DNA Assembly

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Assembling DNA through Affinity Binding to Achieve Ultrasensitive Protein Detection

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Recent advances in DNA assembly and affinity binding have enabled exciting developments of nanosensors and ultrasensitive assays for specific proteins.[1–6] These sensors and assays share three main attractive features:[1,4,7] 1) the detection of proteins can be accomplished by the detection of amplifiable DNA, thereby dramatically enhancing the sensitivity; 2) assembly of DNA is triggered by affinity binding of two or more probes to a single target molecule, thereby resulting in increased specificity; and 3) the assay is conducted in solution with no need for separation, thus making the assay attractive for potential point-of-care applications. We illustrate here the principle of assembling DNA through affinity binding, and we highlight novel applications to the detection of proteins.

1. Assembling DNA through Affinity Binding to the Target

Imagine that a solution contains 1 nM of two complementary strands of short oligonucleotides, each conjugated to an affinity ligand (e.g. antibody and/or aptamer; Figure 1 A). The volume of a sphere containing both molecules would be 1.7 femtoliter (fL), and the radius of this sphere would be $r = (3V/4\pi)^{1/3} = [(3\times 1\,\mathrm{L})/(4\pi\times 1\,\mathrm{nmol}\times 6.023\times 10^{23}\,\mathrm{mol}^{-1})]^{1/3} =$ 734 nm. Now consider binding of the two affinity ligands to a target molecule, such as a protein. This binding brings the two oligonucleotides to the same molecule, with a typical intramolecular distance of less than 10 nm. Confined within a sphere of less than 10 nm in radius, these oligonucleotides have local concentrations of greater than 400 μM ($C = n/NV = n/[N(4\pi r^3/3)] = 1\,\mathrm{mol}/[6.023\times 10^{23}\times 4\pi\times(10\,\mathrm{nm})^3/3]$, where V is the volume of the sphere [approximately 4.2 zeptoliter (4.2×10^{-21} L)] and N is the Avogadro constant. This is a 400 000-fold increase in local concentrations of the complementary oligonucleotides, when compared to the absence of the target binding. This increase in local concentration will enhance the stability of the hybridization between the complementary strands. The concentration enrichment only occurs for the DNA probes bound to the target, while the concentrations of the unbound DNA probes are not affected.

The stability enhancement induced by the target binding can be understood from the following estimates of melting temperatures (Figure 1 B). In principle, two short complementary sequences in 5′-GCCACG$(T)_{20}$-3′ and 5′-$(T)_{20}$CGTGGC-3′ could hybridize and form a duplex. However, this duplex is not stable at room temperature, because the estimated melting temperature (T_m) of this duplex is lower than 10 °C (estimated using the IDT Oligo Analyzer, under the conditions of 1 nM DNA, 1 mM Mg^{2+}, and 50 mM Na^+). If these same sequences are linked together as a single oligonucleotide, 5′-GCCACG$(T)_{40}$CGTGGC-3′, the intramolecular hybridization between the complementary sequences at the two ends forms a stem and the $(T)_{40}$ in the middle forms a loop. This stem–loop structure is much more stable, and the estimated melting temperature of this intramolecular hybrid is 53 °C, significantly higher than that of the intermolecular duplex (< 10 °C).

The increase in local concentration is the underlying reason that raises the melting temperature for the intramolecular hybridization. When the two complementary oligonucleotides are linked together with $(T)_{40}$, the complementary oligonucleotides are constrained in the nanometer

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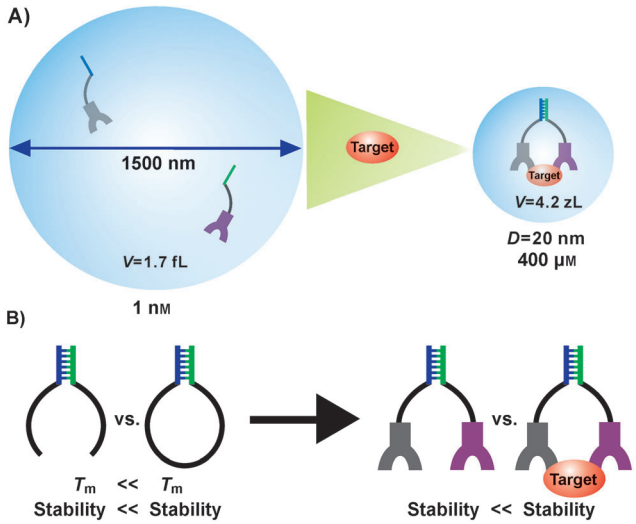

Figure 1. A) Binding of a target molecule to two complementary oligonucleotides substantially increases the local concentrations of the oligonucleotides, thereby increasing the stability of the hybridization. For a 1 nM concentration of two complementary oligonucleotides, the volume of a sphere containing both molecules is 1.7 femtoliter (fL); and the radius of this sphere is 734 nm. Binding of these oligonucleotides to the target molecule brings the two oligonucleotides to the same molecule, resulting in a sphere of less than 10 nm in radius, 4.2 zeptoliter (4.2×10^{-21} L) in volume, and 400 μM in local concentrations. B) The intramolecular duplex has a higher melting temperature and is more stable than the intermolecular duplex under the same temperature. Binding of two affinity ligands to a target molecule brings the complementary oligonucleotides to the same molecule, thereby resulting in stable intramolecular hybridization.

space, which prevents spatial diffusion of the two oligonucleotides and dramatically increases their local concentrations. The increase in local concentration favors the hybridization between two oligonucleotides, thereby giving rise to a substantial increase in the ratio of the duplex to the single-stranded oligonucleotides. It is important to recognize that the hybrids dissociate and rehybridize in solution. In the intramolecular situation, the complementary sequences at the two ends will rehybridize faster after dissociation because of the increased local concentrations.

Likewise, when the two affinity probes are not bound to the target molecule, the duplex between the short oligonucleotides is not stable (Figure 1B). Binding of the target molecule to the two affinity ligands brings the complementary oligonucleotides to close proximity and assembles them on the same molecule, thereby dramatically increasing their local concentrations and enhancing the stability of the hybrids. In contrast, the complementary oligonucleotides in unbound probes remain at a much lower concentration such that they are not able to impact the hybridization of bound complementary oligonucleotides. Therefore, through target binding, the duplex of two short sequences becomes much more stable. This binding-induced DNA hybridization principle can be used to generate binding-induced assemblies of DNA probes containing various motifs and DNA-functionalized nanomaterials.

At least two probes must simultaneously bind to a single target molecule to trigger DNA assembly. Affinity ligands, such as antibodies and aptamers, conjugated to complementary DNA sequences, initiate the events of binding and DNA

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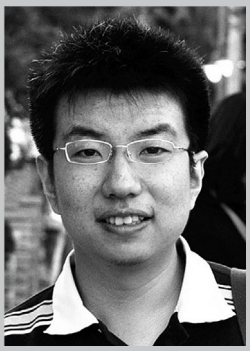

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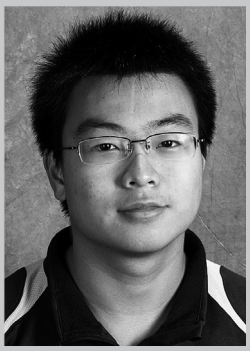

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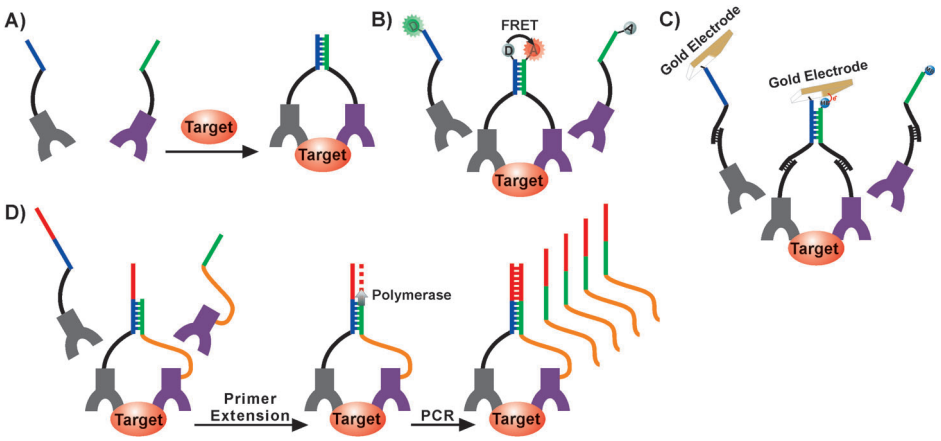

Figure 2. Binding-induced DNA annealing assays. A) General principle of the binding-induced DNA annealing assay.[2,3,7–13] B) Binding-induced DNA annealing assay with FRET as the signal readout.[2,7,9–12] C) Binding-induced DNA annealing assay with electrochemical detection as the signal readout.[13] D) Binding-induced DNA annealing assay with primer extension and PCR amplification.[3]

assembly. These complementary DNA sequences are usually designed in such a way that their hybridization cannot occur (or is not stable) in the absence of the target molecule. The number of bases and base-stacking interaction should be taken into account when designing these complementary DNA sequences, because the stability of a DNA duplex relies on its length and sequence composition. Binding of the target to two or more affinity probes increases the local effective concentration of the probes, thereby stabilizing the assembly of DNA through hybridization of the complementary sequences. Since target binding can induce hybridization of bound DNA probes and this hybridization does not occur for unbound probes, binding-induced assembly of DNA probes can serve the purpose for ultrasensitive detection of specific targets.

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2. Binding-Induced DNA Annealing Assays

A set of simple but effective binding-induced DNA annealing assays have been developed for detecting proteins.[2,3,7–13] These assays take advantage of the drastic increase in the local effective concentration of DNA probes upon target binding to affinity ligands that are conjugated to the DNA probes. A pair of DNA probes is generally designed to have short complementary regions (Figure 2 A), the T_m of which is much lower than the experimental temperature. Such a design minimizes the intermolecular hybridization between the two DNA probes, thereby ensuring a low target-independent response. After incubation of DNA probes with target molecules, binding of two DNA probes to the same target molecule through affinity ligands greatly increases the T_m of the two DNA probes owing to the increase of the local effective concentration, resulting in a stable intramolecular DNA duplex. Thus, the target molecule can be detected indirectly by quantifying the intramolecular DNA duplex.

By using the principle of target-induced DNA annealing, Heyduk and co-workers developed a class of optical sensors based on fluorescence resonance energy transfer (FRET; Figure 2 B).[2,7,9–12] They first designed a pair of DNA probes and conjugated them to a pair of affinity ligands that were able to recognize non-overlapping epitopes of the same target protein. One of the DNA probes was then labeled with a fluorescence donor and the other with a fluorescence acceptor. For efficient FRET, the fluorescence donor and acceptor had to be brought into close proximity (ca. 5 nm), which could not be achieved unless the two DNA probes anneal. Binding of the target molecules to the two probes induced DNA annealing and produced stable DNA duplexes, resulting in FRET. By adopting different types of affinity

ligands, including antibodies, peptides, and aptamers, they successfully achieved sensitive detection of various proteins, for example, thrombin,[2] cardiac troponin I,[9] insulin,[12] and C-reactive protein,[10] with pM detection limits.

Easley and co-workers incorporated the concept of the binding-induced DNA annealing to an electrochemical detection platform and constructed the electrochemical proximity assay (ECPA).[13] As shown in Figure 2C, one DNA probe was attached to a gold electrode through a thiol–gold bond, and the other DNA probe was conjugated to methylene blue (MB). The binding of two DNA probes to the same target molecule through affinity ligands brought MB close to the gold electrode, thus resulting in electron transfer from MB to the gold electrode and the consequent increase in electrical current. The increase in electrical current was proportional to the concentration of the specific target molecule. Aptamer-modified DNA probes were used to construct an ECPA sensor for thrombin detection. Antibody-modified DNA probes were used in the detection of insulin, with a detection limit of 128 fM and a dynamic range of over four orders of magnitude.

The intramolecular DNA duplex formed in the binding-induced DNA annealing assay can also be amplified directly through a DNA-polymerase-mediated primer extension reaction followed by polymerase chain reaction (PCR; Figure 2D).[3] One of the two DNA probes was designed to have a long overhang sequence (red in Figure 2D), serving as a template for the primer extension reaction. In the absence of target molecules, the complementary region of the two DNA probes was too short to form a stable DNA duplex and thus no primer extension reaction could take place. However, in the presence of the target, the binding-induced DNA annealing generated a stable intramolecular DNA duplex, serving as a primer for the primer extension reaction. The newly formed DNA strand from the primer extension reaction (red–green–orange in Figure 2D) was then amplified by PCR to enhance the detection signal. By using this strategy, Liu and co-workers were able to detect as low as 200 zmol of streptavidin. They also developed a multiplexed format that was able to identify ligand–target pairs, with a wide range of affinities, from protein/small molecule libraries.

The intramolecular DNA duplex can also be amplified by rolling circle amplification (RCA). King and co-workers demonstrated binding-induced DNA annealing and RCA for thrombin detection.[8] They used two DNA aptamers: one aptamer was linked to a circular DNA template, and the other aptamer was extended to have a 3′ overhang that could be annealed with the circular aptamer only when both aptamers were bound to the same thrombin molecule. Upon aptamer binding to thrombin, a RCA reaction was initiated from the overhang strand, thereby producing an elongated DNA consisting of repeat units of the template sequence. The linear amplification by RCA led to a modest detection limit of 30 pM thrombin.

3. Proximity Ligation Assay

Proximity ligation assays (PLA) make use of affinity binding to bring oligonucleotide probes into close proximity, enabling subsequent DNA ligation.[1] The detection of a target macromolecule or complex is converted to the detection of new DNA strands that are formed by ligation.[14] A pair of DNA probes, each conjugated to a specific affinity ligand recognizing the target, are brought into close proximity upon two binding events.[1] Two DNA probes then hybridize with a connector oligo, facilitating the enzymatic ligation of the two DNA probes (Figure 3A). Various techniques can then

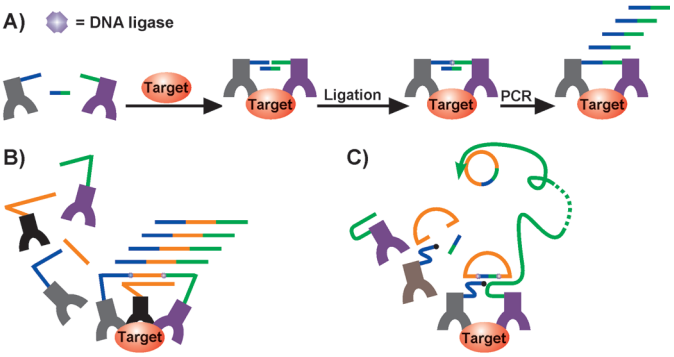

Figure 3. Proximity ligation assay and its variants. A) Principle of the proximity ligation assay. B) 3PLA using an extra affinity ligand bearing the connecting oligonucleotide.[15] C) In situ PLA using a circularizable oligonucleotide for rolling circle amplification.[17]

be used to amplify and detect the new ligated DNA strand.[14] While the concentration of the connector oligo is usually more than 10000-fold higher than the concentrations of the DNA in the affinity probes, hybridization of two DNA probes with the connector oligo only takes place when both affinity probes bind to the same target molecule. Essentially, through affinity binding the local concentrations of both DNA probes are greatly increased to favor stable hybridization with the connector oligo. Stable hybridization of the DNA probes with the connector is required for subsequent ligation to form a new DNA strand for amplification and detection. Background signal is generated from target-independent hybridization of two DNA probes with the connector. Therefore, the length of the connector oligo is crucial for controlling the signal-to-background ratio. Landegren and co-workers first used this strategy in combination with real-time PCR to detect a homodimer of platelet-derived growth factor (PDGF-BB).[1] Aptamers for PDGF-BB served as the affinity ligands and were extended to constitute the DNA probes. They successfully achieved a detection limit of 24000 molecules of PDGF-BB in a 5 μL sample.

Another strategy was introduced later that involved a third affinity probe bearing the connector oligo.[15] This variant was named 3PLA, because it used three affinity probes, in contrast to the original assay (named 2PLA thereafter), which used two affinity probes. In the 3PLA format, three monoclonal or polyclonal antibodies recognizing distinct epitopes of a target molecule were covalently

conjugated to three oligos (two DNA probes and one connector oligo), forming three antibody–oligo probes. Binding of the three affinity probes to the same target molecule facilitated the hybridization of the two DNA probes to the connector oligo and a cassette oligonucleotide to result in two ligation events. The newly ligated DNA strand was then amplified and detected (Figure 3B). The requirement of a target molecule binding to three affinity probes enhanced the specificity and reduced the background signal, thus improving the detection by approximately 100 fold. By using 3PLA, they achieved a detection limit of 60 molecules of vascular endothelial growth factor (VEGF) in 5 μL buffer. Tavoosidana et al.[16] developed a 4PLA assay to detect prostasome, a microvesicle with a mean diameter of 150 nm, which is secreted by prostate cells. This 4PLA required the use of four antibodies to bind to four different proteins on the prostasome surface. Each antibody was conjugated to an oligonucleotide probe. Binding of the four probes to the prostasome vesicle was followed by two ligation events, generating a new DNA strand that was amplified for detection.

PLA has been combined with rolling circle amplification[17] (Figure 3C). Two affinity probes were incubated with fixed cells or tissue, and two binding events triggered the ligation between the circularizable probe and the connector oligo, forming a circular DNA product after ligation. The circular DNA served as the template for RCA to produce hundreds of repeating units that subsequently hybridized with fluorescence probes, generating in situ a signal for direct observation by using a microscope. In situ PLA has been used to detect phosphorylated PDGF receptor β[18] and to screen for inhibitors of PDGF receptor β in cells.[19] In situ PLA has also been applied to study protein–protein interactions, DNA–protein interactions,[20] and RNA–protein interactions.[21] For example, mucin 2 (MUC2) was demonstrated to be a major carrier of the cancer-related sialyl-Tn antigen in gastric carcinoma,[22] and the MUC1 extracellular domain subunit was confirmed to be associated with spliceosomes found in nuclear speckles.[23] Coupling of in situ PLA with flow cytometry was used to study interactions between members of the epidermal growth factor (EGF) receptor family and human epidermal growth factor receptor 2 (HER2) as well as posttranslational modification effects after stimulation by EGF in single cells.[24]

Multiplexed PLA, which has been applied to high-throughput screening, makes use of DNA probes as barcodes.[25–27] These barcode sequences serve as primer sites for subsequent real-time PCR quantification.[25] Solid-phase PLA has also been used for high-throughput analyses[28,29] and could also detect target molecules in complex biological matrix.[30–32] 2PLA has also been modified with a proximity extension assay (PEA), where ligation is replaced with extension.[33] Various formats of PLA have been used to detect proteins,[1,34] protein complexes,[35] and single virus particles;[36] to screen for inhibitors of protein–protein interactions;[37] and to determine the binding specificity of DNA-binding proteins (such as p53, HNF-4α) to different dsDNA sequences.[38]

4. Assays Based on Binding-Induced DNA Assembly

We have developed binding-induced DNA assembly (BINDA),[4,39] enabling the construction of ultrasensitive assays and functional nanostructures. Binding to the target molecule triggers the directed assembly of DNA motifs and DNA-modified nanomaterials. Without the target, no assembly occurs, thus resulting in very little background.

We first used a protein molecule to assemble two DNA motifs that were conjugated to aptamers or antibodies to recognize the target protein.[4,39] Two DNA motifs were constructed in such a way that they assemble only when a specific target binds to the aptamer or antibody (Figure 4A). To achieve this, we designed the DNA motifs to contain short complementary sequences that were used to guide assembly of DNA motifs. The length of the complementary sequences was optimized so that their hybridization was unstable in the absence of target binding. When the two DNA motifs were brought together through target binding, the local concentration of the DNA motifs was dramatically increased, resulting in hybridization of the complementary sequences to form a closed loop structure. DNA ligase was used to join the 3′-end of one motif with the 5′-end of the other motif to form a new DNA sequence that was then detected using real-time PCR.

To further minimize target-independent assembly, we introduced a pair of blocking oligonucleotides to compete with the hybridization between complementary sequences. We designed the blocking oligonucleotides to be five nucleotides longer than the complementary sequences, and we used a much higher concentration of the blocking oligonucleotides than of the DNA motifs, thus resulting in elimination of the target-independent assembly. By eliminating the background arising from target-independent DNA assembly, we were able to detect yoctomole to zeptomole levels of streptavidin, PDGF, and prostate-specific antigen (PSA).[4]

We have also used the BINDA approach to enhance the fluorescence intensity of silver nanoclusters (AgNCs) by facilitating the interaction of AgNCs with guanine-rich DNA sequences (Figure 4B). We have demonstrated an application of this approach to the detection of thrombin.[40] The simultaneous binding of thrombin to two aptamer probes, one conjugated to a AgNC nucleation sequence and the other conjugated to a guanine-rich DNA sequence, resulted in binding-induced hybridization between the two complementary regions in the sequences. This hybridization brought the guanine-rich sequence into close proximity with the AgNCs. The interaction of AgNCs with the guanine-rich sequence significantly enhanced the fluorescence intensity of AgNCs.

In another BINDA approach, we assembled DNA motifs on a gold nanoparticle (AuNP) scaffold and developed a binding-induced molecular translator for the detection of proteins.[5a] The binding-induced molecular translator was designed in such a way that the input target protein could be converted to a predesigned output DNA through the process of binding-induced DNA strand displacement (Figure 4C). This molecular translator was composed of target-recognition and signal-readout elements. Target recognition was achieved by using two affinity ligands that bind to the same target

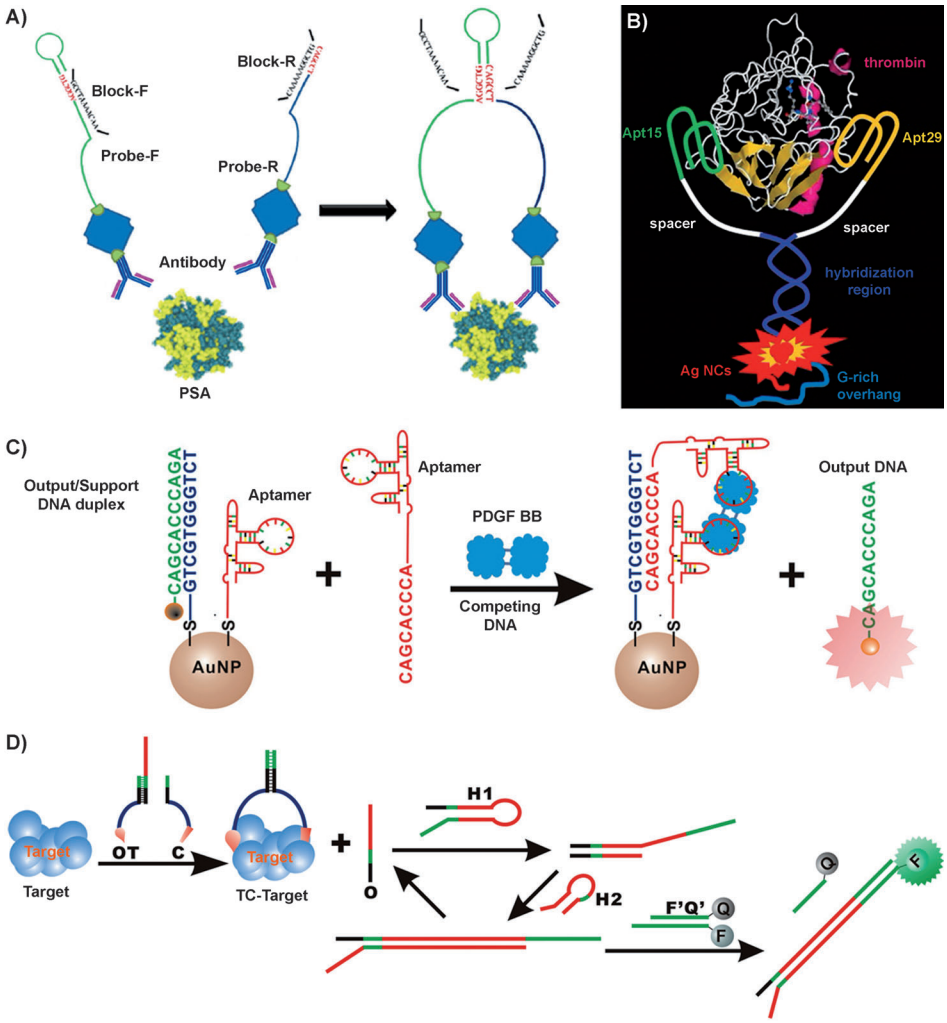

Figure 4. Assays based on binding-induced DNA assembly (BINDA). A) BINDA of a hairpin motif that is ligated and amplified by PCR for protein detection.[4,39] PSA = prostate-specific antigen, Probe-F and Probe-R are DNA motif-tethered affinity ligands which recognize the target protein. Block-F and Block-R are blocking oligonucleotides that are used to minimize target-independent assembly. Reproduced with permission from Ref. [4]. B) BINDA to facilitate the interaction between silver nanoclusters (Ag NCs) and guanine-rich DNA sequences to enhance the fluorescence signal intensity.[40] Reproduced with permission from Ref. [40]. C) Binding-induced strand displacement reaction on a nanoparticle scaffold for a turn-on fluorescent signal readout.[5a] D) Binding-induced strand displacement reaction with the output DNA triggering a subsequent enzyme-free catalytic DNA circuit to amplify the detection signal.[5b] F = fluorophor, Q = quencher, OT = output DNA motif, O = output DNA, C = competing DNA motif, T = support DNA, H1 and H2 = DNA hairpins.

protein. One affinity ligand was conjugated to the AuNP, which served as a scaffold for the molecular translator. The second affinity ligand was conjugated to a competing DNA probe. A fluorophore-labeled output DNA was hybridized to a supporting DNA that was conjugated to the AuNP scaffold. Therefore, the AuNP initially quenched the fluorophore on the output DNA. The competing DNA was also designed to hybridize to the supporting DNA, but was 2–4 nucleotides shorter than the output DNA. Thus, in the absence of target binding, displacement of the output DNA with the competing DNA was minimal. In an example of detecting PDGF-BB, the binding of PDGF-BB to the two aptamer molecules assembled the competing DNA onto the AuNP scaffold and enhanced the local effective concentrations of the DNA probes. This triggered DNA strand displacement between the competing DNA and the output DNA. As a result, the fluorophore-labeled output DNA was released from the AuNP scaffold and the fluorescence signal was turned on. By using this strategy, we were able to detect subnanomolar concentrations of streptavidin and PDGF-BB without any separation steps.

Alternatively, the output DNA can be directed to trigger a subsequent dynamic DNA assembly, resulting in amplification of detection signals in an isothermal and enzyme-free manner.[5b] To construct a binding-induced catalytic DNA circuit (Figure 4D), we have designed a pair of DNA hairpins (H1 and H2) that partially hybridized to each other. The spontaneous hybridization between H1 and H2 was kinetically hindered by caging the complementary sequences in the stems of these hairpins. In the presence of the target, the output DNA (O) was released by binding-induced DNA strand displacement. The released output DNA opened the

stem part of H1 through toehold-mediated strand displacement. The newly exposed stem part of H1 nucleated the sticky end of H2 and triggered a subsequent strand displacement to release the output DNA again. Thus, the output DNA served as a catalyst in the formation of H1–H2 duplexes. This process resulted in the amplification of detection signals and a detection limit of 10 pM streptavidin.[5b]

5. Concluding Remarks

Assembly of DNA probes through target binding is an attractive strategy to extend the tremendous benefit of amplifiable DNA to the detection of other nonamplifiable molecules. This has motivated the development of many ultrasensitive techniques for protein detection. For example, quantitative real-time PCR can be used to detect DNA assembly, providing indirect detection of specific proteins at the yoctomole level.[4,15] These ultrasensitive assays increase the feasibility for detecting low-abundance proteins, which is of great importance for the early detection of disease markers and infectious agents. Recent success of quantifying 35 target proteins in a single solution,[29] by using the DNA assembly strategy in combination with PCR amplification and next-generation DNA sequencing, demonstrates the promising potential for multiplex and high-throughput applications. These techniques complement immuno PCR[41] and single-molecule detection.[42]

The strategies described here rely on interactions of two or more different affinity probes with a single target molecule. Both antibodies and aptamers can serve as affinity probes. Advantages of using aptamers over antibodies include better thermal stability and the ease of incorporation into DNA motifs. However, the use of aptamers has been limited, because few available aptamer pairs can strongly bind to a single protein molecule. The advent of a new generation of aptamers, such as SOMAmers, could overcome this limitation, since high-affinity SOMAmers have been selected to bind to more than 1000 proteins.[43–45]

In the past decade, the assembly of DNA probes through target binding has been applied to the detection of molecules, organelles, and cells, to the study of protein–protein interactions and protein modification, and to the imaging of proteins in fixed cells and tissues.[4,7,14] We foresee that this principle will be further applied to the detection and imaging of proteins in live cells and in the point-of-care diagnostic settings. Nanoparticles and their unique optical properties will be exploited and incorporated in detection schemes, further broadening the utility of the binding-induced assembly. It is also conceivable to construct binding-induced DNA nanomachines and nanodevices, building on recent advances in DNA nanotechnology.

The fundamental principle of using target binding to increase local concentrations of molecules and to facilitate molecular interactions is not limited to the assembly of DNA probes for analytical applications. For example, DNA-templated organic synthesis uses DNA hybridization to increase the local concentrations of reactant molecules and to control reactions.[46–49]

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