Binary Probes for Nucleic Acid Analysis

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Received September 27, 2009

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1. Introduction

Over the last four decades nucleic acid hybridization techniques have been widely used for the detection of specific nucleic acid sequences. In this approach a 15-nucleotide or longer DNA or RNA strand (the probe) forms complementary duplexes with the analyzed nucleic acid (analyte). Since the development of the first hybridization-based procedures by Hall and Spiegeleman[1] and by Bolton and McCarty,[2] oligo- and polynucleotide probes have become routinely used as laboratory tools for nucleic acid analysis. The examples of such techniques include Southern[3] and Northern[4] blots, fluorescent in situ hybridization,[5] and DNA microarrays.[6] Furthermore, the introduction of real-time detection approaches, such as molecular beacon (MB) probes,[7,8] has enabled fast assays, in which the fluorescence change is detected immediately after probe hybridization, thus avoiding the need to separate the probe−analyte hybrid from the excess of the unbound probe. However, low selectivity of the probe−analyte hybridization creates complications in the analysis of single-base differences between two polynucleotides. Indeed, a 15-nucleotide-long probe has a similar affinity to a fully complementary analyte and to an analyte containing a single noncomplementary base.[9]

One general approach for highly selective biopolymer recognition uses two ligands which independently or semi-independently (in the case of cooperative binding) interrogate a protein[10−13] or a nucleic acid of interest (the binary approach). In the case of nucleic acid analysis the general idea of a binary (split or two-component) probe (BP) is as shown in Figure 1. Two oligonucleotides (analyte binding arms in Figure 1) are complementary to the adjoining positions of a nucleic acid analyte. In addition, both parts of the probe bear covalently bound functional groups or oligonucleotide fragments (Figure 1, cyan and yellow semicircles). A detectable signal (green circle) is produced only when the two parts of the probe hybridize to the analyte. Examples of such signals include but are not limited by an increase in fluorescence or luminescence at specific wavelengths and chemical or enzymatic reactions that change the optical properties of the sample. In a majority of BPs the analyte binding arms are DNA, RNA, or peptide nucleic acid (PNA) fragments. The technique that uses a protein fragment

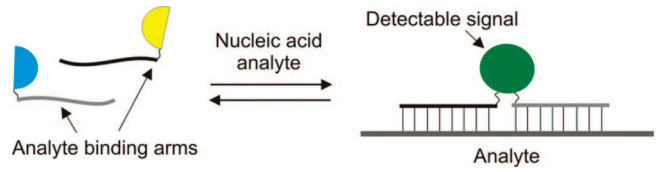


Figure 1. General scheme of a binary probe for nucleic acid analysis. Two parts of the probe produce a detectable signal when hybridized to the abutting fragments of the analyzed nucleic acid.

10.1021/cr900323b
Published on Web 06/28/2010

as the analyte binding arms for reassembly of green fluorescent protein[14−16] is not included in this review. Although promising for intracellular RNA monitoring, this technique lacks the advantage of design simplicity offered by the approaches based on Watson−Crick base-pair formation.

The first BP, which used Förster resonance energy transfer (FRET), was suggested in 1988.[17] Since then, FRET probes have become a routine detection tool in real-time LightCycler PCR technology.[18,19] Along with this, interest in the BP approach has greatly increased in the past decade due to the growing demand for efficient techniques for genome analysis. The aim of this review is to summarize advances in the development of BPs and to point out their improved selectivity in comparison with other hybridization-based techniques. The thermodynamic factors that underlie this advantage are discussed in the first part of the review.

Excellent BP selectivity is relevant to their applications in single-nucleotide polymorphisms (SNP) genotyping, especially in multiplex formats. At present, DNA microarrays is one of the most promising platforms for SNP analysis.[6,20−22] The major advantage of microarrays is high throughput that allows determination of hundreds of thousands of genetic markers in a single experiment. High throughput has eliminated the need for researchers to optimize the conditions for analysis of every polymorphism and dramatically reduced the assay cost.[22] The binary approach has great potential to add to microarray technology by increasing the accuracy of SNP determination. This review discusses BPs in terms of their compatibility with the conventional microarray formats.

Another major advantage of BPs is the possibility to generate signal immediately after probe hybridization (real-time detection). This advantage has applications not only in real-time PCR but also in the monitoring of RNAs in fixed or live cells. In particular, a cluster of works that uses template-assisted chemical reactions has extensively addressed the issue of intracellular RNA imaging. This review does not cover nucleic acid-templated reactions that require gel electrophoresis, mass spectrometry, or chromatography for signal registration. The reader is directed to reviews on these topics.[23−26] The limit of detection (LOD) is an important characteristic in the case of PCR-free detection formats, such as the aforementioned intracellular RNA monitoring. Therefore, when data from the original publications are available, the LOD of the probes is stated.

2. Binary Probes and Hybridization Selectivity

A number of applications for hybridization techniques, such as gene expression studies[27] or detection of polymorphic viral sequences,[28] do not require highly specific binding of the probe to target. In such applications, a wide range of hybridization conditions can be employed for a long oligonucleotide probe to bind tightly to the correct target or a target with a single-base mismatch while rejecting multiple-mismatched targets. SNP genotyping constitutes a significant exception from this set of applications. SNPs represent the most abundant class of variations in the human genome accounting for 80−90% of the differences between the genomes of two individuals.[29−31] SNP analysis has important applications in population-based genetic risk assessment,[32] molecular diagnostics,[33,34] pharmaceutical drug development,[35] linkage analysis,[36,37] and identity testing in forensic applications.[38−40] Allele specific hybridization is used as an SNP typing technique in a variety of platforms including DNA microarrays[21] and real-time PCR,[41,42] among others.[43−47] Hybridization techniques make use of the base paring of thymine with adenine and guanine with cytosine. Simplicity of the probe/assay design is an important advantage of this method. The complexity of alternative SNP genotyping technologies, such as pyrosequencing,[48] invasive cleavage,[49,50] primer extension,[51] or allele-specific ligation,[52] is increased by the use of extra protein-mediated steps for accurate SNP discrimination. Unfortunately, the low selectivity of the probe−analyte hybridization constitutes a challenge for conventional hybridization approaches in SNP analysis. The length of the probe−analyte hybrid is an important parameter that predetermines the hybridization selectivity.[9]

The formation of at least a 15-nucleotide-long probe−analyte hybrid is required to uniquely define a specific fragment in a nucleic acid within a genome. Hybrids of such length are too stable to be sensitive to a base mispairing since a single mismatched unit results in an energetic penalty equivalent to only a small fraction of the total energy gained upon duplex formation[53−55] (Figure 2A, right panel). Suitable conditions for SNP typing require the energy of the probe−analyte dissociated state (DS) to be brought between the energies of the associated states (AS) for matched and mismatched duplexes (Figure 2A right, dashed blue line). Under such conditions the fully matched duplex is formed, while mismatched hybrid is dissociated. In order to achieve such conditions, conventional techniques use buffers with low ionic strength, denaturing agents (formamide),[56] or elevated temperatures (usually 50−65 °C) to destabilize the probe−analyte AS. However, these conditions do not always ensure high specificity, especially if a mismatch is located at the ultimate or penultimate position of the probe−analyte hybrid.[57] Moreover, the optimal hybridization conditions are sequence dependent. Therefore, a specific buffer and temperature can ensure excellent discrimination for one SNP but often fail in discriminating others. In practice, optimization of the multiplex hybridization reaction for DNA microarrays requires substantial capital and time investment.[45,57,58]

Alternatively, to reach an optimal level for SNP typing, the energy of DS can be reduced by allowing the probe to form a competing secondary structure in DS. This approach, introduced initially by Roberts and Crothers as stringency clamping,[59] is accomplished with conformationally constrained probes. Examples of such structured probes include hairpin-shaped oligonucleotides,[7,8,59] partial duplexes,[60−62] and a triple-stem probe.[63,64] The molecular beacon (MB) probe is a well-studied representative of the conformationally constrained probes.[7,8] MBs are oligonucleotide hairpins with a fluorophore and a quencher conjugated to opposite ends of an oligomer (Figure 2B, left). Binding to complementary nucleic acids causes MBs to switch to their elongated conformation, thereby increasing their fluorescence. The reduction of the free energy of DS is achieved due to Watson−Crick base pairing in the stem part of the hairpin. MB probes distinguish mismatches over a wider temperature range than unstructured probes do.[65] However, thermal destabilization of the hybrid is still required for accurate SNP typing.[66,67] MB probes with longer stems have improved mismatch discrimination ability since the energy of the DS is inversely related to the number of base pairs in the stem. However, long stems greatly decrease the probe hybridization rates due to the larger activation barrier.[67]

What mechanisms are behind the high selectivity of BPs? The stabilization of AS can be achieved by both enthalpy

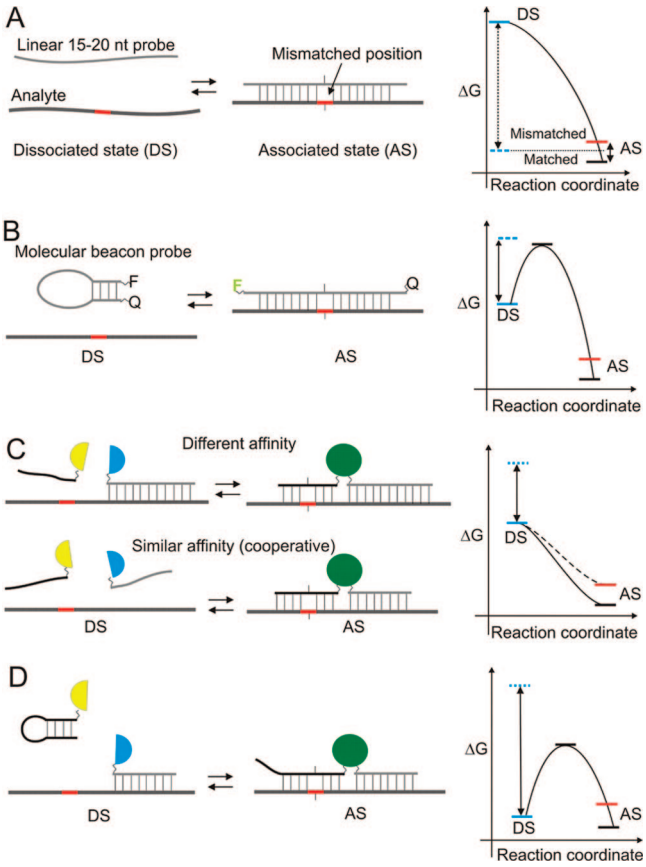


Figure 2. Schemes and energy diagrams for hybridization of 15−25 nucleotide-long probes to a nucleic acid analyte at ambient temperatures in near physiological buffers. "DS" and "AS" represent probe−analyte dissociated and associated states, respectively. (A) Hybridization of a linear oligonucleotide probe. The difference in the energy between matched and mismatched duplexes is much smaller than the energy gap between DS and AS. The energy of DS should be brought between the energy of matched and mismatched duplex to allow SNP discrimination. (B) Hybridization of the molecular beacon probe. The energy of DS is reduced due to the formation of a 4−6-nucleotide-long stem. The energy curve maximum corresponds to the DS when the hairpin structure is not formed. "F" and "Q" indicate a fluorophore and a quencher dye, respectively. (C) Hybridization of binary probes. In different affinity mode, an oligonucleotide with a longer analyte binding arm remains in the complex with the analyte even in DS. In similar affinity (cooperative) mode, the probe−analyte hybrid dissociates into three fragments. The free energy of DS is reduced for both hybridization modes. (D) Deferent affinity mode for conformationally constrained BPs: an analyte binding arm forms a hairpin structure in DS. The DS energy is reduced to the value between the energies of fully matched and mismatched duplexes. All energy graphs represent speculative predictions rather than experimental data.

reduction and entropy gain. In general, the two parts of BPs can hybridize to the analyte with either different or approximately equal affinities (Figure 2C). The later scenario includes probes that hybridize cooperatively. If the affinities are different, one part of the probe remains in complex with the analyte even when the second part is dissociated (Figure 2C, top scheme). This probe−analyte residual hybrid stabilizes the DS. However, in "different affinity" mode this stabilization effect is absent, since the second part of the probe dissociates from the complex (Figure 2C, bottom scheme). In this case, the decrease in the free energy of the DS is achieved due to the entropy increase, as the probe−analyte complex dissociates into three rather than two molecules. This interpretation explains the high mismatch discrimination power of BPs reported in the majority of the original publications reviewed below. The impressive selectivity can be illustrated by an enormous discrimination factor of 3450.[68] In another example, a 5 nM of true analyte was reliably detected even in the presence of a 100 times excess amount of a single-base mismatched oligonucleotide.[69]

BP design allows for the introduction of conformational constraints in the form of stem loops in one (Figure 2D) or both analyte binding arms.[69,70] Therefore, the binary strategy, when combined with the stringency clamping approach, offers almost unlimited potential for hybridization selectivity. The energy of DS can be fine tuned to the ideal dislocation (between the energies of the AS for the matched and mismatched duplexes) for widely variable experimental conditions (Figure 2D, right). Therefore, accurate mismatch discrimination can be achieved by optimization of the probe design rather than by particular hybridization conditions. This property can be applied in multiplex SNP genotyping assays. The spectrum of BPs available to date is reviewed below. Most of the probes, when properly designed, are capable of recognizing SNPs with great selectivity. Therefore, such probe characteristics as limit of detection (LOD), cost, and response time come to the foreground.

3. Binary Probes That Use Resonance Energy Transfer

Early examples of BP used Förster resonance energy transfer (FRET) (Figure 3).[17,71] In FRET the probability of energy transfer depends on the donor to acceptor separation distance with an inverse sixth power law. In binary FRET probes, one part of the probe is conjugated with a donor while another contains an acceptor fluorophore (Figure 3, left). The commonly used concentrations of fluorescent conjugates ($\leq 10^{-6}$ M) are low enough to prevent energy transfer from the randomly diffused donor and acceptor. When hybridized to the target, the oligonucleotides bring the two dyes in close proximity, thus enabling FRET. Upon irradiation of the sample at the donor excitation wavelength the energy transfer can be detected as a decrease in the fluorescence emission of the donor and an enhancement in the emission intensity of the acceptor fluorophore.[17] The ratio of the last to the first emission intensities gives a relative FRET measure, where higher ratios indicate higher energy transfer efficiency.

This strategy was implemented for a quantitative real-time PCR with LightCycler technology (which is referred to also as dual FRET probes).[18,19] A 1−5 base separation between donor and acceptor is recommended to optimize the energy transfer efficiencies.[72] Typical acceptor fluorophores include the cyanine dyes (Cy3 and Cy5), 6-carboxy-4,7,2′,7′-tetrachlorofluorescein (TET), 6-carboxy-*N*,*N*,*N*′,*N*-tetramethylrhodamine (TAMRA), and 6-carboxyrhodamine (ROX). The donor fluorophore is usually 6-carboxyfluorescein.[18] Probes

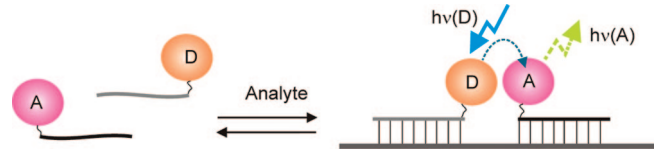
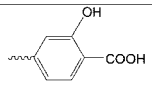
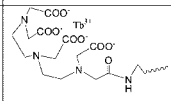
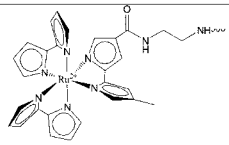
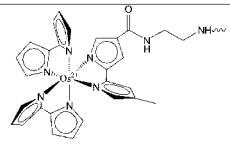
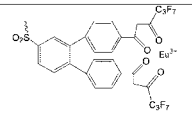
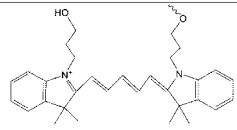
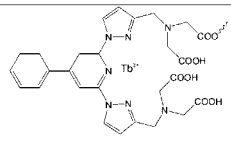
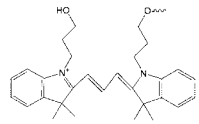


Figure 3. Resonance energy transfer as a means for the detection of BP hybridization. When fluorescent donor (D) is excited at a specific wavelength, it transfers the energy to the acceptor (A) within the tripartite DNA complex (right). The acceptor emission is detected.

Table 1. Donor−Acceptor Pairs for Long-Living RET Binary Probes

Row number	Energy donor	Energy acceptor	Ref.
1	 Salicylate group	 Terbium Diethylenetriaminepentaacetate (DTPA)	76
2	 Ruthenium-tris (2,2'-bypyridyl)	 Osmium-tris (2,2'-bypyridyl)	77
3	 4,4'-Bis (1'',1'',1'',2'',2'',3'',3''-heptafluoro-4'',6''-hexanedion-6''-yl)-sulfo-o-terphenyl (BHHCT)-Eu^{3+}	 2 -{5-[1,3-Dihydro-1-(2-hydroxypropyl)-3,3-dimethyl-2H-indol-2-ylidene]-1,3-pentadien-1-yl}-1-(2-hydroxypropyl)-3,3-dimethyl (Cy5)	79
4	 N,N,N^1,N^1-[2,6-bis(3'-aminomethyl-1'-pyrazolyl)-4-phenylpyridine]tetrakis(acetic acid), (BPTA)-Tb^{3+}	 2-{3-[1,3-Dihydro-1-(3-hydroxypropyl)-3,3-dimethyl-2H-indol-2-ylidene]-1-propen-1-yl}-1-(3-hydroxypropyl)-3,3-dimethyl (Cy3)	80

of this type were shown to be applicable for in vitro transcription monitoring[73] and for direct observation of specific overexpressed mRNA in living cells.[74]

In practice, however, there are several factors that significantly decrease the sensitivity and reliability of the FRET approach. For instance, it is difficult to avoid overlap between the emission spectra of the donor and acceptor fluorophores, which creates high background noise.[75] Moreover, it is not uncommon to observe some fluorescence of the acceptor fluorophore, even in the absence of the target. The acceptor fluorescence is caused by the direct excitation of the acceptor at the donor excitation wavelength.[17] In addition, detection of nucleic acids in living cells is often obscured by a high autofluorescence background.

Long-lived luminescent dyes in combination with time-resolved measurements can help solve the aforementioned problems. Examples of donor/acceptor pairs used for these purposes are summarized in Table 1. Originally Oser and Volet detected emission of a long-living (lifetime 1.58 ms) Tb^{3+} complex (Table 1, first row) at >435 nm excited by the energy transfer from the salicylate group using 400 μs measurement delay.[76] A signal-to-background ratio (S/B) of ~2 was achieved in the presence of ~50 nM DNA analyte. Two other BPs took advantage of the long leaving fluorescence of the Ru^{2+} bipyridyl complex which transferred energy to bipyridyl Os^{2+} chelate (Table 1, second row)[77] or to Cy5.[78] Sueda et al. used an Eu^{3+} complex as the energy donor, which sensitized the emission of the acceptor dye, Cy5 (Table 1, third row).[79] The same authors elaborated on the Tb^{3+} chelate/Cy3 energy transfer pair (Table 1, fourth row).[80] Interestingly, in order to attach the lanthanides to the oligonucleotide probe, the lanthanide complexes were initially coupled with streptavidin followed by conjugation to the biotin-containing oligonucleotide. This allowed for localization of many donor groups near a single acceptor molecule in the probe−target complex. The reported detection limits for these lanthanide-based assays of 0.2[79] and 0.05−0.03 nM[80] are among the lowest for BPs. This impressive LOD was attributed to the low background due to the very sharp luminescent emission profile of lanthanide chelates and a large (>200 nm) Stokes shift.[79]

An alternative approach to reduce the background fluorescence takes advantage of a series of consecutive resonance energy transfer events. Marti et al. described a three-dye FRET BP in which one oligonucleotide was conjugated with a fluorescein (FAM) and a TAMRA moiety while a second oligonucleotide was linked with Cy5.[81] In the probe−analyte tripartite complex, the excited fluorescein transferred energy to TAMRA, which subsequently transferred energy to Cy5. This design enabled minimization of the excitation/readout wavelength overlap: the irradiation at 488 nm was used for excitation of the FAM group, while the ratio of emission values at 669 nm (Cy5) to 583 nm (TAMRA) was used as a measure of FRET (signal). Despite sophisticated design the probe detected only ~5 nM with a signal ~2 times above the background.

Another approach to elevate the problem of high background fluorescence employed intramolecular quenching of either the donor[82] or both the donor and acceptor[83] fluorophores. For example, Santangelo et al. used a pair of MB probes, one with a donor fluorophore and another with an acceptor. The MB probes hybridized to the adjacent regions on the same mRNA target, resulting in FRET.[83] The target detection based on the energy transfer significantly reduced the amount of false positives, thus enabling target mRNA imaging in cells. It was estimated that this approach can reliably detect a few hundred copies of endogenous mRNA per cell. This amount roughly corresponds to a LOD of ~1 nM, based on the assumption that the diameter of a cell is 10 μM. This detection limit matches that of the conventional MB probes.

The sensitivity of resonance energy transfer probes can be increased by covalent cross-linking of the two parts of the probe to each other.[84] Theoretically, the detection limit of this approach can be improved by signal amplification, since one template molecule can catalyze the formation of several RET products. Forty turnovers at 32 °C over a period of 24 h were observed in the presence of 1 nM DNA analyte.[84] When employing functional groups that enable faster cross-linking, it is possible to shorten the incubation time and achieve lower detection limits. However, an intrinsic problem of this approach is product inhibition: longer ligation product has greater affinity to the analyte than each of the two shorter oligonucleotide reactants[26] (see discussion in sections 6.1 and 6.2).

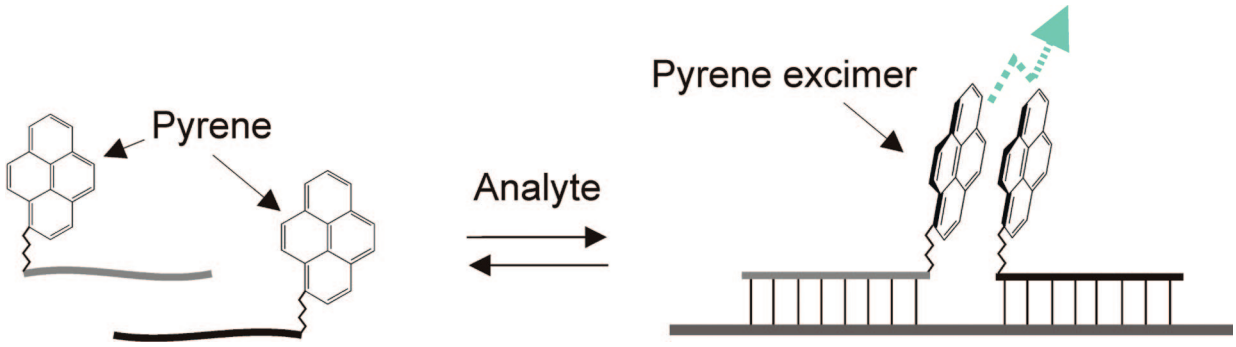


Figure 4. Analyte-dependent excimer formation by two pyrene-conjugated oligonucleotides.[86,87]

4. Excimer and Exciplex Forming Probes

An excimer is a short-living dimeric molecule formed by two molecules of the same species, where at least one is in an electron excited state.[85] Excimer components have attractive interactions in the excited state and repulsive interactions in the ground state. The wavelength of an excimer's emission is longer than that of the excited monomer's emission. The characteristically large Stokes shift can be easily distinguished from the monomer fluorescence, thereby allowing for real-time hybridization assays.

Ebata et al. conjugated two oligonucleotide strands with pyrene residues at the 3′- or 5′-terminal position (Figure 4).[86,87] Upon hybridization of the probe to the analyte, the two pyrene moieties came into close proximity and formed an excimer having an emission band with a maximum at 495 nm. The band was easily distinguished from the emission spectra of monomeric pyrene with an emission below 450 nm. In this method, a 16S rRNA at a concentration of 10 nM was detected. The authors stated that the sensitivity of the pyrene excimer assay was about 1 order of magnitude lower than that of fluorescein-based fluorophores.[87] Indeed, postsynthetic covalent conjugation of pyrene to the oligonucleotide was shown to reduce emission intensity more than 10-fold due to quenching by nucleotide bases.[88] In order to reduce this quenching effect, a significant amount (up to 40%) of DMF was added to the hybridization buffer. The DMF amount of this quantity is incompatible with such applications as real-time PCR or nucleic acid monitoring in live cells.

Paris et al. performed structural optimization of the probe, which helped to solve the quenching problem.[89] First, pyrene-containing nucleotides were incorporated into the 5′ or 3′ end of the BP strands during chemical synthesis of the oligonucleotides. This enabled more rigid organization of the eximer forming groups and, presumably, increased the amount of eximer. In addition, the probe−analyte hybridization positions were varied.[89] The highest excimer emission was observed for the probe that hybridized to the target with partial overlap. The monomer emission band decreased by a factor of 7, while the excimer band increased up to 40-fold. This allowed for detection of the specific target at concentrations below 4 nM. Importantly, there was no need to add DMF or other organic solvents to the reaction buffer.

A remarkable property of the pyrene excimer is its relatively long fluorescence lifetime (30−60 ns in aqueous solutions). Therefore, time-resolved fluorescence spectroscopy, where the signal registration occurs in ∼50 ns upon excitation, can be used to distinguish the pyrene excimer fluorescence from the short-lived fluorescence of biological samples. The usefulness of this property was demonstrated by the detection of specific mRNA externally added to cellular extract.[90] The transcript of sensorin mRNA together with the pyrene excimer forming BP was added to the neuron cell extract. The pyrene excimer emission of the BP was detected in the interval of 30−150 ns after excitation. A signal to background (S/B) of ∼10 was determined at an analyte concentration of 100 nM. This experiment demonstrates that the probe can be used in the absence of organic solvents in a cellular environment and that the excimer fluorescence can be discriminated from the short-living background fluorescence of biological samples. However, the applicability of this approach for the monitoring of endogenous RNAs at their physiological concentrations remains in question.

Exciplexes differ from excimers in the structure of their dimer-forming parts: while excimers are formed from the same molecules, an exciplex fluorescent pair is composed of two molecules from different species. Bichenkova et al. designed a probe that took advantage of the exciplex formation of pyrene and *N*,*N*′-dialkylnaphthalene moieties.[91] The resulting exciplex emitted at ∼480 nm with a Stokes shift similar to that of the pyrene excimer. The exciplex formation depended on the presence of roughly 80% trifluoroethanol. The structure of linkers connecting the exciplex forming partners to the oligonucleotide strands of the probe was shown to be an important factor since it considerably affects the ability of the donor/acceptor interactions. The detection limit of this method was reported to be 400 nM,[92] which is more than an order of magnitude greater than that of excimer forming probes. A possible advantage of exciplex-based probes over probes that employ excimer formation is the possibility of multiplex target detection in one solution. In this hypothetical design, two exciplex forming BPs that contain different fluorophore combinations should emit light at different wavelengths. Overall, the applications of exciplex BPs for nucleic acid analysis might be complicated by the rigorous structural demands for exciplex formation and the requirement of organic solvents for their emission.[93]

5. Analyte-Assisted Complex Formation

Kitamura et al. described formation of luminescent lanthanide complexes upon hybridization of BP to the target (Figure 5).[94−96] The EDTA- and 1,10-phenantroline-conjugated oligonucleotides accommodated lanthanides and provided significant emission only in the presence of the cognate targets. Interestingly, a simple variation of a metal ion in solution (Tb^{3+} or Eu^{3+}) resulted in a different analyte-dependent color change.[95,96] This property can be used to

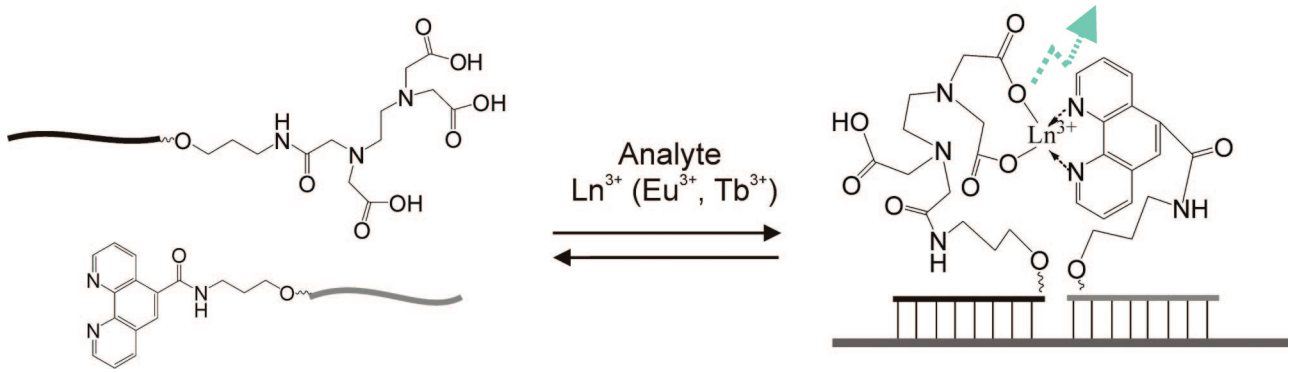


Figure 5. Analyte-dependent luminescent complex formation described by Kitamura et al.[94−96]

discriminate between two[95] or even three[96] alleles during SNP typing. The long-lived lanthanide luminescence enabled reduction of the background, which resulted from the nanosecond-range fluorescence of biological samples. The detection limit of this system was estimated to be ~1 nM.[96]

A sophisticated approach to distinguish guanine from other three nucleotides in a DNA analyte was recently suggested by Ihara et al.[97] A BP strand conjugated with β-cyclodextrin hybridized to the analyte one nucleotide apart from the second unmodified BP strand. Thereby, a gap was formed opposite to the analyzed nucleotide. After addition of a guanine-specific ligand to the probe−analyte tertiary duplex the ligand bound to the complex only if a guanine residue was located in the gap. Subsequently, the signaling moiety of the ligand came in close proximity to the nearby β-cyclodextrin group, which formed a luminous inclusion complex that fluoresced at 443 nm. The relative poor sensitivity of the probe (LOD $\approx$ 400 nM) can be attributed to the low affinity ($K_{as} \approx 2.4 \times 10^5$ M^{-1}) of the G-specific ligand to the oligonucleotide tripartite complex. Besides demonstrating interesting chemistry, this approach does not offer any obvious advantage in SNP analysis when compared with other BPs.

6. Analyte-Catalyzed Chemical Reactions

Approaches described in this section use proximity and orientation effects to catalyze a chemical reaction between the two analyte-bound oligonucleotide conjugates. Hybridization of two reactant-carrying oligonucleotides with the complementary template has been extensively explored to catalyze a broad variety of chemical reactions.[98,99,23−26] Below, only the reactions that result in a change of the optical properties are reviewed. Most of the approaches described in this and the following sections were designed for intracellular RNA monitoring. This application requires biocompatibility for reaction, which should occur under physiological conditions (temperature, pH, aqueous buffers, etc.). In addition, the prereactive groups should be chemically inert toward biological nucleophiles. Peptide nucleic acids (PNAs) were chosen in several studies for the design of analyte binding arms due to their improved resistance to degradation in a cellular environment.[99]

6.1. Chemical Ligation-Based Approaches

Sando and Kool demonstrated that chemical ligation of two template-bound oligonucleotides can be coupled with fluorescent change.[101] In quenched autoligation (QUAL) probes, an S_N2 reaction was used to detach a quencher from a fluorophore-labeled oligonucleotide. The QUAL probe contained a phosphorothioate group at the 3′-end of the one oligonucleotide (Figure 6, left), while the second oligonucleotide was conjugated with both a fluorophore and a quencher dye (F and dabcyl in Figure 6). When the two oligonucleotides bound adjacently, the sulfur atom of the 3′ phosphorothioate group attacked the dabcyl-linked 5′ carbon. The resulting ligation product contained an unquenched fluorophore (Figure 6, right). QUAL probes can be designed for the multicolor identification of several analytes in one solution by employing different fluorophore residues.[102,103] The most developed application of QUAL probes includes in situ rRNA imaging in live cells.[104]

The reaction that causes quencher detachment occurs due to the instability of the C−O bond in the QUAL probe. This makes the bond susceptible to nucleophilic attack, which brings up the stability issue: dabcyl can be easily detached by reaction with other available nucleophiles, such as water or biological molecules, thus resulting in false-positive signals. Moreover, incomplete fluorophore quenching by dabcyl can cause high background. In an attempt to overcome the high background problem, quencher detachment was combined with FRET.[105] In this approach, the phosphothioate-modified oligonucleotide was conjugated with a Cy5 FRET acceptor while the dabcyl-linked probe had a fluorescein residue as a FRET donor. The FRET signal was produced as a result of chemical ligation. Since the Cy5 emission is detected, neither incomplete FAM quenching nor hydrolysis of the C−O bond in solution adds to the fluorescent signal. This method enabled detection of highly abundant mRNAs in cells by fluorocytometry.[105] Further improvement in fluorescence enhancement upon analyte binding is required for detection of moderate to low copy number RNAs. Thus, the sensitivity of this approach is a limiting factor. Moreover, 3−6 h were required for the reaction to generate a high signal, which is not suitable for rapid time scale applications. Stronger nucleophiles, such as phosphorodithioate and phosphorotrithioate, were shown to facilitate the reaction 2−4 times.[106] The detection limit for QUAL probe remains to be determined.

An alternative approach to the ligation of two parts of BP has been suggested by Huang and Coull (Figure 6B).[107] This approach entails ligation of analyte-bound parts of the probe via an aldol-like condensation which resulted in formation of the fluorescent cyanine dye. The potential advantage of this method is reduced background due to low fluorescence of the reactants. However, the aldehyde group may display undesired high reactivity toward biomolecules in cellular environment.

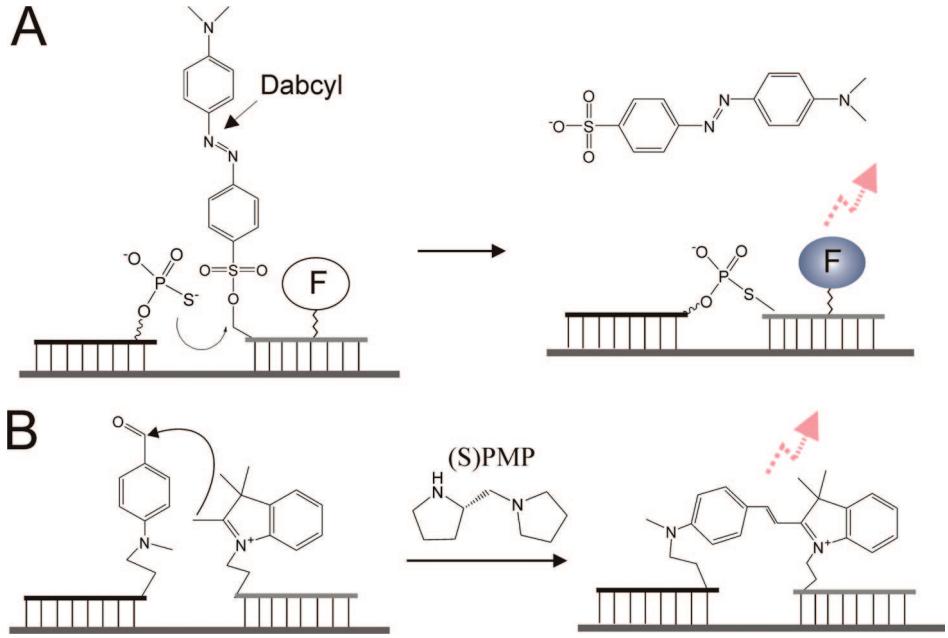


Figure 6. BPs that use chemical cross-linking. (A) Quencher autoligation (QUAL) probe.[101−104] Fluorescence of the fluorophore group can be detected after the detachment of the quencher (dabcyl). The reaction proceeds faster when both reacting oligonucleotides are complementary to the template. Fluorophore (F) can be fluorescein, tetramethylrhodamine (TAMRA), Alexa 350, or Cy5. (B) Hemicyanine dye formation as suggested by Huang and Coull.[107] Oligonucleotide conjugated with 4-formylphenyl undergoes aldol-like condensation with 2,3,3-trimethyl-3*H*-indol-conjugated oligomer to yield hemicyanine fluorescent dye. The reaction catalyzed by (9*S*)-pyrrolidine methylpyrrolidine ((*S*)-PMP) is promoted by hybridization of two reactants carrying oligonucleotides with DNA analyte.

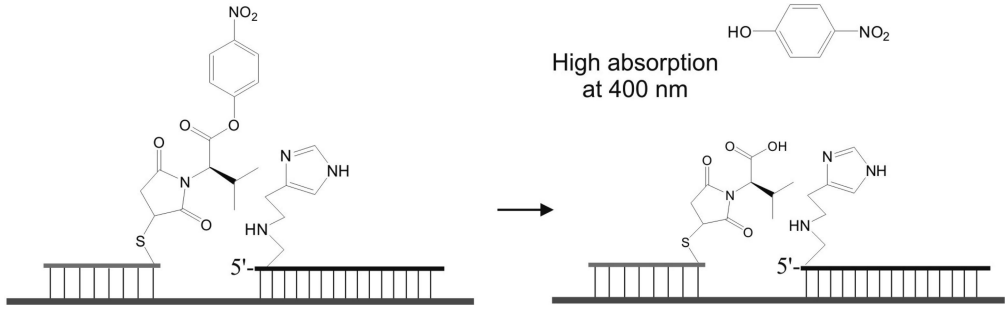


Figure 7. Template-assisted fluorophore activation suggested by Ma and Taylor.[108] The imidazole group catalyzes hydrolysis of the D-valine-*p*-nitrophenol ester, thus releasing colored *p*-nitrophenol (maximal absorbance at 400 nm). To promote the reaction, histamine was conjugated with a 15-nucleotide-long analyte binding arm while *p*-nitrophenyl residue was attached to the opposite terminus of the 8-mer analyte binding arm.

6.2. Ligation-Free Signaling

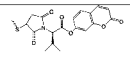
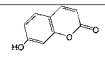
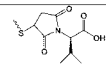
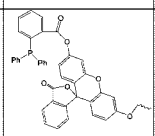
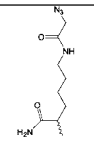
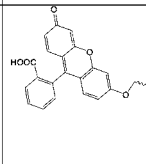
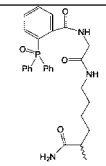
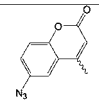
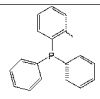
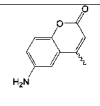
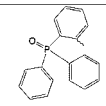
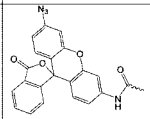
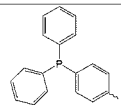
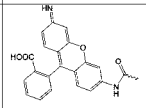
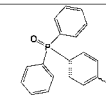
Chemical ligation-based probes produce long oligonucleotide products which tightly bind the analyte and inhibit rapid turnover of the templated reaction.[26] Avoiding chemical cross-linking of the two parts of the BP elevates product release and improves sensitivity.

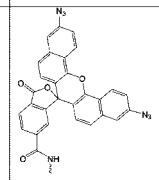
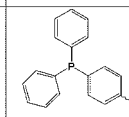
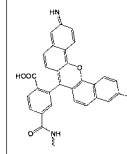
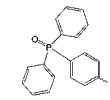
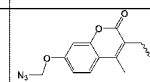
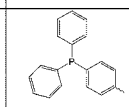
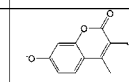
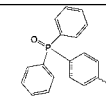
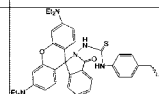
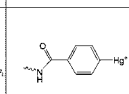
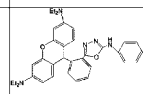
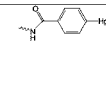
6.2.1. Template-Assisted Profluorophore Activation

Ma and Taylor pioneered a cross-linking-free templated reaction accompanied by the optical change by employing hydrolysis of a *p*-nitrophenyl ester-conjugated probe (Figure 7).[108] Catalysis was provided by the imidazole group of the histamine attached to the second strand of the probe. The imidazole-catalyzed hydrolysis produced *p*-nitrophenol, which was conveniently detected by measuring light absorbance at 400 nm. Importantly, this study demonstrated that the complex of analyte with a histamine-conjugated oligonucleotide acted as a true catalyst. This allowed for the release of *p*-nitrophenol with a k_{cat} of 0.018 min^{-1} and a K_m of 22 μM. However, the release of *p*-nitrophenol is not suitable for diagnostic purposes because of the low sensitivity of absorbance spectroscopy. In an attempt to increase the sensitivity of the assay, Taylor and colleagues suggested imidazole catalyzed release of 7-hydroxycoumarin (Table 2, first row),[109−111] which enabled fluorescent monitoring of the template-assisted reaction at 452 nm (λ_{ex} = 350 nm). Unfortunately, this reaction was slower than that for *p*-nitrophenol release, having a k_{cat} of $\sim 3.7 \times 10^{-3}$ min^{-1} and a $K_m \approx 1$ μM.[111]

A number of alternative reactions have been suggested for template-assisted generation of fluorescent species (summarized in Table 2). An interesting variant was suggested by Cai et al., who employed a Staudinger reduction for activation of a fluorescein precursor (Table 2, second row).[112] One part of the BP was conjugated with a fluorescein acylated by a 2-carboxytriphenylphosphine residue. Fluorescein acylation resulted in a dramatic decrease of its fluorescence. The second part of the BP contained a covalently attached aliphatic azido group. The azide and triphenylphosphine (TPP) moieties underwent a Bertozzi variant of the Staudinger reduction,[113−115] which produced

Table 2. Reactive Groups Attached to BPs That Use Analyte-Assisted Dye Activation and Their Products Formed upon Analyte Detection

	Reactants/catalyst Attached to the BP		Products		Ref.
	Low fluorescent		Fluorescent product	Side product	
1	 N-maleoyl-D-valine coumarin ester	 Histidine amide (catalyst)	 7-Hydroxycoumarin (released in solution)	 N-maleoyl-D-valine	109 - 111
2	 *O*-carboxymethyllfluorescein ester of 2-carboxytriphenylphosphine	 Amide of ε-N-(Azido acetyl) lysine residue	 *O*-carboxymethyl fluorescein group	 Tripenylphosphine oxide	112
3	 7-Azidocoumarin residue	 2- Triphenylphosphine residue	 7-Aminocoumarin residue	 2-Triphenylphosphine oxide residue	116
4	 N-acylazido rhodamine residue	 4-Triphenylphosphine residue	 N-acyl rhodamine residue	 4-Triphenylphosphine oxide residue	117

	Reactants/catalyst Attached to the BP		Products		Ref.
	Low fluorescent		Fluorescent product	Side product	
5	 Diazido naphthorhodamine residue	 4-Triphenylphosphine residue	 Naphthorhodamine	 4-Triphenylphosphine oxide residue	119
6	 7-Azido methoxy 4-methylcoumarin	 4-Triphenylphosphine residue	 4-Methylcoumarin residue	 4-Triphenylphosphine oxide residue	120
7	 Rhodamine B phenylthiosemicarbazide	 *p*-Mercuriobenzoate	 Rhodamine B oxadiazolide	 4-[thiomercuri]-benzoyl amide	121

the bright fluorescence of the deacylated fluorescein molecule. The rate of this reaction increased 188 times in the presence of DNA analyte.[112] It should be noted that Staudinger reduction proceeds very quickly and efficiently even under physiological conditions, which makes such assays particularly valuable for nucleic acid detection in cells. However, one potential disadvantage of the Cai et al. scheme is TPP's susceptibility to oxidation by atmospheric oxygen during probe purification and handling. The strand containing oxidized TPP moiety can still hybridize to the analyte, thus competing with the intact TPP derivative for binding to the analyte. This decreased the overall reaction rate. Moreover, if the fluorescent precursor has a residual fluorescence, the presence of an inactivated second strand may contribute to the undesired high fluorescence background.

Pianowski and Winssinger reported an alternative modification of the Staudinger reaction in which the fluorescent precursor and the TPP moiety were the parts of different oligonucleotides (Table 2, third row).[116] This approach takes advantage of an azide reduction rather than the phosphine oxidation reaction for generation of the fluorescent product. In this probe the PNA oligomer conjugated with 7-azidocoumarine-4-acetic acid was reduced by the TPP derivative of the other oligomer. The resultant 7-aminocoumarin moiety fluoresced 7 times more intensively than the reactant azide. This scheme would enable efficient reduction of the azide group of the pro-fluorophore even in the presence of a partially oxidized TPP-containing part of the BP simply using the excess of the reducer. A LOD of 1 nM for this templated reaction was reported.[116] The relatively low turn-on ratio is a disadvantage of azidocoumarin chemistry.

Another variant of fluorescence turn-on probes that uses the Staudinger reaction was reported by Abe and colleagues.[117] The TPP-mediated reduction of azidorhodamine was shown to increase its fluorescence by ~2000-fold in the wavelength region of 530−550 nm (Table 2, fourth row). Remarkably, the detectable signal was produced in 10−20 min after analyte addition. The reduction can be accomplished by a dithiothreitol[117] or tris(2-carboxyethyl)phosphine[118] conjugated probe. The same redox chemistry was employed to activate the fluorescence of a naphthorhodamine azide derivative at 655 nm.[119] In this case, two TPP residues were required to completely reduce the diazido rhodamine profluorophore (Table 2, fifth row). This can be achieved under conditions of quick exchange with the excess of the TPP-conjugated oligomer. It is likely that this requirement contributed to the somewhat low fluorescence enhancement (≈550) observed for this probe.

Franzini and Kool reported a TPP-mediated reduction of 7-azido methoxy-4-methylcoumarin (Table 2, sixth row),[120] which demonstrated a turn-on ratio of ~29. The reduction of the azidomethoxy group suggested in this work can possibly be applied for activation of other pro-fluorophores with a higher fluorescent enhancement. An original approach suggested by the same authors uses *p*-mercuriobenzoate-catalyzed activation of a rhodamine B derivative (Table 2,

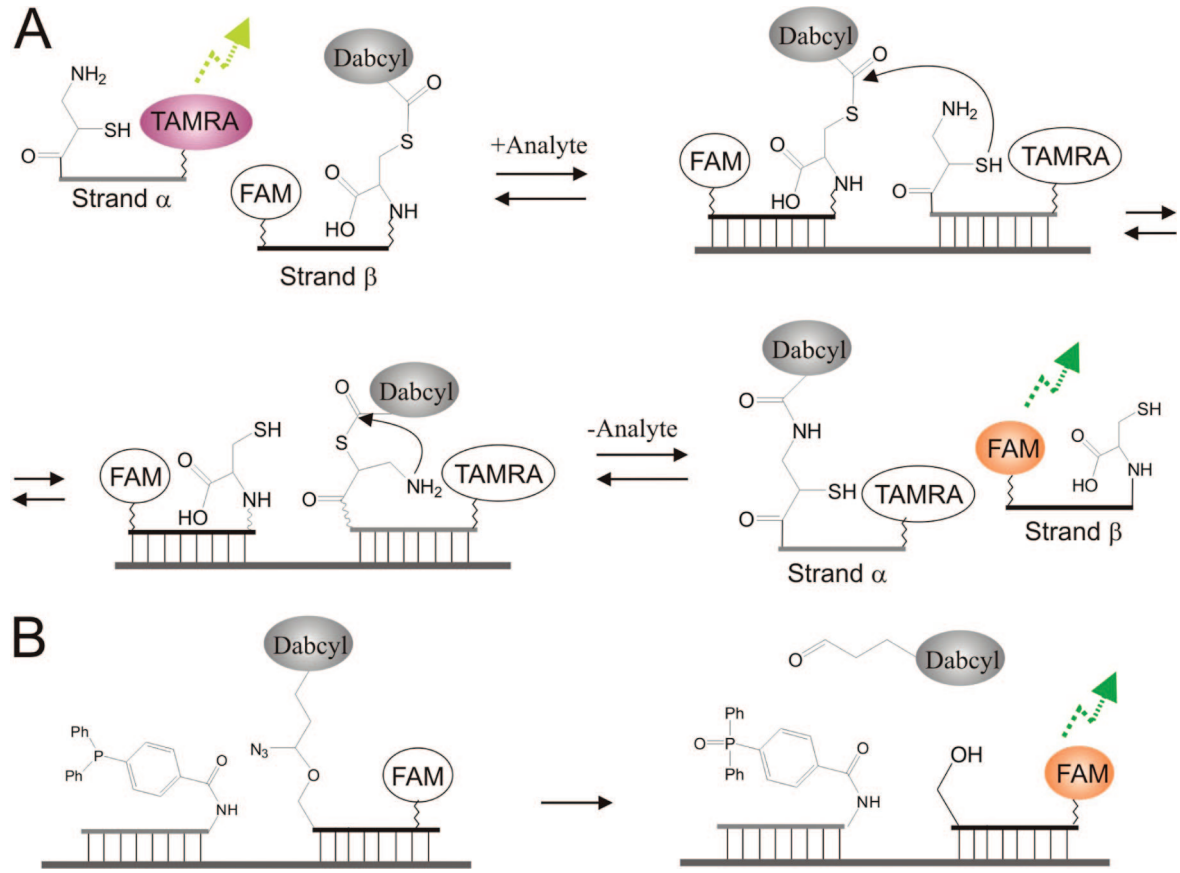


Figure 8. Quencher transfer and detachment probes (A) Quencher transfer probe, as designed by Grossmann and Seitz.[122,123] Both parts of the binary probe are peptide nucleic acids (PNA). FAM, TAMRA, and Dabcyl represent fluorescein, tetramethylrhodamin, and dabcyl groups, respectively. (B) Templated reduction-triggered quencher detachment reported by Franzini and Kool.[124]

seventh row).[121] The fluorescence increase was ~150, which is better than the TPP-promoted 7-azidocoumarin reduction but lower than that of the azidorhodamine and diazidonaphthorodamine reactions.[117,119] BPs that use a TPP-promoted azide reduction hold the greatest promise due to their rapid kinetics, straightforward preparation, and good biocompatibility. The detection limits for these probes, while expected to be lower than for other BPs, remain undetermined.

6.2.2. Quencher Transfer or Detachment Probes

Two alternative ligation-free approaches employ transfer of a quencher group from one part of BP to another or quencher detachment (Figure 8). Grossmann and Seitz reported a PNA-based probe[122,123] which took advantage of quencher transfer according to the following scheme. First, one PNA strand was conjugated with both TAMRA and isocysteine (strand α, Figure 8A). Next, a second PNA strand contained a fluorescein (FAM) and a dabcyl group at the opposite PNA−oligomer termini (Figure 8A, strand *β*). In the absence of the analyte, the TAMRA fluorescence was detected at 593 nm while the fluorescence of the FAM group was quenched by the closely located dabcyl quencher. When both strands hybridized to adjacent positions of the analyte, the thioester group of the *β* strand appeared in the proximity of the thiol group of the isocysteine moiety of the α strand. This configuration promoted a trans-thioesterification reaction. The subsequent S → N-acyl shift (Figure 8A, lower left) stabilized the transfer product, thus making the reaction irreversible. The dabcyl transfer increased FAM fluorescence while simultaneously quenching TAMRA. This allowed reaction monitoring by the intensity ratio F_{FAM}/F_{TAMRA}. It should be noted that dissociation of at least one portion of the probe from the analyte is required to avoid quenching of FAM fluorescence by dabcyl even after the quencher transfer.

To achieve greater sensitivity, the authors altered the structure of the quencher-accepting linker as well as the assay conditions.[123] The optimized probe detected a 0.02 nM synthetic 23 nt DNA analyte concentration after 24 h of incubation with a S/B of ~1.5. The approach suffers from high background, since the quencher can be transferred even in the absence of the analyte. Interestingly, the same chemistry can be applied for the transfer of a pyrene group. In this case, one product of the reaction is an oligomer conjugated with two pyrene residues. Therefore, the presence of the analyte can be detected by the fluorescence of pyrene excimer.

Franzini and Kool suggested a scheme that avoids the need for probe dissociation from the analyte for signal generation.[124] The probe uses a TPP-dependent release of a quencher from a fluorophore- and a quencher-labeled oligomer (Figure 8B). A robust signal in less than 1 h of incubation was registered with 2 nM target. It is likely that analyte at much lower concentrations can be detected using this approach despite noticeable background reaction. A possible modification of this approach can use fluorophore rather than quencher release. This modification avoids the fluorescence quenching by the oligonucleotide nitrogenous bases in the reaction product and thus can lead to a greater turn-on ratio.

The reaction rate is an important issue for all chemical reaction-based BPs. Hybridization of short linear oligonucleotides in solution is accomplished in seconds.[125,126] Therefore,

the chemical reactions should be essentially completed in a comparable time interval or offer other advantages to be competitive with other types of BPs. On the other hand, very high rates of templated reactions should be avoided in the case of SNP typing to allow dissociation of the mismatched complexes prior to the reaction.

7. Aptamer-Based Fluorescent Probes

In all the BP examples mentioned above, chemical conjugations of a dye to the oligonucleotide strands were required. Such conjugates must be thoroughly purified from the fluorescent contaminants to eliminate background fluorescence. Both conjugation and purification steps substantially increase the cost of the probes and potentially limit their applications especially in multiplex formats. Chemical modifications can be avoided by designing BPs from aptamers. Aptamers are the oligonucleotides capable of binding to specific target molecules.[127,128] They are usually created by selecting specific structures from large random DNA or RNA pools using in vitro selection (also known as systematic evolution of ligands by exponential enrichment (SELEX)) approach.

The malachite green aptamer (Figure 9A) is an RNA molecule that has submicromolar affinity to the triphenylmethane dye malachite green (MG in Figure 9B).[129] Upon binding, the aptamer increases the dye fluorescence more than 2000-fold.[130] To design a BP, the aptamer was separated into two strands and nucleic acid binding arms were added to each strand via UU dinucleotide bridges (Figure 9A).[131] In the presence of DNA complementary to the nucleic acid binding arms, the two RNA strands of the probe hybridized to the adjacent positions of the target DNA and reformed the MG binding site (Figure 9A). This complex was able to bind MG from solution, which was accompanied by a fluorescence increase. This probe, composed entirely of RNA, has potential as a light-up tag for mRNA monitoring in living cells.

Sando et al.[132] obtained DNA aptamer (Figure 9C) that can bind and enhance fluorescence (~460 nm) of a substituted bis-benzimidazole dye Hoechst 33258−Hoechst derivative 7− (Figure 9D). Introduction of the two *tert*-butyl groups in the phenyl ring of the dye suppressed its binding affinity to the minor groove of AT-rich double-stranded DNA target. The two-component probe was designed according to the aforementioned scheme for the binary MG probe (Figure 9C). The Hoechst aptamer probe was found to increase fluorescence of the Hoechst derivative 143 times upon binding to a DNA analyte. Taking into account the general high specificity of BPs and the lack of the need for conjugation with organic dyes, the Hoechst binary aptamer represents a promising construct for multiplex SNP typing including DNA microarray platforms.

Recently, Endo and Nakamura reported a BP based on a newly selected Cy3 RNA aptamer.[133] Unfortunately, the fluorescence of Cy3 increased by only ~30% on binding to the RNA sequence, which complicated detection of the signal by fluorescence increase. The assay might be redesigned in FRET format if an aptamer against a fluorophore donor was available. In this assay, one part of the BP contains the full Cy3 aptamer while second part is conjugated with an apatamer that specifically binds a donor fluorophore. Hybridization of the two aptamers to the analyte would bring the apatmer-bound dyes in proximity, thus enabling energy transfer from the donor fluorophore to Cy3. Accordingly,

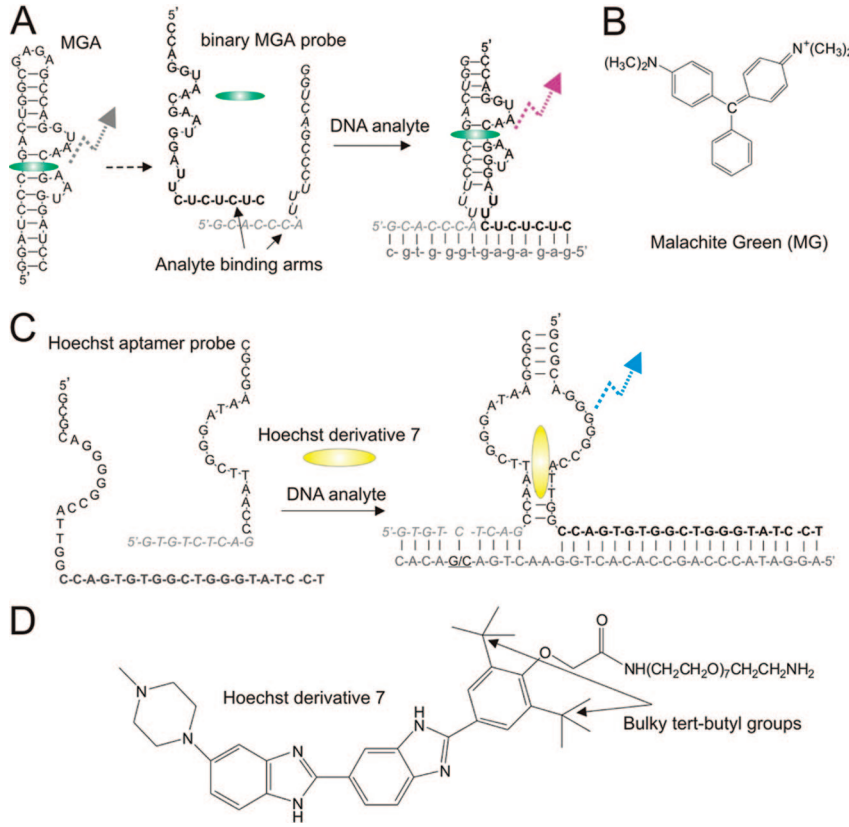


Figure 9. Aptamer-based binary probes. (A) Malachite green aptamer probe.[131] Green ovals represent malachite green molecule. (B) Chemical stricture of malachite green dye. (C) Hoechst dye aptamer-based probe developed by Sando et al.[132] Yellow ovals represent Hoechst derivative 7. (D) Chemical structure of Hoechst derivative 7.

further development of this approach promises to deliver BPs useful in real-time assays.

In all aptamer-based fluorescent probes the affinity of the dye to the probe−analyte complex may limit their sensitivity. For instance, ~100 nM LOD was found for the malachite green aptamer (Kolpashchikov, unpublished data). This value correlates with a K_d for the malachite green−apatamer complex. Therefore, aptamers with high affinities to the dyes are required to impove the sensitivity of the aptamer-based BPs.

8. DNA Junction-Forming Probes

One of the simplest embodiments of the BP concept is hybridization of two deoxyribooligonucleotide strands to an analyte. If this hybridization could be detected in real time, such a probe would have the most straightforward and economical design. Research in this direction has led to the introduction of a BP that forms DNA four-way junction structure, binary DNA probe (BDP).[69] The BDP consists of two DNA strands, α and β, and an MB probe (Figure 10A). Each strand contains a segment complementary to the MB probe (MB binding arms) and a segment complementary to the nucleic acid analyte (analyte binding arms). In the absence of a complementary target all strands remain unbound; the MB is free in the form of a hairpin, and the fluorescence signal is low (Figure 10A, left). Addition of a DNA analyte triggers the formation of a quadripartite complex containing a four-way DNA junction (4J)-like motif (Figure 10A, right). In the 4J, the fluorophore (FAM) is separated from the quencher (Q) in this complex, resulting in a fluorescent signal.

Natural 4Js exist as mixtures of two right-handed antiparallel crosses.[134] The ratio between the two conformers depends on the nucleotide composition at the point of the strands exchange, which in the case of BDP is predetermined by the analyte sequence. In practice, it is important that MB probe acquires an elongated conformation in the quadripartite associate independently of the analyte sequence. This is realized in the BDP quadripartite complex, in which strands α and β contain oligoethylene glycol linkers between analyte binding arms and MB binding arms. In such a design the MB probe and the analyte adopt elongated conformations, while strands α and β are bent at the point of the oligoethylene glycol inserts.

The 4J motifs are naturally occurring well-studied structures. Unlike the three-way DNA junctions, in the 4J all nucleotides form base pairs at the position of strand exchange and, in the presence of Mg^{2+}, are involved in stacking interactions. These features make 4J stable and predictable. Indeed, 4J is the most popular branched structure used by DNA nanotechnology to build DNA-based objects of desired shapes.[135] In BDP, the 4J was used as a basis for the assembly of four DNA strands in a fluorescent complex. This makes BDP a simple prototype probe for application of multistrand probes in nucleic acid analysis.

It should be pointed out that the BDP requires only one MB for analysis of various sequences, so it can be prepared in bulk and utilized efficiently. This characteristic of the BDP potentially reduces the assay cost in the case of multiplex SNP typing. The detection limit for this probe was found to be ~5 nM, which is close to that of MB probes (Kolpashchikov, unpublished data). The BDP design required nonnucleotide linkers between the analyte binding arms and the MB binding arms, which added to the cost of the α and β strands. However, in the solid support-bound format with quencher-free reporter oligonucleotde, the linkers will not be required. Taking in account high selectivity and compatibility with DNA microarray technology, the BDP represents an attractive alternative to the traditional arrays of linear oligonucleotide probes.

Nakayama et al. suggested a protein-assisted three-way junction forming probe.[136] The construct consists of two oligonucleotides, one of which is conjugated with both a fluorophore and a quencher (Figure 10B, left). When the oligonucleotides specifically hybridize to the analyte, a double-stranded restriction endonuclease recognition site is formed (red fragment in Figure 10B). The endonuclease cleaved DNA and induced the quencher removal, thus producing high fluorescence. Not only does this approach demonstrate high selectivity, but it also has a significant potential for signal amplification. However, despite the rather high efficiency of the employed protein enzyme, a 50 nM analyte concentration generated a fluorescence increase of only ~35% after 9 h of incubation. Further optimization of the probe design and reaction conditions may improve the detection limits of this approach.

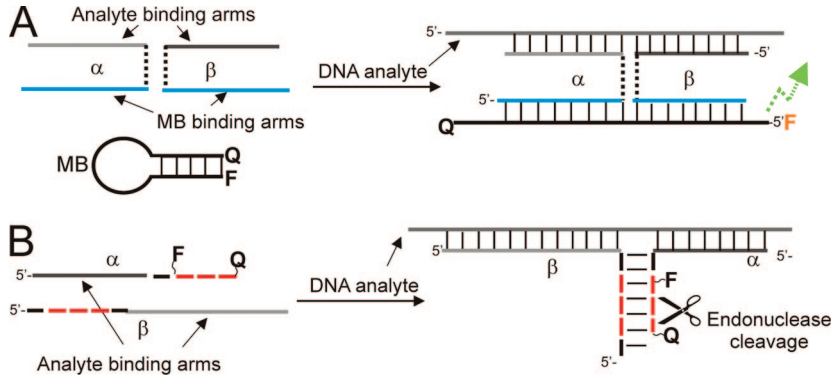


Figure 10. DNA junction probes. (A) Four-way junction-forming probe.[69] The probe utilizes molecular beacon (MB) probes for detection of the hybridization event. A molecular beacon and two synthetic oligodeoxyribonucleotides (α and β) exist in the dissociated state in the absence of a DNA analyte (left). Addition of the analyte results in the formation of a four-stranded associate containing a DNA four-way junction-like structure (right). Triethylene glycol linkers are depicted as dashed vertical lines. (B) Three-way junction-forming probe uses restriction endonuclease for signal generation as reported by Nakayama et al.[136] F and Q represent fluorescein and dabcyl residues, respectively. The 4 bp endonuclease recognition site is marked red.

9. Binary Deoxyribozyme Probes

An ideal sensor must be both specific and sensitive. The binary approach offers excellent specificity. However, most BPs are limited by the detection of analytes at nanomolar or high subnanomolar concentrations. This LOD is not low enough to be of significant practical use in PCR-free diagnostics. For example, the typical concentration of hepatitis C virus in clinical samples is $\sim 10^4-10^6$ copies/mL or $\sim 6 \times 10^{-14}-6 \times 10^{-16}$ M.[137] Therefore, to detect a low-abundant nucleic acid using BPs, the genetic material needs to be PCR-amplified prior to its analysis.

One approach to enhance the sensitivity of the probe is to amplify the signal not the analyte, as demonstrated for probes that use templated chemical reactions. Alternatively, signal amplification can be achieved by employing nucleic acid enzymes, which are capable of inducing a chemical reaction with multiple turnover.[138−140] Deoxyribozymes (DNA enzymes, DNAzymes) are catalytic oligodeoxyribonucleotides derived by in vitro selection.[141,142] DNA enzymes have been considered as promising molecular platforms for sensor design because of high chemical stability, low synthetic cost, biocompatibility, and ease of structural prediction and modification. A split probe based on DNA enzyme that ligates two oligonucleotides can, in general, be designed.[143] However, the ligation product has higher affinity to the DNA enzyme than the substrates. This severally inhibits the substrate turnover, thus making such a probe a less attractive sensor for nucleic acid analysis as compared with the approaches that do not use self-inhibition. BPs based on RNA-cleaving deoxyribozymes and DNAzyme with peroxidase-like activity are reviewed below.

9.1. Probe Based on RNA Cleaving Deoxyribozymes

RNA-cleaving DNAzymes are the most abundant class of catalytic DNAs. They have been widely used for sensing ions, small molecules, proteins, and oligonucleotides (reviewed recently).[138,139] To enable real-time detection of the target, nucleic acid enzymes cleave a double-labeled fluorogenic reporter substrate that has an embedded ribonucleotide phosphodiester bond (Figure 11A).[144,145] The deoxyribozyme-mediated substrate cleavage separates the fluorophore from the quencher, thus enabling fluorescent signal.

The deoxyribozyme E6, one of the first deoxyribozymes selected (Figure 11A),[146] was used to create a binary deoxyribozyme probe.[70] E6 is a Mg^{2+}-dependent DNA enzyme that recognizes the DNA substrate with a single embedded ribonucleotide and hydrolyzes the RNA phosphodiester bond with a catalytic rate of ~ 0.01 min^{-1}.[146] To design a BP, the E6 was divided into two fragments and the analyte binding arms were added to each half of the deoxyribozyme via dithymidine linkers (Figure 11B). In the presence of the analyte the two DNAzyme subunits hybridized to complementary regions of the analyte and reformed the deoxyribozyme catalytic core (Figure 11C). The active enzyme cleaved the reporter substrate and increased the fluorescence. The LOD of this approach was found to be ~ 1 nM. More efficient DNA enzymes can improve the sensitivity of the approach and reduce the assay time. Indeed, when the more efficient catalytic DNA, deoxyribozyme OA-II,[147] and 10−23[148] were used for BP design, the LODs was 0.1 and 0.005 nM, respectively. The last LOD is among the lowest obtained in homogeneous fluorescent assays. Binary deoxyribozyme probes are cost efficient in the case of multiplex analysis, since they require the synthesis of only two short unmodified DNA strands for each new analyte sequence, while the double-labeled reporter substrate is universal for all analytes.

The LOD of such a probe is limited by the value $\sim 10^{-15}$ M because of the general limit of the enzyme-catalyzed reactions.[149] This detection limit might be insufficient for analysis of samples with a low analyte content in PCR-free assays. Deoxyribozyme-based cascades for nonlinear signal amplification may help further lower the LODs of such assays. Deoxyribozymes represent a versatile platform for such cascades, as demonstrated by several 'proof-of-concept' studies.[150−152]

9.2. Split Peroxidase-Like DNA Enzyme

BPs avoid the requirement for precise temperature control for accurate SNP genotyping. However, a fluorometer is needed for signal registration for all the probes described above. At the same time, visually detectable signals may be indispensable in point-of-care settings such as the doctor's office, battlefields, and the first responding sites against bioterrorist attack.[153] As such, BPs were designed based on a peroxidase-like DNA enzyme.[154−158] A hemin binding DNA aptamer (Figure 12A) reported by Sen and colleagues[160−162] demonstrated peroxidase-like activity about 250 times greater than that of hemin alone. On the basis of this DNA enzyme, several sensors for nucleic acids with colorimetric or luminescent readouts were reported (reviewed recently).[140]

We designed a BP based on DNAzyme for visual SNP genotyping in solution. The sequence of the deoxyribozyme

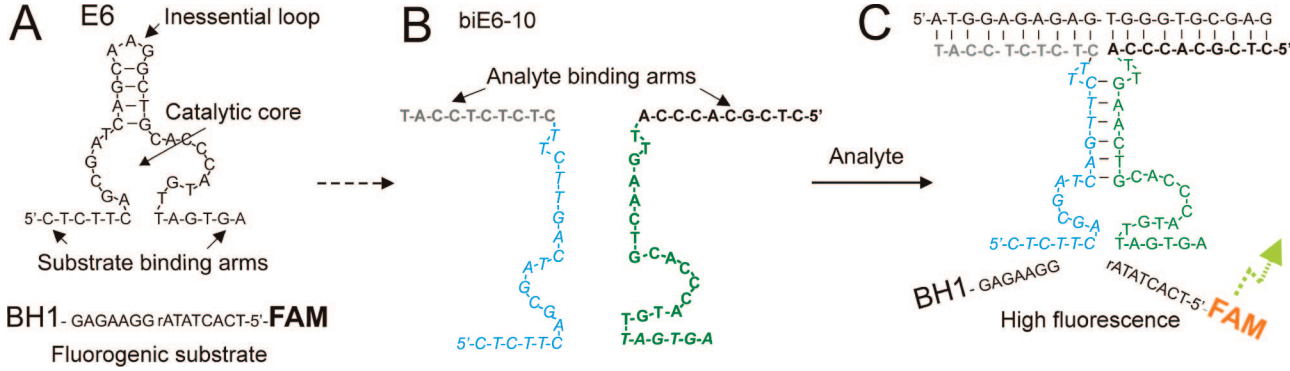


Figure 11. Binary deoxyribozyme probe.[70] (A) Structures of the parent deoxyribozyme E6. (B) Binary deoxyribozymes biE6. (C) Binary deoxyribozyme cleaves a fluorogenic reporter substrate in the presence of the analyte. FAM indicates fluorescein; BHQ1 is Black Hole Quencher 1.

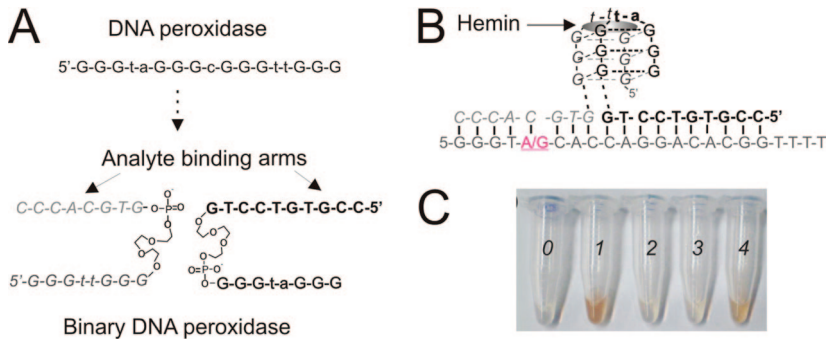


Figure 12. Colorimetric binary probe based on peroxidase-like DNA enzyme.[156] (A) Probe design. (B) Formation of active peroxidase upon hybridization of binary DNA peroxidase strands to the abutting positions of the analyte. (C) Visual detection using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as an oxidizable substrate. Sample *0*, reaction buffer; sample *1*, reaction buffer in the presence of parent DNA peroxidase (positive control); sample *2,* the probe in the absence of the analyte (negative control); sample 3, the probe with a single-base mismatched analyte; sample 4, the probe with the complementary analyte.

was deactivated by splitting it into two halves with analyte binding arms present in each half as shown in Figure 12A (bottom). In the presence of the analyzed DNA, the strands assembled in a catalytically active G-quadruplex-containing complex (Figure 12B). In the presence of hemin, the complex catalyzed oxidation of colorless substrates to colored products, which could be detected visually (Figure 12C).[156] Only a fully complementary analyte changed the color of the solution (compare samples 3 and 4 in Figure 12C). Either DAB (3,3′-diaminobenzidine) or ABTS (2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) can be used as oxidizable substances. The assay was limited by the detection of 10 nM analyte (Kolpashchikov, unpublished data), despite the catalytic action of the DNAzyme. This low sensitivity can be attributed to the moderate affinity of the hemin molecule to the G-quadruplex (∼100 nM); thus, relatively high concentrations of assembled DNAzyme are required for efficient hemin binding. This approach was recently applied for detection of PCR amplified DNA from salmonella and mycobacterium.[159]

An original design for DNA peroxidase-based BP was suggested by Deng et al.[157] The DNAzyme sequence was split into two uneven parts: one part contained three GGG repeats, while the second part contained only one triguanosine sequence. It was recently reported that an optimized probe can detect down to the subnanomolar level.[158] BPs based on peroxidase-like DNAzymes can be adopted to generate chemiluminescent signals when luminol is used as an oxidizable substrate.[154]

10. Conclusion

High sensitivity to a single base mismatch in the hybrid makes BPs promising platforms for SNP genotyping assays of the future. A commonly adopted BP architecture employs the different affinity mode BPs (Figure 2C). In this design, one strand with a longer analyte binding arm (typically ≥12 nucleotides) binds tightly to the position abutting to the SNP site. A second shorter (7−9 nt) analyte binding arm interrogates the SNP site by forming stable hybrid only with the perfectly matched sequence. Introducing conformational constrains (hairpins, for example) in the short analyte binding arm allows fine tuning of the stability of probe−analyte hybrid, which enables excellent assay specificity under broadly variable conditions.

Two-component probes can use a variety of strategies for signal generation: analyte-assisted nonradiative energy transfer; excimer, exciplex, or luminescent complex formation; and chemical reactions between the two components of the probe. Newly designed constructs based on aptamers, DNA junctions, and DNA enzymes offer an opportunity to utilize DNA probes that avoid direct covalent attachment with organic dyes. These cost-efficient sensors might be of particular interest for multiplex SNP genotyping formats.

The mismatch discrimination power of the binary approach can be used in formats that do not involve optical changes upon target recognition. These techniques include electrochemical SNP genotyping[163] as well as ELISA-like assays for PCR-free RNA detection,[164,165] which might be useful in multiplex genotyping formats.

The possibility to detect nucleic acids without separating the hydride from the unbound probe represents a significant advantage of BPs, which has been utilized in real-time PCR by LightCycler technology.[18,19] This property combined with the improved selectivity of the binary approach is essential for monitoring specific RNAs in cells and can enable PCR-free diagnostics of clinical samples. However, the improvement of sensitivity is still required. The lowest LODs (≤0.2[79] and 0.03 nM[80]) for BPs that do not amplify signals were reported for resonance transfer from lanthanide complexes to cyanine dyes. Among signal-amplifying BPs, the ligationless templated reactions, with a LOD of 0.02 nM,[123] and binary deoxyribozyme probes, with a LOD down to 0.005 nM,[150] hold the greatest promise. The sensitivity of some BPs remains to be investigated. Importantly, commonly accepted statistical criteria[166] must be used for LOD determination. LOD values should be revised in a number of reports in which few percent differences between the signal and the background were assumed significant without statistical analysis.

The design of BPs employs self-assembly of more than two nucleic acid components. The same principle is adopted by DNA nanotechnology, which deals with constructing objects and functionally active assemblies from DNA molecules.[135] Devices and structurally sophisticated DNA associates may find application in nucleic acid analysis in the future. For example, "DNA origami" uses hundreds of short oligonucleotide staples to fold a long viral DNA into an arbitrary shape.[167] The staples may be considered as a multicomponent probe for analysis of viral nucleic acid if this shape could be detected efficiently. Thereby, the probes described in this review might be viewed as a step toward application of complex self-assembling probes for DNA/RNA analysis, the full potential of which has yet to be realized.

11. Acknowledgments

Support from the Office of Research and Commercialization, College of Science and Chemistry Department at UCF is greatly appreciated. I am grateful to Yulia Gerasimova and Olga Ozoline for helpful comments and Aaron Hayson for the careful reading of the manuscript.

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