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Diagnostic Applications of Nucleic Acid Circuits

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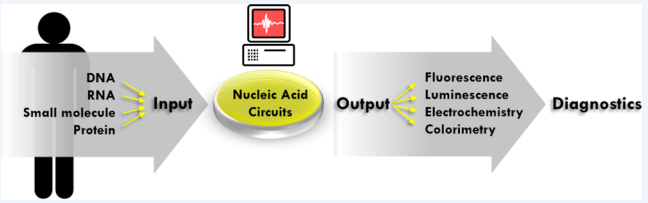
CONSPECTUS: While the field of DNA computing and molecular programming was engendered in large measure as a curiosity-driven exercise, it has taken on increasing importance for analytical applications. This is in large measure because of the modularity of DNA circuitry, which can serve as a programmable intermediate between inputs and outputs. These qualities may make nucleic acid circuits useful for making decisions relevant to diagnostic applications. This is especially true given that nucleic acid circuits can potentially directly interact with and be triggered by diagnostic nucleic acids and other analytes.



Chemists are, by and large, unaware of many of these advances, and this Account provides a means of touching on what might seem to be an arcane field. We begin by explaining nucleic acid amplification reactions that can lead to signal amplification, such as catalytic hairpin assembly (CHA) and the hybridization chain reaction (HCR). In these circuits, a single-stranded input acts on kinetically trapped substrates via exposed toeholds and strand exchange reactions, refolding the substrates and allowing them to interact with one another. As multiple duplexes (CHA) or concatemers of increasing length (HCR) are generated, there are opportunities to couple these outputs to different analytical modalities, including transduction to fluorescent, electrochemical, and colorimetric signals. Because both amplification and transduction are at their root dependent on the programmability of Waston−Crick base pairing, nucleic acid circuits can be much more readily tuned and adapted to new applications than can many other biomolecular amplifiers. As an example, robust methods for real-time monitoring of isothermal amplification reactions have been developed recently.

Beyond amplification, nucleic acid circuits can include logic gates and thresholding components that allow them to be used for analysis and decision making. Scalable and complex DNA circuits (seesaw gates) capable of carrying out operations such as taking square roots or implementing neural networks capable of learning have now been constructed. Into the future, we can expect that molecular circuitry will be designed to make decisions on the fly that reconfigure diagnostic devices or lead to new treatment options.

1. INTRODUCTION

Nucleic acids have been adapted to function as circuits capable of executing algorithms. Although the use of the word "circuit" in general recalls silicon computers using electricity flowing on boards, it can also be applied to virtually any hardware that carries out an algorithm. In the case of nucleic acids, the algorithm is typically embedded directly in the nucleic acid circuit itself, making many nucleic acid circuits "matter computers" whose output is reflected in a changed physical state of the circuit, such as its exposed sequence or conformation. Interestingly, this means that the typical distinction between hardware and software has so far been blurred for nucleic acid circuits, and there is not currently a "software" equivalent for programming with carbon.

The key mechanism that enables nucleic acid circuits to be rationally programmed is known as toehold-mediated strand displacement,[1,2] and the general scheme is described in Figure 1A. In this process, a nucleic acid duplex is composed of an output strand and a substrate strand that is extended with a short single-stranded region known as a toehold. An input strand can initiate binding in the toehold, allowing branch migration to displace the output strand. This simple reaction is the key to many of the more complex toehold-mediated strand displacement reactions described herein. Toeholds can also be exchanged on DNA substrates as part of a toehold-mediated strand displacement mechanism that allows facile equilibration of different nucleic acid species (Figure 1B).

Yurke and co-workers first demonstrated the utility of toehold-mediated strand displacement by creating a simple molecular machine (a tweezer) that underwent particular state changes (opening and closing) in response to the addition of particular nucleic acid substrates.[3] However, these general schemes have since proven to be surprisingly versatile for the creation of a variety of artificial DNA nanomachines,[4−6] logic gates, and nanostructures[7−9] that in turn can perform a variety of tasks, including acting as logic gates; transducing, thresholding, and amplifying signals; and providing feedback and restoration of state.[10−13] As examples, nucleic acid circuits have been shown to be capable of playing tic-tac-toe,[14] calculating the square root of a four-bit binary number,[13] and mimicking neural network computation.[15]

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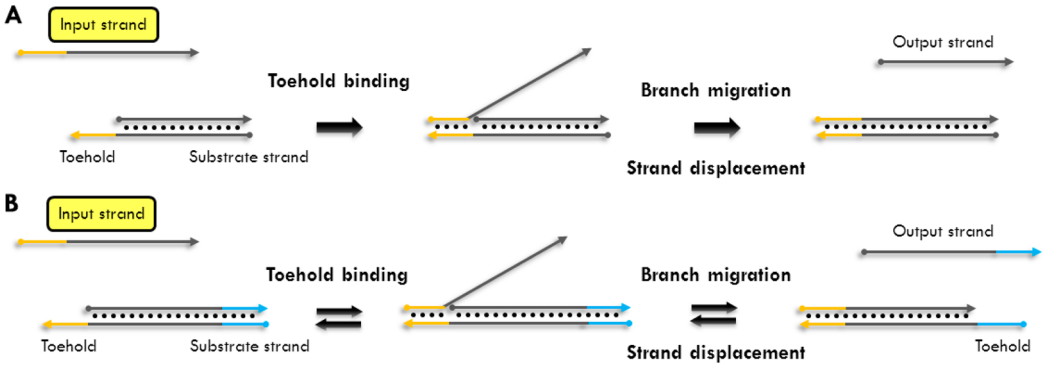

Figure 1. Basic mechanisms of (A) toehold-mediated strand displacement and (B) toehold exchange.

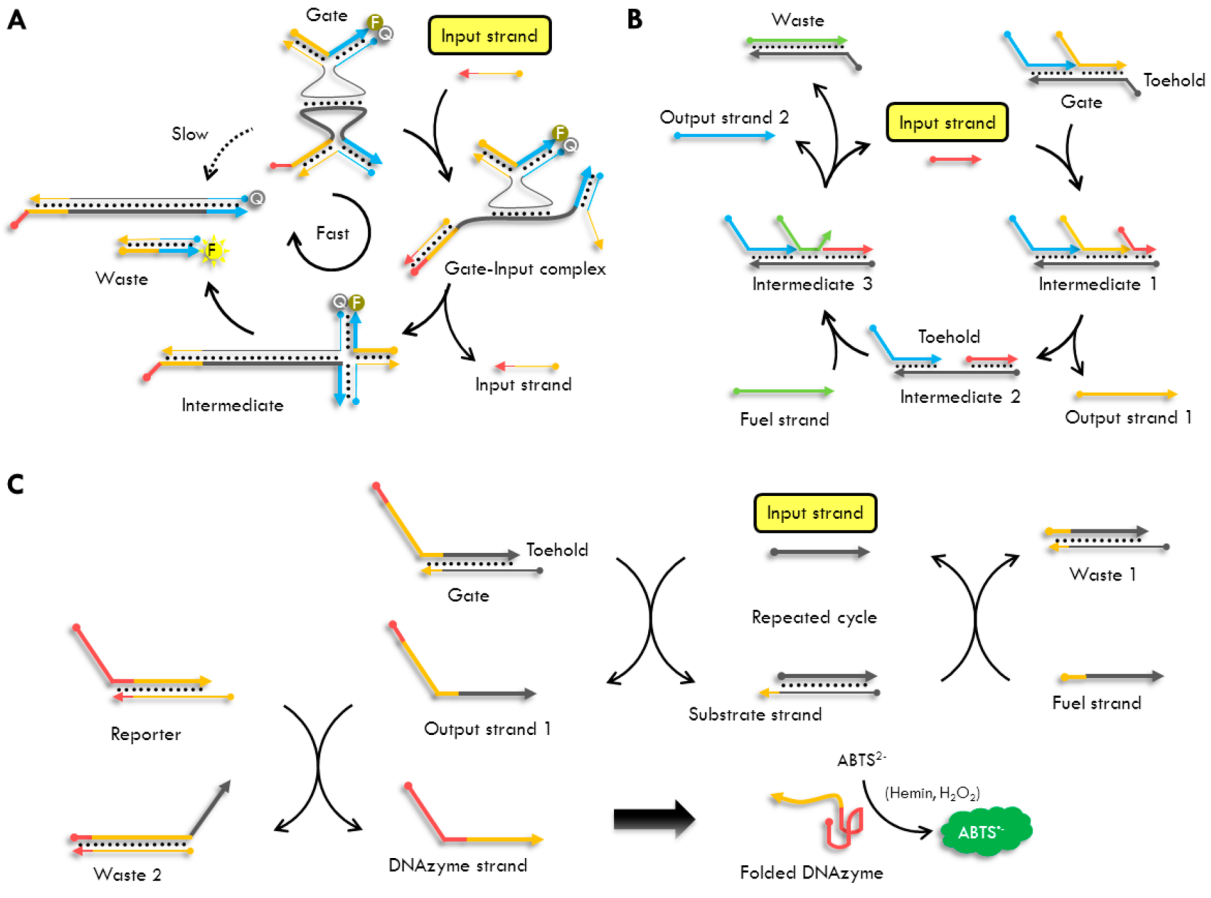

Figure 2. Mechanisms of (A) a catalytic circuit using a metastable kissing-loop structure and (B) entropy-driven catalysis (EDC). (C) Application of EDC to colorimetric detection.

Since medical diagnosis is inherently the process of making decisions about the state of human physiology, it should be possible to adapt the extraordinary capabilities of nucleic acid circuits to diagnostic applications. This is especially true given that nucleic acid circuits can potentially directly interact with and be triggered by some of the most diagnostic molecules in humans, RNA and DNA. In this review, we will attempt to demonstrate both progress in the development of nucleic acid circuits as molecular diagnostics and their future capabilities.

2. TYPES OF NUCLEIC ACID CIRCUITS

Nucleic acid circuits can be artificially divided into classes based on their components and settings. With respect to their components, nucleic acid circuits can operate either completely on their own, with only the nucleic acids themselves carrying out an algorithm, or assisted by protein enzymes. With respect to their settings, nucleic acid circuits can either operate in vitro or they can be embedded within cells or within organisms in vivo.

Most nucleic acid circuits are non-enzymatic and do not rely on protein enzymes for their function. However, there have been several interesting demonstrations of nucleic acid circuits that rely upon proteins for an integral portion of their function. For example, Kim et al. constructed a bistable switch based on cross-repression of promoter units by transcribed RNA molecules.[16] Similarly, Montagne et al. have combined templates, primers, DNA polymerase, a nicking enzyme, and an exonuclease to create a nucleic acid circuit capable of

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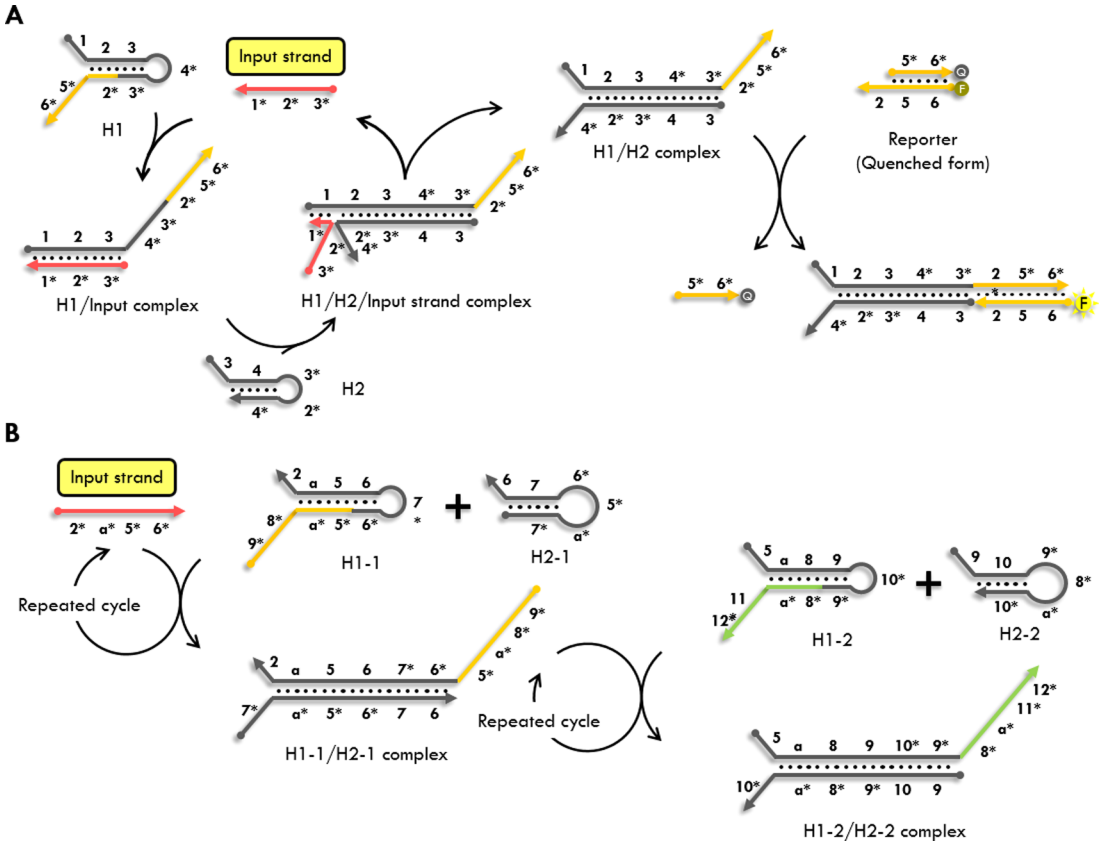

Figure 3. Mechanisms of (A) catalytic hairpin assembly (CHA) and (B) two-layered CHA.

sustained oscillations.[17] To date, nucleic acid circuits that rely on enzymes for some portion of their function have not been widely adapted to in vitro diagnostic applications. This is not because such chimeric circuits cannot eventually prove useful but because of the difficulties in defining rules for (frequently idiosyncratic) protein function that are as simple and robust as those involved in toehold-mediated strand displacement. For similar reasons, most nucleic acid circuits have been implemented in vitro rather than in the much more complex (and enzyme ridden) environment of a cell. This Account will therefore focus on diagnostic applications of enzyme-free nucleic acid circuits in vitro.

3. NUCLEIC ACID AMPLIFIERS

One potential limitation on non-enzymatic nucleic acid circuits for analytical applications is that although they can compute and transduce on the basis of inputs, they may not be able to amplify what are typically quite small biological signals. Thus, it is of particular interest that many of the first applications of non-enzymatic nucleic acid circuits have been as amplifiers. The first amplification circuit that utilized toehold-mediated strand displacement was developed by Turberfield et al. and was later modified by Seelig et al. using metastable kissing-loop structures (Figure 2A).[5,10] Since these early demonstrations, a number of different amplification circuits have been developed and have begun to be adapted to analytical applications.[18]

Non-enzymatic nucleic acid amplifiers can be broadly divided by the mechanisms they utilize. Below, we will consider how the basic toehold-mediated strand displacement mechanism has been utilized to develop complex entropy-driven catalysis (EDC) circuits,[12] so-called seesaw gates,[13] catalytic hairpin assembly (CHA) reactions,[8,19] and hybridization chain reactions (HCR).[20]

3.1. Entropy-Driven Catalysis (EDC)

Zhang et al. initially designed a catalytic circuit in which the addition of a single input strand led to the release of more than one output strand.[12] This reaction is generally driven by the overall entropy of the process and is referred to here as entropy-driven catalysis (EDC; Figure 2B). This mechanism is distinct from the others considered herein, where enthalpy largely drives amplification. In this method, an input strand initiates a series of toehold-mediated strand displacement reactions that lead to catalytic turnover. The input strand first binds to the toehold domain of the gate, displacing an output strand 1. This exposes an additional toehold, to which the fuel strand binds, releasing the output strand 2. The fuel strand further displaces the input strand, and the double-stranded product is waste that does not further react. While the total number of base pairs before and after reaction is not changed, a small amount of free input strand can release a much larger number of free output strands; thus, the overall reaction is driven by a gain in the entropy of the system. This system gave approximately 80- to 100-fold amplification over 24 h with a limit of detection of 10 pM.

Using this basic mechanism, a cascaded circuit was constructed in which the output strand from the first circuit acted as the input strand for the second circuit, leading to approximately 900-fold amplification over 12 h with a 1 pM limit of detection.[12] In addition, by designing the output strand to be the same as the input strand, a catalytic circuit that showed exponential amplification was developed. However, this circuit gave only about 25-fold amplification in 30 min because

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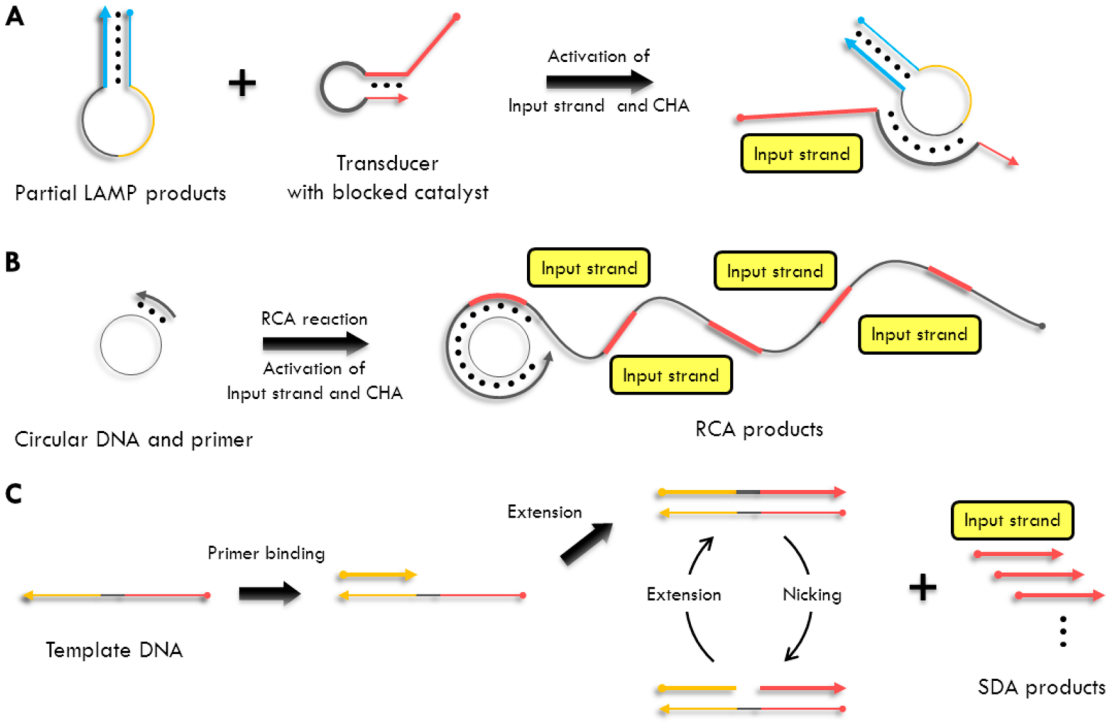

Figure 4. (A) Application of CHA to monitoring LAMP. (B) RCA−CHA and (C) SDA−CHA combinations.

of a high background coupled with exponential growth kinetics.[12]

Our group has previously combined EDC circuits with DNAzyme outputs, thus allowing an input strand to be converted to multiple peroxidase DNAzymes that could in turn convert colorless substrates into a signal that could be readily observed by eye.[21] In this circuit (Figure 2C), an input strand binds to a toehold of the gate. Strand displacement results in the release of output strand 1. Then, a fuel strand further interacts with the substrate strand and ultimately displaces the input strand, which can then interact with additional gates. The released output 1 binds to the toehold of a reporter, displacing a single-stranded oligonucleotide that forms a G-quadruplex structure with peroxidase activity. Although this circuit was a useful signal transducer, it showed only about 4-fold amplification within an hour.

3.2. Catalytic Hairpin Assembly (CHA)

Catalytic hairpin assembly (CHA) differs slightly from the EDC circuits described above, in part in its design (based largely on unimolecular hairpins rather than bimolecular hemiduplexes) and in part on the fact that it is driven by enthalpic as well as entropic considerations. This circuit was originally developed by Pierce and Yin and has proven to be extraordinarily versatile.[8] As shown in Figure 3A, a pair of DNA hairpins (H1 and H2) was designed to be complementary to one another. However, spontaneous hybridization between H1 and H2 is kinetically hindered because the complementary regions are embedded within the hairpin stems. In the presence of an input strand, the stem portion of H1 is opened by the toehold-mediated strand displacement. A newly exposed ssDNA region within H1 can then hybridize to a toehold within H2 and trigger branch migration, ultimately forming a tripartite complex between H1, H2, and input strand. As strand displacement proceeds, this complex will resolve into the most thermodynamically favorable configuration, the H1/H2 duplex, and the input strand will be displaced and be available for additional rounds of toehold-mediated strand displacement. Whereas EDC yields independent output strands, CHA embeds its output within stem structures, making the design of these circuits somewhat more difficult. However, this is offset by much greater synthetic tractability.

The original circuits developed by Yin et al.[8] proved to have higher background than would have been useful for diagnostic applications. We removed so-called clamping domains that were not necessary for the strand exchange reaction and in turn increased the size of each interaction domain from 6 to 8 bp (except for domain 4, which was 11 bp). This simplified design strategy helped to prevent misfolding and thus uncatalyzed hybridization. The total length of the sequences involved in a CHA circuit was also reduced from 75 to 100 bp to 40−60 bp, further decreasing the time required for cycles of design, synthesis, and testing.[19] The improved CHA circuit showed very little background (<0.5 M^{-1} s^{-1}), a good turnover rate (>1 min^{-1}), and 50- to 100-fold signal amplification within a few hours.

Although amplification of a hundred-fold or so is useful, it is not clear that this will enable its use for many diagnostic applications. To improve amplification further, the CHA circuits can be cascaded (Figure 3B). As with a one-layer CHA, the input strand binds to the toehold of H1-1 and leads to the formation of a H1-1/H2-1 complex. However, rather than directly yielding a signal, single-stranded regions on this complex initiate a second layer of CHA. Although the two-layer cascade CHA reaction generates greater amplification, non-specific hybridization among the numerous CHA substrates can again lead to the accumulation of background. By systematically analyzing the origins and characteristics of circuit leakage, we could eventually obtain high-quality CHA circuits that exhibited minimal leakage and 7000-fold signal amplification (Figure 3B).[22] A two-by-two layer cascade (serial transfer between two two-layer cascades) yielded upward of 600 000-fold signal

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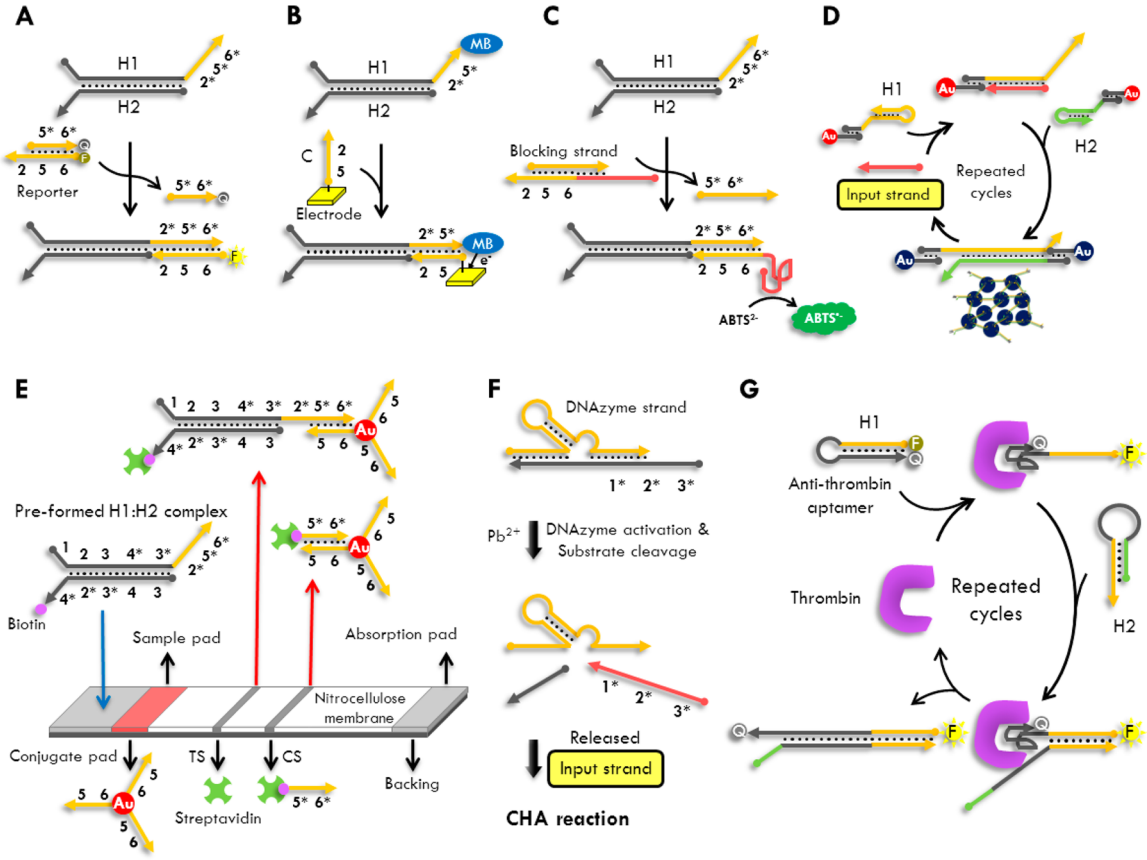

Figure 5. Modularity of outputs and inputs for catalytic hairpin assembly (CHA). Output signaling can include fluorescence (A), electrochemistry (B), and colorimetry either using DNAzymes (C) or gold nanoparticles (D). CHA assays can also be adapted to paperfluidic detection (E). Inputs can include metal ions (actvating DNAZyme cleavage; F) and proteins such as thrombin (G).

amplification. Unfortunately, the second half of the two-layer cascade was relatively slow, and the overall four-layer cascade required 12 h to execute, obviously making it less useful for many diagnostic applications.

Even in the absence of this degree of amplification, it may be that non-enzymatic circuits such as CHA can be used as transducers with more powerful enzyme-based amplification methods. In particular, isothermal amplification assays that proceed through or produce ssDNA products (such as LAMP, RCA, and SDA) might be used to trigger CHA amplifiers/transducers. As an example, CHA has been successfully combined with LAMP.[23] As shown in Figure 4A, the single-stranded loop regions from LAMP were transduced into CHA reporters. One of the LAMP loops opened a hairpin and in turn activated an input strand that triggered the CHA reaction. While most of the amplification is due to enzyme-mediated reproduction of the amplicon, the CHA transducer itself shows a very good signal-to-background ratio, provides several hundred-fold additional amplification within a few hours, and detects less than 17 aM (10 molecules/μl) of a target sequence (M13mp18). More importantly, because of the high sequence specificity of CHA, false-positive signals from parasitic amplicons that arose from primer−primer interactions and non-specific binding to templates were greatly suppressed. In essence, CHA served as the equivalent of a sequence-specific "TaqMan probe" for LAMP reaction. Given that LAMP and many other continuous amplification protocols frequently produce spurious products because of non-specific and often template-independent amplification, CHA circuitry has true utility in redeeming these reactions for diagnostic applications. Further adaptations of CHA probes to real-time and highly specific detection of other isothermal amplification reactions such as RCA and SDA were achieved by engineering a thermostable CHA circuit (Figure 4B,C).[24]

The versatility of CHA circuitry also bodes well for its adoption in a variety of analytical applications. This versatility is best appreciated by examining the variety of outputs and inputs that can be used for CHA circuits. With respect to outputs, CHA cascades have been transduced into various signals including fluorescence, electrochemical, colorimetric, and paperfluidic (strip sensor) methods. In fact, the exact same CHA circuit was adapted to fluorescence, electrochemistry, and colorimetry by simply changing the reporters that were appended to the DNA substrates. To monitor the assembly of H1 and H2 in real time, a fluorescent reporter was constructed by hybridizing oligonucleotides that contained a fluorophore and a quencher. The tail of the H1/H2 complex binds the toehold of the strand containing the fluorophore and displaces the quencher, leading to an increase in fluorescent signal (Figure 5A; similar examples can be seen in Figure 3).[19] For electrochemical signaling, H1 was labeled with methylene blue (MB), and a capture probe (C) that was complementary to the tail of H1/H2 complex was immobilized on a gold electrode. The capture of H1/H2 brought MB closer to the electrode surface, yielding an electrochemical signal (Figure 5B).[19] Colorimetric detection was achieved by having one of the substrates contain a quadruplex DNAzyme peroxidase that catalyzes the oxidation of the colorless substrate, $ABTS^{2-}$, into

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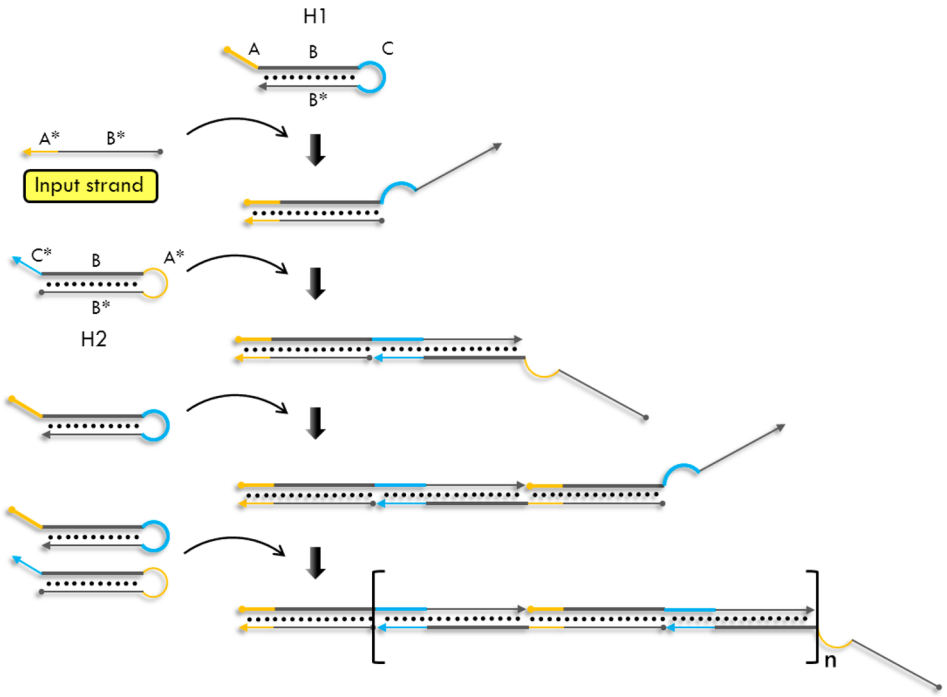

Figure 6. Scheme of the hybridization chain reaction (HCR).

the readily observed green product, $ABTS^{\bullet-}$. Hybridization to a blocking strand denatured the DNAzyme. Input-dependent strand exchange removed the blocker and led to folding of the DNAzyme and thus input-dependent increases in color that could be readily detected by eye (Figure 5C).[19] In other implementations, colorimetric detection was achieved via gold nanoparticle aggregation.[25] Multiple H1 and H2 hairpins were separately conjugated to gold nanoparticles. In the presence of an input strand, the CHA cascade can lead to multiple particles aggregating with one another, in turn leading to a color change (Figure 5D).

The simplicity of CHA circuit design also allows these various detection modalities to be adapted to different analytical or sensor platforms, including media as simple as paperfluidics, either after[26] or during[27] the execution of the circuit. In either case, the detection of CHA reactions on paper strips relies on capture of the reporter strand from the flow stream. For example, the H1/H2 complex includes both a biotin moiety at the 3′ end of H2 and a short overhang at the 3′ end of H1 that leads to the capture of a AuNP−DNA probe at a target site (TS) on the strip sensor (Figure 5E).[26] This probe can also be captured at a control site (CS) even in the absence of reaction. Therefore, two lines at TS and CS appear in the presence of a target, whereas only one line at CS appears in a negative test.

With respect to inputs and as an example of non-nucleic acid detection, a lead-dependent DNAzyme was hybridized to a substrate such that upon lead-induced cleavage an input strand was released for further signal amplification by CHA (Figure 5F). The H1/H2 product was then captured on a strip biosensor. This configuration yielded a detection for lead of 10−100 pM, which is 4 orders of magnitude better than the previously reported strip biosensors without amplification.[26] Similarly, an anti-thrombin aptamer has been used to trigger CHA.[28] As shown in Figure 5G, the anti-thrombin aptamer was embedded into the stem and loop regions of H1. The breathing of H1 presumably allows the thrombin-binding DNA quadruplex to transiently fold. In the presence of thrombin, the quadruplex is stabilized, leading to more persistent presentation of a sequence that can then interact with a toehold on H2. The completion of the CHA cascade again leads to the formation of a H1/H2 complex and the release of thrombin, which can recycle and reinitiate the strand exchange reaction. CHA amplification reduced the detection limit for thrombin to 20 pM, more than 2 orders of magnitude better than similar aptasensors and comparable to conventional ELISA. A similar implementation with the anti-adenosine aptamer was demonstrated. The reaction was coupled to the formation of a G-quadruplex structure that in turn bound the fluorogenic reporter, *N*-methyl mesoporphyrin IX (NMM). This led to a detection limit of 6 μM, an order of magnitude higher than in the absence of amplification.[29]

3.3. Hybridization Chain Reaction (HCR)

The hybridization chain reaction is actually a precursor to catalytic hairpin assembly and was also developed by the Pierce group at Caltech.[20] In HCR, like CHA, two kinetically trapped hairpins (again H1 and H2) are designed to react with one another only in the presence an input strand. However, rather than just forming a dimer, they form a concatenated dimer: a long dsDNA (or dsRNA) that contains hundreds of repeated units (Figure 6). Because there is not a specific sequence output, this mechanism is not typically used in complex circuits. However, the long concatemers make excellent reporters for diagnostics. There is an interesting trade off between the number of inputs and the length of HCR concatemers: because the amount of H1 and H2 probes is fixed, when the input concentration is low the average length of HCR products is longer than in the case of high concentrations of input. Unfortunately, irrespective of the input concentration, HCR concatemers will grow until they exhaust substrates. This makes it difficult to quantitate these reactions unless early time points in the reaction are used.

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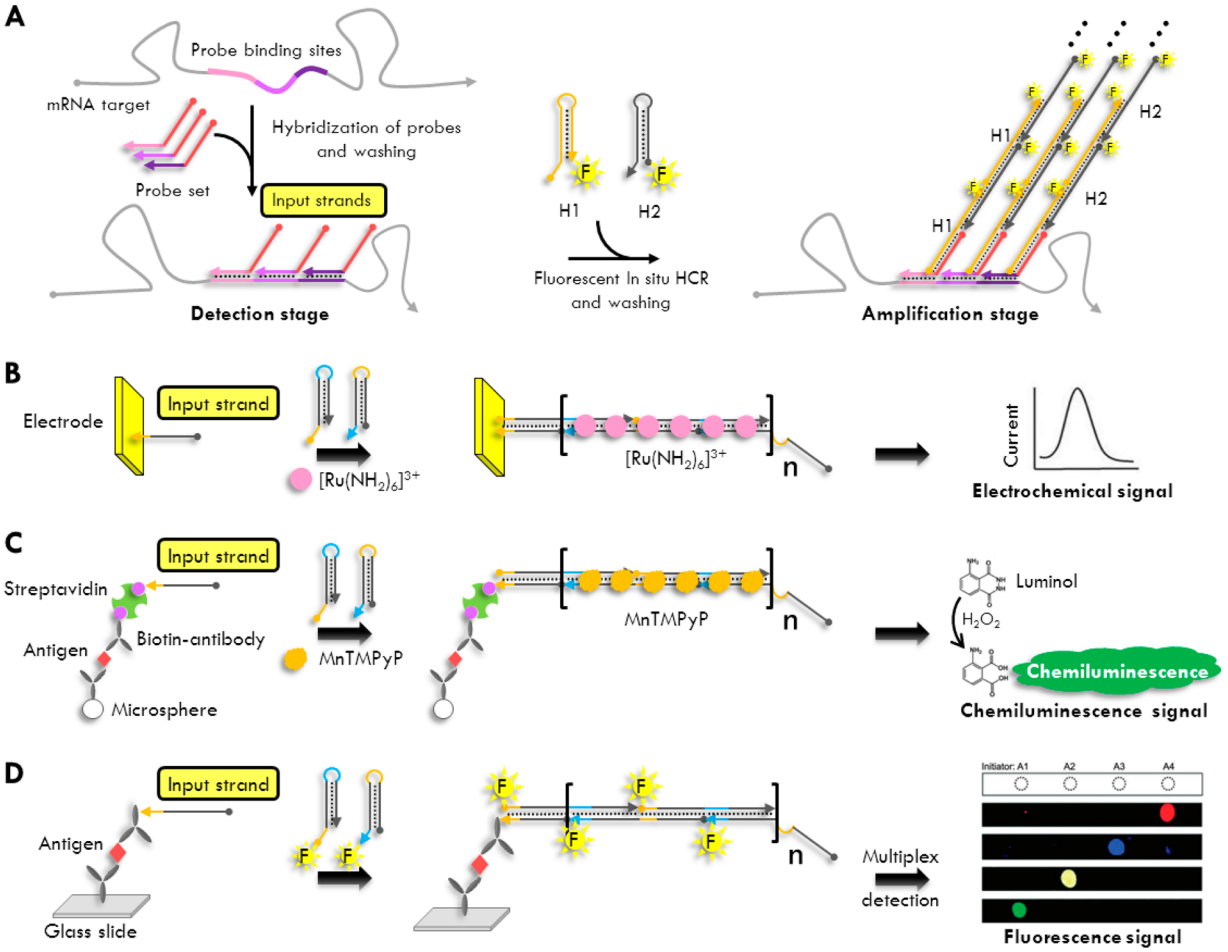

Figure 7. Applications of HCR in solid-state detection. (A) In situ hybridization and fluorescent detection of mRNA targets. (B) Electrochemical detection of DNA. (C) Chemiluminescent detection of antigens. (D) Multiplexed fluorescent detection on glass slides.

HCR has proven to be especially useful for solid-state-based detection methods, as the long concatemer can potentially contain multiple reporters that will be immobilized in one place. The hairpin substrates can once again contain either electrochemical,[30] chemiluminescent,[31] or fluorescent reporters[32] or can contain binding sites for such reporters. For example, the HCR reaction can be used as an excellent way to enhance signals from in situ hybridization and has proven to be extremely useful for visualizing the localization of DNA or RNA within cells and tissues (Figure 7A). Using HCR probes containing different fluorescent reporters, the expression and localization of five different mRNAs could be simultaneously observed in fixed zebrafish embryos.[33] For electrochemical detection, $[Ru(NH_3)_6]^{3+}$ was added to assembled HCR concatemers, bound via electrostatic interactions with the negatively charged phosphate backbone of DNA, and could then serve as a redox indicator for electrochemical signaling (Figure 7B). When used as a transducer for an isothermal amplification reaction, the detection limit was calculated to be 0.02 fM (although only 5-fold came from HCR after 3 h of incubation). For chemiluminescence, a manganese porphyrin (MnTMPyP)/dsDNA complex has been reported to have peroxidase activity, similar to the deoxyribozymes described in the previous section. An antibody-based sandwich assay was used to immobilize an input strand for HCR, and the double-stranded concatemers were detected following addition of MnTMPyp. This assay increased the sensitivity of carcinoembryonic antigen (CEA) detection by about 1 order of magnitude after 1 h (Figure 7C). A similar immuno-HCR reaction was utilized to enhance the detection of secreted cytokines and chemokines from single human mononuclear cells. In this application, HCR probes were labeled with different fluorophores for multiplex analysis (Figure 7D). The sensitivity of detection was improved by an average of 200-fold via the HCR reaction.

HCR can also be adapted to homogeneous solution-based detection methods. As was the case with CHA, conformational changes based on strand exchange can lead to the folding of a G-quadruplex DNAzyme that can oxidize $ABTS^{2-}$ to green-colored $ABTS^{\bullet-}$ (Figure 8A). Because of the large number of DNAzymes activated, as low as 7.5 nM target RNA (input strand) could be detected through a color change induced by DNAzyme.[34]

Because of the bulk dsDNA produced, HCR can also be adapted to more novel homogeneous detection methods (Figure 8B).[35] It has been noted that gold nanoparticles coated with double-stranded DNA aggregate at high salt concentrations, whereas gold nanoparticles coated with single-stranded DNA are dispersed. In the absence of input strand, the tailed ssDNA of each H1 and H2 bind to gold nanoparticles and make them less likely to aggregate. However, in the presence of input strand, the long dsDNA HCR products do not bind to gold nanoparticles and they are therefore more easily aggregated with high salt. Using this simple colorimetric

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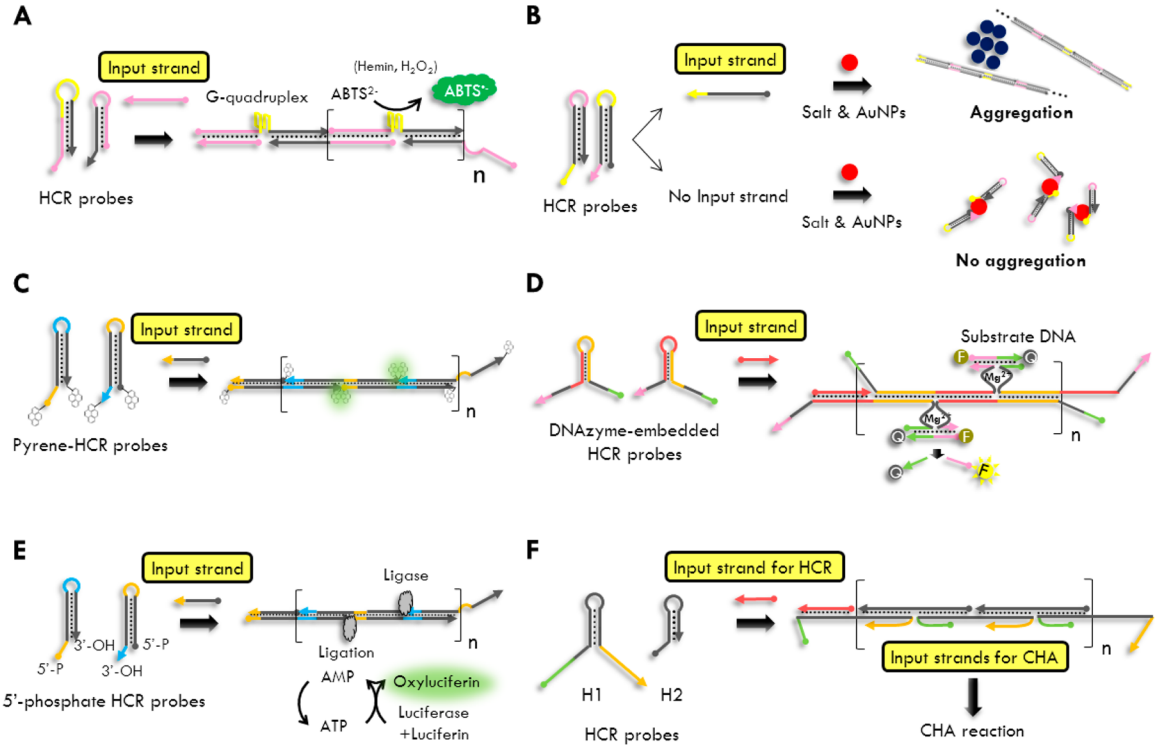

Figure 8. Homogeneous solution based-detection methods for HCR. Conformational transduction and colorimetry via G-quadruplex formation (A) or gold nanoparticles (B). Proximity methods utilizing pyrene-modified HCR probes (C), DNAzyme-embedded HCR probes (D), ligation and ATP recycling (E), or a combination with HCR and CHA (F).

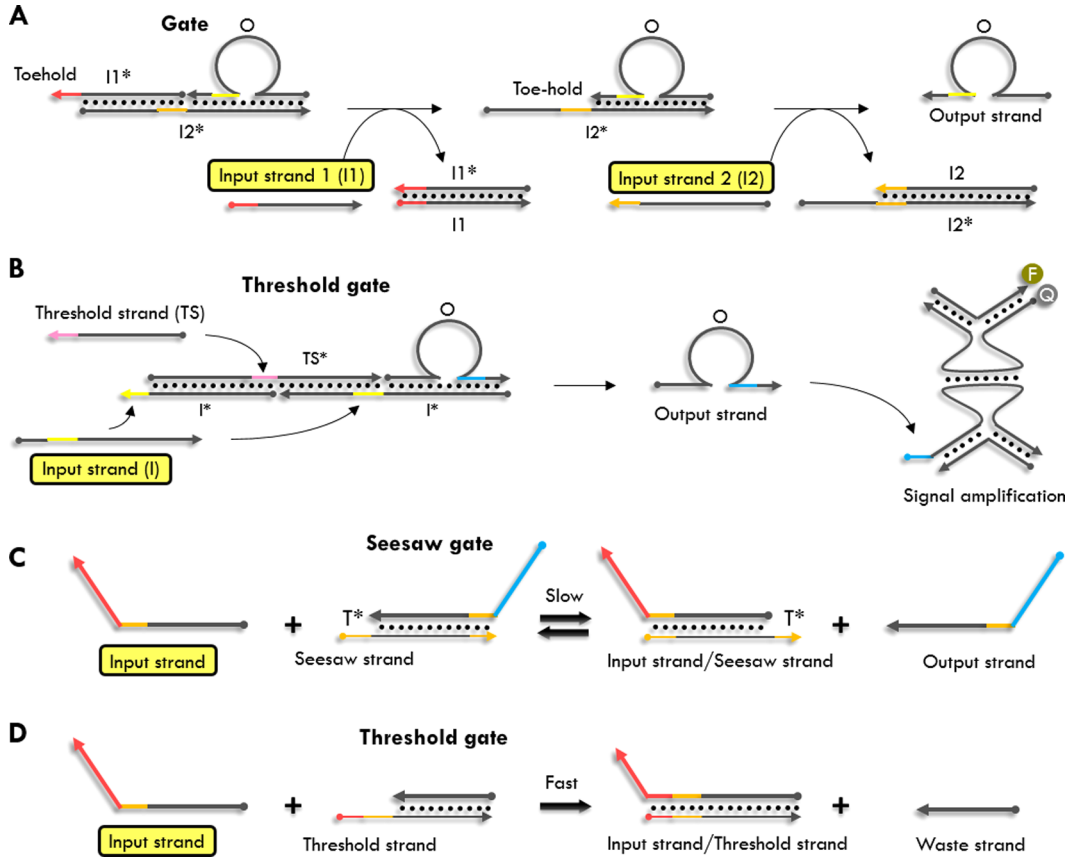

Figure 9. (A) Two-input AND gate. (B) Threshold gate and signal amplification. The basic setup of seesaw gates; seesawing (C) and thresholding (D).

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mechanism, detection limits via instrument and naked eye were determined to be 50 and 100 pM, respectively, about 2 orders of magnitude lower than conventional AuNP-based colorimetric biosensing.

HCR can also be used for homogeneous sensing in solution, primarily by relying on signals that result from the proximity of the multiple H1 and H2 subunits within the longer concatemer. For example, Tan and co-workers modified the ends of H1 and H2 with pyrenes (Figure 8C),[36] and in the presence of input strand, the pyrenes were brought into proximity, forming pyrene-excimers and leading to a fluorescent signal. This method could detect around 250 fM input strand. Similarly, Willner and co-workers incorporated a DNAzyme system into HCR but also utilized proximity approaches by splitting the DNAzyme into two subunits that would only efficiently reassemble upon input strand-mediated concatemer formation (Figure 8D).[37] The DNAzyme was a cleavase that could split apart a fluor/quencher pair on an oligonucleotide substrate, and the combination of HCR and split DNAzyme led to a sensitivity of 10 fM of input strand. Proximity-based DNA ligation has been coupled to the generation of bioluminescence (Figure 8E).[38] The input strand can be quantitatively detected down to a 3 pM limit because of both HCR signal amplification and the recycling of the AMP produced by the ligation assay.

As previously stated, one of the remarkable things about nucleic acid circuitry is its overall modularity, and it has proven to be relatively easy to adapt circuits not only to different inputs and outputs but also to one another. HCR and CHA have been combined (Figure 8F) by designing the H1 substrate for HCR to have single-stranded regions at both its 5′ and 3′ ends so that when these two regions are brought into proximity they can initiate a CHA reaction in solution.[39] In the absence of an input strand for HCR, the two single-stranded inputs for CHA are spatially separated and do not efficiently initiate the CHA cascade. Although this method did provide some additional amplification over CHA alone, the overall sensitivity of detection was not improved because of nonlinearities in the signal response that may be due to the presence of a small fraction of imperfectly formed, chain-terminating HCR substrates.

4. NUCLEIC ACID CIRCUITS MAKING DECISIONS

Because nucleic acid circuits are derived from efforts to establish nucleic acid computation, there are many functions besides amplification where such circuits may prove to be useful in diagnostic assays. Nucleic acid circuits are particularly well-suited to decision making and can include logic gates and thresholding or bandpass elements.

Seelig and Winfree originally constructed enzyme-free DNA circuits that could carry out Boolean logic operations and signal restoration.[11] A two-input AND gate complex consisted of the O, I1*, and I2* oligonucleotides (Figure 9A). The first input strand (I1) binds to the toehold of the gate, and the I1−I1* dsDNA is released via branch migration, exposing a new toehold site where the second input strand (I2) binds. The second input strand displaces the output strand (O), which can also act as an input strand to an additional gate. Using this basic scheme, circuits performing OR and NOT operations were constructed and could again be linked to one another. To buffer circuits against background noise, signal amplification and threshold gates were further introduced into the circuits. As shown Figure 9B, the threshold gate is essentially a three-input AND gate consisting of the input strand (I), threshold strand (TS), and threshold gate. The input strand (I) is used by the gate twice, and the gate is activated only when the input strand (I) concentration is twice as high as that of threshold gate.

Much more complex circuits capable of computation far beyond simple logical decision making have also been constructed. Recently, Qian and Winfree developed a scalable DNA circuit with a simple entropy-driven seesaw gate.[13] Seesaw gates are similar to other entropy-driven circuits (Figure 2B) but are more scalable. In this reversible reaction (Figure 9C), an input strand binds to the seesaw gate via the active toehold site (T*), releasing an output strand by toehold-mediated strand displacement. The resulting input strand/seesaw strand complex has a newly activated toehold site (T*), which is identical to the previous toehold. Therefore, the released output strand can potentially rehybridize to the substrate complex and compete with the input strand. This reversible "seesaw" reaction can be readily tuned, and by adjusting the sequences (and therefore the connectivities) of inputs and outputs, multiple seesaw gates could be readily wired into more complex circuits. The inclusion of a fuel strand for every gate engendered catalysis and amplification and again suppressed background noise by signal restoration via thresholding followed by signal amplification.

Large seesaw networks can be easily constructed that at their root consist of three basic reactions: seesawing, thresholding (Figure 9D), and reporting.[13] Thresholding occurs by modulating toehold-mediated strand displacement rates with different length toeholds. An additional threshold gate is introduced, and the length of the toehold interaction for the threshold gate is made longer than was the case for the seesaw gate. This longer toehold interaction exponentially increases the toehold-mediated strand displacement rate, leading to the seesawing reaction occurring only when the number of output strands exceeds the number of threshold gates. Scale up of DNA circuits has been successfully demonstrated by showing a four-bit square-root circuit that comprises 130 DNA strands and by mimicking neural network computation.[15]

The great advantage of seesaw gates is their simplicity, which allows multiple gates to be prepared in parallel and to be wired to one another in a variety of configurations. However, one drawback to these computational approaches is that the gates are currently built from multistranded DNA complexes, making it more difficult to synthesize and purify components. This drawback may be particularly pronounced for nucleic acid diagnostics, which often have to be very robust for manufacturing and pricing.

5. CONCLUSIONS AND PROSPECTS

We can now compare and contrast the different nucleic acid amplifiers (Table 1). Each circuit has unique features and drawbacks, but this table should assist in identifying features that can be most readily used for different analytes, sensor modalities, or applications.

Nucleic acid amplifier circuits such as EDC, CHA, and HCR show high sensitivity and specificity and can be readily programmed and adapted to different applications. They are particularly useful at background suppression and noise abatement (via thresholding), including for use in real-time applications. The prospect now exists for more complex circuits to act as self-contained diagnostics. It is entirely possible that into the future molecular circuitry will make decisions on the fly that reconfigure the diagnostic device or lead to new treatment options. Indeed, as DNA circuits become ever more complex,

Table 1. Amplifier Circuits

category	EDC	CHA	HCR
circuit structure	multimolecular hemiduplexes	unimolecular hairpin	unimolecular hairpin
recycled component	input	input	nothing (alternating hybridization between H1 and H2)
design flexibility	easy (linear, cascade, exponential)	moderate (linear, cascade)	difficult (linear)
fold-amplification	4–5/h	20–50/h	2–5/h
output type	independent ssDNA	dangled ssDNA on H1/H2 complex	assembled concatemer
connectivity	good	moderate	poor
signal leakage	relatively high	low	low
application type	solution-based	solution-based	surface-based

there will likely come a point at which methods, devices, and platforms begin to be adapted to it rather than the other way around. A future in which DNA "software engineers" help to establish newer generations of analytical "hardware" may mimic the current state of the electronics industry.

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Notes

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