DNA-Mediated Homogeneous Binding Assays for Nucleic Acids and Proteins

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Review

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DNA-Mediated Homogeneous Binding Assays for Nucleic Acids and Proteins

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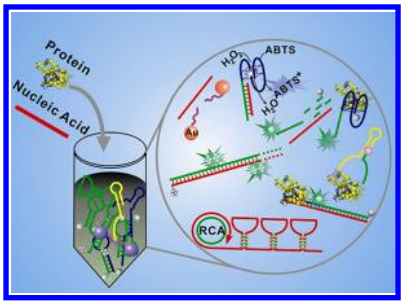

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1. INTRODUCTION

The detection of nucleic acids and proteins is fundamental for studying their functions and for the development of molecular diagnostics. These molecules carry out most biological functions, including but not limited to storing and transmitting genetic information, decoding information in cells, regulating biochemical activities, catalyzing reactions in living organisms, transporting small molecules, and providing mechanical supports.[1,2] Determining these biomolecules in complex systems requires exquisite analytical specificity and sensitivity. To meet these requirements, much effort has been devoted to affinity binding assays incorporating signal amplification. In particular, this review focuses on homogeneous binding assays, which are carried out in solution, without the need for separation, immobilization, or washing steps. Such homogeneous binding assays can be performed in a single tube/vial or in live cells.

The two essential components required for homogeneous binding assays include a target recognition probe and a signal transducer. For biomolecules, such as proteins and nucleic acids, recognition is generally achieved using affinity ligands. The affinity binding of ligands to target molecules is dependent on noncovalent interactions, such as hydrogen bonds, ionic interactions, van der Waals forces, and hydrophobic interactions.[3−6] Substances that are able to bind selectively to target molecules via noncovalent interactions can serve as affinity ligands.

Some common affinity ligands include nucleic acids and antibodies. Nucleic acids (DNA or RNA) are recognized by complementary strands through specific base pairing. The exquisite specificity of base pairing allows perfectly complementary strands to readily hybridize to each other, while a

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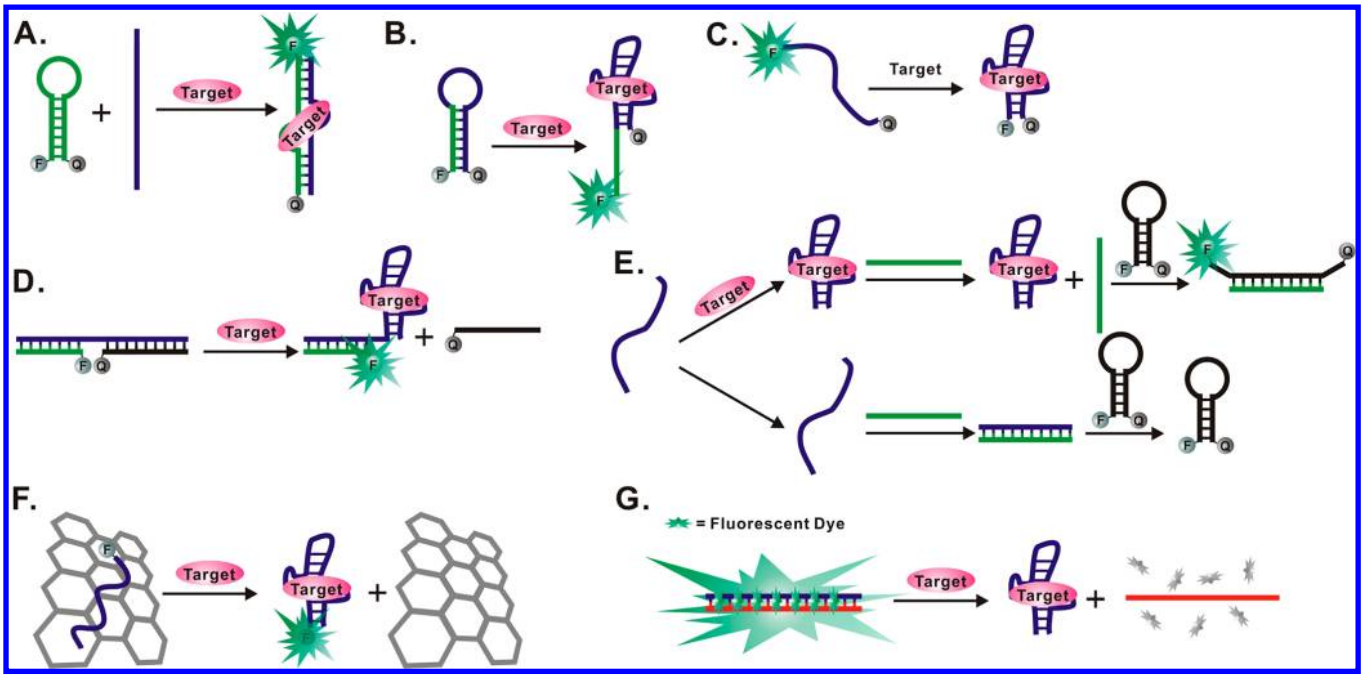

Figure 1. Design strategies for molecular aptamer beacons (MABs). (A) Binding of the target to two oligonucleotides that are modified from the split aptamer sequence results in the formation of a ternary complex and the generation of a fluorescence signal.[50] (B) Binding of the target to a hairpin beacon results in the formation of the preferred aptamer G-quartet structure.[52] (C) Binding of the target induces a conformational change in the aptamer, resulting in fluorescence quenching.[56] (D) Binding of the target to the internally labeled beacon results in structure switching and the release of the quencher.[61,62] (E) Competition-based MAB using an oligonucleotide competitor.[75] (F) Competition-based MAB using nanomaterials, such as single-wall carbon nanotubes, as a competitor.[82] (G) MABs that utilize dye displacement for signal generation in response to target binding.[97]

single-base mismatch between two strands can make hybridization between them less energetically favored.[5,7] To achieve selectivity required for detection of a single mismatch, researchers commonly design nucleic acid probes to contain secondary structures or to require an assembly, e.g., a hairpin structure, or an assembly of multiple nucleic acid strands to recognize a single target strand.[8,9]

Antibodies are commonly used as affinity ligands to recognize proteins. The binding of a specific antibody to its antigen is mediated by hydrogen bonds, van der Waals forces, hydrophobic interactions, and spatial contacts within the antibody-binding sites.[4,10] For many target proteins, the cross-reactivity of antibodies compromises the specificity to the target of interest. Therefore, two antibodies are often required to bind to a single protein target, which enhances the specificity of analysis.

Another affinity ligand commonly used to recognize specific proteins is the aptamer. Aptamers are short, synthetic single-stranded oligonucleotides (DNA or RNA) that can bind to target molecules with high affinity and specificity.[11−15] They are commonly selected from random sequence libraries, using the systematic evolution of ligands by exponential enrichment (SELEX) techniques.[16−22] Advantages of aptamers over antibodies include improved thermal stability, longer shelf life, and ease of modification and conjugation.[23] Aptamers that have sufficient binding affinity and specificity are usually present in their favored secondary structures when bound to the target. The conformational change in the aptamers' secondary structure upon their binding to the target is a useful property which has been incorporated in molecular beacons.[24]

Several signal transduction mechanisms have been developed to convert protein/nucleic acid binding events into detectable signals. Signal transducers are usually designed to report the local environment change that results from binding between the affinity ligand and the target molecule. Optical detection systems are the most common transduction methods used in homogeneous assays. A variety of optical transduction principles have been tested to detect target binding, including fluorescence resonance energy transfer (FRET), fluorescence polarization, the fluorescence lifetime, excimer fluorescence, localized surface plasmon resonance (LSPR), and surface-enhanced Raman scattering (SERS).[9,25−28]

The diverse formats for target recognition and signal transduction have allowed for the development of a wide range of homogeneous binding assays. Here, we aim to review the major advances in the development of nucleic acid-mediated homogeneous assays, where nucleic acids are used as either the recognition component or the signal transduction component for the detection of nucleic acids, proteins, and other molecular targets. Assays that use nucleic acid recognition components for nucleic acid targets, such as molecular beacons and nanomaterial-based assays, have been reviewed elsewhere.[8,9,27−31] The present review mainly focuses on recent advances in aptamer beacons, catalytic beacons, in vitro isothermal amplification techniques, DNA nanostructures, and binding-induced DNA assembly for the detection of specific nucleic acid sequences and proteins. The detection of small molecules and metals has been reviewed elsewhere.[24]

2. BEACON-BASED HOMOGENEOUS BINDING ASSAYS

Molecular beacons (MBs) typically consist of a stem−loop structure with a fluorophore on one end and a quencher on the opposite end.[8,29,32] Fluorescence is quenched in this structure due to efficient energy transfer from the fluorophore to the quencher at close proximity. In the presence of a target that is

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complementary to the loop sequence, hybridization results in the disruption of the stem–loop structure and an increase in separation distance between the fluorophore and quencher. As a result, the fluorophore is no longer quenched and fluorescence is restored. Nanomaterials, such as gold nanoparticles (AuNPs),[33−35] carbon nanotubes (CNTs),[36] graphene oxide (GO),[37−39] and silicon nanowires,[40] have been incorporated into MBs to enhance the quenching efficiency and the sensitivity. MBs provide an excellent signal transduction mechanism, responding to the change of conformation of the beacon as a result of target binding. Additionally, the specificity of nucleic acid hybridization has made MBs an attractive tool for detecting nucleic acids, single-nucleotide polymorphisms (SNPs), and gene mutations.[32,41,42] Other beacon-based strategies have been developed to amplify the signal response as well as to detect non nucleic acid targets.[43,44] Here we review aptamer beacons and catalytic beacons as a means to detect proteins and nucleic acids without the need for separation.

2.1. Assays Using Aptamer Beacons

Molecular aptamer beacons (MABs) have been developed by combining the binding ability of aptamers with the signal transduction mechanism of MBs for the detection of specific biomolecules.[26,45] Upon binding to its target, an aptamer undergoes a conformational change, which usually changes the distance between the fluorophore and the quencher in the MAB, resulting in detectable changes in fluorescence.[46−49] Figure 1 shows several examples of MAB designs that have been reported for detection of proteins.

One of the first MABs was designed for the detection of the HIV-1 Tat protein (Figure 1A).[50] An aptamer for the Tat protein was split into two oligomers. The first oligomer was extended to form a hairpin structure and was labeled with a fluorophore and a quencher at either end. The other oligomer was not modified. In the presence of Tat-1 protein, the two oligomers bind to the protein, forming a ternary complex. The labeled beacon undergoes a conformational change, resulting in an increased distance between the fluorophore and quencher and an enhanced fluorescence signal. In another strategy two aptamers that recognize different epitopes on the same protein were labeled with a fluorescence donor and an acceptor.[51] The presence of the protein would result in the coassociation of the aptamers and FRET signal.

Another strategy to design MABs is to extend an aptamer sequence so that a hairpin structure is formed in the absence of the target (Figure 1B).[52] Hamaguchi and co-workers constructed an MAB for thrombin by adding five nucleotides to the 5′-end of the aptamer for thrombin. The additional nucleotide sequences were complementary to those at the 3′-end of the aptamer, forming a hairpin structure.[52] In the presence of thrombin, the binding induced a conformational change in the aptamer, resulting in the separation of the quencher from the fluorophore, leading to an increase in fluorescence intensity. This approach has also been used to detect myotonic dystrophy kinase-related CDC42-binding kinase α,[53] thrombin,[54] and HIV-1 reverse transcriptase in living cells.[55]

The requirement of the conformational change in MABs upon target binding can be met using aptamers without altering their sequences. For example, the thrombin aptamer has a loose random coil structure in the unbound state and a quadruplex structure when bound to its target (Figure 1C). One nucleotide was added to each end of the thrombin aptamer, which was then labeled with a fluorophore/quencher pair. In the presence of thrombin, the aptamer adopted a bound quadruplex structure, facilitating a decrease in fluorescence.[56] There is no requirement to split the aptamer or design it into a hairpin structure because the conformational change induced upon target binding is sufficient for generating a signal. This approach was also used to detect angiogenin in serum samples and to discriminate between variants of platelet-derived growth factor (PDGF)[57,58] and angiogenin.[59] Excimer-based MABs have also been reported to detect proteins on the basis of the change in conformation of the pyrene-labeled aptamer upon protein binding.[25] Likewise, an MAB was devised by conjugating a thrombin aptamer to a quantum dot (QD) and a metal complex. The binding of thrombin to this MAB led to a conformational change that brought the metal complex closer to the QD, facilitating charge transfer and a corresponding decrease in emission intensity.[60]

For some aptamers, binding to their targets may not result in sufficient conformational changes to be useful for MABs. To confront this challenge, Nutiu and Li introduced "structure switching signaling aptamers" to construct MABs (Figure 1D).[61,62] The aptamer sequence was extended with a sequence that was complementary to a fluorescent oligonucleotide probe. The aptamer sequence was hybridized to an antisense oligonucleotide labeled with a quencher, so that, in the absence of the target, all three oligomers hybridized, resulting in a quenched fluorescent state. When the target was added, the aptamer motif formed a complex with the target, resulting in the release of the quencher strand, which restored fluorescence (Figure 1D). This approach has been used to design MABs for recombinant human erythropoietin,[63] thrombin,[61] and theophylline[66] and as reporters for quantifying enzyme activities.[67] A general design strategy for structure switching signaling RNA aptamers has also been described.[68] Nanomaterials, including AuNPs and QDs, have been incorporated into structure switching signaling aptamers to enhance the fluorescent energy transfer for the detection of thrombin,[69,70] human recombinant erythropoietin-α,[71] mucin 1,[72] and PDGF.[73]

MABs can also be designed for competitive assays.[74,75] Examples of competitors include nucleic acids,[74−76] DNA-binding proteins,[77] and nanomaterials.[69,78−92] Figure 1E shows an example of an MAB in the competitive format for the detection of human neutrophil elastase.[75] The competitor was designed to be complementary to the aptamer for human neutrophil elastase and to the molecular beacon probe. Binding of the target to the aptamer prevented the hybridization of the competitor to the aptamer, leaving the competitor available to hybridize to the molecular beacon. The hybridization of the competitor to the molecular beacon opened the hairpin structure and resulted in a detectable fluorescence signal. In the absence of the target protein, the competitor oligonucleotide hybridized to the aptamer and was not available to open the hairpin structure, and thus, no fluorescence signal was generated. The competitive format obviates the need for labeling or modifying the aptamers.

Nanomaterials, e.g., single-wall carbon nanotubes (SWCNTs) and AuNPs, can also act as competitors and quenchers on the basis of the preferential adsorption of single-stranded DNA (ssDNA) to the surface of nanomaterials over the adsorption of bound aptamers.[36,80,82] For example, SWCNTs have been used as a competitor and quencher to develop an MAB for thrombin (Figure 1F).[82] In the absence of

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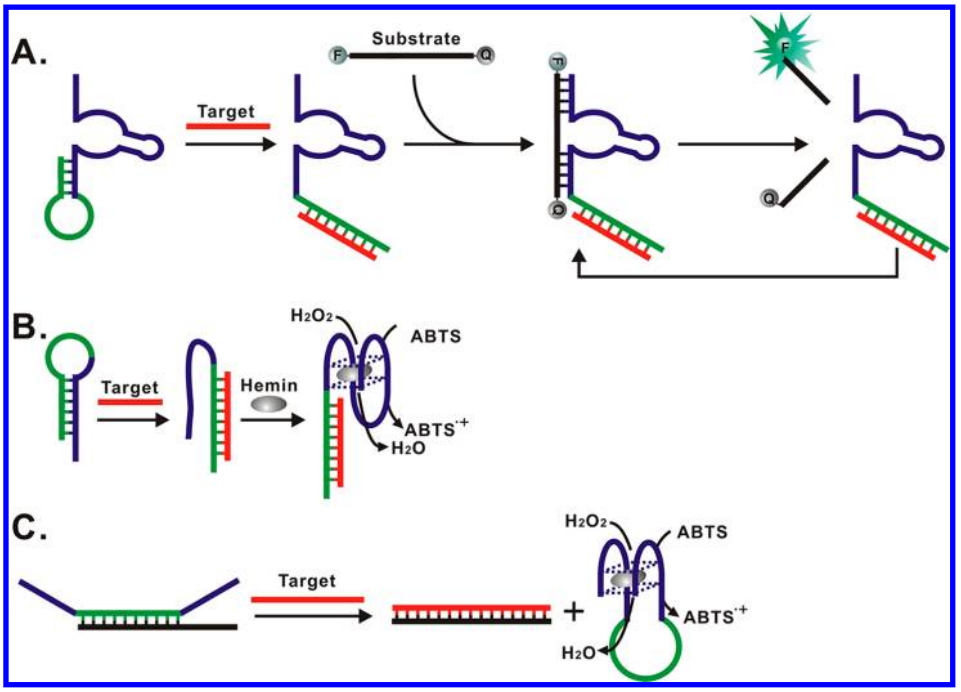

Figure 2. Strategies for DNAzyme-based catalytic beacons: (A) caged strand-cleaving DNAzyme;[114] (B) caged horseradish peroxidase (HRP)-mimicking DNAzyme;[119] (C) assembly of a split HRP-mimicking DNAzyme.[124]

thrombin, the fluorescently labeled aptamer probe interacted with the SWCNT via $\pi-\pi$ stacking interactions so that the fluorescence signal was quenched. When the aptamer bound to the thrombin, the aptamer−thrombin complex had a weakened interaction with the SWCNT so that the fluorescence was not quenched. Detection of 1.8 nM thrombin was achieved. Other nanomaterials displaying fluorescence quenching properties and adsorption interactions with ssDNA have also been used in developing competitive MABs. These nanomaterials include graphene,[83,84] mesoporous carbon nanoparticles,[85] poly(*m*-phenylenediamine),[86] carboxylic carbon nanoparticles,[87] and AuNPs.[69] Additionally, due to the nonselective adsorption nature of the unbound aptamers onto these nanomaterials, MABs making use of these quenchers have demonstrated multiplexing capabilities.[88,89] One consideration is the physical adsorption mechanism of DNA to the nanomaterials. For example, chemical interactions of DNA with AuNPs have slower desorption kinetics compared to those with graphene oxide and SWCNTs where $\pi-\pi$ stacking and hydrophobic interactions are predominant.[93,94]

The preferential binding of ssDNA to AuNPs can also be used to design a colorimetric detection platform. In the absence of the target, the ssDNA probe adsorbed onto the AuNP surface to prevent salt-induced aggregation of AuNPs, so the solution remained red. Conversely, when the target was present, the ssDNA probe formed a complex with the target and was not able to prevent the salt-induced aggregation of AuNPs. The aggregation of AuNPs turned the solution blue.[95] This approach has been applied to the detection of thrombin.[80,96]

Strategies that do not rely on the labeling of any component are desirable because they save time and costs and avoid potential problems of altering the binding affinity by the labeling. For this purpose, dyes that have differential binding affinities and optical properties toward bound and unbound aptamers have been used as reporters for binding events (Figure 1G). For example, ethidium bromide (EB) intercalated into the duplex formed by an aptamer and its antisense sequence, generating a strong fluorescence signal.[97] The binding of the target, e.g., thrombin, to the aptamer resulted in the disruption of the duplex, and therefore, EB was exposed to the solvent, reducing the fluorescence signal. Likewise, a molecular light switching dye, $[Ru(phen)_2(dppz)]^{2+}$, had no luminescence in solution but displayed strong luminescence when intercalated into duplex DNA.[98,99] This approach was used to detect abrin toxin through its binding to its respective aptamer to displace $[Ru(phen)_2(dppz)]^{2+}$, resulting in a decrease of luminescence. This approach was able to detect immunoglobulin E (IgE) and PDGF-BB, with detection limits of 100 pM and 1 nM, respectively.[100] TOTO (1,1′-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)-bis-4-(3-methyl-2,3-dihydro(benzo-1,3-thiazole)-2-methylidene) quinolinium tetraiodide) has been used in a similar way in MABs to detect PDGF-BB with a detection limit of 0.1 nM.[102]

2.2. Assays Using Catalytic Beacons

A very useful strategy to improve the sensitivity of beacons is to amplify the signal response through coupling the beacon recognition to enzymatic activity. These catalytic beacons commonly make use of DNAzymes, DNA sequences that display specific catalytic activities.[24] Biochemical reactions that DNAzymes can catalyze include cleavage of the nucleic acid glycosidic bond,[103−106] DNA self-modification,[107−109] porphyrin metalation,[110] and H_2O_2-mediated oxidation.[111,112] Sequences of DNAzyme beacons can be obtained through either rational design or in vitro selection.[113] Here we discuss the construction of catalytic beacons for homogeneous binding assays.

One common strategy to construct catalytic beacons involves inactivation of DNAzymes through caging them into a hairpin structure or using a blocker strand. The binding of nucleic acid targets can restore the activity of the DNAzyme by opening the hairpin structure or releasing the blocker strand. Figure 2A

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shows a catalytic molecular beacon that makes use of a DNAzyme with RNase activity to cleave a single ribonucleotide site.[114] The DNAzyme was modified by adding a hairpin structure that contained a target recognition domain. The partial substrate binding site was caged in the stem of the hairpin structure so that catalytic cleavage of DNAzyme was inhibited in the absence of the target DNA. The binding of the target DNA liberated the DNAzyme from the hairpin structure, enabling cleavage of the labeled substrate and turning on fluorescence. Similar catalytic beacons were further designed for detection of microRNA[115] and thrombin.[116] The catalytic beacon for thrombin was designed by incorporating an aptamer beacon in a hairpin structure into the DNAzyme. Likewise, binding of thrombin to the aptamer opened the hairpin structure and restored the catalytic activity of the DNAzyme.[117]

A similar strategy was applied to fabrication of catalytic beacons based on the horseradish peroxidase (HRP)-mimicking DNAzyme. In the presence of hemin, the HRP-mimicking DNAzyme can form a quadruplex structure and catalyze H_2O_2-mediated oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or luminol to produce a colorimetric or chemiluminescence signal, respectively.[118] Willner and co-workers developed a catalytic beacon for the detection of DNA by adapting a hairpin structure that contained a sequence complementary to the DNA target of interest in the loop region and a partial DNAzyme sequence embedded in the stem structure (Figure 2B).[119] Upon target DNA binding to the loop region, the hairpin structure opened up, resulting in the liberation and activation of the DNAzyme, which catalyzed the oxidation of ABTS to a colored product. This catalytic beacon was extended to the detection of telomerase activity by masking the DNAzyme sequence in one end of the hairpin structure and attaching a telomerase primer at the other end. In the presence of a telomerase and deoxyribonucleotide triphosphate (dNTP) mixture, telomerase extended the primer, resulting in an extended telomere that opened the beacon structure to activate the DNAzyme hidden within the stem region. This catalytic beacon permitted the detection of telomerase activity in HeLa cells with a detection limit of 500 HeLa cells. This approach was also applied to the detection of lysozyme[120] and ligases.[121] In both cases, an HRP-mimicking DNAzyme sequence was caged in the stem region of the hairpin structure.

Willner and co-workers further conjugated this catalytic beacon to a QD so that, in the presence of the target DNA, the DNAzyme was activated in the presence of hemin and H_2O_2 to oxdize luminol, resulting in a chemiluminescence signal.[122] Chemiluminescence resonance energy transfer (CRET) signal to the QD was then monitored in response to target binding and the subsequent catalytic events. This approach could facilitate multiplex detection by exploiting CRET signals generated by differently sized QDs. Similarly, HRP-mimicking activity of the thrombin−aptamer−hemin complex could produce a CRET signal with a conjugated QD to detect 200 pM thrombin.[123]

Another DNAzyme inactivation approach involves the use of a DNA strand to block the formation of an active DNAzyme.[124,125] The beacon was designed by splitting a DNAzyme sequence into two portions which were then linked with additional nucleotides in the middle. The middle sequences were hybridized with a blocker DNA that was complementary to the target DNA (Figure 2C). Assembly of the DNAzyme was inhibited due to the formation of a rigid duplex in the middle of the probe.[124] The blocker DNA dissociated from the beacon in the presence of the target DNA, which regenerated the formation of a catalytically active DNAzyme. Lysozyme has also been detected using this format, by designing the blocker DNA to contain a lysozyme aptamer.[125] Alternatively, a catalytic beacon was designed by dividing an HRP-mimicking DNAzyme into two subunits that are inactive on their own, but assemble into an active DNAzyme in the presence of hemin. Subunits were modified with sensor arms that recognize the target. The presence of the target inhibited the assembly of two subunits and led to no bioluminescence signal. In the absence of the target, the DNAzyme could form and a signal could be generated.[126]

The design of multicomponent nucleic acid enzymes (MNAzymes) represents another important strategy to construct catalytic beacons (Figure 3).[127−133] The fabrication

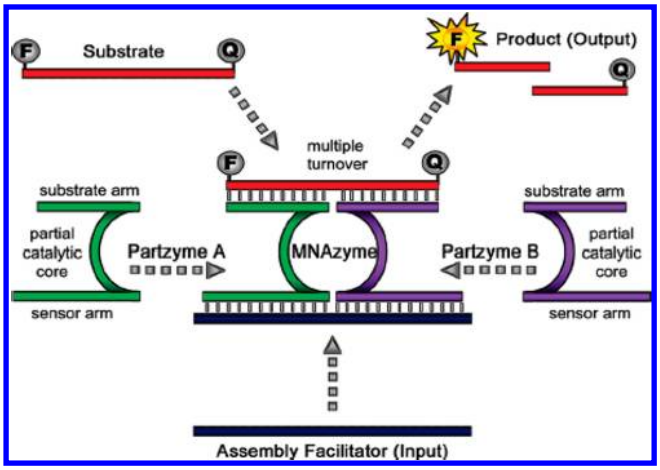

Figure 3. DNAzyme-based catalytic molecular beacons that depend on assembly. The presence of a target facilitates the assembly of "partzymes" A and B to form the active MNAzyme. Reprinted from ref 128. Copyright 2010 American Chemical Society.

of MNAzymes starts with splitting a parent DNAzyme sequence into two subunits. Each subunit contains a partial catalytic core sequence and a portion of the substrate binding domain. The subunits themselves do not show catalytic activity. A sensor arm that can recognize the target DNA is then incorporated into each subunit. In the absence of the target DNA, the substrate binding sequence of each subunit is so short that substrates cannot hybridize with two subunits. When the target DNA is introduced, two subunits self-assemble together through the hybridization of two senor arms with a single target DNA, re-forming and activating the DNAzyme. The MNAzymes were initially constructed by rational design utilizing specific tertiary structures within DNAzymes such as G-quartets or hairpins. Mokany and co-workers demonstrated a general approach where partial enzyme pairs were generated by splitting the catalytic core domain of a DNAzyme at different sites.[128] Each pair was then used to fabricate MNAzymes, and the partial enzymes that lead to the highest activity of MNAzymes were usually chosen. This strategy does not necessarily require the knowledge of functional domains of the DNAzymes.

DNAzymes that have been used to build MNAzymes include the HRP-mimicking DNAzyme[130−133] and substrate-cleaving DNAzymes, such as Mg^{2+}-dependent,[127,129] 10−23 and 8−17 DNAzymes.[128] DNAzyme-cleaving substrates are ssDNA probes that are modified with a fluorophore−quencher pair at

the 3′- and 5′-ends. The cleavage of substrates by MNAzymes results in the separation of the quencher and fluorophore to enhance the fluorescence signal. Diaminodenzidine tetrahydrochloride was used as the substrate for the MNAzyme with peroxidase activity to generate a colorimetric readout signal in the presence of a target DNA.[130] Willner and co-workers designed a quasi-circular DNA as the substrate which contained two blocked HRP-mimicking DNAzymes.[131] The cleavage of the substrate releases HRP-mimicking DNAzymes that can then generate a colorimetric signal.

MNAzymes can be applied as biosensors for the homogeneous detection of both RNA and DNA. The sensitivity of this approach is dependent on the activity of the MNAzymes, substrate designs, and turnover rate. Under optimized conditions MNAzymes can detect 1 pM DNA.[127,131] The MNAzyme-based biosensors can discriminate between perfectly matched targets and targets with a single mismatch.[129,130,132,133] This approach has been used for the detection of DNA from *Salmonella* and *Mycobacterium* DNA[134] and from genetically modified organisms.[135] However, one drawback of MNAzymes is their lower turnover rate, therefore requiring 1−7 h to complete, as compared to the use of analogous protein enzymes (∼1 h).

Similar to DNAzymes, protein-based enzymes can also be designed into catalytic beacons. These catalytic beacons usually involve conjugation of enzymes with a DNA strand that is linked to an enzyme inhibitor. In the absence of a DNA target, the interaction between the enzyme and its inhibitor forms an analogous hairpin structure, inhibiting the activity of the enzyme. The binding of a DNA target leads to the opening of the hairpin structure, restoring the activity of the enzyme. For instance, Saghatelian and co-workers introduced an intrasterically regulated semisynthetic enzyme that contains a covalently associated inhibitory ssDNA to inhibit enzymatic activity.[136] In the presence of the target DNA, hybridization results in a change of conformation of the inhibitory DNA so that it is liberated from the enzyme's active site (Figure 4). This

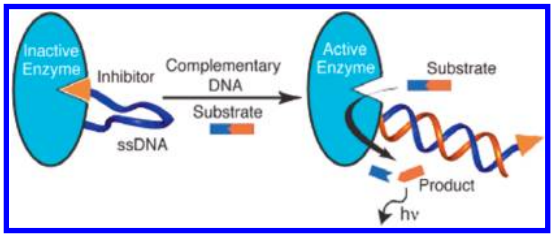

Figure 4. Enzyme-regulated catalytic beacons. Regulation of *cereus* neutral protease through a conformational change of the DNA inhibitor.[136] Reprinted from ref 136. Copyright 2003 American Chemical Society.

activates the protease activity of the enzyme to cleave the labeled substrate. Activity is monitored by an increase in fluorescence to detect as little as 10 pM DNA. This work pioneered the development of sensors that functioned on the basis of regulating the enzymatic activity through an ssDNA-conjugated inhibitor to detect analytes that would bind to the ssDNA.

Willner and co-workers developed a novel "beacon-type" sensor that consisted of thrombin and a DNA-linked thrombin aptamer that inhibits the enzymatic activity of thrombin.[137,138] The catalytic activity of thrombin for hydrolyzing nonfluorescent bis(*p*-tosyl-Gly-Pro-Arg) to fluorescent rhodamine110 was inhibited by complexation with the aptamer. Upon hybridization with the target DNA, the double-strand duplex distorted the thrombin−aptamer complex, resulting in the activation of thrombin for the hydrolysis of the substrate and an enhanced fluorescence signal. This approach has also been used to modulate the natural coagulation activity of thrombin. DNA detection was monitored by the clotting of fibrinogen, which did not require any chemical modification.[139,140] Using this technique, non nucleic acid targets such as insulin[141] and IgE[142] have also been detected.

3. HOMOGENEOUS BINDING ASSAYS INCORPORATING ISOTHERMAL AMPLIFICATION

The use of nucleic acids as probes in homogeneous binding assays allows for amplified detection by using polymerases and nucleases to catalyze reactions involving nucleic acid substrates. Polymerases achieve the amplification of the detection usually by producing repeated target units, while nucleases produce amplified signals often by cyclic cleaving of signal-reporting probes. Although real-time polymerase chain reaction (RT-PCR) is one of the most widely used amplification methods for detection of nucleic acids, PCR requires thermal cycling to achieve amplification. In this section, we focus on homogeneous binding assays that are conducted under isothermal conditions.

3.1. Rolling Circle Amplification for Homogeneous Binding Assays

Rolling circle amplification (RCA) is a powerful isothermal DNA amplification method which has been widely used as a signal amplification tool for DNA or RNA genotyping and protein microarrays.[143−148] RCA is able to extend a primer with a circular DNA template to form a long ssDNA product with thousands of repeated units complementary to the circular template. Figure 5A shows the use of RCA for DNA analysis by using padlock probes.[143] Both ends of a DNA padlock probe are juxtaposed by the specific hybridization to a target DNA sequence and joined together by a DNA ligase to form a circular DNA. The resulting circular DNA serves as the template for an RCA reaction to produce a long ssDNA. Direct monitoring of the RCA products in real time, without separation, has been achieved using molecular beacons,[149,150] molecular zippers,[151] and intercalating fluorescent dyes.[143,152]

In addition to DNA analysis, RCA has also been applied to the homogeneous analysis of microRNA.[153−155] Several strategies have been developed to enhance the specific binding between microRNA and DNA padlock probes. For example, Fan and co-workers developed a dumbbell-probe-mediated RCA strategy for highly sensitive microRNA detection (Figure 5B).[154] The dumbbell probe contained a stem−loop structure in the middle of the padlock. The stem−loop structure could enhance sensitivity by intercalating the multiple SYBR Green (SG) I dyes to each stem unit. It also facilitated high specificity by imbedding high conformational constraint in the padlock probe. In the presence of a target microRNA, the two ends of the dumbbell probe were brought together by the target and ligated to form circular DNA that was amplified by RCA and detected by monitoring the fluorescence signals from the SG dyes. A dynamic range from 1 fM to 100 nM was achieved for has-miR-21, a potential biomarker for colon cancer. Other RCA strategies for the homogeneous detection of microRNA include the use of T4 RNA ligase[153] and chemical ligation.[155]

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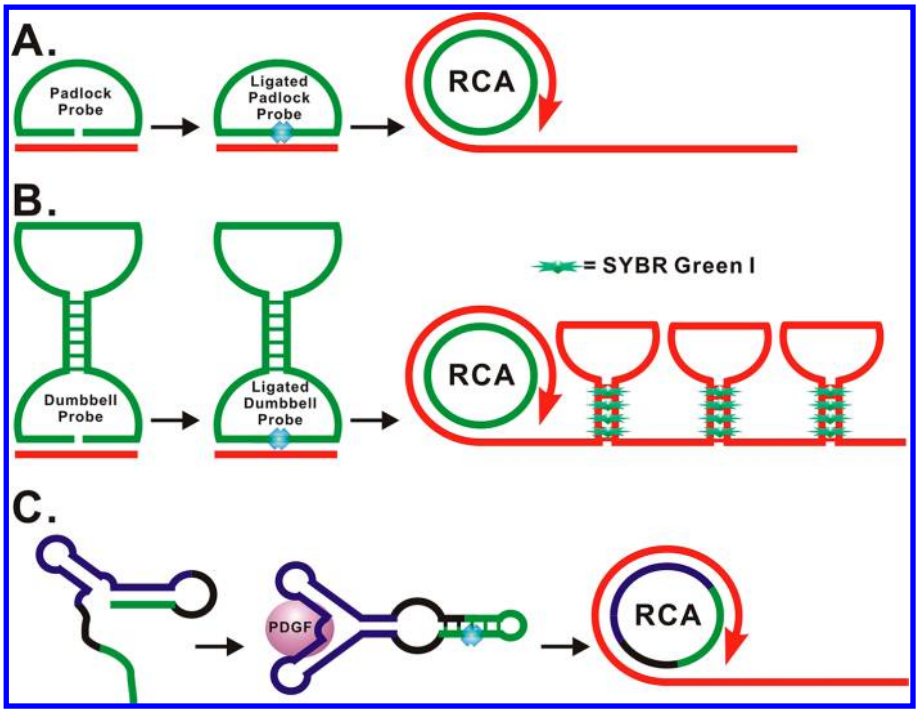

Figure 5. Homogeneous binding assays using rolling circle amplification (RCA): (A) padlock probe for RCA analysis of DNA;[143] (B) dumbbell padlock probe for RCA analysis of microRNA;[154] (C) binding of platelet-derived growth factor (PDGF) to its aptamer switches the aptamer conformation and triggers RCA for analysis of PDGF.[157]

Applications of RCA to protein detection have been achieved by incorporating protein binding to nucleic acid probes.[156−158] Ellington and co-workers designed a conformation-switching aptamer to facilitate the detection of PDGF using real-time RCA (Figure 5C).[157] The conformation-switching aptamer circularized upon the binding with a PDGF molecule. The circular DNA served as the template for the subsequent RCA reaction. Detection of the amplified DNA was translated to the ability to detect as little as 0.4 nM PDGF.

3.2. Strand-Displacement Amplification for Homogeneous Binding Assays

Strand-displacement amplification (SDA), as an isothermal, in vitro DNA amplification technique, was first described by Walker et al.[159,160] SDA is driven primarily by the primer extension polymerization reaction, and large numbers of repeated DNA units can be produced through the SDA process. Detection signals can be generated and amplified by monitoring the formation of the extended DNA using intercalating fluorogenic dyes,[161−163] Taqman probes,[164] molecular beacons,[165−172] catalytic beacons, or nanomaterials.[173−175] When combined with DNA-nicking enzymes, the detection signal can be further enhanced, making it possible to achieve exponential amplification without thermal cycling.[161,166] Because of the amplification power of SDA, many efforts have been made to adapt SDA into homogeneous binding assays for nucleic acids and proteins.

Wang and co-workers demonstrated the use of SDA for the homogeneous detection of nucleic acids (Figure 6A).[166] They combined SDA with a molecular beacon. In the absence of a target, the stem part of the molecular beacon was unable to anneal with the primer to induce a polymerization reaction, and no SDA was initiated. However, in the presence of a target DNA, the stem sequences of the molecular beacon were separated upon hybridization to the target DNA sequence, and

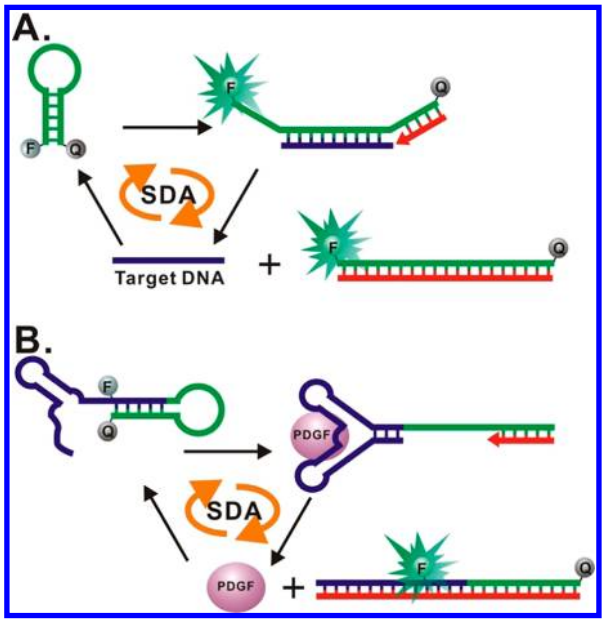

Figure 6. Homogeneous binding assays using non-nicking strand-displacement amplification (SDA): (A) detection of nucleic acids using non-nicking SDA;[166] (B) detection of platelet-derived growth factor (PDGF) using non-nicking SDA.[171]

the fluorescence signal was turned on. Along with this signal generation, the open stem of the beacon was then annealed to a primer. The primer extension polymerization reaction resulted in the displacement of the target DNA. The displaced target then triggered another round of the SDA reaction, and the detection signal was amplified during this process. A detection limit of 6.4 fM was achieved for nucleic acid. Zhang and co-workers measured telomerase activity in cancer cells using the same detection format except that telomerase was used for SDA

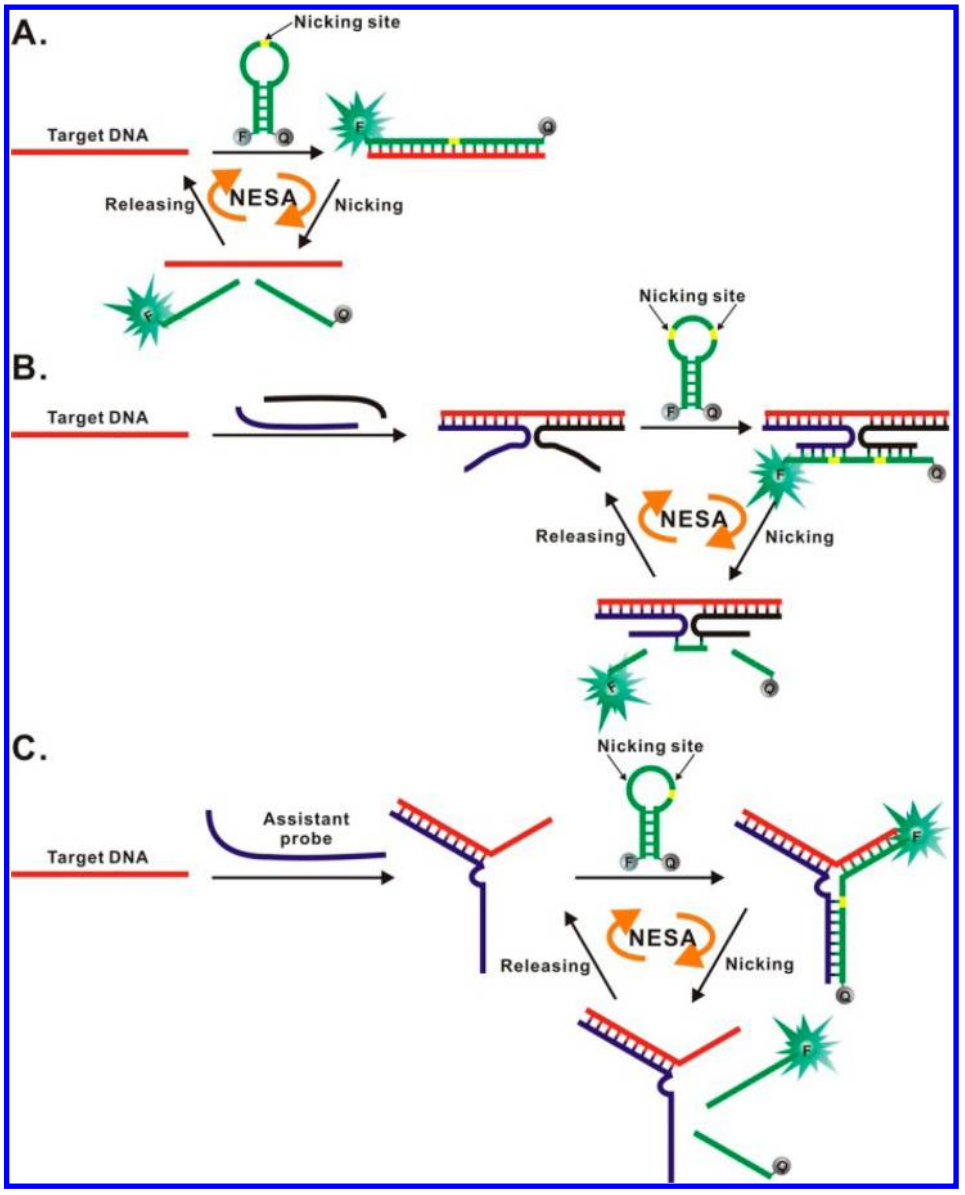

Figure 7. Nicking endonuclease-assisted amplification assays: (A) nicking enzyme signal amplification (NESA) assay using a molecular beacon as the signaling probe;[180] (B) introduction of the binary probe to the nicking endonuclease-assisted amplification assay for DNA;[190] (C) introduction of the Y-shaped junction probe to the nicking endonuclease-assisted amplification assay for DNA.[191]

instead of polymerase.[168] When DNAzyme catalytic beacons were used instead of molecular beacons, colorimetric signals were able to be generated and amplified by SDA for the homogeneous detection of nucleic acids, with a detection limit of 1 pM.[124]

SDA has also been applied to protein detection by using structure-switching aptamers (Figure 6B). Yu and co-workers[171] built a fluorescently labeled aptamer beacon for PDGF. Binding of PDGF to the aptamer induced a conformational change of the aptamer and opened the stem part of the aptamer beacon. The open stem sequence of the aptamer beacon was annealed with a primer to trigger the subsequent SDA reaction. By monitoring the fluorescence generated from the SDA reaction, subnanomolar levels of PDGF could be detected.

Many nanomaterials can be used as universal quenchers for multiple fluorescent dyes, enabling their applications to multiplexed homogeneous binding assays. For example, a highly sensitive assay for multiple microRNA was developed using GO as the quencher and using SDA for signal amplification.[175] Three ssDNA probes for the target microRNA were fluorescently labeled with three different dyes and then mixed with GO. DNA probes were strongly adsorbed by GO, and fluorescence signals were quenched. In the presence of the target microRNA molecules, the hybridization between the targets and the probes released the dye-labeled DNA probes from GO by forming the DNA−RNA duplex and thus turned on the fluorescence signal. The formation of the DNA−RNA duplex also exposed a short complementary sequence at the end of the DNA probe for primer binding. An SDA reaction was then triggered from the primer regionn and the target microRNA was released from the DNA−RNA duplex. The released RNA could enter another round of hybridization and SDA. The regeneration of the target RNA molecules after each round of SDA resulted in the linear amplification of detection signals. Other nanomaterials, including AuNPs, carbon nano-

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tubes, and carbon nanoparticles, can potentially be used to combine with SDA for multiplexed assays.

3.3. Nicking Endonuclease-Assisted Amplification Assays

Nicking endonucleases (NEases) recognize specific nucleotide sequences in double-stranded DNA (dsDNA) and catalyze the cleavage of only one strand of a dsDNA at fixed position relative to the recognition sequence.[176−179] This unique cleaving feature of NEase has been adopted to develop homogeneous binding assays for nucleic acids and proteins.

3.3.1. Assays for Nucleic Acids. NEase-assisted amplification assays have been developed mainly for homogeneous detection of DNA. Assays commonly use NEases to cyclically cleave a signaling probe and recycle the target DNA, resulting in amplified detection signals. A DNA signaling probe is usually designed to comprise three major components: a target-recognizing element, a signaling element, and a partial NEase recognition sequence. The hybridization of the target DNA to the signaling probe leads to the formation of a complete NEase recognition sequence, triggering the cleavage of the probe by NEase. The NEase cleavage of the probe induces the signaling element to generate detection signals, and the target DNA is concurrently recycled for the next round of events. Therefore, a single target DNA can result in cleavage of many probes, achieving amplified DNA detection.

Xie and co-workers[180] developed a nicking enzyme signal amplification (NESA) assay using a molecular beacon as the signaling probe (Figure 7A). The loop of the molecular beacon is designed to consist of a complementary sequence of the target DNA and one strand of the NEase recognition sequence. When the molecular beacon hybridizes with the target DNA that contains the other strand of the NEase recognition sequence, a complete NEase recognition site is formed, initiating the NEase cleavage of the loop of the molecular beacon. The nicking cleavage of the loop destroys the hairpin structure of the molecular beacon, separating the fluorophore from the quencher and restoring the fluorescence. In the meantime, the NEase cleavage also regenerates the DNA target from the target−probe complex, resulting in a cycling amplification. The assay was able to detect as low as 6.2 pM DNA target. The assay was further combined with RCA to detect targeted-induced products of RCA. The detection limit was further improved to 85 fM.

In addition to molecular beacons, catalytic beacons were also used as signaling probes. Catalytic beacons cage a DNAzyme into a hairpin structure, therefore deactivating the function of the DNAzyme.[114,119] Similar to the molecular beacon design, the loop of catalytic beacons was designed to comprise one strand of the NEase recognition sequence and a sequence complementary to the target DNA.[181−183] Target-induced NEase cleavage liberates the DNAzyme from the hairpin structure, restoring the function of the DNAzyme. A HRP-mimicking DNAzyme was often used to generate a colorimetric signal by catalyzing the H_2O_2-mediated oxidation of $ABTS^{2-}$ (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)).[126,184,185]

Oligonucleotide-modified AuNPs have been applied to colorimetric DNA detection.[27,186,187] The color change from red to purple can be observed upon analyte-guided aggregation of AuNPs. This AuNP-based DNA detection system was utilized to detect DNA target-induced NEase cleavage.[188] A DNA linker that can direct the aggregation of oligonucleotide-modified AuNPs was designed to comprise a complementary sequence of the target DNA. The hybridization of the target DNA triggers NEase cleavage of the DNA linker. The cleavage of the DNA linker lessens AuNP aggregation, which can be observed by a color change. The FRET between QDs and a fluorophore was also used to detect the cleavage of the DNA linker in a similar approach.[189]

The above NEase-assisted amplification assays have a common drawback, which is the need to include an NEase recognition sequence in the target DNA. Therefore, the application of these assays is limited. Probes with branched DNA structures were devised to overcome this shortcoming. A binary probe was designed to consist of two DNA strands, each containing a reporter-binding arm and a target-binding arm (Figure 7B).[190] A molecular beacon was used as the signal reporter. The hybridization of the target DNA to the probe leads to the formation of a quadripartite complex, in which two DNA strands of the probe serve as adaptors to connect the target DNA and molecular beacon. Because each reporter-binding arm contains an NEase recognition sequence, the formation of the quadripartite induces NEase cleavage of the molecular beacon, generating a fluorescence signal. This binary probe was applied to detecting both DNA and RNA. A Y-shaped junction structure was also applied to serve as the signal probe (Figure 7C).[191] The junction probe was composed of a molecular beacon and an assistant probe. The junction probes operate via a concept called the template-enhanced hybridization process, where the molecular beacon and the assistant probe do not hybridize but can be made to anneal to each other in the presence of a target DNA.[192] The assistant probe was designed to contain an NEase recognition sequence so that the addition of a target DNA results in formation of a ternary complex, enabling NEase cleavage of the molecular beacon.

NEase-assisted amplification assay can be used to detect DNA products of other amplification techniques to further improve the detection sensitivity.[180,193,194] Zhou and co-workers developed an ultrasensitive DNA assay involving a cascade enzymatic signal amplification of Afu flap endonuclease and NEase.[194] This cascade enzymatic signal amplification strategy consisted of three steps: Afu flap endonuclease-assisted cycling amplification, flap ligation, and NESA. A downstream probe and an upstream probe were designed specific to the target DNA. The hybridization of the target DNA with the two probes formed a ternary complex in which the upstream probe−target DNA duplex overlapped the downstream probe−target DNA by one base pair. The 5′ flap of the downstream probe was then cleaved by Afu flap endonuclease, thereby dissociating the cleaved downstream probe from the target DNA. Another intact downstream probe could then hybridize with the target DNA, leading to the next cleavage by Afu flap endonuclease. Consequently, one target DNA could result in several thousand flaps. Then the loop region of a molecular beacon was used as a template for the ligation of the flap with a predesigned 5′-phosphorylated oligonucleotide, triggering the process of NESA. By utilizing two steps of amplification, this assay detected femtomolar target DNA concentrations.

3.3.2. Assays for Amino Acids and Proteins. The application of NEase-based amplification to amino acid and protein detection is still limited. One strategy for developing amino acid and protein assays focused on constructing DNAzyme-based catalytic beacons and aptasensors that release the DNA target of NESA upon the addition of analytes. An L-histidine-dependent DNAzyme was used to design a catalytic beacon, in which the DNAzyme is linked with its substrate.[195] When L-histidine is introduced, the DNAzyme catalyzes the

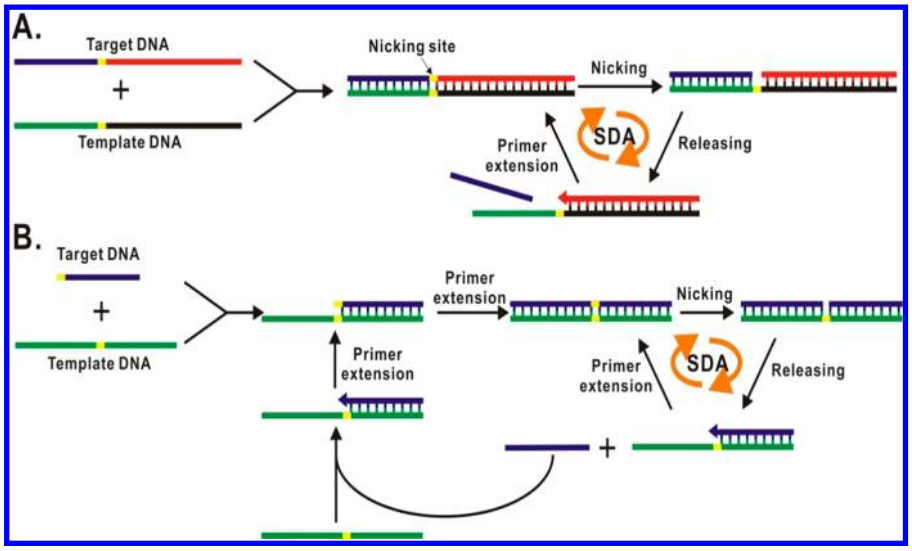

Figure 8. Homogeneous binding assays based on nicking-assisted strand-displacement amplification (SDA): (A) linear amplification using nicking-assisted SDA;[161] (B) exponential amplification using nicking-assisted SDA.[161−163]

cleavage of its substrate. The cleaved partial substrate is then released from the catalytic beacon, serving as the target DNA for NESA. Therefore, L-histidine-induced cleavage by the DNAzyme is amplified and detected by NESA. To detect thrombin by NEase-based amplification, a thrombin aptasensor was constructed into a hairpin structure.[196] The binding of thrombin opens the hairpin structure, releasing a DNA domain that can hybridize to the molecular beacon of NESA. Therefore, NESA is triggered, enabling the amplified detection of thrombin. A detection limit of 2 pM was achieved for thrombin.

3.4. Homogeneous Binding Assays Making Use of Both SDA and NEase

NEases have been combined with SDA to develop isothermal DNA amplification techniques (nicking-assisted SDA). This is achieved by incorporating NEase to regenerate primers for multiple rounds of SDA reactions.[159,161,167] For example, Galas and co-workers[161] developed a class of isothermal reactions for amplifying DNA both linearly (Figure 8A) and exponentially (Figure 8B) by coupling the SDA with NEase. A template DNA sequence was designed to hybridize with the target DNA and to afford a specific single-strand nicking site in the middle of the target DNA. The use of the NEase and the DNA polymerase allowed the cycles of SDA to continue. With each cycle producing a new copy of DNA, the detection signal was amplified linearly by monitoring the formation of newly extended DNA with SYBR Green (Figure 8A). To achieve the exponential signal amplification, a unique DNA template was designed to contain two copies of the same DNA sequence in tandem that were complementary to the primer, but separated by the complement of the NEase recognition site and a four-base spacer. Upon cleaving by the NEase, two primers were generated at the same time, each of which was able to trigger a second round of primer extension reaction separately. As a result, detection signal was amplified exponentially. The authors were able to achieve a 10^8-fold enhancement within a few minutes. Li and co-workers applied this exponential amplification strategy for microRNA analysis, and a detection limit of 0.1 zmol was achieved.[162] Zhang and co-workers applied the same strategy to PDGF analysis by using the structure-switching PDGF aptamer. A detection limit of 1 pM was achieved with a dynamic range of 5 orders of magnitude.[163] This strategy has also been applied to promote the aggregation of DNA-functionalized AuNPs, enabling colorimetric detection of DNA with ultrahigh sensitivity.[174]

For many homogeneous binding assays adapting nicking-assisted SDA, molecular beacons and catalytic beacons were used to generate fluorescence or colorimetric detection signals.[167,169,197,198] A beacon-assisted amplification strategy was developed for DNA analyses by combining the use of nicking-assisted SDA with molecular beacons.[167,168] The binding of the target DNA to its complementary sequence in the molecular beacon opened the stem region of the beacon to generate the fluorescence signal and initialize an SDA reaction from the primer binding region at the 3′-end. This SDA reaction regenerated the target DNA and also produced a long DNA duplex beacon containing a specific nicking site. The target DNA was released to trigger another round of SDA, and the duplex beacon was cleaved by NEase to release a newly formed ssDNA containing the same sequence of the target DNA. Since two cycles of SDA were generated simultaneously, exponential amplification was achieved. Willner and co-workers developed a colorimetric assay for the detection of M13 phage ssDNA.[197] A G-quadruplex-based catalytic beacon was produced as an end product of SDA, and as low as 10 fM M13 phage DNA was detected. The displaced DNA from SDA has also been used as a promoter for the aggregation of AuNPs, also enabling the colorimetric detection of nucleic acids. To enhance the detection specificity for nucleic acids, T4 DNA ligase was used to facilitate the nicking-assisted SDA assays.[198] Jiang and co-workers developed a ligation-mediated SDA assay for label-free genotyping of SNPs.[173] The presence of the target DNA triggered a ligation of two DNA hairpins and further induced a nicking-assisted SDA which released thousands of copies of HRP-mimicking catalytic beacons enabling label-free chemiluminescence detection.

3.5. Exonuclease III-Assisted Amplification Assays

As an enzyme of the 3′−5′ exonuclease family, exonuclease III (Exo III) exhibits 3′ to 5′ exodeoxyribonuclease activity.[199] Exo III shows many unique properties: (i) Exo III catalyzes stepwise removal of mononucleotides from the 3′-end hydroxy of duplex DNA, thus enabling selective hydrolysis of one single-stranded DNA in duplex DNA; (ii) DNA strands with a blunt or

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recessed 3′-end are preferred for Exo III cleavage, while single-stranded DNA and the 3′-protruding end of double-stranded DNA are resistant to cleavage; (iii) Exo III does not require a specific recognition site to function. These properties have evoked the use of Exo III to develop a variety of amplification platforms for homogeneous detection of nucleic acids and proteins.

3.5.1. Assays for Nucleic Acids. The Exo III-based amplification assays for DNA detection usually involve biocatalytic recycling of the target DNA by Exo III. A DNA signaling probe is first designed to hybridize with the target DNA. The hybridization of the target DNA induces the enzymatic cleavage of the probe by Exo III. The cleavage of probes thus generates the detection signal and concurrently regenerates the target DNA for the next cleavage. The cycling cleavage of probes leads to amplified DNA detection.

The development of Exo III-based assays for DNA detection was mainly focused on the design of different signaling probes. Plaxco and co-workers[200] developed an assay termed Exo III-aided target recycling that uses a molecular beacon as the signaling probe (Figure 9A). The molecular beacon was modified with a fluorophore at its 5′-end and a quencher at an internal position. The formation of the hairpin structure placed the fluorophore in close proximity to the quencher, leaving a single-stranded overhang at the Exo III-resistant 3′-protruding end. As a result, Exo III is not active on the molecular beacon. When the molecular beacon encountered the DNA target, it formed into a double-stranded structure with a blunt 3′-end, thereby enabling the selective cleavage of the molecular beacon from its 3′-end. This cleavage was able to liberate the fluorophore from the quencher and recycle the target DNA. The target DNA could then hybridize with another molecular beacon, starting cyclic cleavage. When the assay was conducted at 37 °C, a 10 pM detection limit was achieved within 30 min. However, significant digestion of free molecular beacons by Exo III was found, which increased the background fluorescence and greatly undermined the detection limit of the assay. To circumvent this problem, the same group incorporated digestion-resistant locked nucleic acids (LNAs) in the 5′ stem sequence of the molecular beacon.[201] The detection limit of the assay was further improved to 30 fM by using this chimeric LNA−DNA molecular beacon.

In addition to the traditional molecular beacons, a linear molecular beacon was recently reported to serve as the signaling probe.[202] The linear molecular beacon was prepared by conjugating a fluorophore and quencher to the 3′-terminal and penultimate nucleotides of a single-stranded oligonucleotide, respectively. However, the absence of a hairpin structure in the linear molecular beacon could diminish the specificity of analysis compared to the use of a traditional molecular beacon. A DNA-displacing probe was also used to develop an Exo III-based amplification assay.[203] A displacing probe is a stable DNA duplex formed by two complementary oligonucleotides with different lengths.[205] A fluorophore and a quencher were attached to the 5′-end of the longer strand and 3′-end of the shorter strand, respectively, allowing quenching of fluorescence. To avoid the Exo III cleavage, the 3′-end of the quencher strand was extended to form an Exo III-resistant protruding end. In the presence of the target DNA, the quencher strand was displaced and the target DNA hybridized with the fluorophore strand, inducing the selective cleavage of the fluorophore strand by Exo III and regenerating the target DNA.

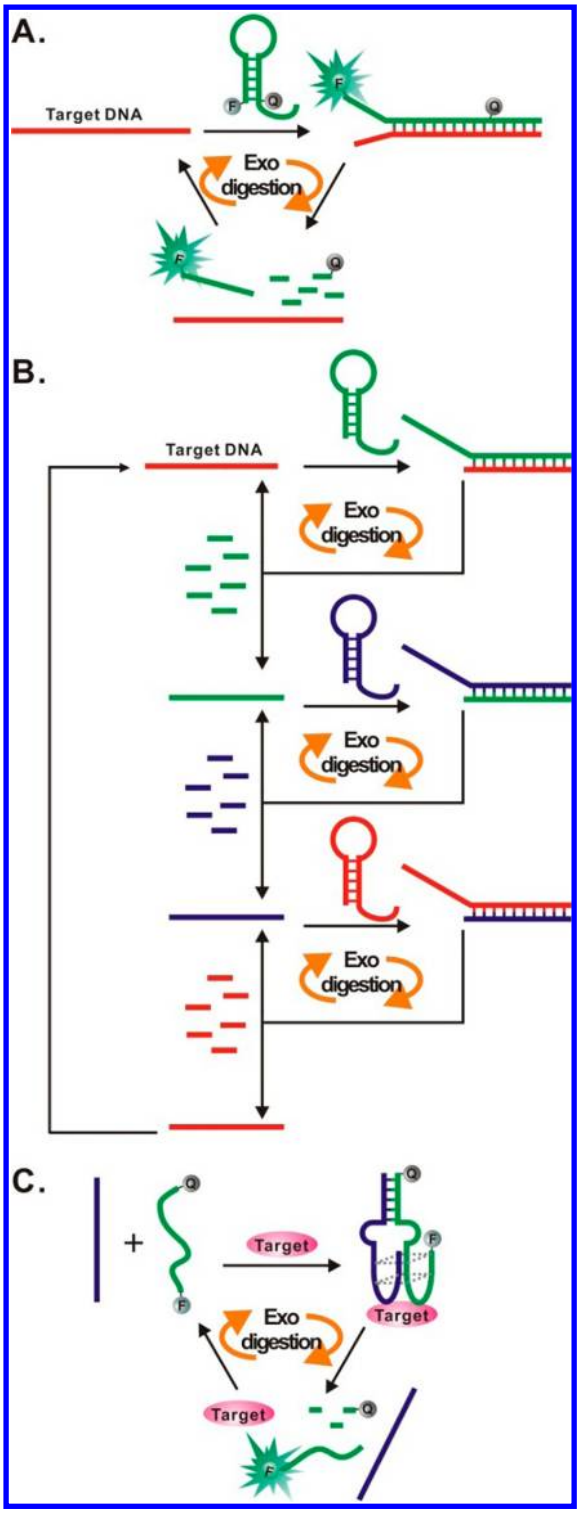

Figure 9. Exonuclease III-assisted amplification assays: (A) Exo III-assisted target recycling assay using a molecular beacon as the signaling probe;[200] (B) Exo III-assisted cascaded recycling assay for label-free DNA detection;[219] (C) Exo III-assisted aptamer sensor for thrombin detection.[225]

The use of a displacing probe allowed the detection of picomolar DNA with improved selectivity.

In addition to organic fluorescent dyes, QDs functionalized with quencher-conjugated oligonucleotides were used as probes to generate a fluorescence signal.[206] The binding of the target DNA to the probes led to the cyclic cleavage of quencher-

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conjugated oligonucleotides, resulting in a corresponding increase in the fluorescence of the QDs. Multiplexed detection of DNA was also demonstrated, taking advantage of size-controlled luminescence properties of QDs.[207−209]

Recently, SWCNTs and GO have been recognized as efficient quenchers for a variety of fluorophores.[37,38,82,210,211] In addition, SWCNTs and GO have a much higher binding affinity to ssDNA than to single nucleotides. Therefore, SWCNTs and GO were utilized to quench the fluorescence of fluorophore-conjugated ssDNA, serving as effective signaling probes for Exo III-based amplification assays.[212−214] An ssDNA was first conjugated with a fluorophore at its 5′-end or at an internal position. SWCNTs or GO was then introduced to bind with the fluorescently labeled ssDNA, and fluorescence was quenched. The hybridization of the target DNA induced the enzymatic cleavage of fluorescently labeled ssDNA into single nucleotides, placing the fluorophore away from the SWCNTs or GO. The use of SWCNTs or GO makes probe designing easy and greatly decreases the background due to efficient quenching.

An ssDNA labeled with a fluorophore at its 5′-end was used as the signaling probe,[216] taking advantage of the effect of the molecular size on fluorescence polarization.[215] The hybridization of the target DNA with the probe selectively triggered cleavage of the probe from its 3′-end, liberating the fluorophore with several nucleotides. The significant shortening of the fluorophore-labeled oligonucleotide resulted in a substantial decrease in fluorescence polarization values, which are used for quantitation.

A DNA linker that can guide the aggregation of oligonucleotide-functionalized AuNPs was used for colorimetric DNA detection.[204] The hybridization of the target DNA with the DNA linker triggered the cyclic cleavage of the DNA linker by Exo III. Oligonucleotide-functionalized AuNPs were applied to the detection of the cleavage of the DNA linker, which can be observed by a color change.

Label-free probes have also been devised by deactivating functional DNA in a secondary structure or through hybridization with a blocker DNA.[217−219] The target-induced enzymatic cleavage released the functional DNA from the probes, thereby restoring its activity and generating a detection signal. Bi and co-workers designed three kinds of unlabeled molecular beacons (MB1, MB2, and MB3) (Figure 9B).[219] MB1 contained an HRP-mimicking DNAzyme sequence, and the activity of the DNAzyme was inhibited by the formation of a hairpin structure. MB2 and MB3 served as transduction components to enable cascade recycling amplification. In the presence of a perfectly complementary target, MB1 was opened and subsequent Exo III cleavage released the DNAzyme sequence. The DNAzyme sequence then served as a trigger for cleavage of MB2, releasing another DNA sequence as the trigger for cleavage of MB3. The cleavage of MB3 then released the target DNA, accomplishing a cascade recycling amplification where the released DNAzyme was used to generate a signal for detection. Another label-free probe was designed using the blocker DNA to prevent the functional DNA from forming the G-quadruplex.[217] The hybridization of the target DNA with the probe enabled Exo III to digest the blocker DNA, activating the G-quadruplex. *N*-Methylmesophorphyrin IX (NMM) was then used to selectively interact with the G-quadruplex, giving rise to a fluorescence enhancement.

3.5.2. Assays for Proteins. Exo III-based amplification assays for proteins are mainly limited to the detection of DNA-binding proteins and determining the activity of enzymes. Assays for DNA-binding proteins were developed on the basis of the capability of DNA-binding proteins to protect DNA-signaling probes from digestion by Exo III. The DNA-signaling probes are usually composed of two complementary oligonucleotides conjugated with different labels for signal generation. For example, Wang and co-workers developed an Exo III protection assay to detect DNA-binding proteins.[220] A FRET probe containing two complementary oligonucleotides was labeled in the middle with a donor and acceptor pair. The hybridization of the two oligonucleotides placed the donor and acceptor into close proximity, exhibiting a high FRET signal. The probe harbored one complete target protein binding site at either side of the FRET pair. In the presence of the target protein, the binding of the protein to the two binding sites inhibited Exo III digestion of the probe. In the absence of the target protein, the probes were not protected and were digested by Exo III; thus, the close proximity of the FRET pair was destroyed, leading to a low FRET signal. This assay could detect nanomolar concentrations of four different proteins. Alternatively, conjugation of two complementary oligonucleotides with a fluorophore and quencher pair or AuNPs was employed to construct signaling probes for detection of DNA-binding proteins.[221,222] Upon addition of protein, the fluorophore and quencher pair resulted in a decrease in fluorescence signal, while the AuNP conjugates led to a purple-to-red color change. Leung and co-workers designed a label-free luminescent switch-on probe for detection of transcription factor NF-κB.[223] The assay took advantage of a luminescent transition ruthenium complex which was switched on in the presence of dsDNA.

The development of Exo III-based assays for detection of enzymes was based on the impact of enzymatic activity on the DNA-signaling probes. Plaxo and co-workers designed a molecular beacon as a signaling probe for DNA detection and further applied it to the detection of telomerase activity.[201] The assay for telomerase detection was composed of two steps: elongation of telomeres by telomerase and the detection of elongated telomeres. A primer oligonucleotide was first subjected to elongation by telomerase, and the elongated telomeres were then hybridized with molecular beacons, triggering the Exo III cleavage of the molecular beacons from the 3′-end. The increase in fluorescence was used to measure the activity of telomerase. Min and Lee designed a signaling probe to directly detect the activity of Exo III. The signaling probe was prepared by conjugating a fluorophore to the 5′-end of a hairpin DNA structure. Because GO preferentially binds to ssDNA over dsDNA, the binding of the signaling probe to GO was limited. The presence of Exo III started the cleavage of the probes from the 3′-end, generating fluorescently labeled ssDNA that could be adsorbed to GO, quenching the fluorescence signal.[224]

Willner and co-workers developed an Exo III-based aptasensor for the amplified detection of thrombin (Figure 9C).[225] The thrombin aptamer was split into two subunits. Subunit 1 was labeled with a fluorophore and a quencher at its 3′- and 5′-ends, respectively. In the absence of thrombin, the two subunits existed in single-stranded configurations and subunit 1 was resistant to Exo III cleavage so that the fluorescence was quenched. Upon addition of thrombin, the thrombin binding assembled the two subunits into a G-quadruplex structure. The formation of the G-quadruplex structure led to hybridization of four complementary base pairs

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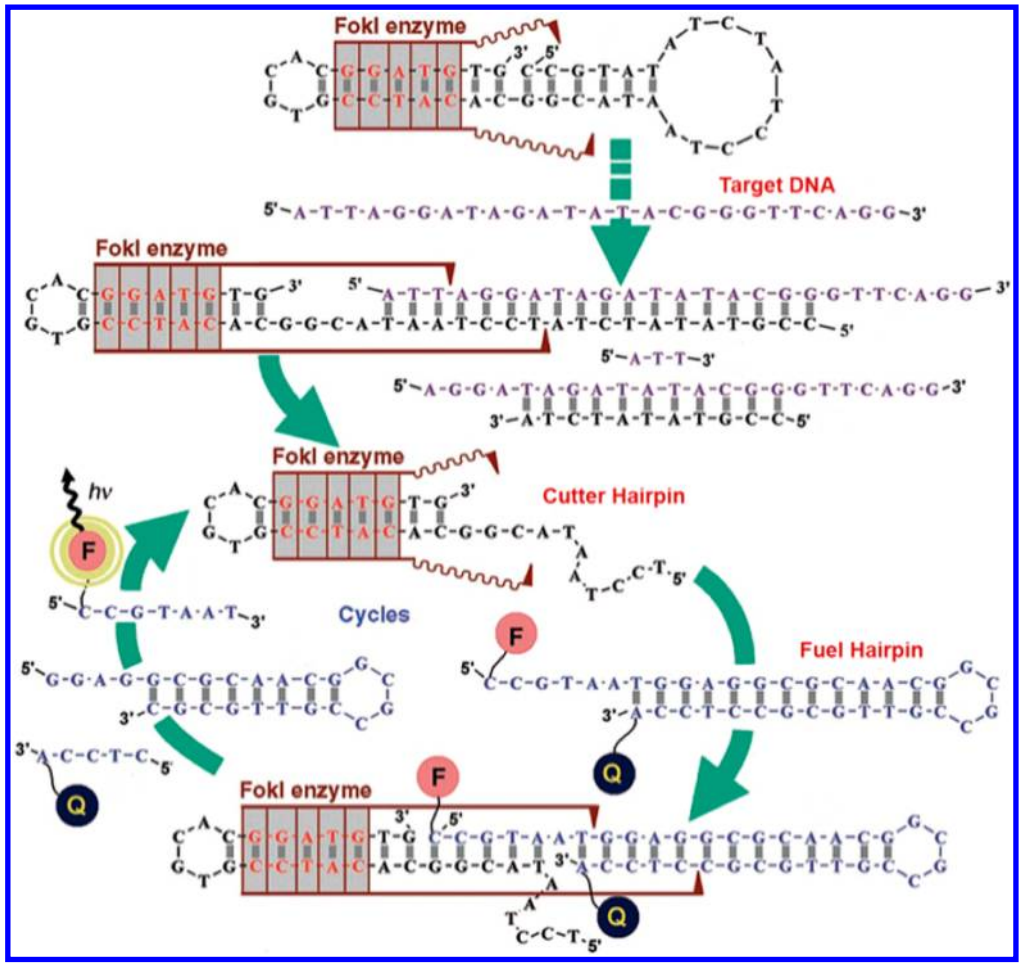

Figure 10. DNA machine making use of the *Fok*I enzyme for amplified DNA detection.[227] *Fok*I–DNA is used as a biocatalytic template for the cleavage of a fluorophore–quencher substrate (fuel) to yield a fluorescent product (waste). Reprinted with permission from ref 227. Copyright 2006 John Wiley & Sons, Inc.

starting at the 3′-end of subunit 1 and the 5′-end of subunit 2 into a duplex structure, which triggered the Exo III cleavage of the 3′-end of subunit 1. Exo III cleaved the quencher from subunit 1 to restore the fluorescence and destroyed the thrombin–aptamer subunit complex to regenerate thrombin for the next cleavage. This recycled cleavage reaction was repeated approximately 1000 times within 12 min, resulting in the detection of 89 pM thrombin.

3.6. Amplification Assays Based on Other Nucleic Acid-Cleaving Enzymes

In addition to NEase and Exo III, other nucleic acid-cleaving enzymes, including restriction endonucleases, RNase H, and apurinic–apyrimidinic-based endonuclease IV, were also applied for recycling targets and generating cyclic amplification. Restriction endonucleases are enzymes that catalyze the cleavage of specific double-stranded nucleotide sequences known as restriction sites. *Fok*I is a type II restriction endonuclease that catalyzes the cleavage of double-stranded DNA at a precise distance from the recognition site.[226] Willner and co-workers fabricated a "*Fok*I-based DNA machine" that enabled the amplified detection of target DNA (Figure 10).[227,228] A DNA sequence was designed to contain a hairpin structure at either end. The stem sequence of the hairpin at the 3′-end contained the restriction site for *Fok*I, and the hairpin at the 5′-end contained a target recognition domain. The hybridization of the 5′-end hairpin with the target DNA yielded a double-stranded complex which was then cleaved by *Fok*I. The *Fok*I cleavage resulted in a 3′-end hairpin with a single-stranded overhang at the 5′-end, which served as the cutter of the *Fok*I–DNA machine. A fluorophore–quencher-functionalized hairpin was designed to have a single-stranded overhang at the 3′-end, acting as fuel for the machine. Hybridization of the two overhangs triggered the cleavage of the stem sequence of the fuel hairpin by *Fok*I, and the cleavage restored the fluorescence and regenerated the cutter hairpin for the next cleavage. Therefore, the DNA machine allowed the amplified detection of the target DNA through the cyclic cleavage of the fuel to give rise to the fluorescence signal readout. In addition, the fuel sequence can be designed so that, after cleavage, the cleaved sequences could generate a new cutter for the DNA machine, facilitating further amplification. The machine was applied to the detection of a DNA mutant of the Tay–Sachs genetic disorder, and a detection limit of 10 fM was obtained. Another *Fok*I-based DNA machine was designed using an embedded HRP-mimicking DNAzyme to generate a chemiluminiscence signal for the amplified DNA detection.[228]

Y-shaped junction probes were designed as substrates for a restriction endonuclease.[192,229] The introduction of a nucleic acid target assembled a fluorophore–quencher-labeled reporter probe and an assistant probe into a ternary Y-shaped junction structure. The arm formed between the assistant probe and reporter contained a cleavage site for the *Bfu*CI restriction

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endonuclease. Enzymatic cleavage separated the fluorophore and quencher to generate a signal and also disassembled the junction structure, regenerating the target for more cyclic cleavage reactions. This approach has been applied to the amplified detection of both DNA and RNA targets.[192,229]

Another amplification assay using a restriction endonuclease involves two hairpin probes.[230] Probe A was designed to have a "toehold" (i.e., a sticky end consisting of typically 5−8 nucleotides in a DNA duplex) at the 3′-end, and probe B had a toehold at the 5′-end. The 5′-end of probe B was labeled with a fluorophore. Hairpin probes A and B coexisted in the absence of a target DNA and were adsorbed to the surface of a cationic polymer, resulting in efficient FRET from the polymer to the fluorophore. In the presence of a target DNA, hybridization of the target DNA with probe A triggered the hybridization with probe B through toehold-mediated strand displacement. Concurrently, the target DNA was released, resulting in the cyclic hybridization of probes A and B. The hybridization of probes A and B formed a complete restriction site of the *Hae*III restriction endonuclease, enabling the cleavage of the A−B duplex. Cleavage produced short fluorescently labeled DNA fragments that moved away from the surface of the polymer, thereby reducing the FRET signal. The decrease in FRET was used to detect the target DNA.

Unlike restriction endonucleases, RNase H is an endoribonuclease that specifically catalyzes the cleavage of the phosphodiester bonds of RNA in the DNA−RNA heteroduplex.[231] Therefore, the use of RNase H for amplified DNA detection requires designing of RNA-based or RNA-containing probes. Both RNA and chimeric DNA−RNA−DNA molecular beacons have been used as probes.[232,233] The hybridization of a target DNA with a loop of molecular beacons formed a DNA−RNA heteroduplex, leading to the cleavage of the loop of molecular beacons and recycling of the target DNA. Therefore, the target DNA resulted in repeated cleavage of many molecular beacons. In addition to fluorophore−quencher-labeled molecular beacons, label-free molecular beacons have also been designed for detection of the target DNA. The label-free molecular beacon converted the cleavage into initiating the translation of a reporter gene encoding luciferase. The translated luciferase was then used to catalyze the oxidation of luciferin, generating strong luminescence readout.

Zhao and co-workers developed an apurinic−apyrimidic (AP) probe-based endonuclease IV signal amplification assay for DNA detection.[234] Endonuclease IV recognizes and cleaves AP sites, leaving 3′-hydroxyl and 5′-deoxyribosephosphate ends. More importantly, endonuclease IV strongly prefers to cleave the AP site in double-stranded DNA and has very limited activity with the AP site in single-stranded DNA.[235] An AP-site-containing probe was labeled with a fluorophore and quencher pair. The hybridization of the target DNA with the AP probe triggered the cleavage of the probe at the AP site by endonuclease IV, generating a fluorescence signal. Meanwhile, the target DNA was regenerated to initiate another cleavage reaction.

4. HOMOGENEOUS BINDING ASSAYS USING DNA NANOSTRUCTURES

Over the past 30 years, DNA molecules have been assembled to build a variety of nanostructures and nanodevices, and potential applications have emerged in many fields, including biomimetic systems, computing systems, photonics, diagnostics, and therapeutics.[236−240] Here, we summarize the applications of assembled DNA nanostructures and nanodevices as promising signal generation and amplification tools in homogeneous binding assays.[196,205,241−271] Important features include high sensitivity, high specificity, isothermal amplification, no requirement for protein enzymes, and no requirement for any separation steps.

4.1. Assays Based on Toehold-Mediated DNA Strand-Displacement Reactions

As one of the most powerful strategies to construct and control dynamic DNA nanostructures and nanodevices, DNA strand-displacement reaction is the process through which two strands with partial or full complementarity hybridize to each other, displacing one or more prehybridized strands in the process.[238] Different from enzyme-driven DNA strand-displacement and enzyme-driven DNA machinery as we mentioned in the previous sections, the strand-displacement reaction that we describe in this section is initiated at complementary single-stranded domains termed "DNA toeholds" and progresses through a branch migration process. A toehold in a double-stranded DNA refers to a sticky end consisting of typically 5−8 nucleotides. By varying the length and sequence of the toeholds,[272] the rate of the toehold-mediated strand-displacement reactions can be enhanced by a factor of 10^6. Toehold-mediated strand-displacement reactions have been successfully applied to homogeneous binding assays.

Li and co-workers demonstrated the use of the toehold-mediated strand-displacement beacons for nucleic acid detections.[205] As shown in Figure 11A, two complementary DNA strands were labeled with a quencher and a fluorophore, respectively. One of the strands was a few nucleotides longer than the other, leaving a toehold part for target binding. In the absence of a target nucleic acid, a stable DNA duplex was formed and the fluorescence signal was quenched. Upon target binding, the toehold-mediated strand-displacement reaction resulted in the release of the shorter DNA strand and turned on fluorescence. This strand-displacement beacon showed two distinct advantages over the commonly used molecular beacons. One was the sharper transition observed in the melting curve of the strand-displacement beacon than observed in a variety of molecular beacons. The other advantage was a wider detection window between the perfectly matched and single-mismatch targets. These observations suggest the potential use of strand-displacement beacons to detect SNPs with higher sensitivity and specificity.

Indeed, by establishing a theoretical framework for the analysis of nucleic acid hybridization specificity, Yin and co-workers have designed toehold exchange probes that were able to achieve high hybridization specificity across a wide range of temperatures, salinities, and oligonucleotide concentrations (Figure 11B).[241] Two toeholds were designed on the same strand-displacement beacon probe at the 3′- and 5′-ends (Figure 11B). The hybridization of the probe to the correct target was initiated at the 3′ toehold region and completed via the spontaneous dissociation of the 5′ toehold region to release the ssDNA that was labeled with the quencher. The two toeholds allowed both forward and reverse reactions to proceed with fast kinetics, and the standard free energy of the forward reaction was close to zero. This process mimicked the hybridization behavior of the probe at close to its melting temperature, which was commonly used as the optimal condition to discriminate the fully matched target DNA from the single-base mismatched DNA. Using the toehold exchange

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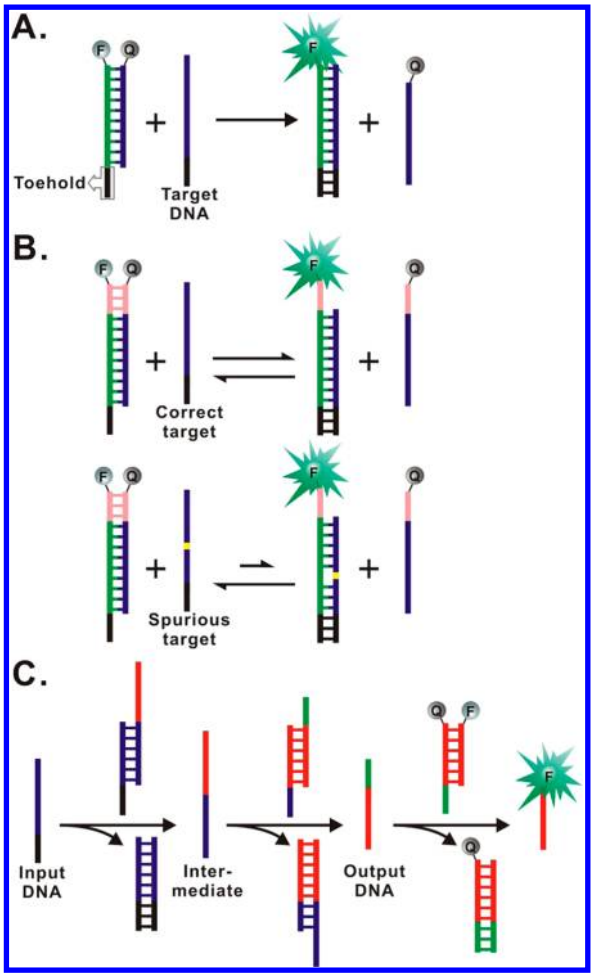

Figure 11. Homogeneous binding assays using toehold-mediated strand-displacement reactions: (A) strand-displacement beacon for nucleic acid detection;[205] (B) toehold exchange reactions for detection of a specific single-nucleotide polymorphism;[241] (C) universal molecular translators for nucleic acid analyses using multiple strand-displacement reactions.[242]

probes to test five different DNA targets against 55 single-base mismatched spurious analogues, the authors achieved discrimination factors between 3 and over 100 (median 26) under a wide range of conditions.[241]

Multiple toehold-mediated strand-displacement reactions can be programmed to generate signals for detection of nucleic acids.[242,243,245] For example, Ghadiri and co-workers have developed universal translators that were able to convert any target nucleic acid input into a unique output molecule by combining multiple strand-displacement reactions (Figure 11C).[242] In the presence of a target nucleic acid, an intermediate DNA was released through a toehold-mediated strand-displacement reaction. The intermediate DNA triggered a secondary strand-displacement reaction to release a universal output DNA, which was then detected by a strand-displacement beacon. Through this translator, several biologically relevant input DNA sequences were successfully translated into a unique output DNA in 5 min at room temperature without any separation steps.

Many nanomaterials have been used to enhance the detection signal of toehold-mediated strand-displacement beacons.[245−248] Mirkin and co-workers have developed DNA-functionalized AuNP probes (nanoflares) to visualize and quantify RNA in living cells.[245,246] Fluorescently labeled DNA reporter probes were initially hybridized to DNA-functionalized AuNPs, and the fluorescence of these reporter probes was quenched by AuNPs. The presence of a target RNA then displaced and released the reporter probes from AuNPs through a toehold-mediated strand-displacement reaction, and the fluorescence signal was turned on (Figure 12). By attaching

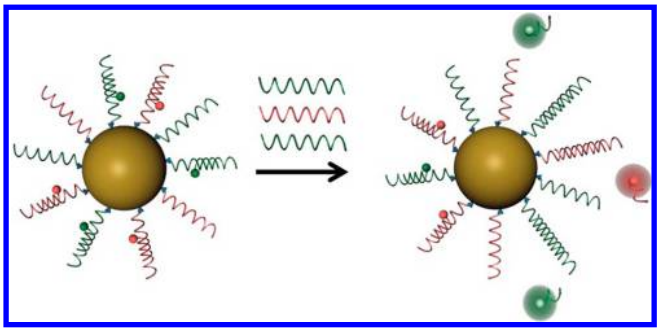

Figure 12. Multiplexed nanoflares.[245,246] The multiplexed nanoflares bind different target nucleic acids (shown in red and green), displacing the corresponding flare. Once the flare is released, the labeled fluorescent dye is no longer quenched by gold nanoparticles, and an increase in fluorescence can be measured. The ratio of two targets can be determined in cells using two different fluorophores. Reprinted from ref 246. Copyright 2012 American Chemical Society.

two different types of fluorescent reporter probes on a single AuNP, the authors designed a multiplexed nanoflare capable of detecting two distinct mRNA targets simultaneously inside a living cell. Other unique features of nanoflares include direct cellular uptake without the need for transfection agents,[249] the ability to regulate cell function,[250] enhanced enzymatic stability of DNA probes,[251] and favored strand-displacement kinetics on AuNPs.[252]

4.2. Assays Based on Hybridization Chain Reactions

Dynamic DNA nanostructures can be programmed through a cascade of strand-displacement reactions to amplify the detection signal for homogeneous binding assays.[253−265,273] One of the most widely used strategies is known as the hybridization chain reaction (HCR).[253−259] In HCR, DNA molecules can be assembled into nicked dsDNA structures similar to alternating copolymers from a cascade of hybridization events triggered by a single target nucleic acid molecule. The detection signal can be amplified by monitoring the long double-stranded DNA with hundreds of repeated units.[253−259] Pierce and Dirks introduced the concept of HCR in 2004 and then explored its potential as a label-free biosensor and as an amplification probe for multiplexed imaging of mRNA expression.[253,254] Soon after the discovery of HCR, many efforts have been made to apply it as a signal amplification strategy for homogeneous binding assays.[255−259]

For a typical HCR (Figure 13A), two DNA hairpins (H1 and H2) are used. H1 and H2 are stable in the absence of the target DNA, but in the presence of the target DNA, a toehold-mediated strand displacement is initiated at the sticky end of H1, opening its hairpin. The newly exposed end of H1 nucleates at the sticky end of H2 and opens the hairpin to expose the end of H2, which is identical in sequence to the target. Hence, each copy of the target DNA can propagate a chain reaction of hybridization events between alternating H1 and H2 hairpins to form a nicked double helix, amplifying the

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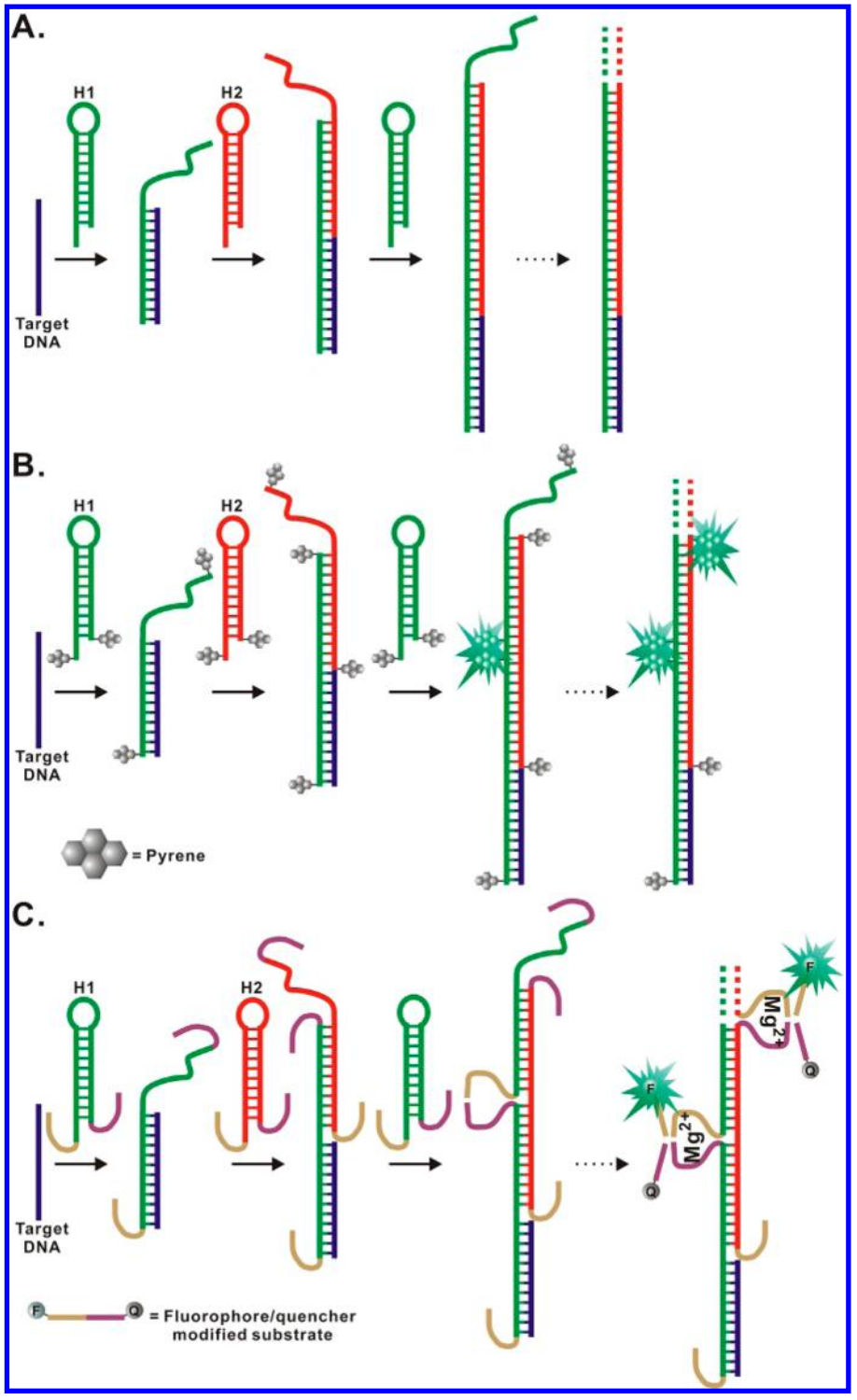

Figure 13. Homogeneous binding assays using hybridization chain reactions (HCRs): (A) typical process of the HCR using two hairpin probes (H1 and H2);[253] (B) HCR using a pyrene excimer as a fluorescence turn-on probe;[255] (C) HCR using a DNAzyme as a signal amplification probe.[256]

signal of the target DNA. To monitor the HCR product, Pierce and Dirks developed a fluorescence quenching assay using the adenine analogue 2-aminopurine (2AP). 2AP fluoresced when incorporated in the single-stranded form, but was significantly quenched when in a stacked double-helical conformation.[253]

To develop a fluorescence turn-on assay for nucleic acids using HCR, Tan and co-workers designed pyrene-excimer-based HCR probes.[255] As shown in Figure 13B, the DNA hairpins for HCR were dual-labeled at both ends with pyrene moieties. In the absence of the target DNA, the two pyrene moieties on each hairpin were spatially separated by the extra length of the sticky end and only emitted at a low wavelength range (375 and 398 nm). In the presence of the target DNA, the HCR process was triggered. A pyrene moiety on one probe was brought into close proximity to a pyrene moiety on the neighboring probe, forming pyrene excimers which turned on the fluorescence signal at 485 nm. Numerous pyrene excimers

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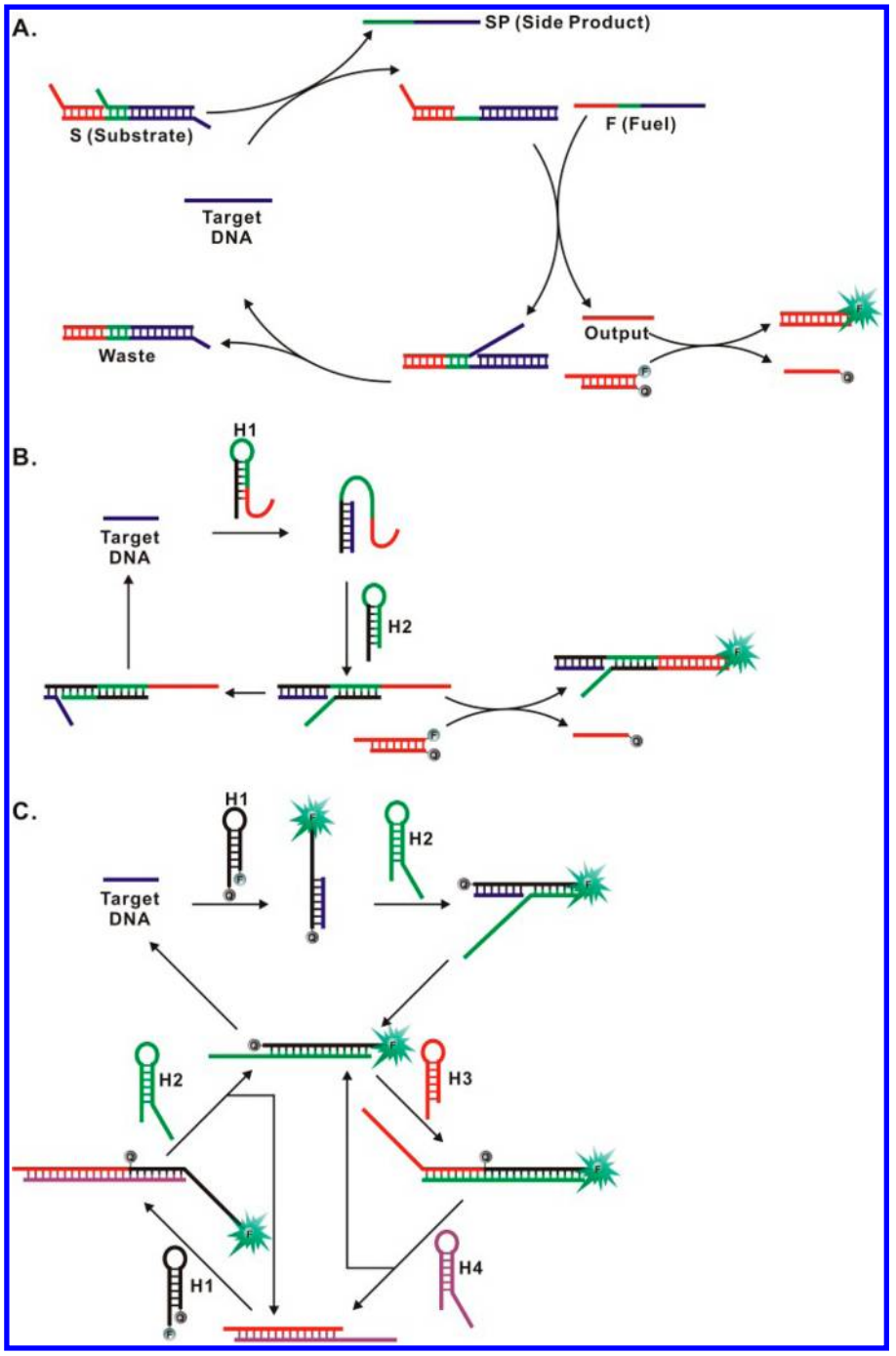

Figure 14. Homogeneous binding assays based on catalytic DNA circuits: (A) linear amplification using an entropy-driven DNA circuit;[261] (B) linear amplification using a double-hairpin DNA circuit;[264] (C) exponential amplification using multiple catalytic DNA circuits.[263]

were formed by HCR, and the target DNA was detected at the subpicomolar range.

Willner and co-workers further improved the HCR signal amplification for homogeneous binding assays by incorporating DNAzymes into the HCR product.[256,257] For example, a Mg^{2+}-dependent DNAzyme was split into two subunits, each of which was imbedded in a DNA hairpin for HCR (Figure 13C).[256] In the presence of the target DNA, HCR was triggered and two DNAzyme subunits were brought into close proximity to form a complete DNAzyme. The DNAzyme catalyzed the cleavage of the fluorophore−quencher-modified substrates, turning on fluorescence. A detection limit of 10 fM was achieved for DNA detection. A colorimetric detection method was also developed by the same group for DNA analyses when an HRP-mimicking DNAzyme was incorporated in HCR.[257]

4.3. Assays Based on Catalytic DNA Circuits and DNA Machines

The recent advances in the field of dynamic DNA nanotechnology have also yielded enzyme-free DNA catalytic circuits that can be adapted to amplify signals for homogeneous binding assays.[260,261,263−266,273] Winfree and co-workers designed an entropy-driven DNA circuit that has potential to be used as a signal amplification tool for homogeneous binding assays.[260−262] As shown in Figure 14A, the target DNA was used to catalyze the reaction between the fuel strand (F) and

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the substrate (S). The target DNA was first used to displace a side product DNA (SP) through a toehold-mediated strand-displacement reaction. Through the second toehold on the substrate, the fuel (F) displaced both the output DNA (O) and the target DNA by forming the F−S duplex. The target DNA was used again to trigger another round of reaction, and the output DNA was detected through a displacement beacon. This target-catalyzed DNA circuit was thermodynamically driven forward by the entropic gain of the liberated molecules. As low as 1 pM target DNA could be detected after amplification.

Pierce, Yin, and co-workers described another DNA circuit based on catalyzed hairpin assembly in their seminal work on programming DNA self-assembly pathways.[263] Chen, Ellington, and co-workers further adapted this signal amplification strategy with a strand-displacement beacon and explored its modularity to multiple detection methods commonly used in bioanalysis.[264] As shown in Figure 14B, a pair of DNA hairpins (H1 and H2) were designed to potentially hybridize partially to each other. However, the spontaneous hybridization between H1 and H2 was kinetically hindered by caging complementary regions in the stem parts of the hairpins. In the presence of the target DNA, the stem part of H1 was opened by the toehold-mediated strand-displacement reactions initiated from the sticky end of H1. The newly exposed sticky end of H1 nucleated at the sticky end of H2 and triggered another strand-displacement reaction, forming an H1−H2−target complex. This complex was inherently unstable, and target DNA dissociated from the H1−H2 complex, completing the reaction and allowing the target to act as a catalyst to trigger the formation of other H1−H2 complexes. By coupling to a strand-displacement beacon, the detection signal was amplified linearly by 20−50-fold with negligible background. Using the same DNA circuit but a different read-out format, Yang and co-workers developed a colorimetric assay for homogeneous detection of nucleic acids by releasing an HRP-mimicking catalytic beacon as the end product.[273]

It is possible to engineer a DNA circuit capable of amplifying the detection signal exponentially by combining multiple catalytic DNA reactions.[263,265] For example, Pierce, Yin, and co-workers designed a cross-catalytic DNA circuit that has shown exponential kinetics by using four metastable DNA hairpins (Figure 14C).[263] Using this strategy, they amplified and detected fluorescently 5 fM target DNA.

Toehold-mediated strand-displacement reactions can also be applied to construction of DNA machines for amplified detection of nucleic acids.[266] For example, Willner and co-workers have fabricated an autonomous ligation DNAzyme machine that combines the function of a Zn^{2+}-dependent ligation DNAzyme with a DNA strand-displacement reaction for enzyme-free DNA detection (Figure 15).[266] A Zn^{2+}-dependent ligation DNAzyme was modified to respond to a target DNA by including a target recognition domain in a hairpin structure. In the absence of the target DNA, the stem part of the hairpin structure blocked the formation of the complete ligation DNAzyme. However, in the presence of the target DNA, the hybridization of the target DNA with the recognition domain opened the hairpin structure, enabling the assembly of the two substrate components to form a complete DNAzyme. As a result, two substrate components were ligated into a new DNA sequence as the product. This DNA product was designed to contain a DNA toehold capable of displacing the target DNA from the target recognition part on the hairpin structure. This toehold-mediated strand-displacement reaction

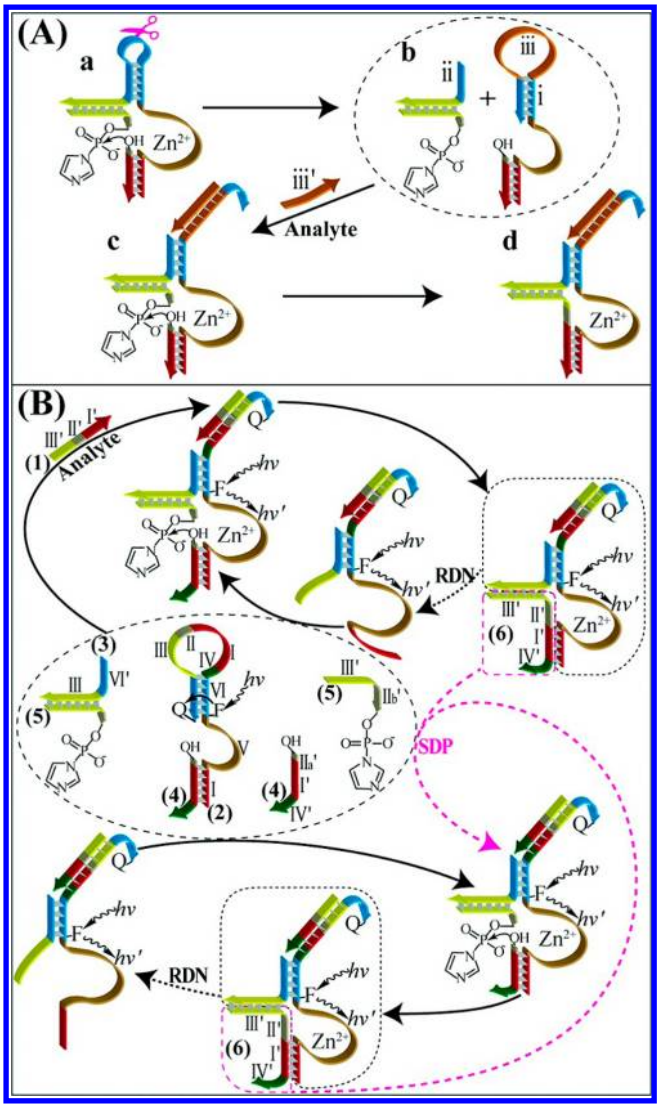

Figure 15. Homogeneous binding assay based on an autonomous ligation DNAzyme machine driven by toehold-mediated strand-displacement reactions:[266] (A) general design of the Zn^{2+}-dependent ligation DNAzyme for target detection; (B) autonomous signal amplification by the Zn^{2+}-dependent ligation DNAzyme upon the target binding and the autonomous assembly of the DNAzyme nanostructures. Reprinted from ref 266. Copyright 2012 American Chemical Society.

regenerated both the target DNA and the catalytic region on the DNAzyme. Therefore, one target DNA was able to trigger multiple rounds of ligation reactions, amplifying the detection signals. To monitor this signal amplification process, the hairpin structure was functionalized with a quencher at the 3′-end and a fluorophore at the complementary part of the internal position. Thus, fluorescence signals were turned on upon the binding of the target DNA or the newly generated ligated DNA to the hairpin portion of the DNAzyme. This ligation DNAzyme machine was applied to detection of the Tay−Sachs genetic disorder mutant, and a detection limit of 10 pM was achieved.

4.4. Assays Based on DNA Nanobarcodes

As we mentioned in the previous sections, dynamic DNA assembly processes have been applied to generate or amplify detection signals for homogeneous binding assays. Assembled

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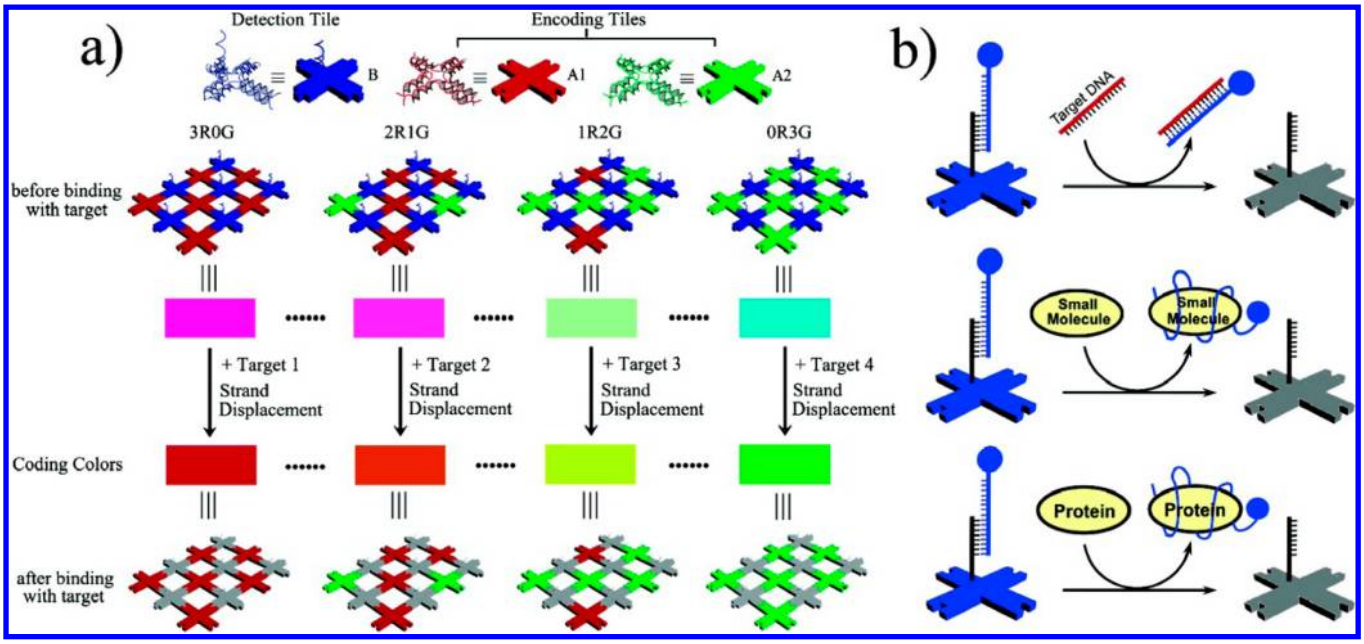

Figure 16. Homogeneous binding assays based on DNA tiles as nanobarcodes.[271] (a) Two subgroups of DNA tiles, A1 modified with a red fluorescent dye (Cy5) and A2 modified with a green fluorescent dye (Rhodamine Red-X), serve as encoding tiles. DNA tile B carries a detection probe that is labeled with a blue fluorescent dye (Alex Fluor 488) and acts as the detection tile. A series of differently colored detection arrays can be generated by mixing the A1 and A2 tiles at different ratios. (b) Target binding with the probe displaces the detection probes that are labeled with blue fluorescent dyes, causing a color change of the nanobarcode. Analytes of interest include nucleic acids, proteins, and small molecules. Reprinted from ref 271. Copyright 2007 American Chemical Society.

DNA nanostructures, recognized as DNA nanobarcodes, have also been applied as biosensors for multiplexed DNA and protein analyses.[267−271] Because multiple termini of DNA motifs can be modified with fluorescent dyes, the colors of DNA nanostructures, including dendrimer-like DNA nanostructures and DNA tiles, can be tuned precisely by controlling the ratios of different fluorescent dyes within each structure unit.[268−271]

Inspired by the synthesis of dendrimer-like DNA, Luo and co-workers developed a multiplexed detection strategy for pathogen DNA using DNA-based fluorescence nanobarcodes.[267,268] They designed Y-shaped DNA structures that had two ends carrying a combination of fluorescent dyes or a fluorescent dye and an affinity probe, leaving a sticky end as a point for ligation.[268] Mixing these Y-shaped DNA structures together with DNA ligase produced the desired DNA nanobarcode. Multicolor DNA nanobarcodes could be achieved by tuning the type and number of the fluorescent dye, allowing for the possibility of tens of thousands of distinct color ratios that could each correspond to a molecular probe. Dendrimer-like DNA nanobarcodes were coupled to polystyrene microspheres and enabled the multiplexed detection of several different pathogenic DNA molecules using fluorescent microscopy, with an attomole detection limit.

Yan and co-workers developed DNA nanobarcodes based on DNA tiles and applied them to the multiplexed detection of nucleic acids and proteins.[271] The 2-D DNA tiles used in this study were constructed by self-assembly of cross-shaped double-crossover DNA building blocks. As shown in Figure 16a, each DNA nanobarcode was constructed with many pieces of detection tiles and encoding tiles. The detection tile contained detection DNA probes labeled with blue fluorescent dyes (Figure 16b), and the encoding tiles were labeled with either green fluorescent dyes or red fluorescent dyes. By tuning the ratio among three different tiles, the authors constructed DNA nanobarcodes with different colors, each of which could represent a different target. In the presence of the target, the detection probes labeled with the blue fluorescent dyes were released from the detection tiles as a result of the target binding. The release of blue fluorescent dyes resulted in an overall color change of the DNA nanobarcodes. Multiple targets, including nucleic acids, proteins, and small molecules, could be detected using this DNA nanobarcode system by monitoring their color changes using a fluorescence microscope.

5. HOMOGENEOUS ASSAYS BASED ON BINDING-INDUCED DNA ASSEMBLY

The previous section described strategies that relied on the assembly of DNA to generate amplified signal responses. Binding of a target analyte to nucleic acid-containing probes, leading to the development of binding-induced DNA assembly (BINDA), further expands these strategies to diverse applications beyond nucleic acids. DNA assembly is dependent on hybridization of complementary DNA sequences. The melting temperature is usually used to describe the stability of the hybridized complementary sequences, and the stability is affected by the concentration of the sequences. Binding of two DNA tethered-affinity ligands to a single target molecule can remarkably increase the local effective concentrations of DNA sequences.[51,274−276] The increase in local concentrations can be utilized to favor the assembly of DNA sequences that would otherwise be difficult to achieve at low concentrations.[276] This DNA assembly can only be induced by target binding events, which have been used to develop various homogeneous assays for proteins and other molecules.

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5.1. Proximity Ligation Assays

The proximity ligation assay (PLA) was initially developed by Landegren and co-workers in 2002 (Figure 17A).[274] Two proximity probes were prepared by conjugating affinity ligands, such as antibodies and aptamers, with oligonucleotides.[274,277] When two probes were bound to a single target molecule, the free ends of the oligonucleotides were brought into close proximity, enabling cooperative hybridization to an added connector oligonucleotide. The connector oligonucleotide served as the ligation template to juxtapose the ends of the oligonucleotides of the two probes. The enzymatic ligation was then used to join the ends of the oligonucleotides, forming a new DNA strand. The ligation products were amplified and detected by RT-PCR.

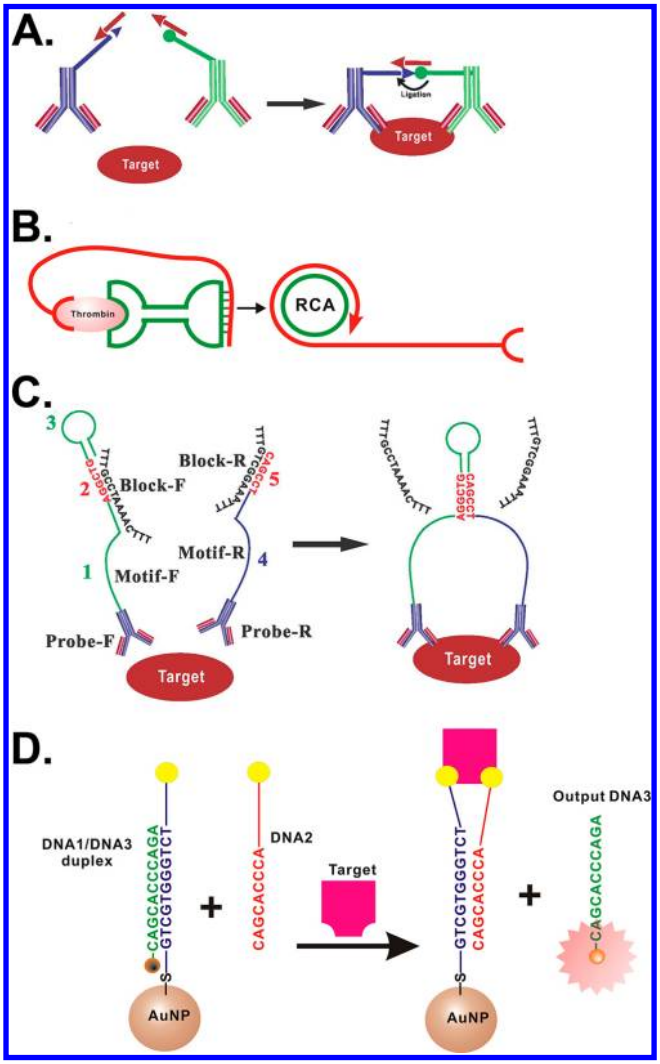

Figure 17. Homogeneous assays based on binding-induced DNA assembly. (A) Proximity ligation assay.[274] (B) Binding-induced DNA annealing assay using rolling circle amplification (RCA).[156] (C) Binding-induced DNA assembly assay making use of sophisticated DNA motifs. Reprinted from ref 276. Copyright 2012 American Chemical Society. (D) Binding-induced molecular translator, converting the input protein to output DNA signals. Reprinted with permission from ref 298. Copyright 2012 John Wiley & Sons, Inc.

In addition to the use of two probes, triple-binder PLA (3PLA) was developed, in which a third proximity probe was introduced to simultaneously bind with the same target molecule.[278] The oligonucleotide conjugated in the third probe was used as a template for enzymatic ligation of the two other oligonucleotide probes. Although 3PLA further improved the detection limit of 2PLA, the requirement of three binding events to a single target molecule could limit its application. Typically, the connector oligonucleotide contained the same number of complementary bases for each of the two probes. The use of an asymmetric connector resulted in an improvement of the PLA dynamic range by 2 orders of magnitude.[279]

The use of real-time PCR enabled PLA to be highly sensitive, and the detection of low femtomolar or attomolar proteins has been demonstrated.[280,281] PLA has been employed in a variety of applications, including detection of proteins and pathogens,[282−284] study of protein interaction and modification,[285−288] and biomarker development.[289] Several reviews have focused on the topic of PLA.[280,281]

5.2. Assays Based on Binding-Induced DNA Annealing

Binding-induced DNA annealing requires the binding of two affinity probes to a single target molecule.[290] Affinity probes are prepared by conjugating short complementary oligonucleotides to affinity ligands via nanometer scale flexible linkers. An oligonucleotide or non-DNA spacer can be used as the linker.[51,291] The length of complementary oligonucleotides is controlled such that very little association between the oligonucleotides occurs in the absence of a target. The binding of two affinity probes to a single target molecule places two complementary oligonucleotides into a nanosized sphere, dramatically increasing the local effective concentration of oligonucleotides. Consequently, two complementary oligonucleotides hybridize to each other, resulting in binding-induced DNA annealing. The detection of the binding-induced DNA annealing provides an indirect measure of target molecules.

Heyduk and co-workers developed fluorescence sensors by using the FRET principle to detect the binding-induced DNA annealing.[290,292] A fluorescence donor and acceptor were labeled at the respective free ends of complementary oligonucleotides. The binding-induced DNA annealing brought the donor and receptor into close proximity, generating a FRET signal. These fluorescence sensors have been used for the detection of proteins, specific antibodies, nucleic acids, and whole cells.[290,293,294] Liu and co-workers achieved the detection of a femtomolar concentration of proteins using PCR to detect the binding-induced DNA annealing.[275,295] Two ligands were tethered with two oligonucleotides, one at the 5′-end and the other at the 3′-end. The oligonucleotide with the free 3′-end was designed to have a complementary sequence at its free end, while the oligonucleotide with the free 5′-end contained a complementary sequence at an internal region. Therefore, binding-induced DNA annealing allowed enzymatic extension of the 3′-end of the complementary sequences by using the other oligonucleotides as a template. PCR was then used to amplify and detect the products of enzymatic extension. This assay has been applied to the identification of ligand−target pairs from libraries of ligands and targets and is therefore termed interaction-dependent PCR.[295] A similar approach was also used for the detection of low-abundance proteins in human blood.[296]

Besides PCR, RCA has also been used to amplify the detection signal from the binding-induced DNA annealing. King and co-workers demonstrated this strategy for thrombin detection by using two thrombin-binding DNA aptamers

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(Figure 17B).[156] The first aptamer (binding to the heparin exosite II of thrombin) was extended to form a circular dumbbell and served as both an affinity ligand and a circular template for RCA reaction. A second aptamer (binding to the thrombin exosite I) was designed to have a 3′ tail that was long enough to bridge the circular aptamer only when both aptamers were bound to one thrombin molecule. In the presence of thrombin, the binding of the two aptamers to the same thrombin molecule initiated an RCA reaction. The detection of the RCA product provided an indirect measure of thrombin, with a detection limit of 30 pM.

5.3. Assays Based on Binding-Induced DNA Assembly

Our group explored the use of binding events to generate assembly of DNA motifs with more sophisticated structures and to assemble DNA-functionalized nanomaterials. We term this binding-induced DNA assembly because such an assembly is preferentially formed only when a specific target triggers a binding event. BINDA has been applied to the development of different homogeneous assays for proteins.

We first described the principle and technique in a patent application[297] filed in August 2009 and then demonstrated applicability of BINDA to the development of homogeneous assays with extreme sensitivity.[276,297] As shown in Figure 17C, DNA motif-F consisted of a spacing sequence 1 that linked the affinity probe-F to the complementary sequence 2. Sequence 2 was linked to a hairpin sequence 3 and was partially hybridized to block-F that was designed to minimize the nondesirable target-independent DNA self-assembly. DNA motif-R consisted of a spacing sequence 4 that linked the affinity probe-R to the complementary sequence 5. Sequence 5 was partially hybridized to block-R that was used to reduce target-independent DNA assembly. The length of two complementary sequences was designed such that the self-assembly between the two strands was unstable. When two affinity probes were bound to a single target molecule, motif-F and motif-R assembled through hybridization of complementary sequences 2 and 5. The assembly of the two motifs brought the 3′-end of the hairpin sequence 3 next to the 5′-end of complementary sequence 5. Enzymatic DNA ligation joined sequence 3 together with sequence 5, forming a new DNA sequence that could be detected using real-time PCR. We demonstrated that the use of these DNA motifs led to elimination of DNA self-assembly while maximizing BINDA. The elimination of the target-independent DNA self-assembly overcame the background problem commonly encountered in the detection of minute amounts of molecular targets, enabling homogeneous detection of streptavidin, PDGF, and prostate-specific antigen (PSA) at the yoctomole to zeptomole levels.

We further explored the benefit of a AuNP scaffold, combined with the BINDA technique, to construct a binding-induced molecular translator for homogeneous protein analysis. The binding-induced molecular translator uses the AuNP as the scaffold for target recognition and signal transduction (Figure 17D).[298] Target recognition was achieved by binding of two affinity ligands to the target protein. One affinity ligand was conjugated to AuNP via DNA1. The second affinity ligand was linked to DNA2, serving as competing DNA. A fluorescently labeled DNA3 was designed to hybridize with DNA1, forming a stable DNA1−DNA3 duplex. Therefore, the fluorescence of DNA3 was quenched by the AuNP. To minimize target-independent strand-displacement between DNA2 and DNA3, we designed the three DNA sequences in such a way that the complementary sequences between DNA1 and DNA3 were 2−4 nucleotides longer than the complementary sequences between DNA1 and DNA2. Upon binding of the target molecule to the two affinity ligands, DNA2 was brought into close proximity to the DNA1−DNA3 duplex, accelerating the strand-displacement reaction between DNA2 and DNA3. This binding-induced strand-displacement reaction led to the assembly of DNA2 with the DNA1-conjugated AuNP and released DNA3 from the scaffold. The release of DNA3 turned on fluorescence for detection. This molecular translator enabled the detection of picomolar concentrations of streptavidin and PDGF-BB.

A binding-induced fluorescence turn-on assay was also developed,[299] in which BINDA was used to enable interaction between silver nanoclusters (AgNCs) and guanine-rich DNA sequences, resulting in fluorescence enhancement of the AgNCs. Thrombin and its two aptamers were chosen to demonstrate the proof-of-concept. One aptamer was extended with a complementary sequence and a AgNC nucleation sequence at the 5′-end, and the second aptamer was extended with another complementary sequence and a G-rich overhang at the 3′-end. The AgNC nucleation sequence allowed the growth of site-specific AgNCs. The binding of two aptamers to a single thrombin led to binding-induced hybridization of two complementary sequences, which therefore placed the G-rich overhang close to the AgNCs, enhancing the fluorescence of the AgNCs. A detection limit of 1 nM thrombin was achieved by using this homogeneous assay.

The BINDA strategy is currently being explored for the construction of nanostructures and nanodevices. It is also applied to the development of assays for DNA damage, carbohydrates, and specific proteins, toward the goal of point-of-care diagnostics.

6. CONCLUSIONS AND OUTLOOK

The development of bioanalytical techniques that are highly sensitive, specific, multiplexed, and robust, with a reasonable cost and minimum requirement for instrumentation and trained personnel, will contribute greatly to medical diagnostics and health research.[300] The various homogeneous binding assays as described in this review represent examples of recent advances in bioanalytical chemistry, aimed at achieving this goal. These developments demonstrate the proof-of-principle and hold promising potential for biochemical and clinical applications.

Homogeneous assays are attractive because conducting an assay in a single tube reduces time and minimizes contamination-prone steps. Obviating the immobilization of affinity ligands and/or enzymes on solid surfaces, homogeneous assays benefit from usually faster binding processes in solution than in a solid phase. Homogeneous assays are conducted without the need for separation or washing steps, simplifying assay procedures. However, this same feature makes it challenging to deal with the adverse effects of an often complicated sample matrix. To detect a specific target in a biological sample of a complex matrix, homogeneous assays have stringent demands on specificity of target recognition. Thus, homogeneous assays often require the use of affinity probes of high specificity and/or the use of multiple affinity probes to recognize a single target.

Heterogeneous assays, such as enzyme-linked immunosorbent assays (ELISAs), have been well established and applied to biomedical research and clinical diagnostics.[301−304] The recent advances in microarray technology have further improved the

Table 1. Summary of Homogeneous Binding Assays for the Detection of Nucleic Acids and Proteins Showing the Range of Detection Limits and Key Design Features

technique	detection scheme	target	typical LOD (M)	general features	refs
molecular aptamer beacons	FRET	protein	10^{-12}	assembly-based	50, 51
	FRET	protein	10^{-7}	hairpin-based	52, 53
	FRET	protein	10^{-12}–10^{-9}	conformation-switching	56, 57
	FRET	protein	10^{-10}–10^{-8}	structure-switching signaling aptamers	61–70
	FRET	protein	10^{-12}–10^{-11}	competition-based	74, 75, 77
	FRET	protein	10^{-12}–10^{-9}	competition-based (nanomaterials)	82–87
	fluorescent	protein	10^{-11}–10^{-9}	dye displacement	92, 97, 99–102
catalytic beacons	FRET	nucleic acid	10^{-10}	caged, strand-cleaving	114
	colorimetric	nucleic acid	10^{-8}	caged, HRP-mimicking	119
	CRET	nucleic acid	10^{-9}	caged, HRP-mimicking	122
	colorimetric	nucleic acid	10^{-9}	blocked DNAzyme, HRP-mimicking	125
	luminescent	nucleic acid	10^{-8}	inactivated upon assembly, HRP-mimicking	126
	FRET	nucleic acid	10^{-12}–10^{-9}	MNAzyme, strand-cleaving, high specificity	127–129
	colorimetric	nucleic acid	10^{-12}–10^{-9}	MNAzyme, HRP-mimicking, high specificity	130–135
	FRET	nucleic acid	10^{-12}	protein-based catalytic beacon, protein enzyme amplification	136
	thrombin activity	nucleic acid	10^{-9}	protein-based catalytic beacon, protein enzyme amplification	139
RCA	fluorescent	nucleic acid	10^{-15}–10^{-14}	isothermal linear amplification, requires polymerase	152–154
	fluorescent	protein	10^{-10}	requires specific aptamer	157
SDA	fluorescent	nucleic acid	10^{-15} –10^{-14}	isothermal linear amplification, requires polymerase	166, 175
	colorimetric	nucleic acid	10^{-12}	isothermal linear amplification, requires polymerase	124
	fluorescent	protein	10^{-10}	requires specific aptamer	171
nicking endonuclease-assisted	colorimetric	nucleic acid	10^{-12}–10^{-11}	isothermal linear amplification	182, 183, 188
	fluorescent	nucleic acid	10^{-12}–10^{-10}	isothermal linear amplification	180, 189–191
	fluorescent	nucleic acid	10^{-15}–10^{-12}	combing two isothermal linear amplifications	180, 193, 194
	fluorescent	protein	10^{-12}	requires specific aptamer	196
nicking-assisted SDA	fluorescent	nucleic acid	10^{-15}–10^{-14}	isothermal linear and exponential amplification	161, 162, 167, 169
	colorimetric	nucleic acid	10^{-14}–10^{-13}	requires both polymerase and nicking endonuclease	173, 174, 197
	fluorescent	protein	10^{-12}	requires specific aptamer	163
exonuclease III-assisted	colorimetric	nucleic acid	10^{-11}	isothermal linear amplification	204
	fluorescent/ luminescent	nucleic acid	10^{-13}–10^{-11}	isothermal linear amplification	200–203, 206, 212–214, 216–219
	colorimetric	protein	10^{-8}	limited to DNA-binding proteins	222
	fluorescent/ luminescent	protein	10^{-14}–10^{-8}	limited to DNA-binding proteins and enzymes	201, 220, 221, 223, 224
	fluorescent	protein	10^{-13}	requires specific aptamer	225
based on other cleaving enzymes	colorimetric	nucleic acid	10^{-8}	isothermal linear amplification	232, 233
	fluorescent	nucleic acid	10^{-14}–10^{-11}	isothermal linear amplification	192, 229, 230, 234
	fluorescent	nucleic acid	10^{-14}	isothermal exponential amplification	227, 228
strand-displacement beacon	fluorescent	nucleic acid	10^{-10}–10^{-9}	high specificity	205, 242–248
HCR	fluorescent/ luminescent	nucleic acid	10^{-14}–10^{-13}	isothermal linear amplification, enzyme-free, long incubation time	253–259
catalytic DNA circuit	fluorescent	nucleic acid	10^{-15}–10^{-12}	isothermal linear or exponential amplification	260–266

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Table 1. continued

technique	detection scheme	target	typical LOD (M)	general features	refs
	colorimetric	nucleic acid	10^{-11}	enzyme-free	273
PLA	RT-PCR	protein	10^{-16}–10^{-13}	exponential amplification	274, 277–289
based on binding-induced annealing	FRET	protein	10^{-11}–10^{-9}	without amplification	51, 290–294
	fluorescent	protein	10^{-11}	isothermal linear RCA amplification	156
	RT-PCR	protein	10^{-15}–10^{-13}	exponential amplification	295, 296
BINDA	fluorescent	protein	10^{-10}–10^{-9}	without amplification	298, 299
	RT-PCR	protein	10^{-16}–10^{-15}	exponential amplification	276, 297

feasibility and throughput of the heterogeneous assays for detection of nucleic acids and proteins.[304−308] Compared to homogeneous assays, heterogeneous assays can adopt a wider range of signal-transduction platforms, including many forms of electrochemical detection. Heterogeneous assays, however, usually require immobilization, separation, and washing steps, making the assays time-consuming. The use of a solid surface often leads to the problem of nonspecific adsorption of probes, thereby compromising the specificity and sensitivity of the assay. Binding of the target to recognition probes that are immobilized on a solid phase can be hindered by steric effects.

Complementing heterogeneous assays, homogeneous binding assays have quickly evolved in recent years, incorporating new advances in affinity binding, nanotechnology, and DNA technology. Developing homogeneous binding assays requires accomplishing both target recognition and signal transduction in the solution. We chose to focus on homogeneous binding assays that are mediated by nucleic acids because nucleic acids have attractive properties serving both target recognition and signal transduction purposes. The exquisite specificity of Watson−Crick base-paring enables differentiation of nucleic acid sequences and SNPs. Structures of nucleic acids are predictable, making it useful to design nucleic acid probes that change structures upon binding to the target. This binding-induced conformational change, resulting in signal transduction, is represented in molecular beacons and aptamer beacons. Nucleic acids are amplifiable, giving rise to a variety of amplification techniques for highly sensitive detection. The isothermal amplification techniques described in this review represent diverse amplification mechanisms for nucleic acids. Nucleic acids are programmable, making them unique templates for constructing versatile and dynamic nanostructures. These nanostructures can function as various probes for desired signal transduction and amplification. Nucleic acids are easy to modify, tailoring to various applications. Specific nucleic acid sequences, such as aptamers and DNAzymes, can function as affinity probes and/or catalysts for target recognition, signal transduction, and/or signal amplification purposes

The topic we reviewed here demonstrates applicability of nucleic acids to the development of various homogeneous binding assays. Features of nucleic acids that have been explored impact these assays. Incorporating the binding ability of aptamers with the signal transduction mechanism of MBs leads to the development of MABs, extending the detection of nucleic acids (by MBs) to the detection of proteins (by MABs), with a detection limit on the order of picomolar to nanomolar. The incorporation of DNAzymes into MBs forms catalytic beacons, improving the sensitivity for nucleic acids (picomolar to nanomolar detection limits). Because DNAzymes have lower enzymatic activities than protein enzymes, the application of isothermal amplification techniques using protein enzymes further increases the sensitivity of the assays over catalytic beacons, enabling detection of femtomolar to picomolar nucleic acids and proteins. The use of programmed DNA nanostructures and nanodevices enables amplified detection of nucleic acids without using any enzymes. Binding-induced DNA assembly coverts protein binding into assembled DNA structures, which can then be detected by using various DNA amplification techniques, such as RT-PCR, thereby dramatically improving the detection limits to femtomolar or attomolar. A comparison of the various techniques, with the range of detection limits and main design features, is summarized in Table 1.

Several challenges are faced by the development and routine applications of nucleic acid-mediated homogeneous binding assays. Newly developed assays are slow to be commercialized and approved in clinical laboratories. Several issues need to be addressed. First, most of the assays were developed and tested in well-controlled buffer systems, not in biological fluids or cells. Because there are no separation steps in homogeneous binding assays, sample matrix effects, e.g., from human serum or plasma, need to be evaluated carefully. Further optimization or modification should be made when nucleic acids and enzymes are used in routine analysis, because they may become unstable in the presence of a particular sample matrix. Second, most of the nucleic acid-mediated homogeneous assays, e.g., catalytic beacons, RCA, nicking endonuclease amplification assays, SDA, and catalytic DNA circuits, were designed and optimized for nucleic acid analyses. The similar signal generation and amplification strategies have been adapted to only a few proteins with well-understood aptamer structures, including human thrombin, PDGF-BB, HIV1 reverse transcriptase, and human IgE. There is much need to develop universal molecular translators that can convert the information of other clinically relevant proteins to an amplifiable and detectable DNA signal output. Third, it is difficult to adapt current homogeneous binding assays to the determination of multiple targets simultaneously. Different from array-based techniques, homogeneous assays generally do not provide spatial resolutions for multiple targets. In addition, simultaneous detection of multiple targets using homogeneous binding assays requires minimum or no cross-reaction between the multiple affinity ligands and the various targets when the multiple affinity ligands are present in the same test solution.

Confronting these challenges presents new opportunities for further research and development on homogeneous binding assays, in particular better selection of affinity ligands and innovative applications of nanomaterials with rational design of nucleic acid probes. A recently developed technique for selection of a slow off-rate modified aptamer (SOMAmer)

from modified oligonucleotide libraries has the potential to generate aptamers for almost any protein target.[309] Containing modified nucleoside deoxyuridine triphosphate (dUTP) mimicking amino acid side chains, aptamers (SOMAmers) selected for 813 proteins have shown high specificity and binding affinity, with dissociation constant (K_d) values ranging from $10^{-11.5}$ to $10^{-7.5}$ M. Application of modified SELEX techniques,[18−21,320] such as those based on capillary electrophoresis separation, microchannel and magnetic bead separation, and single-cycle selection, could accelerate the generation of SOMAmers for more proteins.

Application of these new aptamers to homogeneous binding assays could overcome the limitation of many current homogeneous binding assays that focus on DNA detection, by broadening the scope to the detection of proteins. The dramatic increase in the number of aptamers available for proteins should open up opportunities for developing assays for multiple proteins. Another opportunity could come from the second-generation DNA sequencing technology, which could be adapted to developing nucleic acid-mediated homogeneous binding assays. This technology has already been used to facilitate the aptamer selection process[310] and to develop a solid-phase proximity ligation assay for multiple proteins in human plasma.[289]

Rational designs of nucleic acid probes incorporating unique properties of novel nanomaterials could overcome some of the problems encountered by nucleic acid-mediated homogeneous binding assays. For example, AuNPs have been demonstrated to protect nucleic acids from enzymatic degradation in biofluids.[251] QDs can be engineered to emit in the near-IR region, avoiding autofluorescence from a clinical sample matrix.[311] SERS from gold and silver nanoparticles can be used for detection of multiple targets.[312] Nanomaterials with extraordinary photonic, electronic, and catalytic properties will contribute to improving assay performances. Many of these nanomaterials have already been applied to homogeneous binding assays to generate and amplify detection signals.[187,210,313−317]

Nucleic acid-mediated homogeneous binding assays can also be applied to in vivo imaging or live cell imaging, with two potential advantages. First, nucleic acids can be engineered to construct structure-switching probes, e.g., aptamer beacons and DNA nanodevices and nanostructures, that can provide a detectable image when triggered by the target in live cells or tissues. Recently, the spatiotemporal pH changes in a multicellular living organism have been successfully mapped using a pH-sensitive DNA nanodevice.[318] By engineering such nanodevices or nanostructures with structure-switching aptamers as affinity ligands, it is possible to map other functional biomolecules in the living cells and organisms. Second, structure-switching nucleic acids can be assembled onto nanomaterials to monitor the function of such materials in living systems. Many nanomaterials, e.g., AuNPs, QDs, and assembled DNA nanostructures, have been used to regulate cell functions or deliver drugs, and it would be desirable to sense the functions of these materials in live cells and organs.[240,245,249,319] Several successful examples have recently appeared in the literature, including nanoflares and aptamer-functionalized QDs,[245,319] demonstrating the potential of imaging applications using nucleic acid-mediated homogeneous assays.

Homogeneous binding assays are most promising for point-of-care applications. These assays can be performed in a single tube containing the specimen and all reagents, obviating any separation or washing steps and minimizing the chances of contamination. The nucleic acid probes, e.g., aptamers, have the advantage of improved stability over proteins, e.g., antibodies. Assays making use of these more stable and robust reagents will be suitable in resource-limited settings. For these reasons, nucleic acid-mediated homogeneous binding assays have great potential for molecular diagnostics, on-site analysis, and point-of-care testing.

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The authors declare no competing financial interest.

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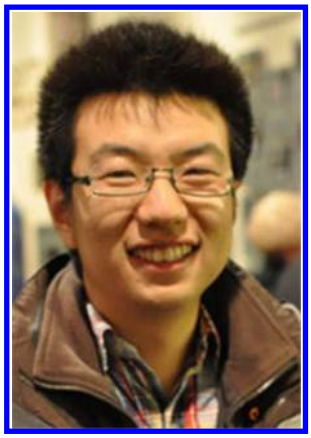

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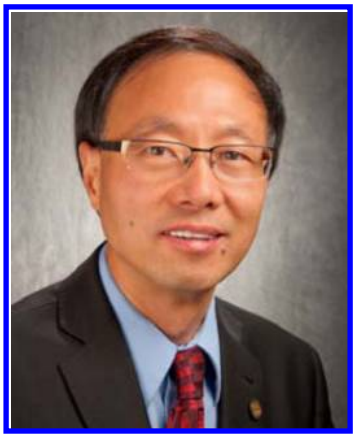

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