CHAPTER 4

STEM-LOOP OLIGONUCLEOTIDES AS HYBRIDIZATION PROBES AND THEIR PRACTICAL USE IN MOLECULAR BIOLOGY AND BIOMEDICINE

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Abstract: As originally designed, stem-loop (SL) hybridization probe is a single-stranded oligonucleotide containing a sequence complementary to the target that is flanked by inverted repeats forming self-complementary termini, and harbors a fluorophore–quencher pair at the 5′- and 3′-ends. When the target is missing, these molecules form closed pan handle-like structures in which the fluorophore and quencher are located in a close proximity, due to 5′- and 3′-termini spatial neighborhood. Such a quencher proximity represses fluorescence. In contrast, in the presence of the target, probe forms a complex with it, which spatially separates the fluorophore from the quencher. Once the fluorophore and quencher are dissociated, the fluoresce increases, thus enabling quantitative measuring of signals with the threshold value evidencing presence of the target. Alternatively, SL probe can be fluorescence resonance energy transfer (FRET)-labeled, with the fluorescence shift message being monitored in the latter case. SL probes are currently in use in a number of applications, primarily for specific nucleic acid motif detection and in real-time polymerase chain reaction (PCR). Also, SL probes can be utilized in protein–DNA interaction studies, and even for specific inorganic ion detection. This quickly evolving group of methods gave rise to at least 40 international patents and is cited in ~50 peer-reviewed papers annually.

Keywords: Stem loop, hybridization probe, molecular beacon, mismatched target, secondary structure, linear probe, thermodynamic limitations, fluorophore, quencher, fluorescence resonance energy transfer, FRET, real-time, mismatch, mismatch discrimination, accuracy, sensitivity, nanoparticle, multiplex, scorpion, DNAzyme, catalytic molecular beacon, DNA probe, mispaired base, immobilized.

Abbreviations: cDNA, complementary DNA; dNTP, deoxyribonucleotidetriphosphate; DABCYL, 4-{[4′-(dimethylamino)phenyl]azo}benzoic acid; FRET, fluorescence

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resonance energy transfer; mRNA, messenger RNA; PDGF, platelet-derived growth factor; PCR, polymerase chain reaction; RT, reverse transcription; SL, stem-loop.

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1. INTRODUCTION

Naturally occurring stem-loop (SL)-forming RNA and DNA structures have important regulatory roles in normal cellular functioning (Cech 1993; Pearson and Sinden 1998; Sinden et al. 2002). Apart from recognition by proteins (Varani 1995) and specific gene silencing (Meins et al. 2005), SL structures exhibit some advantageous characteristics in nucleic acids hybridization as compared with other conformations (reviewed by Broude (2002)) and other authors (Fang et al. 2002; Tsourkas and Bao 2003; Drake and Tan 2004; Goel et al. 2005; Santangelo et al. 2006). The use of SL-forming oligonucleotides for the *selective polymerase chain reaction (PCR) suppression* effect, which gave rise to a multitude of useful PCR-based experimental approaches, is reviewed in the Chapter 2 of this book and will not be considered here. In this chapter, we focus on the use of SL oligonucleotides as hybridization probes. The advantage of SL-forming oligonucleotides was clearly demonstrated when analyzing hybridization of linear and SL (also termed *molecular beacon*) DNA probes has revealed significantly higher specificity of the latter (Bonnet et al. 1998, 1999). By analyzing free-energy phase diagrams of molecular beacons in solution with matched and mismatched targets, the authors showed that structurally constrained probes can generally distinguish mismatches over a wider range of temperatures than unstructured probes can do (Bonnet et al. 1998; Broude 2002).

The probable explanation could be a lower free energy of SL probe annealing provided by smaller number of configurations (and, consequently, decreased entropy of hybridization) (Roberts and Crothers 1991). The loop regions, which are generally complementary to the targets, are better exposed for hybridization, as compared with linear probes. It should be mentioned, however, that secondary structures of SL probes dictate kinetic and thermodynamic limitations: pan handle-like structure formation proceeds somewhat slower (but is still fast), and results in products with lower melting temperatures and free energies (Bonnet et al. 1998; Broude 2002). Nevertheless, the beauty of structured probes lies in their ability to better discriminate mismatches in different applications (Riccelli

et al. 2001). SL structures may be hybridized in solution (and thus used as capture devices in many applications, e.g. as a specific probe in a quantitative real-time PCR (Yesilkaya et al. 2006)), or being attached to a solid support so that the loop is immobilized on a surface and dangling ends are used for hybridization (Riccelli et al. 2001; Broude 2002).

In this chapter, we review the recent developments in using SL probes for DNA microarrays and molecular beacon approaches.

2. MOLECULAR BEACONS

As originally designed, a molecular beacon is a single-stranded oligonucleotide probe containing a sequence complementary to the target that is flanked by inverted repeats forming self-complementary termini, and harbors a fluorophore–quencher pair at the 5′- and 3′-ends (Tyagi and Kramer 1996; Tyagi et al. 1998) (Figure 1). When the target is missing, these molecules form closed pan handle-like structures in which the fluorophore and quencher are located in a close proximity, due to 5′- and 3′-termini spatial neighborhood. Such a quencher proximity represses fluorescence. In contrast, in the presence of the target, the molecular beacon forms a complex with it, which spatially separates the fluorophore from the quencher (like commonly used 4-{[4′-(dimethylamino)phenyl]azo}benzoic acid (DABCYL)). Once the fluorophore and quencher are dissociated, the fluoresce increases, thus enabling quantitative measuring of signals with the threshold value evidencing presence of the target (Figure 1) (Broude 2002).

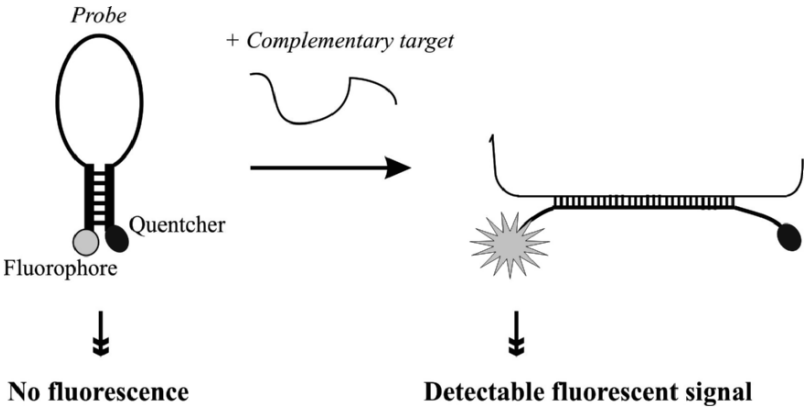

Figure 1. Principle of action of the first generation molecular beacons. The probe, which forms stem-loop structure due to intramolecularly base pairing of the inverted repeats on its termini, is labeled with fluorophore and quencher at the opposite ends. When a complementary target is added, a stronger complementary pairing between target and molecular beacon destroys the initial stem-loop structure and the fluorophore label may produce fluorescence due to a greater distance from quencher.

Alternatively, molecular probes utilizing fluorescence resonance energy transfer (FRET) can be adopted for using in molecular beacon format (Figure 2). Excitation energy is sometimes transferred from one fluorescent dye to another by resonance energy transfer. After excitation by the absorption of a photon, the donor or D dye can transfer its excited state energy to a second chromophore or dye (the acceptor or A) nonradiatively (i.e. without photon emission). The efficiency of this transfer depends on, among other things, the extent of overlap of the D emission and A absorption spectra, the relative orientation of the transition dipoles of D and A and – most importantly – the distance between D and A. FRET can measure distances between 20 and 100 Å (Stryer 1978), as well as detect conformational changes and determine their magnitudes (Johnson 2005). FRET is most widely used for protein interaction studies, but is also of a great usefulness for DNA hybridization probe design (Baker et al. 2006; Kim et al. 2006; Okamura and Watanabe 2006; Wahlroos et al. 2006). In the case of molecular beacons, one attaches donor and acceptor dyes to two different probe termini forming the stem of the pan handle-like structure. When both dyes are colocalized, resonance transfer may occur and the respective fluoresce peak (typical of the acceptor) will be observed; in contrast, when the SL probe conformation is

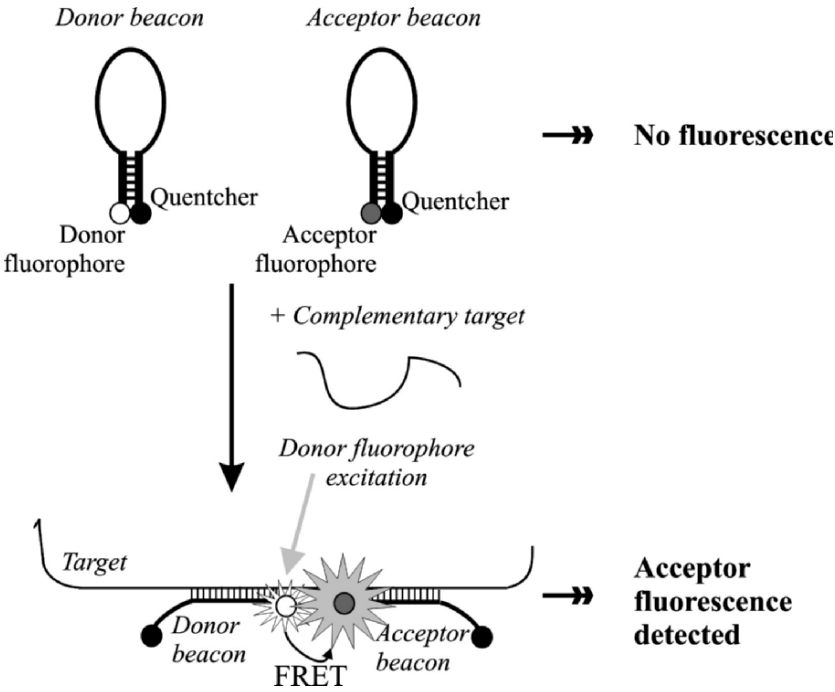

Figure 2. Example of the technique utilizing FRET-labeled molecular beacons.

altered, donor and acceptor fluorophores are too far to enable an efficient energy transfer, and a donor peak will be registered.

Molecular beacons have rapidly found many applications because of the relative simplicity of the assay and the option of real-time process monitoring (Fang et al. 2002). Because of their superiority in mismatch discrimination, DNA molecular beacons were used to detect single nucleotide polymorphisms (Kostrikis et al. 1998; Piatek et al. 1998; Marras et al. 1999), in quantitative PCR (Tyagi et al. 1998; Vet et al. 1999), as DNA microarray-immobilized probes (Liu and Tan 1999; Liu et al. 2000; Steemers et al. 2000), and as complementary probes for detecting RNA targets *in vivo* (Sokol et al. 1998; Broude 2002). Several improvements and further developments of molecular beacon technology have been reported. For example, several targets may be detected simultaneously in one assay by using differently colored molecular beacons (Tyagi et al. 1998; Vet et al. 1999). The method turned to be very sensitive in many applications, including pathogenic viral or bacterial strain detection (Gore et al. 2003; Henry et al. 2006; Patel et al. 2006).

In another approach, the method accuracy and sensitivity was substantially increased by using gold nanoparticles as a quencher due to the superior quenching ability of gold clusters (Dubertret et al. 2001). When fluorescently tagged oligonucleotides are located near metal surfaces, their emission intensity is impacted by both electromagnetic effects (i.e. quenching and/or enhancement of emission) and the structure of the nucleic acids (e.g. random coil, hairpin, or duplex) (Stoermer and Keating 2006). The use of metal nanoparticles in the molecular beacon strategies opens up bright perspectives for creating a variety of quenchers with very different properties, by changing the shape, size, or composition of the metal cluster. A variety of metal-based quenchers with different peculiarities may be used in the future for simultaneous detection of multiple different targets in a single tube (Dubertret et al. 2001). Alternatively, this problem of multiplexing target identifications may be solved by using wavelength-shifting molecular beacons (Tyagi et al. 2000). Nevertheless, highly sensitive multiplex molecular beacon assays using probes labeled with different fluorophores became practice in our days (Sinsimer et al. 2005; Balashov et al. 2006; Gubala and Proll 2006).

Whitcombe and colleagues combined both molecular beacon and a PCR primer in a single SL probe termed "scorpion" (Whitcombe et al. 1999). In this approach, the PCR primer is designed to have a 5′-extension that has all the attributes of a beacon: a loop region complementary to a target flanked by the self-complementary stems, and a fluorophore–quencher pair at the 5′- and 3′-ends of the extension, respectively (Figure 3) (Broude 2002). The SL extension is linked to the PCR primer via a linker, which stops DNA polymerase from replicating the SL. During PCR, when the primer is extended and the target is synthesized, the SL unfolds and the loop sequence hybridizes intramolecularly with its target, which increases fluorescence (Figure 3). Thus, the scorpion–primer approach uses a monomolecular mechanism of probe–target hybridization,

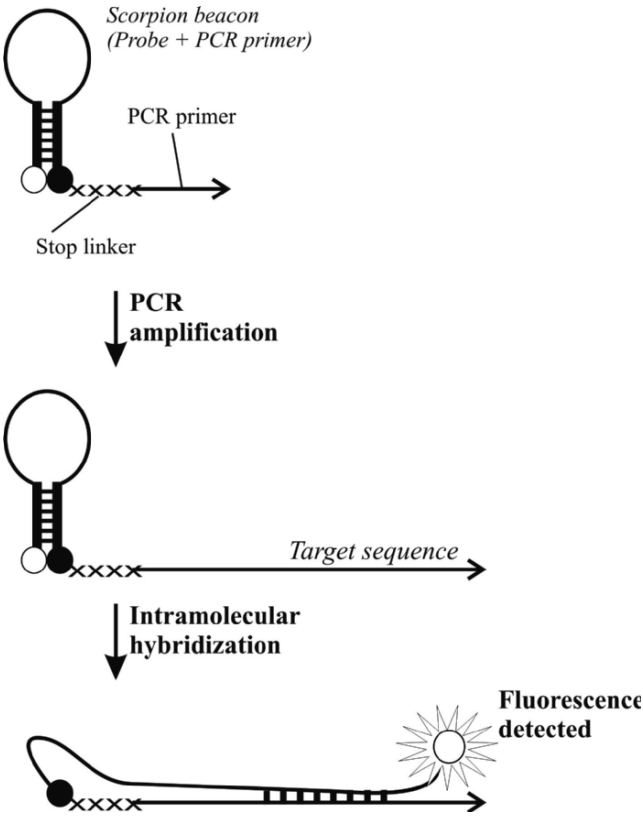

Figure 3. So-called "scorpion" molecular beacon probe. Scorpion probe has both molecular beacon and PCR primer modules. When the target sequence is amplified using scorpion primer, beacon unfolds and fluorophore–quencher pair is separated, thus allowing fluorescence.

compared with the bimolecular recognition in the traditional molecular beacon assay. This provides faster kinetics and greater stability of the probe–target complex (Thelwell et al. 2000; Broude 2002).

Another promising technique utilizes catalytic DNAs (DNAzymes) in combination with the molecular beacon approach. In this case, the PCR primer contains a sequence complementary to a DNAzyme (Todd et al. 2000). During PCR, an active amplicon is synthesized that cleaves the fluorescent beacon-shaped substrate included in the reaction mixture, increasing the fluorescence. This approach can be generalized so that one generic DNAzyme and one corresponding beacon substrate are used to detect different genomic targets (Broude 2002).

Catalytic molecular beacons represent a next generation of molecular probes with the potential to amplify signals and to detect nucleic acid targets without PCR amplification. In this case, a complex double-stranded DNA probe is engineered

that combines the features of a molecular beacon and a hammerhead-type DNAzyme with RNase activity; these are located on two opposite DNA strands (Stojanovic et al. 2001) (Figure 4). In the absence of a target, the DNAzyme module is blocked being hybridized with the beacon module. When the target is added, the beacon module changes its conformation and allows the DNAzyme to hydrolyze substrate, which is a linear oligonucleotide having fluorophore and quencher at the opposite ends. The DNAzyme cuts the substrate at a cleavable ribonucleotide, which releases fluorophore from quencher and results in increasing fluorescence. The digested substrate dissociates, and the DNAzyme module hybridizes with the beacon module again, and the cycle is repeated. Although the fluorescence response from the substrate turnover was several times smaller than the response caused by the opening of the conventional SL

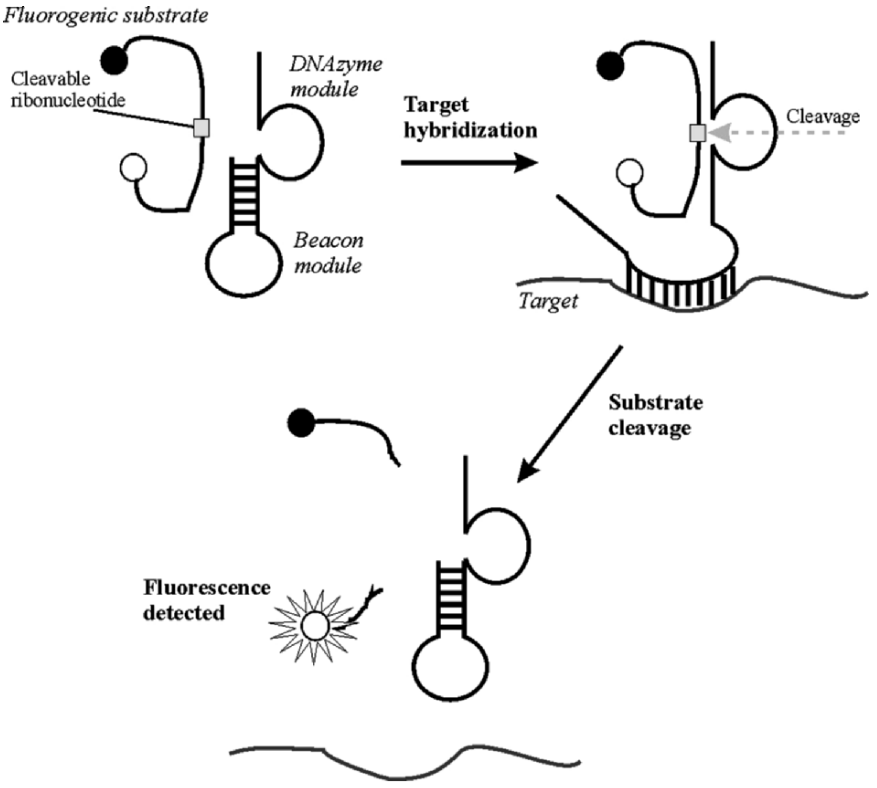

Figure 4. Molecular beacon acting as the DNAzyme. In the absence of a target, the DNAzyme module is blocked being hybridized with the beacon module. When the target is added, the beacon module changes its conformation and allows the DNAzyme to hydrolyze substrate, which is a linear oligonucleotide having fluorophore and quencher at the opposite ends. The DNAzyme cuts the substrate at a cleavable ribonucleotide, which releases fluorophore from quencher and results in increasing fluorescence.

molecular beacon (Stojanovic et al. 2001), this approach initiated catalytic events (Broude 2002). In contrast to the classical molecular beacon method based on binding, these methods utilize catalytic cleavage to release the fluorophore for detection and quantification, making it possible to take advantage of catalytic turnovers for signal amplification.

Unlike classical molecular beacons that detect only nucleic acids, catalytic molecular beacons can be applied to different DNAzymes to detect a broad range of analytes. The methods aimed at the metal ion recovery (e.g. bivalent lead (Chang et al. 2005)) are based on the finding that almost all known transcleaving DNAzymes share a similar structure comprised of a catalytic DNAzyme core flanked by two substrate recognition arms. Using a typical DNAzyme called the "8–17" DNAzyme as an example, the design of highly sensitive and selective Pb^{2+} sensors became practice. The initial design employs a single fluorophore–quencher pair in close proximity, with the fluorophore on the 5′-end of the substrate and the quencher on the 3′-end of the enzyme, with an additional quencher attached to the 3′-end of the substrate (required to improve the efficiency of quenching and to suppress background fluorescence) (Liu and Lu 2006). The dual quencher method allows the sensor to perform at ambient temperatures with a high signal-to-noise ratio.

If the structure of the catalytic beacon is optimized for faster substrate turnover, this approach might be a good alternative to amplification-based detection of nucleic acid targets, or even of inorganic ions. However, currently, this method is still in the development stage and is far from being applied routinely.

Interestingly, molecular beacon can be delivered directly into the living cells, by using standard transfection reagents, or, more efficiently, by attaching a cholesterol unit to a free terminus of the hairpin stem. Such a molecular probe that penetrates cellular membrane may be employed as an excellent biosensor for *in vivo* monitoring in the real time of gene expression at the RNA level and studying further transcript fates (Santangelo et al. 2006; Seo et al. 2006).

3. STEM-LOOP DNA PROBES ON MICROARRAYS

It is not a secret that the accuracy of conventional linear-probe microarrays at detecting mutations is often insufficient (Hacia 1999), and alternative approaches may be advantageous (reviewed in Chapter 9). SL DNA probes, with their enhanced fidelity in mismatch discrimination, have also found applications in DNA microarray technology to improve the array sensitivity by discriminating mispaired bases in a better way. Hairpin probes were attached to surfaces in topologically different manners, depending on the experimental design. For example, capturing probes with single-stranded overhangs were immobilized through the loop (Broude et al. 2001; Riccelli et al. 2001; Zhao et al. 2001), whereas the molecular beacons were tethered through the 5′- or 3′-end (Ortiz et al. 1998; Steemers et al. 2000), or through the linker attached to the stem (Figure 5) (Liu and Tan 1999; Broude 2002). The probes may be immobilized

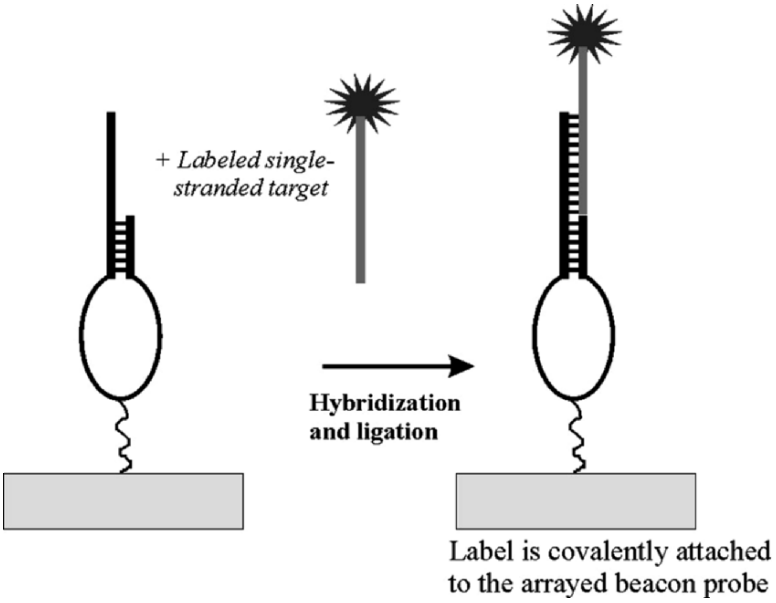

Figure 5. Hybridization–ligation approach for target detection using arrayed molecular beacons.

with either streptavidin–biotin binding (Ortiz et al. 1998; Steemers et al. 2000; Riccelli et al. 2001), or can they be covalently bound to the surfaces with different chemicals (Broude et al. 2001; Zhao et al. 2001; Wang et al. 2005).

Immobilized DNA or DNA–peptide nucleic acid (PNA) chimeric beacons preserve the ability to detect label-free complementary probes (e.g. cRNA, cDNA, or PCR amplicons) (Ortiz et al. 1998; Du et al. 2005). SL DNA probes with dangling overhangs can be used as capture devices, thus showing a twofold increase in hybridization rates and greater stability of the probe–target complex than the corresponding linear probes (Riccelli et al. 2001). An elegant technique combining hybridization and enzymatic ligation using SL DNA probes was recently developed for mutation detection in single-stranded DNA targets (Figure 5) (Broude et al. 2001). It should be noted here that the arrays with SL DNA probes are still at the early development stage.

4. CONCLUSIONS

It is clear now that SL DNA probes already have a wide range of applications in essentially all aspects of molecular biology and biomedicine. Two major factors that are responsible for such broad applications of the SL constructs are: (1) enhanced specificity of the probe–target recognition and (2) the possibility of one-tube formats and real-time reaction monitoring with molecular beacons.

There is no doubt that in the future, new applications will be developed with the sensitivity of future arrays being crucial.

Techniques that combine DNA SL probes with other molecular devices (e.g. ribozymes and DNAzymes, inorganic nanoparticles) and technological developments (e.g. microarrays, advanced optic techniques) promise to become sensitive and robust, high-throughput research and diagnostic methods. Finally, molecular beacon strategies, especially those coupled with aptamer-based bioassays, provide instrument for DNA–protein interaction studies. For example, FRET-labeled molecular-beacon aptamer may bind to a specific protein biomarker, platelet-derived growth factor (PDGF) (Vicens et al. 2005). This bioassay is compatible with pH, temperature, and monovalent cation levels typically encountered in biological samples, and phosphorothioate backbone-modified aptamer is able to exhibit specific FRET. With minimal sample processing and without optimization, the assay is able to detect as little as 10 ng PDGF per microgram of serum proteins from cell culture media (Vicens et al. 2005).

Overall, the success of SL approaches, as well as their universality, versatility, high reproducibility, and ubiquity make us believe in their bright perspectives of being widely useful for many future and current biomedical applications (~50 PubMed citations per year, 40 international patents to November 2006).

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