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Photo-regulatable DNA isothermal amplification by template-mediated ligation

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By combining azobenzene-tethered oligonucleotides as modulators and poly(L-lysine)-*graft*-dextran (PLL-*g*-Dex) a chaperone polymer to facilitate strand displacement, we successfully developed a photo-regulatable DNA isothermal amplification method. By alternating UV and visible irradiation, linear amplification was achieved. The method enables photo-regulatability and mismatch discrimination in linear amplification of DNA target.

DNA- or RNA-templated amplification reactions are crucially important for copying the original oligonucleotides and rapid and precise nucleic acid sequence sensing.[1-4] By attaching two different chemical groups to one of the two nucleic acid substrates, the reaction can be predictively triggered by hybridization of substrate strands and template strands. Kool and co-workers have reported several strategies to achieve effective and rapid DNA/RNA detection and imaging in which fluorescence is induced by templated transformational reactions[5, 6] or chemical ligations and the signals are amplified by DNA strand displacement.[7, 8]. A problem to employ chemical ligation is that non-natural linkages, which are used to conjugate oligonucleotides, inhibit the further templating process and recognition by enzyme. Moreover, as the turnover generated by strand displacement for amplification requires rather unstable hybridization,[9, 10] sacrificing the duplex stability by introducing a non-natural linker and repeating optimization of conditions are necessary.

In our group, light has been applied to externally regulate the formation and dissociation of DNA duplexes without contaminating the nano-environment.[11] Photo-responsive azobenzene moieties are introduced into the DNA strand via a D-threoninol scaffold and light-induced molecular isomerization allows reversible control of duplex formation. With this method, several photo-driven DNA nanomachines have been developed including a photoresponsive DNA tweezer,[12] a photo-controllable RNA scissor based on a DNAzyme,[13] and a DNA seesaw.[14] Recently, we have reported a photo-driven DNA strand displacement reaction that performs in a toehold-less fashion.[15] In all these systems azobenzene-tethered DNA is the engine that drives the motions of nanodevices. We have also shown that poly(L-lysine)-*graft*-dextran (PLL-*g*-Dex), developed by Maruyama's group,[16-18] serves as a chaperone polymer that facilitates strand displacement to increase the efficiency of the nucleic-acid-based machines. We reasoned that a photo-driven DNA strand displacement mechanism could be used to regulate DNA isothermal amplification assays. Since our method does not contain heating process like PCR, thermally unstable enzymes are available. Isothermal amplification is also applicable in the presence of thermally unstable molecules or proteins. Unlike changing temperature, switching light and/or changing wavelength will be much faster and easier. Hence, we believe that our method provides the alternative solution for copying nucleic acids.

The photo-regulatable DNA amplification reaction we developed is shown in Scheme 1a. The system consists of a template strand, an azobenzene-tethered modulator that is complementary to template strand, and two substrate strands each of which is complementary to half of the template. One of the substrate strands is labelled with a TAMRA-group at the 3' end and has a phosphate group at the 5' end. Initially, the azobenzene moieties in the modulator strand are in *trans*-form, and this allows hybridization with the template. Once irradiated with ultraviolet light (UV, 365 nm), the azobenzene moieties isomerize to the *cis*-form, inducing dissociation of the duplex between template and modulator thereby allowing the template to hybridize with the two substrate strands. Enzymatic ligation then occurs to produce a product strand. The system is then irradiated with visible light (465 nm), and the azobenzene

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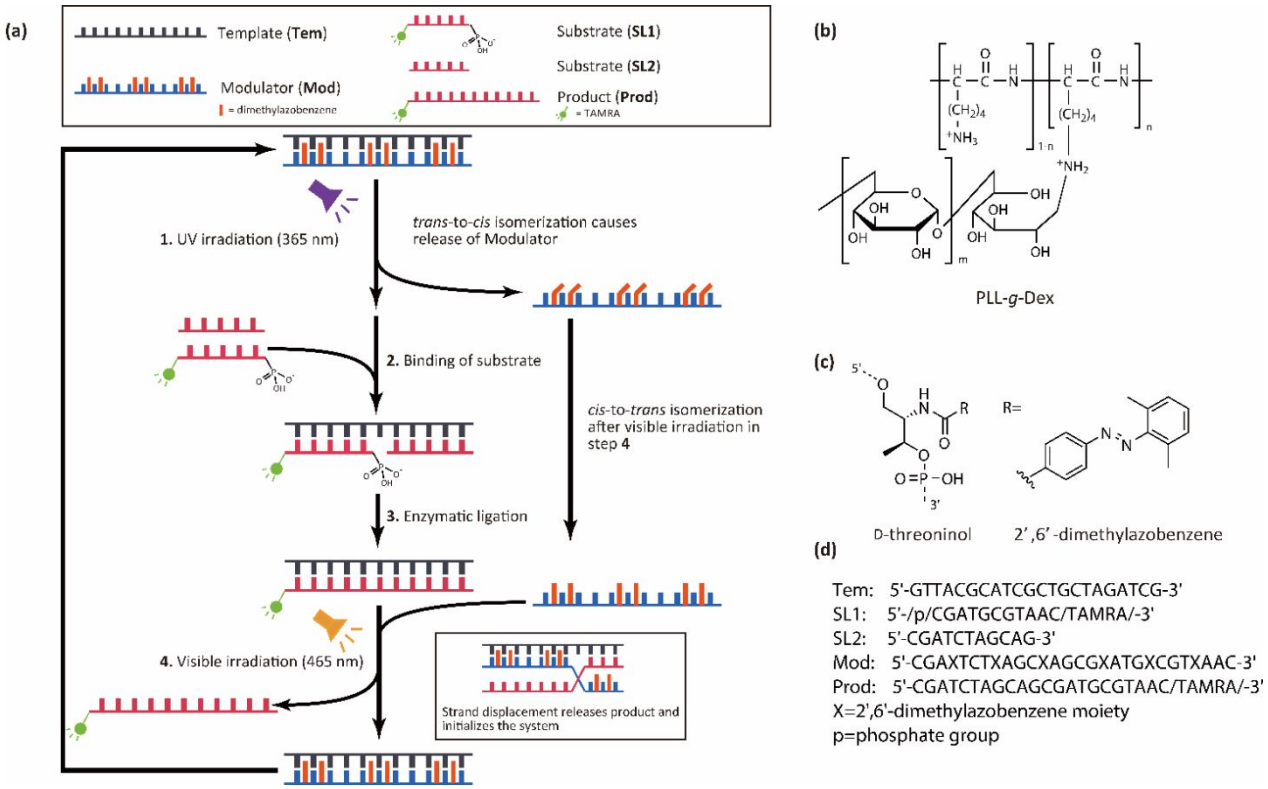

Scheme 1. (a) Strategy of photo-regulatable DNA amplification. The template strand (Tem) is in **black**, the modulator strand (Mod) is in **blue** with azobenzenes in **orange**, and the substrate strands (SL1 and SL2) are in red. (b) Chemical structure of chaperone polymer PLL-*g*-Dex. (c) Chemical structure of D-threoninol scaffold and 2',6'-dimethylazobenzene. (d) Sequences used in experiments.

moieties revert to the *trans*-form. This induces the strand displacement so that the modulator binds to template releasing the ligated product and initializing the system. In this way, by alternating irradiation with UV and visible light, amplification of the product can be precisely, quantitatively, and repetitively controlled. Note that ligation product does not involve any non-natural linkage so that it does not reduce duplex stability to the complementary strand. Furthermore, unlike PCR, we do not have to change the temperature to regulate hybridization of oligonucleotides.

In the system characterized here, six 2',6'-dimethylazobenzene moieties were incorporated into the modulator strand through D-threoninol as a scaffold because previous study revealed that incorporation of the moieties at every three nucleotides was most efficient for strand displacement reaction.[15] Incorporation of dimethyl-modified azobenzene instead of non-modified azobenzene enhanced the stability of DNA duplex due to increased hydrophobicity and stacking effect,[19, 20] which, as previously described,[15] facilitated the strand displacement process. Moreover, addition of the previously characterized cationic comb-type copolymer, PLL-*g*-Dex,[16, 18] stabilized the DNA duplex so that improved thermodynamics and kinetics of the strand displacement. According to previous investigations, this acceleration is the result of spontaneous interaction between PLL-*g*-Dex and DNA to form interpolyelectrolyte complexes, which promotes the intermediate formation during strand displacement by alleviating the counterion condensation effect.[17] In this method, we choose enzymatic ligation reaction instead of a chemical reaction to ligate the substrates so that a natural linkage is produced for further application.

Each step in Scheme 1 includes hybridization, dissociation and strand displacement processes. Therefore, in order to validate our strategy, we measured melting temperatures (T_ms) of all the duplexes in the amplification system before conducting amplification experiment, which are shown in Tab. S1 and Fig. S7. Since T_ms of both **Tem/SL1** and **Tem/SL2** are lower than **Tem/Prod**, amplification does not occur without initializing the reaction system by the ***trans*-Mod**. Note that T_m of **Tem/*trans*-Mod** is higher than that of **Tem/Prod**, demonstrating strand displacement is thermodynamically favourable. With the aid of PLL-*g*-Dex, strand displacement occurs efficiently without toehold. In the previous paper,[15] we quantitatively investigated photo-triggered strand displacement reaction from the fluorescence change and proved efficient and repetitive displacement by *trans-cis* isomerization. Concentration of substrates (**SL1** and **SL2**) was set as 50-fold higher than that of template (**Tem**) for amplification of ligation and preferential displacement of product with substrates.

In this method, PLL-*g*-Dex is employed to enable the efficient strand displacement reaction. In order to select suitable ligase that works in the presence of PLL-*g*-Dex, we tested conventional T3, T4 and T7 ligases for five cycles amplification to compare

their compatibility with PLL-*g*-Dex. The result is shown in Fig. S1. All three ligases successfully facilitated the amplification in the presence of PLL-*g*-Dex under alternating irradiation strategy. Since the concentration ratio of template and substrate is 1:50, there should be theoretically 2% of substrate to be ligated in a single cycle. Here we define the Degree of Amplification (**DA**) value to evaluate the extent of amplification reaction (for the definition see Experimental Section in Supporting Information). For instance, 2.0 **DA** indicates that the amount of ligated substrate, i.e. product, is 2 times higher than that of template, which is 400 nM. Use of T3 ligase resulted in the highest **DA**, approximately 3.6 after five cycles of amplification; while the theoretical **DA** is 5.0. This result is coincident with strand displacement efficiency in our previous study.[15] We also confirmed that the activity of T3 ligase persisted despite irradiation (Fig. S2).

We next conducted a series of control experiments to verify that the amplification attained was template-dependent and could be controlled obediently by light irradiation. In all control experiments, the repetitive amplification was conducted for five cycles, after which products were visualized by electrophoresis and **DA**s were calculated by quantifying band intensity. PAGE images are shown in Fig. S3, and **DA**s are plotted in Fig. 1. In the presence of template, **DA** was approximately 3.1 when the maximum theoretical **DA** was 5.0 under the conditions employed. In contrast, the amplification did not occur at all in the absence of template, which indicated the predominant template-dependency. There was no amplification in the absence of PLL-*g*-Dex after five rounds of alternating irradiation, either, suggesting that the chaperone polymer is crucial for achieving efficient strand displacement. It should be noted that amplification did not occur without light irradiation even in the presence of photoresponsive modulator. Native modulator without azobenzene did not amplify ligation at all, clearly demonstrating that strand displacement caused by light-driven *trans-cis* isomerization boosted the repetitive amplification. We also tested our strategy with poly(*L*-lysine)

Template	+	-	+	+	+	+
PLL-*g*-Dex	+	+	-	+	+	+
Modulator	+	+	+	-	-	+
Native-Mod	-	-	-	-	+	-
Irradiation	+	+	+	+	+	-

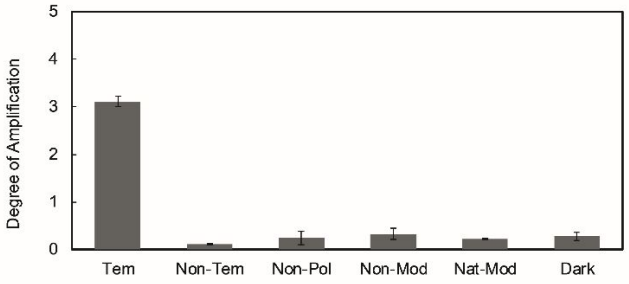

Figure 1. Template, PLL-*g*-Dex, modulator, photoswitches and light irradiation are necessary for amplification. Degree of Amplification value was calculated from fluorescence intensity of bands in PAGE (Fig. S3). Conditions: 200 nM Template if present, 10 μM each Substrate, 2 μM Modulator if present, PLL-*g*-Dex at the N/P ratio of 1.5 based on the previous study[15] if present (N/P ratio refers to the ratio of amino groups of the copolymer to phosphate groups of DNA), 16 U/μL T3 ligase, performed in 1 x T3 reaction buffer at 37 °C. For all samples, 5 cycles of amplification were conducted. Irradiation protocol is described in Supplementary Material.

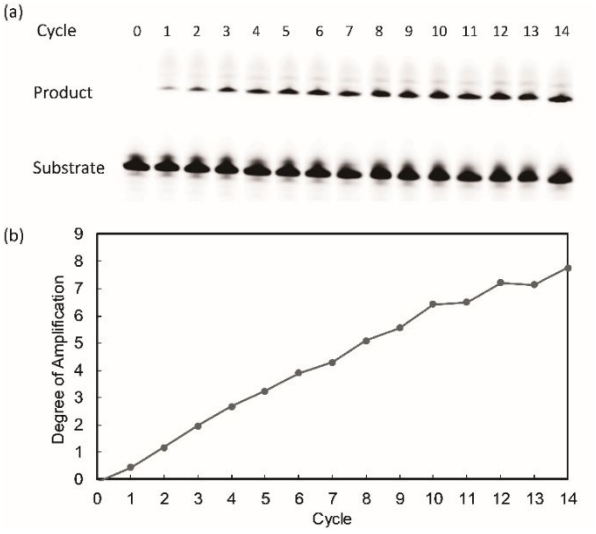

Figure 2. Amplification is almost linear over 14 irradiation cycles. (a) PAGE image of 14-cycle photo-regulatable DNA amplification. Lane 0, before adding of T3 ligase; lanes 1-14, products of first to fourteenth cycles. (b) **DA** after each cycle calculated from fluorescence intensity of bands in PAGE image shown in panel a. Conditions: 200 nM Template, 10 μM each Substrate, 2 μM Modulator, PLL-*g*-Dex at N/P = 1.5, 16 U/μL T3 ligase, performed in 1 x T3 reaction buffer at 37 °C. Irradiation protocol is described in Supplementary Material.

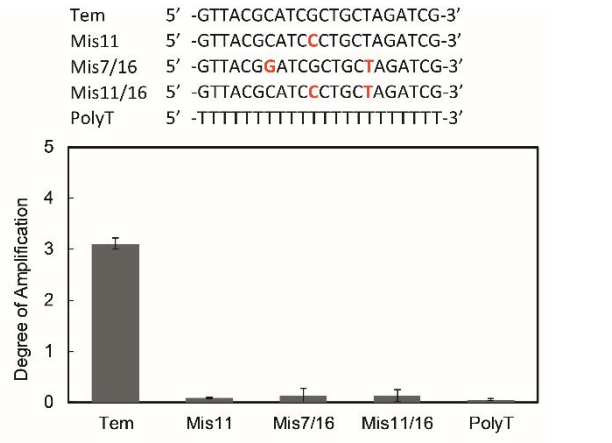

Figure 3. Photo-regulatable DNA amplification is sequence-specific. Degree of Amplification value was calculated from fluorescence intensity of bands in PAGE (Fig. S6). Conditions: 200 nM indicated template, 10 μM each Substrate, 2 μM Modulator, PLL-*g*-Dex at N/P ratio of 1.5, 16 U/μL T3 ligase, performed in 1 x T3 reaction buffer at 37 °C. For all samples, 5 cycles of amplification were conducted. Irradiation protocol is described in the Supplementary Material.

(PLL) which does not contain the grafted dextran chain shown to enhance the interaction with DNA.[21] Amplification was not observed in the presence of PLL (Fig. S4), indicating that the hydrophilic dextran chain is necessary for assisting the strand displacement. Moreover, the non-modified azobenzene-tethered modulator did not facilitate amplification (Fig. S5) either, revealing that 2',6'-dimethylation of azobenzene particularly promoted the strand displacement very efficiently, which is in agree with previous research.[15]

Next, we examined if our method could be photo-controlled quantitatively and flexibly. Each cycle included a 5-min irradiation with 365 nm UV light, a 30-min incubation in the dark, and a 5-min irradiation with 465 nm visible light. As the number of light irradiation cycles increased, the intensity of the ligated product band increased (Fig. 2a). This is the direct evidence that product was successfully amplified during the alternating irradiation by UV and visible light. As shown in Fig. 2b, the yield increased almost linearly over 14 cycles. The slight decrease in last several cycles is probably caused by emulative inhibition derived from the decrease in substrate concentrations and the increase of product concentration.

Finally, we investigated the dependence of the reaction on complementarity between template and substrate strands. We evaluated amplification when there were one or two mismatches in the template strand (Fig. 3). Amplification was not observed when templates contain one and two mismatches, and the polyT template did not facilitate the amplification reaction (Fig. 3 and S6). Apparently, the decreasing stability of duplex generated from mismatches prevented the ligation thus the photo-driven strand amplification was not triggered. This result indicates that the strategy achieves accurate amplification of a specific target sequence.

Conclusions

We have developed a photo-regulatable and isothermal DNA amplification method that relies on light-induced isomerization of an azobenzene in modulator oligonucleotide and chaperone polymer PLL-*g*-Dex. The amplification increased almost linearly over 14 cycles of UV and visible light alternating irradiation. We also demonstrated a competitive discriminability of mismatches in template strand. The photo-responsivity revealed by this method offers the possibility of precise control of an amplification process, which so far has been consecutive and uninterruptable in other isothermal amplification methods such as rolling circle amplification.[22] It also provides an alternative solution for designing new diagnostic tool of genetic diseases, though the condition should be further optimized to demonstrate the detection limit. We will focus on development of a strategy that allows photo-regulatable amplification in an exponential yield.

Conflicts of interest

There are no conflicts to declare.

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