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An isothermal cascade reaction that exponentially amplifies pre-designed, single-stranded DNA was developed as a sensor and signal amplifier module for DNA-based computing and molecular robotics. Taking advantage of the finding that locked nucleic acid can suppress problematic *ab initio* DNA synthesis, up to million-fold amplification rates and concurrent hybridization were achieved at a physiological temperature in a single reactor. Although the effect of locked nucleic acid introduction to the templates was complicated, undesired leak DNA amplification was generally suppressed in the amplification reaction for distinct DNA sequences. The present reaction that senses one DNA as input and generates a large amount of another DNA as output, exhibiting high correlation between molecular concentration and amplification time, is applicable for nucleic acid quantification.

Introduction

Molecular logic operation and computing are increasingly gaining attention, especially for chemical sensing, intelligent diagnostics, and molecular robotics.[1,2] Among various molecules, DNA is a prominent material for computing owing to its inherent capability to bind with its complement in a sequence-specific manner according to the Watson–Crick base-pairing rule. Since the pioneering work on the solutions to combinatorial problems via common molecular biology techniques,[3] many studies have reportedly implemented advanced computing devices, including state machines comprising DNA and enzymes[4,5] as well as logic gates utilizing DNAzymes.[6]

Cascading DNA computing elements were then explored to allow modular architecture and scaling up of computation by utilizing single-stranded DNA (ssDNA) output from an upstream element as an input signal to a downstream element in the construction of enzyme-free DNA logic circuits,[7,8] DNA-directed operation of a state machine,[9,10] and a molecular toolbox encoding non-linear dynamics.[11,12] For achieving a reliable DNA-based computing cascade, ssDNA amplification is an essential function for restoring the signal level.[7,13] Furthermore, for the construction of an autonomous DNA-based robotic system,[14] it is essential to develop an ssDNA amplification reaction module that enables the integration of a DNA-based computing cascade with DNA-directed molecular motors.[15–17] A high amplification rate would be necessary for supplying a large amount of ssDNA to direct a swarm of motors in response to a tiny amount of molecular stimuli.

There is thus an emergent demand for a cascadable DNA amplification reaction that efficiently senses an input DNA, generates an output DNA in single-stranded form, and allows the generated DNA to stably hybridize with its pre-designed target in one pot. Such a DNA amplification reaction should avoid the short amplified sequences and high-temperature conditions that melt away the hybrid formed by the generated DNA and its target. Although a number of isothermal DNA amplification reactions have been developed for biomolecule detection,[18–21] those achieving exponential amplification and concurrent hybridization of ssDNA under low-temperature conditions remain rare. In this context, high or low temperature is defined as the temperature that exceeds or falls below, respectively, the melting temperature (T_m) of the generated DNA.

In the present study, we constructed a DNA amplification reaction that can sense one ssDNA and exponentially amplify another ssDNA at a physiological temperature. With the use of locked nucleotides, up to million-fold DNA amplification was achieved by suppressing leak amplification, which inevitably occurs only with natural nucleotides. The amplified DNA promptly hybridized with its target and sustained hybridization during amplification in one pot.

Scheme of DNA amplification

Our low-temperature amplification (L-TEAM) reaction comprises two DNA species used as templates, called the

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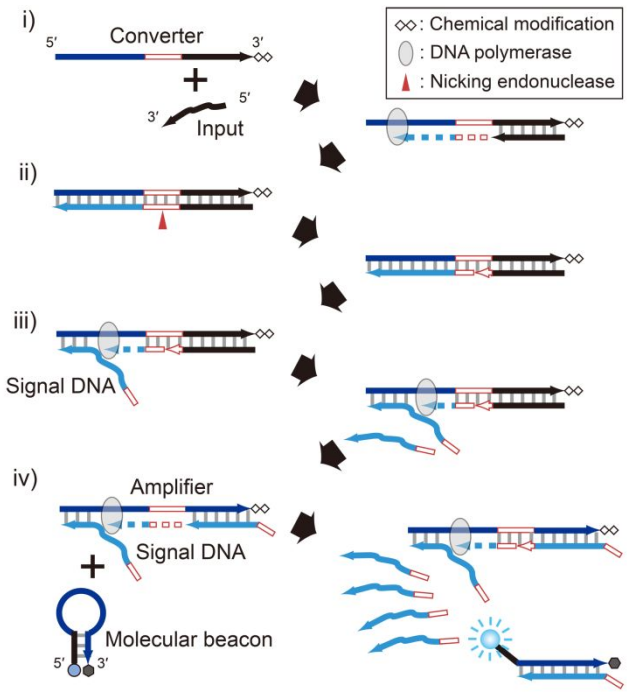

Fig. 1 The scheme of the L-TEAM reaction. Arrows and vertical lines indicate DNA strands and base pairs, respectively. The converter and amplifier are chemically modified at their 3′ ends to avoid unexpected extension by the DNA polymerase. Red open rectangles represent the recognition site of the nicking endonuclease. A portion of the signal DNA shown in light blue represents the pre-designed signal sequence; portions of the converter, amplifier, and molecular beacon shown in blue represent the sequences complementary to the signal sequence. Circles and hexagons attached to the 5′ and 3′ ends of the molecular beacon indicate fluorophores and quenchers, respectively. See details of the reaction in the main text.

converter and amplifier; one primer DNA as an input; a DNA polymerase with strand displacement ability; and a nicking endonuclease (Fig. 1). Upon binding of the input to the converter, the DNA polymerase extends the input and transforms a recognition site of the nicking endonuclease to the double-stranded form (step i in Fig. 1). The nicking endonuclease then nicks the extended input and generates a DNA strand of a fixed length as a signal (step ii). The signal DNA bound on the converter is displaced by the DNA polymerase when it again extends the nicked strand from the cut point (step iii)). The signal DNA strands are continuously generated and released from the converter via iteration of DNA extension and nicking. Here, the input sequence is converted to the pre-designed signal sequence and amplified almost linearly with reaction time.[22]

The signal DNA further binds as a primer to the amplifier that has sequences complementary to the signal DNA at both sides of a recognition site for the nicking endonuclease. Iteration of DNA extension and nicking also occurs on the amplifier, and the generated signal DNA successively binds to the remaining intact amplifier and triggers the iterative reaction (step iv). As a consequence, exponential amplification of the signal DNA is achieved.

L-TEAM is characterized by its design features of a relatively long signal sequence and low reaction temperature, which have been avoided in conventional reactions specialized for rapid amplification. The use of tandemly aligned pairs of sequences in template DNA was previously reported by van Ness *et al*. in the development of the exponential amplification reaction (EXPAR).[23] In EXPAR, the signal DNA spontaneously dissociated from the template due to high temperature (60.0°C) and the use of a short signal sequence of 11 nucleotides (nt), whose T_m becomes significantly lower than the reaction temperature.[23] In contrast, dissociation of the signal DNA fully depends on the strand displacement by the DNA polymerase in the L-TEAM reaction.

This feature that enables hybridization of the amplified DNA to its target, concurrent with DNA amplification in a single reaction vessel, is essential for establishing signal transduction in DNA-based computing and molecular robot control. It also allows real-time monitoring of DNA amplification via sequence-specific hybridization of the signal DNA with a fluorophore-modified probe, such as a molecular beacon.[24] The T_m of the hybrid formed between the signal DNA and the molecular beacon was predicted to be 55.4°C under the present experimental conditions using the DINAMelt software;[25] this T_m is significantly higher than the reaction temperature of 37.0°C. Upon hybridization, a fluorophore and a quencher attached to the molecular beacon separate, resulting in increased fluorescence (step iv in Fig. 1). Here, the reaction system senses the input and generates a sufficient amount of the signal DNA to direct the output operation of the molecular beacon as a fluorescence-emitting nanodevice.

Experimental

DNA

Template DNA strands, primer DNA, and the molecular beacon used in the experiment were commercially synthesized and HPLC-purified by Gene Design, Inc. (Ibaraki, Japan). Template DNA strands were further purified by ion-exchange HPLC as needed. To avoid unexpected extension reactions, the 3′ ends of the template DNA strands were chemically modified with two consecutive inverted deoxythymidines (idT). The 5′ and 3′ ends of the beacon DNA were modified with the fluorescent dye 6-carboxyfluorescein (6-FAM) and the quencher Dabcyl. DNA concentrations were determined by measuring absorbance with a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific K. K., Tokyo, Japan, or NanoVue; GE Healthcare Japan Corporation, Tokyo, Japan).

Fluorescence measurements

For the exponential DNA amplification reaction, except for amplification of the three distinct input sequences, we prepared 25 µL reaction mixtures of NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT [pH 7.9 at 25°C]) (New England BioLabs Japan, Tokyo, Japan), each containing a 5 nM converter, a 20 nM

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amplifier, a 100 nM molecular beacon, and an input of a certain concentration. The reaction mixture also contained 0.2 mM dNTP, 0.16 units/μL Bst DNA polymerase, Large Fragment (New England BioLabs Japan), and 0.1 units/μL Nb.BbvCI (New England BioLabs Japan). The reaction was incubated at 37°C in a real-time PCR system (CFX96; BioRad Japan, Tokyo, Japan), and fluorescence was scanned 120 times, approximately every 70 s. For amplification of the three distinct input sequences, a 2 nM converter and a 50 nM molecular beacon were added, and fluorescence was scanned 150 times.

The fluorescence intensity (FI) measured for each sample in triplicate was normalized by setting the minimum value to 0 and the maximum value to 1, except for the samples in which increased FI saturation was not observed. For such non-amplified samples, including those without the input, the maximum FI values of samples containing the identical templates with the 1 nM input were used for normalization. Finally, the normalized FIs of the triplicate samples were averaged. In the analysis of correlation between the point of inflection (POI) and input concentration, the POI was determined as the monitoring point exhibiting the maximum FI incremental difference.

Table 1 DNA sequences used in the experiment. Blue and red letters represent the sequences complementary to the signal sequence and the recognition site of Nb.BbvCI, respectively. LNA positions are indicated by the superscript letter L. I, F, and D in parentheses represent modifications with inverted deoxythymidine, 6-carboxyfluorescein (6-FAM), and dabcyl, respectively, at the terminal ends.

Converter	5′ AGCCCTGTACAATGCCCTCAGC CTGTTCCT^{L}GCTGAACT^{L}GAGCCA-(I)-(I) 3′
Converter (2)	5′ AGCCCTGTACAATGCCCTCAGC GAAACCCAG^{L}CAGACAAT^{L}GTAGCT-(I)-(I) 3′
Converter (3)	5′ AGCCCTGTACAATGCCCTCAGC TCAACATC^{L}AGTCTGAT^{L}AAGCTA-(I)-(I) 3′
Amplifier	5′ AGCCCTGTACAATGCCCTCAGC AGCC^{L}CTGT^{L}ACAA^{L}T^{L}-(I)-(I) 3′
Amplifier 3-6	5′ AGCCCTGTACAATGCCCTCAGC AGCCCTGT^{L}ACA^{L}AT-(I)-(I) 3′
Amplifier 2-3-6	5′ AGCCCTGTACAATGCCCTCAGC AGCCCTGT^{L}ACA^{L}A^{L}T-(I)-(I) 3′
Amplifier 2-3-6-10	5′ AGCCCTGTACAATGCCCTCAGC AGCC^{L}CTGT^{L}ACA^{L}A^{L}T-(I)-(I) 3′
Input	5′ TGGCTCAGTTCAGCAGGAACAG 3′
Input (2)	5′ AGCTACATTGTCTGCTGGGTTTC 3′
Input (3)	5′ TAGCTTATCAGACTGATGTTGA 3′
Beacon	5′ (F)-AGCCCTGTACAATGCGGCT-(D) 3′

Results and discussion

Leak-free DNA amplification

The reaction design of L-TEAM, requiring only two template DNA species, might reduce erroneous amplification caused by undesired hybridization between mismatched sequences. However, this alone cannot prevent leak amplification occurring in the absence of the input (Fig. S1 (A) in ESI). It is well known that DNA amplification reactions using a thermophilic DNA polymerase generally suffer from template/primer-independent DNA synthesis, called *ab initio* DNA synthesis.[26,27] *Ab initio* DNA synthesis is drastically accelerated with the use of restriction or nicking endonucleases, even under high-temperature conditions,[28–31] which would reduce mishybridization. Although the details of *ab initio* DNA synthesis remain unknown, the proposed mechanism involves anomalous interaction and extension of *de novo* synthesized oligonucleotides.[27] A novel means of suppressing *ab initio* DNA synthesis is expected to be developed as a solution to this common problem.

A locked nucleic acid (LNA) is an artificial nucleic acid that contains a bridged structure in the ribose portion (Fig. 2 (A)).[32,33] LNA is often introduced for raising the specificity and stability in hybridization and the resistance to nucleases.[33,34] The introduction of LNA to the primer also affects the efficiency of DNA polymerization depending on the introduced position.[35] In this study, we investigated the effect of LNA introduction to the templates on the undesired DNA polymerization resulting in *ab initio* DNA synthesis.

We performed the signal DNA amplification and hybridization to its target at a physiological temperature with the use of a nicking endonuclease, Nb.BbvCI, and template DNA strands containing LNA. To avoid affecting the proper templated polymerization and nicking reactions, we introduced LNA only into the 3′-side regions at least 4 nt away from the nicking endonuclease recognition site of the converter and amplifier (Table 1, Table S1, Table S2). Reaction samples were prepared in triplicate and incubated at 37.0°C in a real-time PCR system, and fluorescence was monitored. The obtained fluorescence intensity (FI) was normalized (Fig. S2) and averaged for each triplicate sample (Fig. 2 (B)).

The signal DNA generated after the nicking reaction by Nb.BbvCI, which nicks the DNA strand within the recognition site, contained an additional 5 nt sequence at its 3′-end (Fig. 1). The predicted T_m of the hybrid formed between the signal DNA and the amplifier was 63.3°C. At a reaction temperature significantly below the T_m, the signal DNA generated on the converter or the amplifier should be efficiently displaced to achieve exponential amplification. The FI plot shown in Fig. 2 (B) exhibits sigmoidal curves typical of exponential DNA amplification probed via hybridization with the molecular beacon. To illustrate deviations in the triplicate samples, the normalized FI plot before averaging the sample values to generate the mean-normalized FI plot in Fig. 2 (B) is also shown in Fig. S2. FI in the samples of input concentrations ranging from 100 fM to 1 nM increased faster in samples with higher input concentrations. Saturation of the FI increase in the sample containing the 100 nM beacon that had been triggered by the 100 fM input was observed at approximately 80 min. We confirmed million-fold amplification and targeted hybridization of the signal DNA at 37.0°C.

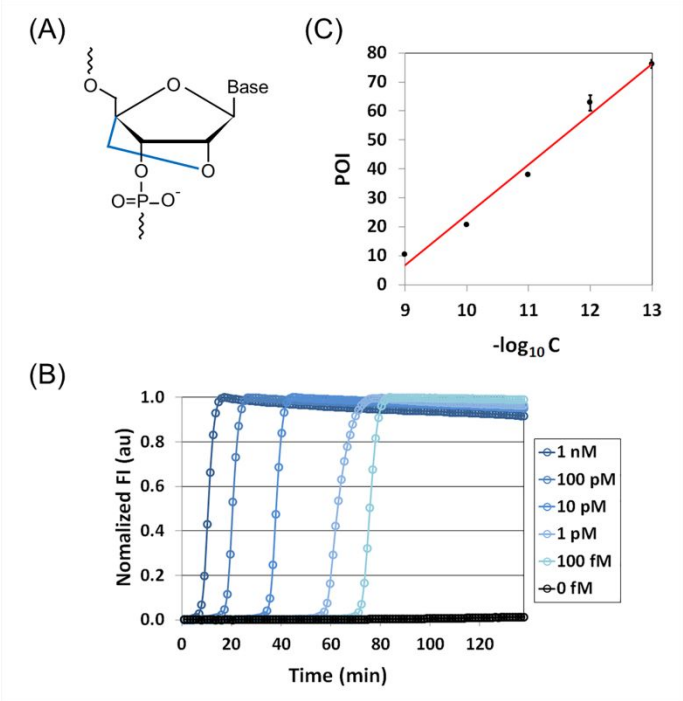

Fig. 2 (A) The bicyclic LNA structure, having a methylene link between the 2′-*O* and 4′-*C* of ribose, shown by light blue lines. (B) Resulting mean-normalized FI plot of L-TEAM reactions using Converter and Amplifier in the absence and presence of the input at concentrations from 100 fM to 1 nM. (C) Plot of the point of inflection as a function of the logarithm of input concentration. Error bars show the standard deviation of measurement in triplicate. The red line represents the linear regression curve.

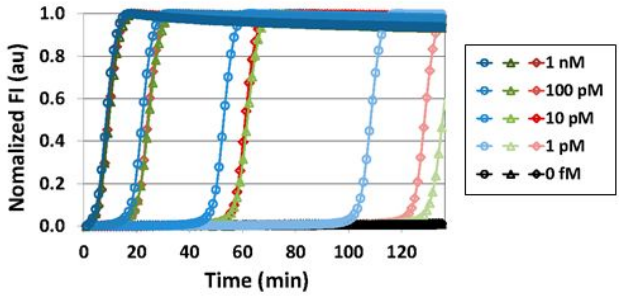

Fig. 3 Resulting mean-normalized FI plot of L-TEAM reactions using Converter and Amplifier 3-6 (○), Amplifier 2-3-6 (Δ), or Amplifier 2-3-6-10 (◊) in the absence and presence of the input at concentrations from 1 pM to 1 nM.

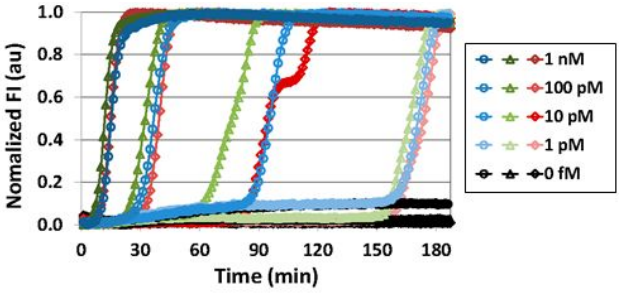

Fig. 4 Resulting mean-normalized FI plot of L-TEAM reactions for Input (○), Input (2) (Δ), and Input (3) (◊), each using Converter, Converter (2), or Converter (3), respectively, and with Amplifier 3-6 in the absence and presence of the respective input at concentrations from 1 pM to 1 nM.

In addition, the FI increase in the absence of the input was completely suppressed for more than 2 h. We showed a novel utility of LNA in the suppression of leak amplification attributed to *ab initio* DNA synthesis. Since LNAs were introduced arbitrarily at multiple positions in the templates, we then investigated the effect of LNA position in the amplifier and confirmed different suppression levels of leak amplification (Fig. S3, Fig. S4, Fig. S5). We found that LNA introduction at the sixth position from the 3′ terminal end in the amplifier markedly delayed the amplification reaction (Fig. S3 (F)). However, the effect of the combinatorial LNA introduction was not the simple synthesis of the effect of single LNA introduction. There seemed no clear rule, such as a relation between the effective position and the predicted secondary structure, determining the suppression level. We further explored the combinatorial LNA introduction including the sixth position in the amplifier. By reducing the number of LNAs from four to two, leak-free DNA amplification up to hundred-thousand-fold within 2 h was achieved with Amplifier 3-6 (Fig. 3).

Next, to validate the generality of introducing LNA to allow leak-free DNA amplification, we performed the L-TEAM reaction for three distinct input sequences with the three corresponding converters, each containing LNA at identical positions, and the common Amplifier 3-6 (Fig. 4). The sequences of the used inputs are same as those of human microRNAs (hsa-miR-24-3p, hsa-miR-221-3p, and hsa-miR-21-5p)[36] (Table 1). Note that the partially-deformed mean-normalized FI plot for Input (3) at the concentration of 10 pM is due to the relatively large deviation of the amplification time among triplicate samples seen in the normalized FI plot before averaging, as shown in Fig. S6 (C). The relatively slower amplification in the L-TEAM reactions with Converter, Amplifier 3-6, Input, and Beacon shown in Fig. 4 compared with those with the same DNA strands in Fig. 3 is attributed to the lower concentrations of Converter and Beacon. We confirmed the suppression of leak amplification and comparable amplification performance independent of the input sequences. The present results indicate that a laborious optimization of the LNA positions is not required for the input sequence by sequence.

Conversion of input concentration to amplification time

The physiological temperature of the L-TEAM reaction, which prevents protein denaturation, has great advantages. Integration of L-TEAM with a DNA-directed motor protein for the construction of a molecular robot is feasible. L-TEAM will be useful not only in DNA-based computing and molecular robotics but also in nucleic acid measurement using blood samples, because it can eliminate the need for laborious sample purification due to blood clotting under high-temperature conditions. The point of inflection (POI), which is the time corresponding to the maximum slope in the FI curve of Fig. 2 (B), is plotted against the input concentration (Fig. 2

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(C)). The regression equation is $Y = -149.6 - 17.38 \cdot \log_{10}C$, with a correlation coefficient of 0.9913, where Y and C represent the POI and input concentration, respectively. High correlation between the POI and input concentration indicates that information conversion between molecular concentration and amplification time is possible. L-TEAM would be applicable to isothermal nucleic acid quantification, similar to quantification by real-time PCR, based on the correlation between the threshold cycle of FI increase and the target concentrations.[37]

Conclusions

In this study, we developed an isothermal reaction in which an ssDNA molecule is efficiently amplified and transduces an operational signal via hybridization to the pre-designed target in one pot. We experimentally showed that sensing of the tiny amount of input by the converter triggered signal DNA amplification and directed fluorescence emission of a vast number of molecular beacons. In addition, we found a novel utility of LNA in circumventing the common leak problem in DNA amplification reactions. The present results indicate that LNA might provide a novel means for probing the mechanism of *ab initio* DNA synthesis. However, the mode of suppressing endonuclease-enhanced leak amplification has not yet been clarified. The present L-TEAM reaction achieves up to million-fold exponential DNA amplification at a physiological temperature and could connect DNA-based computing to DNA-directed actuators by bridging the gap in molecular concentrations. Moreover, the use of biological nucleic acids as primers would allow the application of L-TEAM in the quantification of short nucleic acids such as microRNA for medical testing or processing biomolecule information by molecular robots.

Conflicts of interest

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