodiment, the biosensor is used to detect prostate-specific antigen, which is an issue of great relevance in early diagnosis of cancer
US7135563 [93]	Compositions for detecting target sequences	Boston Probes, Inc., Dako A/S	The present patent describes a method of forming a PNA probe triplex of diagnostic relevance	The present invention relates to methods, kits, and compositions suitable for the improved detection, quantitation and analysis of nucleic acid target sequences using probe-based hybridization assays
US20110123988 [37]	Antisense compounds and methods for diagnostic imaging	Geneseen Llc	This patent application describes compounds comprising a diagnostic moiety conjugated to a PNA that is targeted to the transcripts from a gene of interest. In addition this structure is also conjugated to at least one targeting moiety specific for an extracellular receptor or other cell-surface molecule for specific internalization. Binding of the PNA to its target RNA transcript retains the compound within the cell	The PNA can be designed to bind to a predetermined nucleic acid sequence from an RNA transcript, for example a sequence mutated or overexpressed in a pathological state. Two levels of specificity in the imaging protocol have been reached, the first based on receptor-ligand interactions, the second based on hybridization to the target RNA

Table 3. Partial list of enterprises involved in recent patents or patent applications on PNAs relevant to molecular diagnosis (continued).

Patent or patent application	Title	Original assignee or co-assignee	Short description (claims)	Validity, significance, and biomedical applications
US20110111416 [94]	Peptide nucleic acid probes, kits and methods for expression profiling of microRNAs	Panagene	The patent describes PNA probes capable of specifically binding to a target miR through complementary to 3 – 10 base sequences in 5′ seed of the target miR	The present patent application relates to PNA probes for expression profiling of miRs. This strategy can be applied to molecular diagnosis of circulating miRs
WO2009125934 [95]	PNA probes, kits and methods for cytochrome P450 genotyping	Panagene	Described in this patent are PNA probes capable of genotyping a cytochrome P450 2C9, 2C19, and/or 2D6	The invention relates to the development of PNA-based probes for analysis of the expression of genes closely associated with drug metabolism
US20080248461 [96]	PNA probes, kits, and methods for detecting genotypes of human papillomavirus	Panagene	In this patent application PNA probes are described capable of specifically binding with selected genotypes of Human Papillomavirus (HPV) DNA	Applications are focused on the development of methods for detecting HPV genotypes by using the kits, which enables the accurate detection of all 24 genotypes of HPV found in cervix. The relevance of this method is related to the possible diagnosis of combined infection with more than one HPV genotype
US20080233557 [97]	PNA probes, kits, and methods for detecting Lamivudine-resistant Hepatitis B viruses	Panagene	Disclosed in this patent application are PNA probes to detect lamivudine-resistant mutants of hepatitis B virus (HBV). They can accurately detect mutations of rtL180 M, rtM204 V, rtM204I, and rtV2071 within B and C domains of HBV DNA polymerase gene, the main cause of lamivudine resistance, as well as mixed mutants of more than one mutant	The present patent application relates to molecular diagnosis of HBV, which causes acute and chronic hepatitis; the methods proposed allows the development of kits for detecting lamivudine-resistant HBV, based on PNA probes with high specificity and sensitivity
WO2011093606 [98]	Method and kit for detecting BRAF mutant using real time PCR clamping on the basis of PNA	Panagene	The present patent application relates to a method for detecting mutants using PNA probes which bind specifically to the wild type of codon 600 in BRAF gene	The present invention can examine tumors (such as malignant melanoma, ovarian cancer, colorectal cancer, and thyroid cancer) quickly and accurately in the early stages to enable effective treatment through early diagnosis of cancer. Similar patent applications have been filed such as WO2011105732 (Method and kit for detecting EGFR mutant detection using PNA-based real time PCR clamping) [99], WO2011049343 (Method and kit for detecting K-RAS

Table 3. Partial list of enterprises involved in recent patents or patent applications on PNAs relevant to molecular diagnosis (continued).

Patent or patent application	Title	Original assignee or co-assignee	Short description (claims)	Validity, significance, and biomedical applications
				mutant detection using PNA-based real time PCR clamping) [100], and WO2012064035 (Method and kit for detecting BCR-ABL fusion gene mutation using PNA-based real time PCR clamping) [101]. The publications of these similar patent applications demonstrate the flexibility of this approach in molecular diagnosis applications
US20040043383 [102]	Methods and probes for the detection of chromosome aberrations	Dako A/S	In this patent application, chromosome aberrations are detected by *in situ* hybridization using at least two sets of hybridization probes, at least one set comprising one or more PNA probes	The method may be used for detecting chromosome aberrations in the form of breakpoints
US7981599 [103]	Non-nucleic acid probes, probe sets, methods and kits pertaining to the detection of individual human chromosomes X, Y, 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 16, 17, 18, and 20 as 13/21 as a pair	Boston Probes, Inc.	The non-nucleic acid probes, probe sets, methods, and kits of this patent are particularly well suited for use in multiplex ISH and FISH assays, which are possible because two or more of the probes used in the assay are labeled with one or more independently detectable labels	The methods of this patent are particularly well suited for automated analysis, including slide scanner-based systems, microscopes, and CCD cameras or flow cytometers. Furthermore, this invention is useful for detection and identification of chromosome abnormalities. Applications considered are very important and include preimplantation diagnosis and prenatal screening
US20120082980 [104]	PNA probes, probe sets, methods and kits pertaining to the detection of Candida	Life Technologies Corp.	This patent application is related to novel PNA probes, probe sets, methods, and kits pertaining to the detection of one or more species of *Candida* yeast	The PNA probes, probe sets, methods, and kits of this invention can be useful for the rapid, sensitive, and reliable detection of *Candida* yeast in food, beverages, water, pharmaceutical products, personal care products, dairy products or for the analysis of environmental samples. In addition, this invention can be useful for the detection of *Candida* yeast in clinical samples and clinical environments. Altogether, the claims sustain the high level of flexibility of this approach

Table 4. Partial list of enterprises involved in recent patents or patent applications designed for alterations of gene expression relevant for gene therapy.

Patent or patent application	Title	Original assignee or co-assignee	Short description (claims)	Validity, significance, and biomedical applications
US20100292471 [105]	Peptide nucleic acid oligomers comprising universal bases, preparation methods thereof, and kits, devices, and methods for the analysis, detection, or modulation of nucleic acids using the same	Panagene	Disclosed is a PNA oligomer with increased solubility in water and specificity upon hybridization with nucleic acids	This patent application relates to PNA oligomers with remarkably increased specificity upon hybridization with nucleic acids, allowing development of kits, devices, and methods. This permits not only highly efficient analysis and detection of nucleic acids, but also modulation of gene expression
WO2009113828 [106]	Peptide nucleic acids with good cell penetration and strong affinity for nucleic acid	Panagene	The present invention provides a novel class of PNA derivatives showing good cell penetration and strong binding affinity for nucleic acids	This patent application relates to one of the major issues in using PNAs and proposing them for molecular therapy. The claims are highly significant in the field of gene therapy
US6165720 [107]	Chemical modification of DNA using peptide nucleic acid conjugates	Gene Therapy Systems, Isis Pharmaceuticals, Inc.	Complexes comprising a nucleic acid molecule and a conjugated PNA are described in this patent. The PNA may be labeled or conjugated to proteins, peptides, carbohydrate moieties, or receptor ligands	The complexes described in this patent are used to transfect cells with plasmids, to affect their biodistribution, to promote nuclear localization, to induce transcriptional activation. The claims are highly significant in gene therapy, since the described complexes increase the efficiency of the final expression of a therapeutic gene
US5700922 [108]	PNA-DNA-PNA chimeric macromolecules	Isis Pharmaceuticals, Inc.	Macromolecules are provided that have increased nuclease resistance and binding affinity to a complementary strand, and that activate RNase H. The macromolecules are PNA-DNA-PNA structures, where the DNA portion is composed of subunits of 2′-deoxy-erythro-pentofuranosyl nucleotides	The molecules described in this patent are useful for diagnostics and other research purposes, including modulation of protein expression in organisms. In particular the claims are significant, since PNA-based antisense molecules do not activate RNase H
WO2002031166 [68]	Artificial transcriptional factors and methods of use	Crosslink Genetic Corp.	Artificial transcription factors (ATFs) are proposed having a non-peptidic-DNA binding domain, a flexible linker and a short synthetic effector domain	The ATFs described in this patent application are highly potent transcriptional modulators *in vitro* and *in vivo*. Methods for targeted

Table 4. Partial list of enterprises involved in recent patents or patent applications designed for alterations of gene expression relevant for gene therapy (continued).

Patent or patent application	Title	Original assignee or co-assignee	Short description (claims)	Validity, significance, and biomedical applications
				manipulation of gene expression and development of new class of pharmaceuticals are also provided. Further validation studies are necessary to understand the real impact of the proposed approach
US20100240058 [52]	miR antisense PNAs, compositions comprising the same, and methods for using and evaluating the same	Panagene	Disclosed is a PNA oligomer with increased solubility in water and specificity upon hybridization with nucleic acids. In detail, miR antisense PNAs are described, capable of inhibiting activity or function of miRs, and a method for evaluating the effectiveness of the treatment	This patent application is related to "miRNA Therapeutics" for the development of methods for inhibiting the activity or function of miRs involved in human pathologies. This field is of particular interest, considering the impact of miRs in human pathologies
WO2009101399 [43]	Treatment of muscular dystrophy using PNA	Panagene and Isis Innovation Ltd.	Proposed is a method based on a PNA comprising a sequence capable of targeting a region responsible for exon skipping in the mutated pre-mRNA at an exon to be skipped or included. The PNA inducing exon skipping or inclusion is able to correct the expression of mutated pre-mRNAs	The patent application relates to a method of correcting expression of a gene in human cells having a muscular dystrophy (MD) phenotype, wherein without correction the gene fails to express a functional protein due to one or more mutations. The impact and significance of the claims are relevant, as also firmly demonstrated by the fact that the exon skipping strategy for MD is the basis of recent clinical trials
WO2011112516 [109]	Treating and preventing Hepatitis C Virus infection using c-RAF kinase antisense oligonucleotides	Panagene and iCO Therapeutics Inc.	The invention relates to raf antisense oligonucleotides for the treatment or prevention of HCV infection in a patient	In the proposed approach, the raf antisense oligonucleotides are proposed in combination with other agents or treatments having activity against HCV. The claims are highly significant in the field of gene therapy and expected to be translated to clinical trials, as in the case of

Table 4. Partial list of enterprises involved in recent patents or patent applications designed for alterations of gene expression relevant for gene therapy (continued).

Patent or patent application	Title	Original assignee or co-assignee	Short description (claims)	Validity, significance, and biomedical applications
				DNA-based antisense molecules. Similar patent applications are US20110224283 (Antisense modulation of nuclear hormone receptors) [110] and US20110289608 (Antisense modulation of interleukins 17 and 23 signaling) [111]. The publication of these patent applications demonstrates the flexibility of this approach
US20120095079 [81]	Treatment of transcription factor E3 (TFE3) and insulin receptor substrate 2 (IRS2) related diseases by inhibition of natural antisense transcript to TF3	Opkocorna, Llc	The present invention relates to antisense oligonucleotides that modulate the expression of and/or function of Transcription Factor E3 (TFE3) and/or Insulin Receptor Substrate 2 (IRS2) polynucleotides	This patent application relates to the identification of antisense oligonucleotides and their use in treating diseases associated with the expression of TFE3 and/or IRS2. Similar patent applications are US8153606 (Treatment of apolipoprotein-A1 related diseases by inhibition of natural antisense transcript to apolipoprotein-A1) [112], US20120252869 [Treatment of sirtuin (sirt) related diseases by inhibition of natural antisense transcript to a sirtuin (sirt)] [113], US20120095081 [Treatment of paraoxonase 1 (pon1) related diseases by inhibition of natural antisense transcript to pon1] [114], WO2011146674 [Treatment of bcl2-like 11 (bcl2l11) related diseases by inhibition of natural antisense transcript to bcl2l11] [115], and US20130035373 [Treatment of fibroblast growth factor 21 (fgf21) related diseases by inhibition of natural antisense transcript to fgf21] [116]. The publications of these similar

Table 4. Partial list of enterprises involved in recent patents or patent applications designed for alterations of gene expression relevant for gene therapy (continued).

Patent or patent application	Title	Original assignee or co-assignee	Short description (claims)	Validity, significance, and biomedical applications
				patent applications demonstrate the flexibility of this approach
US8357664 [117]	Antisense antiviral compound and method for treating influenza viral infection	AviBiopharma, Inc.	The antisense antiviral compounds proposed in this patent are molecules having a nuclease-resistant backbone (including PNAs), 12 – 40 nucleotide bases, and a targeting sequence of at least 12 bases in length that hybridizes to a target region of Influenza virus A, Influenza virus B, and Influenza virus C	The invention provides antisense antiviral compounds and methods of their use and production in inhibition of growth and infection of viruses of the *Orthomyxoviridae* family. The compounds are particularly useful in the treatment of influenza virus infection. Similar patents are US6828105 (Antisense antiviral agent and method for treating ssRNA viral infection) [118] and US8329668 (Antisense antiviral compound and method for treatingpicornavirus infection) [119]. The publication of these patents demonstrates the flexibility of the proposed approach
US7625873 [120]	Antisense antibacterial method and compound	AviBioPharma, Inc.	A method and antisense compound for inhibiting the growth of pathogenic bacterial cells are disclosed. The compound contains no more than 12 nucleotide bases and has a targeting nucleic acid sequence of no fewer than 10 bases in length complementary to region downstream of the translational start codon of a bacterial mRNA encoding a protein essential for bacterial replication	The compound binds to a target mRNA with a T_m of between 50 and 60°C. The relatively short antisense compounds are substantially more active than conventional antisense compounds having a targeting base sequence of 15 or more bases. This short length appears to be a key feature of antibacterial DNA mimics
US20110224283 [110]	Antisense modulation of nuclear hormone receptors	AviBiopharma	This patent application describes antisense oligonucleotides and other agents that target and modulate nuclear hormone	The claims are significant in the field of gene therapy and do apply to selected human pathologies. NHR modulators

Table 4. Partial list of enterprises involved in recent patents or patent applications designed for alterations of gene expression relevant for gene therapy (continued).

Patent or patent application	Title	Original assignee or co-assignee	Short description (claims)	Validity, significance, and biomedical applications
			receptors (NHRs) such as the glucocorticoid receptor (GR), compositions that comprise the same, and methods of use thereof	may be useful in treating NHR-associated diseases. Modulators of NHRs can be useful in the treatment of inflammatory and immune diseases and disorders such as osteoarthritis, rheumatoid arthritis, multiple sclerosis, asthma, inflammatory bowel disease, transplant rejection, and graft vs host disease
US20110289608 [111]	Antisense modulation of interleukins 17 and 23 signaling	Sarepta Therapeutics, Inc. and AviBiopharma	This patent application describes antisense oligonucleotides and other agents that target and modulate IL-17 and/or IL-23 signaling activity in a cell	Therapeutic applications might involve immune-mediated inflammatory diseases, chronic inflammatory diseases as well as acute inflammatory diseases. The claims are highly significant in the field of gene therapy, even considering the specificity of the selected molecular targets
US6284538 [121]	Antisense inhibition of PTEN expression	Isis Pharmaceuticals, Inc.	Antisense compounds, compositions, and methods are provided for modulating the expression of PTEN. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding PTEN. Methods of using these compounds for modulation of PTEN expression and for treatment of diseases and conditions associated with expression of PTEN are provided	The method described in the patent can be directed to pathological conditions including diabetes and hyperproliferative conditions. Similar patents by Isis Pharmaceuticals are US6383808 (Antisense inhibition of clusterin expression) [122], US6033910 (Antisense inhibition of MAP kinase kinase 6 expression) [123], US6043091 (Antisense modulation of liver glycogen phosphorylase expression) [124], and US5789573 (Antisense inhibition of ICAM-1, E-selectin, and CMV IE1/IE2) [125]. The publications of these similar patents demonstrate the flexibility of this approach, as well as its efficacy toward different molecular targets

Table 4. Partial list of enterprises involved in recent patents or patent applications designed for alterations of gene expression relevant for gene therapy (continued).

Patent or patent application	Title	Original assignee or co-assignee	Short description (claims)	Validity, significance, and biomedical applications
US20120183538 [126]	Sparc antisense compositions and uses thereof	Abraxis Bioscience, Llc	The invention provides SPARC antisense oligonucleotides and methods for their use in proliferative diseases such as cancer and hepatic fibrosis. Secreted protein acidic and rich in cysteine (also known as osteonectin, BM40, or SPARC) (hereainfter "SPARC") is a matrix-associated protein highly expressed in several aggressive cancers, while it is absent in the corresponding normal tissues (e.g., bladder, liver, ovary, kidney, gut, and breast)	Biomedical applications include proliferative diseases such as cancer, restenosis, fibrosis, osteoporosis, and inflammatory diseases including arthritis or exaggerated wound healing. Despite being innovative, further validation studies are necessary to understand the real impact of the proposed approach

been developed by the group led by Nicholas Winssinger, who has demonstrated how nucleic acid-template reactions leading to a fluorescent product represent an attractive strategy for the detection and imaging of cellular nucleic acids. In particular, they developed an approach based on a Staudinger reaction to promote the reduction of profluorescent azidorhodamine. The use of two cell-permeable guanidinium peptide nucleic acid (GPNA) probes, one labeled with the profluorescent azidorhodamine and the other with trialkylphosphine, enabled the detection of the mRNA encoding O-6-methylguanine-DNA methyltransferase in intact cells [174,175]. Similar approaches have been the basis of a patent focusing on the detection "*in vivo*" of analytes in living cells, tissues, or organisms [176]. Cells can be obtained from cell cultures or from test animals or patient sources; cells include bacterial cells, mammalian cells, embryonic or somatic stem cells, spermatocytes, yeast cells, erythrocytes, and leukocytes.

In addition to the proposed use of profluorescent PNA analogs and similar reagents to allow imaging of mRNA/miR in live cells, other approaches have been proposed generating PNAs able to permit visualization of gene expression in live cells and intact tissues. For instance, a very interesting application of PNAs has been proposed by Wickstrom *et al.* to detect the onset of activated oncogene expression during the earliest stages of cancer *in vivo* using a noninvasive approach, avoiding surgical interventions with a significant reduction of mortality. These authors proposed that Tc-99m-PNA-peptides are internalized by human cancer cells, hybridize to complementary mRNA targets, permit scintigraphic imaging of oncogene mRNAs in human cancer tissues, and ultimately allow imaging of oncogene mRNAs in human tissues even in the absence of other indications of the disease. Experiments performed in mouse cancer xenograft demonstrated that Tc-99m PNA-peptides designed to bind to IGF1 receptors on malignant cells are taken up specifically and concentrated in nuclei. Furthermore, IGF1-specific Tc-99m-PNA-peptides were prepared employing PNA sequences that specifically hybridize to mRNAs for overexpressed cyclin D1, ERBB2, and c-MYC oncogenes, for activated K-RAS mutated in the 12th codon, and for mutant tumor suppressor p53. Proof-of-concept experiments demonstrated that this method allows detection of mRNA sequences in intact cells, suggesting that this approach might lead to noninvasive detection of gene expression in living cells and tissues [177]. In addition to Tc-99m, preferred radioactive metal isotopes for scintigraphy include ^{64}Cu, ^{67}Ga, ^{68}Ga, ^{87}Y, ^{99m}Tc, and ^{111}In [37,178-180].

7. PNAs in gene therapy: targeting promoters and transcription factors

Figure 4 summarizes the possible use of PNAs for alteration of gene expression and for the development of protocols to be eventually used in therapy. Sections 7 and 8 will consider this specific issue.

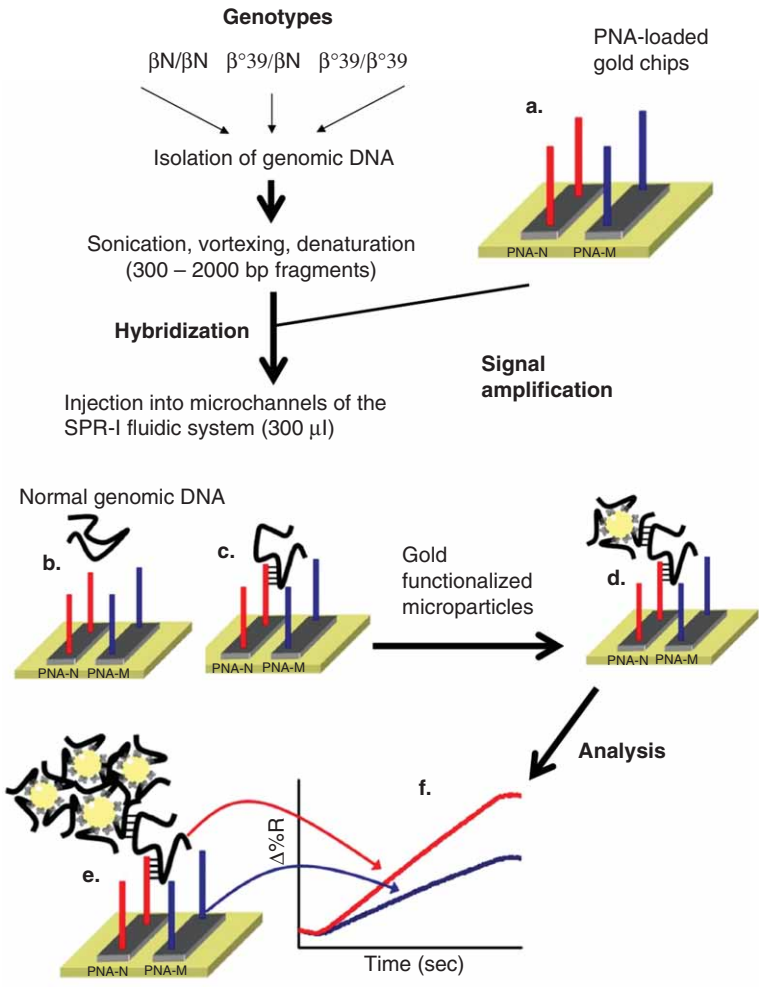

Figure 3. Scheme outlining the nanoparticle-enhanced SPR-Imaging (SPR-I) strategy used to detect the normal βN/βN, heterozygous β°39/βN, and homozygous β°39/β°39 genomic DNAs in PCR-free detection of β-thalassemia mutation [146]. PNA probes recognizing the normal (PNA-N) or mutated (PNA-M) human β-globin gene sequences are loaded on gold chips (**a**). Genomic DNAs from normal βN/βN, heterozygous β°39/βN, and homozygous β°39/β°39 subject can be isolated, shared, denatured, and hybridized (**b,c**). To simplify the pictorial representation only specifically adsorbed DNA is shown. Nonspecifically adsorbed DNA is also present on the surface and contributes to generate the surface plasmon resonance imaging-detected signal [146]. After amplification of the signal using functionalized gold nanoparticles (**d**), analysis is performed (**f**).
Adapted from [146].

7.1 Triple-helix formation by PNA targeting gene promoters

The ability of some PNAs to bind to dsDNA has also promoted attempts to use them in an "antigéne" approach to block transcription from DNA to mRNA. PNAs are able to target the promoter through triple-helix formation; moreover, the high efficiency of PNAs in generating complexes with double-stranded target DNA sequences, deeply altering their functions, is known as "strand-invasion" [54-58]. As a representative example, using a nuclear localization signal peptide, a PNA directed antigéne c-myc oncogene was delivered to the nucleus, and an antigene effect was shown to occur, a mechanism rarely observed for other modified oligonucleotides [56]. The coupling with compounds able to interact with specific cellular receptors, such as dihydrotestosterone, was shown to be an efficient method for cellular/nuclear delivery for an antigéne PNA, which was specifically targeted to prostatic carcinoma cells [58]. After these key studies, other applications of the antigéne strategy have been described. This type of approach can greatly benefit from the availability of PNAs carrying modified bases, which allow targeting of dsDNA in a more efficient way. It was recently shown that antigéne PNAs directed against the N-Myc DNA can have a dramatic effect on growth of human tumor cell lines responsible for neuroblastoma [36].

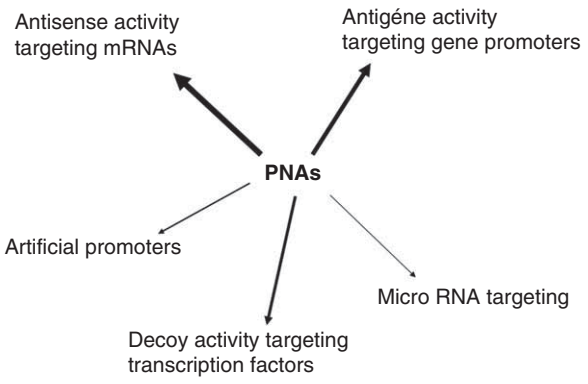

Figure 4. Biological activity of PNAs. The thickness of the arrows are related to the numbers of papers available in the literature.

7.2 PNAs and transcription factor decoy strategy

The transcription factor decoy (TFD) approach is based on the competition for trans-acting factors between endogenous cis-elements present within the regulatory regions of the target gene and exogenously added decoy molecules (for instance, double-stranded DNA) mimicking the specific *cis*-elements [78,79]. The objective of this molecular intervention is to cause an attenuation of the authentic interactions of trans-factors with their *cis*-elements, leading to a removal of the trans-factors from the endogenous cis-element inside the cell [78]. The TFD could be a very useful approach to develop antitumor agents. In fact it is well known that a variety of transcription factors (TFs) are involved in neoplastic cell growth and tumor onset and development, such as Sp1, GATA-1, NF-Y, GATA-4 and GATA-6, NF-κB, CRE-binding proteins, Ets1, TTF-1, AP-1, AP-2, and ERE [180,181]. These TFs are highly expressed in a variety of tumors, including breast cancer, thyroid tumors, hematopoietic tumors, and ovarian tumors. Some of the most interesting examples appeared in the recent literature on the decoy-based approach for gene therapy employ as target TF NF-κB, estrogen receptor, Stat6, CRE, RF-X, NF-Y, and E2F. Applications of TFD to breast cancer have been described by our research group [181]. By treating MCF7 ERα-positive breast cancer cells with a specific PCR decoy molecule belonging to canonical ERα promoter (named DNA-120, -3258/-3157) we obtained a marked reduction of ER mRNA. By opposite, using a PCR decoy molecule belonging to upstream ERα promoter (named DNA-102, -3258/-3157) we obtained not only an increase of ERα RNA in these cells, but also a reactivation of ERα gene transcription in MDA-MB-231 ERα-negative breast cancer cells. Few reports are available on the possible use of PNA-based double-stranded molecules to target TFs [79]. This is due to the fact that double-stranded PNA/PNA and PNA/DNA hybrids exhibit structural features significantly different from those of DNA/DNA hybrids [18,23]. This feature affects direct binding of TFs to target PNA-based molecules, as well as stability of the generated complexes. In fact, PNA/PNA and PNA/DNA duplex are not suitable for TFD. PNA/PNA duplex do not recognize TFs, as recently reported [182], employing sequences recognized by the nuclear factors belonging to the NF-κB and Sp1 superfamily. On the contrary, PNA-DNA-PNA chimeras (PDP) are DNA molecules composed of a part of PNA and a part of DNA. Interestingly, the size of both major and minor grooves and the turn of double helix of PDP/PDP hybrids are much more similar to DNA/DNA hybrids than to PNA/DNA or PNA/PNA hybrids. Accordingly, PNA-DNA-PNA chimeras were found to be active as TFD decoy reagents. These are the first observations that PNA-based molecules could be proposed for TFD pharmacotherapy. As it was found for PNAs, PNA-DNA chimeras also are resistant to exonucleases (both 5′->3′ and 3′->5′), endonucleases, and when incubated in the presence of serum or cellular extracts. In addition, the resistance of these molecules to enzymatic degradation could be improved after complexation to liposomes and microspheres [23].

7.3 Patents on PNAs targeting promoter elements and transcription factors

Patents describing PNA-based molecules suitable for triple-helix formation and strand invasion have been reported first, in consideration of this peculiar activity of PNAs. For example, in a recent patent a method is described for the determination of nucleic acids, which is highly specific and simple, also based on PNAs [59]. The method can be used to differentiate between nucleic acids having a single-base difference in sequence. This approach might lead to therapeutic effects as suggested in several papers [54-58]. A patents application reporting biological effects (i.e., inhibition of transcriptional activation) and describing compositions and methods for inhibiting gene expression in biological systems via inhibition of the binding of TFs to DNA using PNA-based oligomers able to perform strand invasion has been submitted [66]. The target region reported in the patent is the homopyrimidine strand invasion surrounding the NFκB site of the IL-2Rα gene. Inhibition of transcription following strand invasion was demonstrated. In addition to these strategies aimed at targeting promoter elements and clearly obtaining the expected inhibitory effects on transcription, other patent applications [68,69] describe the employment of PNAs targeting promoters in obtaining an opposite effect, being able to behave as a true artificial TF. This strategy was based on the approach originally reported by Mollegard *et al.* [67]. Finally, based on several reports showing *in vitro* and *in vivo* biological activities of decoy oligomers targeting TFs, a recent patent describes DNA- and PNA-based decoys targeting NFATc1 and able to induce apoptosis of primary osteoclasts, suggesting an application of these reagents on bone-related diseases (including bone metastasis) due to osteoclast hyperactivity [80]. In another interesting patent TF3 was targeted either by an antisense therapy against its mRNA, or by decoy molecules [81].

8. PNAs in gene therapy: RNA targeting with antisense molecules

In nonviral gene therapy, the antisense approach targeting specific RNA molecules is the most successful approach. This is demonstrated by the high number of studies, patents, companies including RNA therapeutics in their pipelines (for instance MiRNAtherapeutics, Miragentherapeutics, Santarispharma, Retro-sense, and Antisense Ldt.) and, finally, clinical trials. Recent review articles, papers, and patents related to the antisense approach available and already cited [31-42]. Clinical trials based on RNA targeting with antisense molecules are several and some of them very promising [183-186]. For instance, in the field of the development of antitumor protocols, the main objective of the clinical trial "A Phase I/Ib Study of AZD9150 (ISIS-STAT3Rx) in Patients With Advanced/Metastatic Hepatocellular Carcinoma" (lead sponsor AstraZeneca; main collaborator ISIS Pharmaceuticals; ClinicalTrials.gov Identifier: NCT01839604) is to assess the safety, tolerability, pharmacokinetics, and preliminary antitumor activity of the antisense oligonucleotide AZD9150 in patients with metastatic hepatocellular carcinoma [183]. While the majority of the clinical trials are in the field of oncology [184], important trials have been proposed for other human pathologies, as is the case of a clinical trial on the safety and efficacy of antisense oligonucleotides in Duchenne Muscular Dystrophy (title: "Restoring Dystrophin Expression in Duchenne Muscular Dystrophy: A Phase I/II Clinical Trial Using AVI-4658"; lead sponsor: Imperial College London, UK; ClinicalTrials.gov Identifier: NCT00159250) [185]. Despite the fact that these examples do not employ PNAs, we like to underline that in the case of PNAs, their employment in antisense strategy has been reported in several papers and is one of the most robust applications of these molecules in gene regulation [187-189].

8.1 mRNA targeting

The endpoint of this approach is inhibition of translation or RNase H-mediated degradation in the case of DNA-PNA chimeras [31]. In this context, PNAs are flexible reagents leading to simple inhibition of translation (or RNA processing) or RNAseH-dependent RNA cleavage; for instance, it was shown by Malchère *et al.* that in PNA-based molecules a short phosphodiester window is sufficient to direct RNaseH activity [31]. An extensive analysis of this issue is not among the objectives of the present review. However, few examples of antisense-mediated inhibition of gene expression can be reported based on PNAs targeting galanin receptor type 1 [32], MDM2 [33], and E-cadherin [34]. Lentivial transcripts were also targeted by antisense PNAs [35]. It should be underlined that antisense PNAs can be designed not only to inhibit gene expression, but also to correct altered mRNAs, as in the case of exon skipping [38-41]. For instance, Duchenne muscular dystrophy (DMD) is a lethal disease caused by mutations in the dystrophin gene that results in the absence of the essential muscle protein dystrophin. Among many different approaches for DMD treatment, exon skipping, mediated by antisense oligonucleotides, is one of the most promising methods for restoration of dystrophin expression [185]. PNAs were found to be very efficient tools to induce exon skipping of the DMD mRNA [40].

8.2 PNAs targeting microRNAs and miRNA therapeutics

The issue of targeting microRNAs (miRNA Therapeutics) appears to be one of the most relevant fields of applied biomedicine. microRNAs (http://microrna.sanger.ac.uk/sequences/) are a family of small noncoding RNAs that regulate gene expression by sequence-selective targeting of mRNAs [190-194], inducing translational repression or mRNA degradation, depending on the degree of complementarities between miRs and the target sequences [191]. These miRs/mRNAs interactions lead to the regulation of very important biological processes, such as differentiation, cell cycle, and apoptosis [193]. In view of the role of miRs in epigenetic regulation of gene expression, miRs have been proposed as possible candidates for drug targeting with the objective of interfering with their biological functions, altering the expression of the mRNAs specifically regulated by the targeted miRs [195-198]. Accordingly, an increasing number of reports describing targeting of the miR biogenesis have demonstrated that this has deep impact on specific phenotypes and even on pathological conditions [197]. These effects on miR metabolism were first reported *in vitro*; however, it has been firmly demonstrated that miRs can be antagonized *in vivo* by oligonucleotides composed of high-affinity nucleotide mimics [199]. The effects of PNAs against miR have been the object of very recent studies. Of course, the endpoint of treatment of target cells with PNAs against selected miRs is the alteration of miR-regulated genes. In this respect, the reports available demonstrate that PNAs targeting specific miRs lead to de-repression of the major endogenous mRNA targets of miRs. For instance, increase in Aldolase A mRNA levels was found by Fabani *et al.* [11] following treatment with PNA targeting miR-122. In a second study, a PNA against miR-155 was used, demonstrating deregulation of the target mRNA Bat5, Sfp1, and Jarid2 [200]. These results are very encouraging, since they allow to propose PNAs as possible gene-expression modifiers. This might have important therapeutic applications, in consideration of the involvement of miRs in important human diseases. In our laboratory, we analyzed the effects of PNAs targeting miR-210 and miR-221. In the first paper, Fabbri *et al.* [48] demonstrated high efficiency of the PNA targeting miR-210 in inhibiting miR-210-controlled biological effects; in detail, the anti-miR-210 PNA was found to fully reproduce the inhibitory effects on erythroid differentiation and γ-globin mRNA induction previously reported by

Bianchi *et al.* [201] using a commercially available antagomiR and the human leukemia K562 cell system. In a second work, Brognara *et al.* [49] used a PNA directed against miR-221 and demonstrated that the expression of miR-221 is strongly hampered in PNA-treated MDA-MB-231 cells; at the same time increased expression of the miR-221 target p27-kip1 was observed, both at mRNA and protein levels.

8.3 Patents on antisense PNAs: from the laboratory bench to clinical settings

In the field of PNA-based antisense therapeutics, no overarching patents exist, but a number of patents on specific gene sequences and therapeutic applications. Relevant assignees are Panagene, Optco Curna LLC, Sarepta Therapeutics, Inc., ISIS Innovation Ltd, and iCO Therapeutics, Inc. (**Table 4**). As examples of patent applications concerning antisense PNAs targeting mRNAs, we like to discuss two studies [36,37]. The first teaches how to selectively inhibit the expression of human N-Myc gene in tumors [36]. As known since many years, n-myc mRNA sequences have been implicated in human cancer, such as neuroblastoma, and reagents inhibiting at different levels, the N-Myc gene can have a dramatic effect on growth of human tumors both *in vitro* and *in vivo* [36]. The second study describes antisense compounds, including PNA-based molecules, targeting mRNAs, demonstrating, by diagnostic imaging approaches, their ability to interact with targets in intact cells [37]. The DNA-PNA chimeras for RNaseH-mediated target mRNA degradation are also described in an important patent focusing on antisense strategy for modulating gene expression [77]. The issue of targeting miRs with PNAs in miRNA Therapeutics is covered by a recent patent describing antisense PNAs [52]. The capacity of miR antisense PNAs to inhibit the activity or function of miRs, and a method for evaluating the effectiveness of the treatment are described in detail. This strategy is expected to bring novel protocols for regulating miR expression and, therefore, regulating miR targets.

9. Delivery of PNAs to target cells

One of the most important issues in PNA technology, as known and reported in several papers, is the uptake by target cells. To solve this drawback, several approaches have been considered, including the delivery of PNA analogs with liposomes and microspheres [21-23,202]. In addition, a large variety of PNA analogs have been developed [203-206].

9.1 PNA analogs or modified PNAs for efficient delivery

One of the possible strategies for PNA delivery is to link PNAs to polylysine (K) or a polyarginine (R) tails, based on the observation that this cell-membrane-penetrating oligopeptides are able to facilitate uptake of conjugated molecules [203]. Since their discovery, many modifications of the original PNA backbones have been proposed in order to improve performances in terms of affinity and specificity. Modification of the PNA backbone with positively charged groups has also been demonstrated to enhance cellular uptake and thus PNA efficiency [34,204]. In respect to the delivery issue Fabani and Gait administered anti-miR PNAs by electroporation [11]. In the second set of experiments, Fabani and Gait showed that miR inhibition can be achieved without the need for transfection or electroporation, by conjugating the PNA to the cell-penetrating peptide (CPP) R6-Penetratin, or merely by linkage to just four Lys residues, highlighting the potential of PNAs for future therapeutic applications as well as for studying miR function [200]. In a parallel work, Oh *et al.* described the effectiveness of miR targeting by PNA-peptide conjugates, using a series of CPPs as carriers, including R6 pen, Tat, a four-Lys sequence, and transportan [205]. They found that best conditions were obtained with cationic peptides, and in particular the Tat-modified peptide RRRQR RKKRR. In this study, cells were transfected with plasmid containing a luciferase gene carrying a target site for each miR tested. Inhibition of the miR activity was monitored by expression of the luciferase gene. Inhibition of miR-16, which regulates Bcl-2 expression, and miR-21 activity could be monitored in this way. In a recent study we evaluated the activity of a PNA targeting micro-RNA 210 [48]. The major conclusion of our study was that a PNA against miR-210 and conjugated with polyarginine peptide i) is efficiently internalized within target cells, and ii) strongly inhibits miR-210 activity. Unlike commercially available antagomiRs, which need continuous administrations, a single administration of the PNA conjugated with the polyarginine peptide was sufficient to obtain the biological effects. Interestingly, cellular uptake was found to be crucial in order to obtain biological activity (also *in vivo*), since the PNA lacking of the poly-arginine tail, despite being able to hybridize to target nucleotide sequences, displayed very low activity on cells [48].

9.2 Delivery of PNA-DNA chimeras

As far as the delivery is concerned, the PNA-DNA chimeras are able to take advantage from the possibility of conjugating cell-penetrating and nuclear-localizing peptide moieties to the PNA stretch, therefore allowing cells targeting. On the other hand, unlike PNAs, PNA-DNA chimeras could be suitable for delivery mediated by liposomes and microspheres. We have recently studied the complexation of PNA-DNA chimeras to liposomes [23] and microspheres, demonstrating that these biomolecules efficiently interact with cationic liposomes and microspheres, just as the corresponding ODN-based TFD molecules.

10. Patents based on PNAs: examples of applications to therapeutic intervention

10.1 Antiviral PNAs

One example of patents concerning antisense PNAs describes PNA-based antisense antiviral compounds and the relative methods of use for treating influenza viral infection [117]. Similar patents are available ("Antisense antiviral agent and method for treating ssRNA viral infection" and "Antisense antiviral compound and method for treating picornavirus infection") (**Table 4**) [118,119].

10.2 PNAs altering bacteria growth and viability

PNA-based methods and compositions for killing or inhibiting the bacterial growth are reported in several patents [120,206,207]. The methods described in two of these patents comprise the use of PNAs that are targeted to mRNA and/or rRNA sequences essential to the viability of bacteria. Furthermore, the patents describe a possible protocol in which the PNA-based antisense molecules are delivered together with one or more separate antibiotics. The method was extended to *in vivo* treatment following administration of one or more PNA-based compounds, also with concurrent treatment with an antibiotic [206,207].

10.3 Anticancer PNAs

The approaches aimed at controlling viral and bacterial infections were facilitated by the several already demonstrated activities of antisense molecules on several pathologies caused by virus infection. On the other hand, in a very interesting patent a PNA-based approach was presented describing a composition comprising a PNA polymer and an excipient or liposome delivery complex (or, alternatively, a covalent linkage to a polypeptide sequence that enhances cellular uptake of the PNA polymer) with the aim of modulating mammalian telomerase activity in a cell [208]. This approach is of interest, considering that telomerase activity is involved in the onset and progression of cancer cells. In the field of PNA-based anticancer agents and of the delivery of PNA-based anticancer drugs, a patent describes a class of antisense agents having a distributed GPNA backbone which has excellent uptake into mammalian cells, can bind to the target DNA or RNA in a highly sequence specific manner, and can resist to nucleases and proteases both outside and inside the cell(s) of interest [209]. To test this approach, either systemic or intratumoral administration of antisense "EGFR" GPNA molecules were reported to downmodulate EGFR levels, reducing head and neck squamous cell carcinoma tumor growth [209].

10.4 PNAs targeting mitochondria

PNA-based approaches are also directed against other defects, as recently reported in studies and patents concerning gene therapy for mitochondrial DNA (mtDNA) defects. For instance, a PNA-based approach is described which can be employed ideally to treat patients with heteroplasmic mtDNA defects by selectively inhibiting the replication of the mutant mtDNA by sequence-complementary PNAs [210]. If this inhibition was maintained for a sufficient period of time then levels of wild-type mtDNA would increase relative to those of the mutant mtDNA. The replication of human mtDNA may give a unique opportunity for such a strategy since it is initiated at two different origins of replication and this results in the formation of single-stranded mtDNA during much of the replication process. Thus, during the single-stranded phase of mtDNA replication there is the opportunity for binding of sequence-specific PNAs which inhibit replication. These PNAs might be attached to or linked to a mitochondrial-targeting peptide in order to transfer the bioactive PNA sequences into mitochondria. One example of mitochondria-targeting peptide comprises an N-terminal region of human cytochrome c oxidase subunit VIII (a nuclear-encoded inner mitochondrial membrane protein) [210].

11. Expert opinion

11.1 Strength and weaknesses of the research field based on peptide nucleic acids

PNAs have been discovered and firstly described in 1991 by Peter Nielsen [16]. Therefore, these molecules cannot be considered as "novel reagents." However, since their discovery, PNAs have been proven to be very important reagents in molecular diagnosis, allowing the achievement of unmet objectives after comparison with diagnostic protocols carried out with standard reagents. We would like to underline that PNAs cannot be considered more efficient tools with respect to LNAs, morpholinos, mixmers, gapmers [7-15,46,211-214], and other DNA mimics extensively employed in diagnostic and therapeutic strategies (comparison in specific applications are needed for reaching conclusive information), but are certainly an interesting option. Key findings are the demonstration that PNAs are very efficient in hybridizing with DNA and RNA [19,21], are stable in biological fluids [23], and can perform strand invasion of DNA [17] allowing the development of novel diagnostic and therapeutic protocols. These conclusions are very solid and anticipate the expectation that PNAs will allow the developments of extremely efficient, reproducible, and sensible diagnostic methods, suitable for performing genomic and transcriptomic analyses using very low amounts of cells. The summary of the diagnostic applications of PNAs reported in **Table 3** gives just an idea of the potential of these reagents in diagnosis. On the other hand, PNAs have been proven to be very active and specific for alteration of gene expression, despite the fact that solubility and uptake by target cells can be a limiting factor. Accordingly, the studies and patents on PNAs have taken in great consideration the delivery strategy, which is a very important parameter [22,23]. The stability of the PNA molecules *in vivo* is very high; however, issues related to long-term toxicity and/or possible effects on innate and adaptive immune response have not

been conclusively explored and should be carefully considered. In any case, PNAs have been shown to be, as some other reagents (such as mixmers and gapmers) [11,46,211-214], more efficient than control DNAs in targeting RNA molecules (for instance, mRNAs, ribosomal RNAs, and miRs in several biomedical oriented applications) [18,20]. This issue should be considered for novel therapeutic interventions, such as those recently proposed concerning i) block of activated non-canonical cryptic splicing sites or ii) induction of exon skipping to restore in abnormal mRNA a correct reading frame. Despite the fact that *in vivo* data on PNA treatments are available in the literature [200], no therapeutic clinical trials have been activated. On the contrary, as a further support to the conclusion that PNAs are very important reagents in diagnostics, clinical trials in the field of molecular diagnosis and based on PNAs (employed in PNA-clamping and PNA-FISH methodology) have been reported [215,216].

11.2 Potential and ultimate goals of PNA-based research

It is expected that PNAs, coupled with a variety of signal enhancement strategies, will allow PCR-free (and reverse transcription PCR-free) diagnostic protocols, therefore introducing key simplification in the technical steps [146]. In the near future we are expecting that the use of PNA-based diagnostics will move from *in vitro* cell-free assays to analysis at the cellular level, even considering live cells or intact tissues. This is a fascinating issue and many examples are already available [161-163,165-176]. Moreover, full-body diagnostics are expected to be dramatically improved using suitably modified PNAs as recently reported [177-179,217].

In the field of gene therapy, PNA-based molecules, if demonstrated to be safe to the patients, can be considered of great interest. In this field, it is of particular interest the recent demonstration that PNAs are strong inhibitors of miR activity both *in vitro* [46-49] and *in vivo* [200] and therefore might be proposed as key reagents in "miRNA Therapeutics" [51,194,199]. In fact, miRs are becoming novel targets for therapeutic interventions in view of their key roles in normal biological functions on one hand, and human diseases on the other [192,193].

11.3 Key areas of investigation and future perspectives

The key area of investigation on PNAs is certainly the validation of the therapeutic potential in *in vivo* experimental systems. This is a prerequisite to propose PNAs in clinical trials aimed at correcting pathological states. On the other hand, we expect novel and exciting data in research areas in which great difficulty still exists in using standard DNA- and/or RNA mimics. For instance, a field of great relevance is the so-called homologous recombination, a strategy aimed at correcting gene defect without the need of viral-based gene therapy [218]. When this technology is combined with the generation of induced pluripotent stem cells (iPS), it allows to generate corrected iPS cells lines from patients carrying important genetic diseases (for instance thalassemia) to be employed in regenerative medicine [218]. Unfortunately, homologous recombination is still not efficient [219]. For this reason, the possible applications of PNAs in homologous recombination could be of great interest to increase efficacy. PNA-based protocols for homologous recombination have been reported, but still need further controls and validation steps to understand their limits and advantages [220-222]. In this respect, two papers were published on homologous recombination based on the use of PNAs [220,221]. Chin *et al.* have designed a series of triplex-forming PNAs that can specifically bind to sequences in the human β-globin gene and demonstrate that these PNAs, when cotransfected with recombinatory donor DNA fragments, can promote single base-pair modification at the start of the second intron of the β-globin gene, the site of a common thalassemia-associated mutation [220], demonstrating that these PNAs were effective in stimulating the modification of the endogenous β-globin locus in human primary hematopoietic progenitor cells [220]. This approach was confirmed by Rogers *et al.*, who recently reported a method allowing an increased efficiency of PNA-based gene corrections, employing the conjugation of a triplex-forming PNA to the transport peptide, antennapedia (Antp) [221]. This strategy allows the successful *in vivo* chromosomal genomic modification of hematopoietic progenitor cells, while still retaining intact differentiation capabilities. Finally, it has been already reported that the homologous recombination can be increased by inducing a double-strand break at target site, including protocols employing artificial restriction DNA cutter (ARCUT), composed of Ce(IV)/EDTA complex (molecular scissors) and two strands of PNA, without proteins, as demonstrated by the group of Komiyama [63-65]. ARCUT for desired homologous recombination is easily and straightforwardly designed and synthesized and promotes the targeted homologous recombination [71].

When these innovative approaches are considered together with more validated strategies-based antisense and antigéne properties of PNAs, as a conclusive remark, we are expecting in the near future relevant clinical trials based on PNAs, when the issues related to tolerability and toxicity will be suitably approached. In this respect, in order to improve clinical parameters, many patents suggest the use of the PNA molecules in combination with drugs already employed in therapy. At present, however, the most relevant and promising clinical trials using PNA-based approaches are active only in the field of diagnosis and/or prognosis [215,216]. The most interesting example, in our opinion, is based on the demonstration that protocols based on PNA clamping are very important to detect point mutations as described by Thiede *et al.* [223]. This strategy was more recently applied to detect mutations of the EGFR in non-small-cell lung cancers [132,224]. Interestingly, this approach was found to be useful to predict the responsiveness of patients to the therapy based on gefitinib [225-227]. The same strategy was the object of recent patent applications [99,227] and of the clinical trial "Comparison of

Sequencing and PNA Clamping of EGFR Gene in Patients With Non-Small Cell Type Lung Cancer" (Lead Sponsor: Chonnam National University Hospital; Collaborator: Astra-Zeneca; ClinicalTrials.gov Identifier: NCT01767974) [215]. This is probably the best example concerning the transfer of basic research on PNAs, to patent applications and clinical settings.

Declaration of interest

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