

- double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl Acad. Sci. USA* 98, 9742–9747
- 5 Brummelkamp, T.R. *et al.* (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296, 550–553
  - 6 McManus, M.T. *et al.* (2002) Gene silencing using micro-RNA designed hairpins. *RNA* 8, 842–850
  - 7 Paddison, P.J. *et al.* (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16, 948–958
  - 8 McCaffrey, A.P. *et al.* (2002) RNA interference in adult mice. *Nature* 418, 38–39
  - 9 Lewis, D.L. *et al.* (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.* 32, 107–108
  - 10 Winston, W.M. *et al.* (2002) Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science* 295, 2456–2459
  - 11 Gitlin, L. *et al.* (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* 418, 430–434
  - 12 Jacque, J.M. *et al.* (2002) Modulation of HIV-1 replication by RNA interference. *Nature* 418, 435–438
  - 13 Novina, C.D. *et al.* (2002) siRNA-directed inhibition of HIV-1 infection. *Nat. Med.* 8, 681–686
  - 14 Lee, N.S. *et al.* (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat. Biotechnol.* 20, 500–505
  - 15 Brummelkamp, T.R. *et al.* (2002) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* 2, 243–247
  - 16 Pan, D. *et al.* (2002) Biodistribution and toxicity studies of VSVG-pseudotyped lentiviral vector after intravenous administration in mice with the observation of *in vivo* transduction of bone marrow. *Mol. Ther.* 6, 19–29
  - 17 Ohkawa, J. and Taira, K. (2000) Control of the functional activity of an antisense RNA by a tetracycline-responsive derivative of the human U6 snRNA promoter. *Hum. Gene Ther.* 11, 577–585

## PNA and LNA throw light on DNA

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**In some aspects, homogeneous (all-in-solution) nucleic acid hybridization assays are superior to the traditionally used heterogeneous (solution-to-surface) alternatives. Profluorescent probes, which reveal fluorescence enhancement or fluorescence polarization upon their binding to DNA and RNA targets, are a paradigm for the real-time sequence-specific homogeneous detection of nucleic acids. A variety of such DNA or RNA-derived probes of different constructs has already been developed with numerous applications. However, the recent additions to the field – locked nucleic acids (LNAs) and peptide nucleic acids (PNAs) – significantly increase the potential of profluorescent probes and provide a robust impulse for their new uses.**

The DNA complementarity principle was discovered 50 years ago, along with the famous double-helical DNA structure [1]. This discovery soon led to several hybridization assays to detect and isolate specific nucleic acid sequences [2–4] – keystone procedures in molecular biology and biotechnology. Originally, the hybridization process was done as a homogeneous reaction in solution but the separation of hybrid molecules from unhybridized probes required either ultracentrifugation or chromatography and filtration: those who have worked with these early semi-homogeneous methods know how cumbersome and tedious those procedures are.

Consequently, a major advance was achieved when methods for immobilization and transfer of DNA to membrane or filter were developed [5–7]. One of the heterogeneous ‘on surface’ hybridization assays, Southern blotting [7], revolutionized the field and dominated for two decades. However, the need to immobilize hybrids on a solid surface

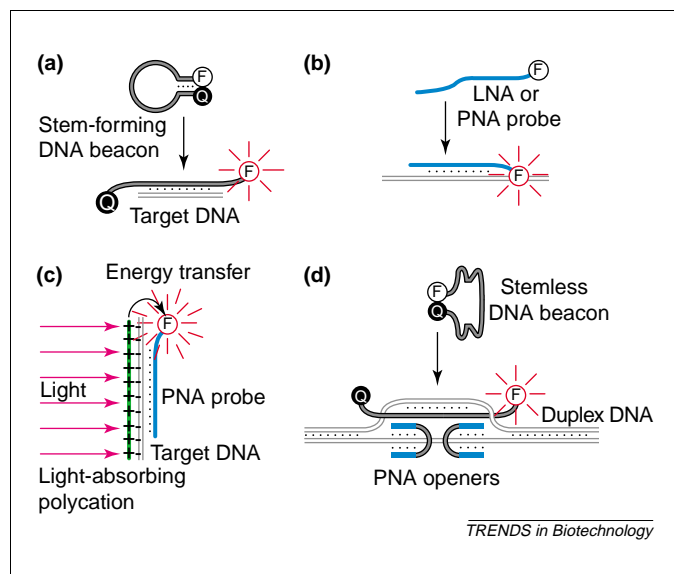
inherent in the solid-phase hybridization formats limits their sensitivity (owing to non-specific adsorption of the probe) while the requirement to wash out unhybridized probes before detection of specific sequences prevents real-time monitoring.

These factors, and other drawbacks of non-homogeneous assays, drew attention to solution-based homogeneous hybridization formats. The study led to the first generation of profluorescent probes – DNA-derived molecular beacons (Fig. 1) [8,9], that turn brightly radiant under light after binding the target. These dual-labeled fluorogenic probes provide the means for truly homogeneous diagnostic assays and enable high-throughput multiplex analyses. But nothing is perfect: the novel hybridization tools are also not free from limitations such as insufficient stability, difficulties in the double labeling and lack of further signal amplification. The international search for better probes has recently led to new developments.

### Singly labeled conformationally restricted fluorogenic probes

It is evident that singly labeled probes are more practical than dual-labeled probes because minimal DNA modification eliminates common problems caused by tagging two DNA sites, including low yield, high cost and singly labeled impurities. In the recent study from Caliper Technologies Corp. (Mt. View, CA, USA) the backbone-modified LNA (locked nucleic acid)-based probes were 5′ end-labeled with either rhodamine or hexachlorofluorescein and their hybridization to target DNAs was followed by measuring the fluorescence polarization (FP) of these dyes [10]. LNA, or locked nucleic acid, is a synthetic RNA derivative in which the ribose moiety in sugar-phosphate backbone is structurally constrained by a methylene bridge between

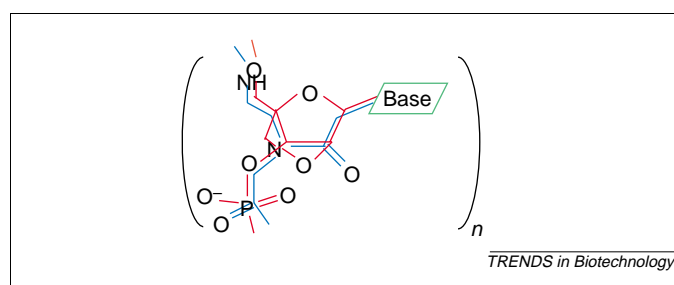
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**Fig. 1.** Various types of fluorogenic probes and different formats of their hybridization (Q and F, quencher and fluorophore tags, respectively). (a) Dual-labeled stem-forming DNA beacons represent the first generation of probes that brightly fluoresce on hybridization. (b,c) Singly labeled locked nucleic acid (LNA) or peptide nucleic acid (PNA)-based probes are more economical and allow further signal amplification by using light-harvesting polycationic 'helpers'. (d) PNA openers locally expose the duplex DNA target for stable or transient binding the stemless DNA beacon.

the 2'-oxygen and the 4'-carbon atoms (Fig. 2). The link 'locks' the sugar ring in the fixed 3'-endo conformation, which is preferable for the formation of hybrids with complementary DNA or RNA sequences. This feature gives the LNA probes very high binding affinity but does not compromise their sequence specificity [11,12]. For DNA diagnostics it is important that LNA is stable against cleavage by nucleases, which might contaminate biogenic DNA analytes.

Simeonov and Nikiforov exploited these properties of LNA probes to develop an original sequence-specific method of DNA detection in real time [10]. They found that hybridization to correct DNA targets of six- or seven-mer LNA probes mono-tagged with fluorogenic dye resulted in significant FP increments (Fig. 1b), whereas the presence of single mismatches between a probe and the target caused only very small changes in FP, if any. The notable increase in FP of dyes following probe-to-target binding is a result of a change in rotational mobility of the target-fastened fluorophores as compared with free probes. The researchers believe that the high sensitivity



**Fig. 2.** Chemical structures of locked nucleic acid (LNA; red) and peptide nucleic acid (PNA; blue). Note that LNA has the sugar-phosphate backbone carrying negative charges. In PNA, nucleobases are attached to the uncharged protein-like (polyamide) backbone thus yielding a chimeric pseudopeptide-nucleic acid structure.

and relative simplicity of this approach give it potential for future use in high-throughput genotyping experiments, including the multiplex analyses of human single nucleotide polymorphisms. Moreover, the very short length of the LNAs used enables a unique opportunity to construct the complete and universal library of a few thousand fluorescently labeled probes for homogeneous testing of all possible single-base genome variations.

### A change of backbone plus use of light-harvesting 'helpers'

Besides LNA, peptide nucleic acid (PNA) is another promising synthetic imitator of natural nucleic acids [13,14]. However, unlike LNA comprising a slightly modified RNA backbone, the PNA backbone is radically different, although it is structurally homomorphous to nucleic acid forms (Fig. 2). Similar to LNA, PNA exhibits excellent biochemical stability along with high binding affinity and high recognition specificity. In addition, owing to an electrically neutral pseudopeptide backbone, PNA provides a wider range of hybridization conditions and has an exceptional ability to invade duplex DNA. Perception of these PNA advantages stimulated the use of a variety of PNA-based probes, including the fluorogenic ones [15,16]. Recently, Kubista and co-workers from Lundberg Institute and Chalmers University of Technology (Göteborg, Sweden) came out with the original concept of mono-tagged light-up probes for nucleic acid detection [17].

The light-up probe consists of a PNA oligomer to which thiazole orange, an asymmetric cyanine dye, is tethered. It was found that although free light-up probes have low fluorescence, it might increase almost 50-fold on probe binding to complementary DNA targets owing to the large fluorescence enhancement of cyanine dye in the hybridized state (Fig. 1b). The binding of light-up probes to DNA is highly sequence-specific: a single mismatch in a ten-mer target sequence is readily distinguished. Consequently, these singly labeled profluorescent probes are well suited for rapid and specific homogeneous detection of PCR products in real time [18], similar to dual-labeled molecular beacons. Notwithstanding the great practical potential of light-up probes, they nevertheless have one drawback: in general, the fluorescent response is probe sequence-dependent and elimination of this unwanted effect requires elevated temperatures [19]. Furthermore, the light-up PNA probes, as well as the FP-responded LNA probes, provide no means for further signal amplification.

The latest report by the Bazan-Heeger group (University of California at Santa Barbara, CA, USA) takes an important step forward by introducing a new PNA-based homogeneous hybridization approach, which is relatively simple and highly sensitive [20]. The group designed a sandwich-like system in which PNA-to-DNA sequence-specific binding triggers a single fluorescent molecule (fluorescein) attached to the PNA strand to powerfully emit light (Fig. 1c). In this system, the light-absorbing cationic-conjugated polymer is used as an energy-harvesting 'helper' to effectively sensitize the emission of a probe-linked dye. Electrostatic interactions bring the positively charged polymeric helper and hybrid complex of neutral

PNA probe and the negatively charged DNA target within short distances required for efficient energy transfer. Thus, the signal transduction is strictly controlled by hybridization and light is emitted much more (>25 times) brighter compared with the standard method of direct dye excitation. Consequently, the technique can be used to detect target DNA at concentrations down to 10 pM with a standard fluorometer. Note that the brief report by Gaylord *et al.* [20] presents only a proof-of-principal study and several issues, such as sequence specificity of the assay, its multiplexity potential and independence of fluorescent signal on probe sequence, should be addressed before this elegant approach will be put into practice.

### Combing stemless DNA beacons with PNA 'openers'

One more twist in the story of PNA-assisted fluorescent DNA detection, now with probing the DNA methylation status by stemless DNA beacons, came very recently from the study of a Japanese group at Kyoto University and Japan Science & Technology Corporation [21]. Site-specific DNA methylation is involved in the regulation of gene expression and gene silencing, and is believed to cause a significant number of transition mutations responsible for human genetic diseases and cancer. Therefore, methods are needed to find out whether or not the particular site in genomic DNA is methylated. To this end, Okamoto *et al.* proposed a simple and direct method for discrimination of cytosine and 5-methylcytosine in DNA duplexes that combines the PNA-assisted hybridization of profluorescent probe with treatment by restriction enzymes [21]. The method is based on the advances reported at the beginning of last year by Kuhn *et al.* (Boston University and Boston Probes Inc., Bedford, MA, USA) [22], who pioneered the use of PNA openers [16] for hybridizing a variety of molecular beacons directly to duplex DNA (Fig. 1d).

Although Okamoto *et al.* do not explain clearly the rationale behind their experiments [21], it appears that they use the transient formation of unstable strand-displacement complexes of stemless DNA beacon and double-stranded DNA target selectively exposed by PNA openers to enzymatically cut the beacon-type probe in the methylation-dependent manner. The stemless DNA beacon is a simplified type of molecular beacons, which has the compact non-fluorescent form in a free state (Fig. 1d). For unstable hybridization, only a slight increase in the background beacon fluorescence is expected. However, even transient binding allows the DNA beacon to be cut in half with a restriction enzyme at the corresponding enzyme-recognition site, thus converting some fraction of beacons into the singly labeled truncated probes lacking a quencher and hence able to fluoresce even in a free state. Such a transformation of the DNA beacon is inhibited if the chosen DNA site is methylated because restriction enzymes are normally poorly active on the hemi-methylated substrates (note that only one DNA strand of the beacon-target complex is methylated).

Therefore, it was possible for Okamoto *et al.* to distinguish between modified and non-modified DNA sites: they observed, in the best of presented examples, that after the treatment the unmethylated substrate become brightly fluorescent whereas the methylated substrate remained

dark [21]. Notwithstanding the value of this idea, two awkward oversights in its realization are worth mentioning. First, obviously being unaware of this very rare activity of few restriction enzymes, the researchers unintentionally used those nucleases that can efficiently cut single-stranded DNA as well. As a result, these enzymes gradually degraded free probes, thus raising additional background fluorescence and yielding poor discrimination of methylated versus non-methylated DNA sites in other cases [21; supplementary materials]. Second, considering that DNA is normally only partially methylated, it will be more practical to generate optical signal from the modified sites but not from the intact ones, as demonstrated in the proof-of-principal study [21].

The concept is attractive although its true realization requires further work to finally shape it up. Compared with currently available methods for evaluating the methylation status of DNA, a major advantage of a newly proposed approach is that site-specific DNA methylation can be detected optically, as well as avoiding duplex denaturation and electrophoresis procedures, although its general use is limited owing to the sequence restrictions imposed on the target sequences by enzyme-recognition and PNA-binding sites.

### Concluding remarks

Notwithstanding great progress that microarrays brought to the area of heterogeneous, solution-to-surface nucleic acid hybridization assays, the homogeneous approaches have certain important advantages. At present, a variety of fluorogenic DNA or RNA-based probes was developed that allow the real-time sequence-specific optical DNA detection in solution. As shown here, the recent advent in the field of LNA and PNA oligomers significantly enlarges the potential of profluorescent probes and gives a robust impulse for their new uses. Note that in some cases of DNA sensing LNA-based probes could be more convenient than PNA-based probes [10]. Also, the singly labeled fluorogenic PNA or LNA probes make it possible to develop simpler, less expensive DNA diagnostics with enhanced signal amplification. Furthermore, the PNA openers could be useful for hybridization-dependent conversion of dual-labeled probes to the singly labeled probes to enable the probing of site-specific DNA modifications.

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### References

- 1 Watson, J.D. and Crick, F.H.C. (1953) Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. *Nature* 171, 737–738
- 2 Hall, B.D. and Spiegelman, S. (1961) Sequence complementarity of T2-DNA and T2-specific RNA. *Proc. Natl Acad. Sci. USA* 47, 137–146
- 3 Bautz, E.K.F. and Hall, B.D. (1962) The isolation of T4-specific RNA on a DNA-cellulose column. *Proc. Natl Acad. Sci. USA* 48, 400–408
- 4 Winocour, E. (1965) Attempts to detect an integrated polyoma genome by nucleic acid hybridization. I. 'Reconstruction' experiments and complementarity tests between synthetic polyoma RNA and polyoma tumor DNA. *Virology* 25, 276

- 5 Gillespie, D. and Spiegelman, S. (1965) A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* 12, 829–842
- 6 Noyes, B.E. and Stark, G.R. (1975) Nucleic acid hybridization using DNA covalently coupled to cellulose. *Cell* 5, 301–310
- 7 Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98, 503–517
- 8 Tyagi, S. and Kramer, F.R. (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnol.* 14, 303–308
- 9 Vet, J.A.M. *et al.* (2002) Molecular beacons: colorful analysis of nucleic acids. *Expert Rev. Mol. Diagn.* 2, 77–86
- 10 Simeonov, A. and Nikiforov, T.T. (2002) Single nucleotide polymorphism genotyping using short, fluorescently labeled locked nucleic acid (LNA) probes and fluorescence polarization detection. *Nucleic Acids Res.* 30, e91
- 11 Koshkin, A.A. *et al.* (1998) LNA (locked nucleic acid): an RNA mimic forming exceedingly stable LNA:LNA duplexes. *J. Am. Chem. Soc.* 120, 13252–13253
- 12 Braasch, D.A. and Corey, D.R. (2001) Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. *Chem. Biol.* 8, 1–7
- 13 Nielsen, P.E. *et al.* (1991) Sequence selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* 254, 1497–1500
- 14 Demidov, V.V. (2002) PNA comes of age: from infancy to maturity. *Drug Discov. Today* 7, 153–155
- 15 Demidov, V.V. (2002) New kids on the block: emerging PNA-based DNA diagnostics. *Expert Rev. Mol. Diagn.* 2, 89–91
- 16 Demidov, V.V. (2001) PD-loop technology: PNA openers at work. *Expert Rev. Mol. Diagn.* 1, 343–351
- 17 Svanvik, N. *et al.* (2000) Light-up probes: thiazole orange-conjugated peptide nucleic acid for detection of target nucleic acid in homogeneous solution. *Anal. Biochem.* 281, 26–35
- 18 Isacson, J. *et al.* (2000) Rapid and specific detection of PCR products using light-up probes. *Mol. Cell. Probes* 14, 321–328
- 19 Svanvik, N. *et al.* (2001) Free-probe fluorescence of light-up probes. *J. Am. Chem. Soc.* 123, 803–809
- 20 Gaylord, B.S. *et al.* (2002) DNA detection using water-soluble conjugated polymers and peptide nucleic acid probes. *Proc. Natl Acad. Sci. USA* 99, 10954–10957
- 21 Okamoto, A. *et al.* (2002) Site-specific discrimination of cytosine and 5-methylcytosine in duplex DNA by peptide nucleic acids. *J. Am. Chem. Soc.* 124, 10262–10263
- 22 Kuhn, H. *et al.* (2002) Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. *J. Am. Chem. Soc.* 124, 1097–1103

## Letters

## Safe choice of metals in food bioprocess enzyme-mimicry?

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The association of metals with proteins confers greatly enhanced biocatalytic activity. For example, powdered iron can trigger a rapid decomposition of hydrogen peroxide into water and oxygen. However, such a reaction is a form of enzyme mimic and catalase does it  $\sim 10^5$  faster. The search for full understanding of the enhanced role of the protein moiety is a laudable application of *in silico* molecular modelling to complement 'real lab' studies of enzyme specificity and efficacy [1].

Iron is frequently present in the active site of enzymes, including catalases, peroxidases, cytochrome P450 [2] and the bacterial superoxide dismutase (SOD). In animals, SOD in cell cytosol contains copper and zinc [3] and in mitochondria contains manganese. Electron-shift redox is crucial to the enzymic mechanism [4]. For example, in the case of cytochromes P450 (EC 1.14.14.1: oxygen monooxygenase) the substrate is initially bound by FeIII ions (within the protohaematin IX) and an electron is supplied by the electron transport chain from NADPH (in animal tissues) via molecules of cytochrome P450 reductase flavoprotein to form FeII(S)O<sub>2</sub>. An additional electron, supplied by cytochrome b2, promotes the formation of a perferryl FeV complex with oxygen, [FeO]<sup>3+</sup> – the working 'oxygenase facilitator'. What else does the iron do [5]? Mechanistic understanding requires the recognition of the low-spin to high-spin orbital shift, which accentuates the catalytic ability of FeIII. Unfortunately, this shift to a

very high  $k_{cat}$  lowers the thermal stability of the enzyme: this 'trade-off' is finely balanced and FeIII is competent in this role.

Such choice of 'best metal' should be explored *in silico* (and in 'real-lab' studies [1]) before committing a biotechnology-based process to an enzyme-mimic containing, for example, the ruthenium common in cytochromes P450 complex mimics. Indeed, a quick-test benchmarking system is required for metal redox-competency in mimicry of many common enzymes, such as the SOD metals copper, zinc and manganese relative to the iron-containing isoform in bacteria. Indeed, studies on the loss of efficacy (and on the catalytic constant  $k_{cat}/K_m$ ) and temperature- and pH-rate and stability definition, should be put into the *in silico* databank for would-be designers of metal-defined enzyme mimics. Moreover, the redox-potential of all metals capable of valency shift should be included. Unfortunately, one-electron recycling by a particular metal (or in coupled metal systems) can regenerate a superoxide anion that damages the biomolecules it encounters [6].

Enzyme mimics in foods or for use in enzyme therapy must be non-toxic. Enzyme mimics that contain only iron are likely to be acceptable for food bioprocessing because the addition of powdered iron to many foodstuffs is acceptable as a mineral supplement even if it is accompanied by traces of chromium, which is now thought to protect against diabetes mellitus type II. Other forms of dietary