

Using photons to manipulate enzyme inhibition by an azobenzene-modified nucleic acid probe

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The ability to inhibit an enzyme in a specific tissue with high spatial resolution combined with a readily available antidote should find many biomedical applications. We have accomplished this by taking advantage of the *cis-trans* photoisomerization of azobenzene molecules. Specifically, we positioned azobenzene moieties within the DNA sequence complementary to a 15-base-long thrombin aptamer and then linked the azobenzene-modified cDNA to the aptamer by a polyethylene glycol (PEG) linker to make a unimolecular conjugate. During the photoisomerization of azobenzene by visible light, the inhibition of thrombin is disabled because the probe hybridizes with the cDNA in the *trans*-azobenzene conformation so that the aptamer cannot bind its target thrombin. However, when UV light is applied, melting of the hairpin structure (duplex) is induced via *trans-to-cis* conversion, thereby changing conformation of the aptamer and making the aptamer free to bind to and inhibit its target thrombin. By using standard clotting assays, we measured the IC₂₀₀ of various probe designs in both states and concluded the feasibility of using photon energy to temporally and spatially regulate these enzymatic reactions. Thus, we can report the development of DNA probes in the form of photon-controllable (thrombin) inhibitors, termed PCIs, and we expect that this approach will be highly beneficial in future biomedical and pharmaceutical applications.

anticoagulation | aptamer | enzyme inhibitor | photo controllable | thrombine

Over the last decade, extensive research has been devoted to the study of phototransformable molecules based on photochromism chemistry. This science has been applied to photooptical technology and such photomodulated devices as eyeglasses called Transitions (1–4). A photochromic compound has 2 molecular states: stable and relatively unstable. The 2 states are interchangeable by the effect of irradiation using different wavelengths and differ from one another in terms of both physical and chemical properties. The photoconversion mechanism can largely be divided into 3 transformation types: photochromic tautomerism, *cis-trans* isomerization, and photocyclization. Briefly, photoisomerization is a process in which molecular structural change between isomers is caused by photoexcitation. Therefore, because isomerization causes a conformational change that can change the overall structure of a molecule, *cis-trans* isomerization is an intriguing mechanism that can be used to regulate mechanical devices and biological reactions (5–8).

As one of the most popular phototransformable molecules in use today, azobenzene and its derivatives belong to the *cis-trans* isomerization category and are composed of 2 phenyl rings linked by a N=N double bond (Fig. 1) (9). The 2 isomers can be switched with particular wavelengths of light: UV light at 365 nm, corresponding to the *trans-to-cis* conversion, and visible light at 465 nm, corresponding to the *cis-to-trans* isomerization. There are reports that demonstrate the possible applications of such a feature in the development of sensors (10), nanomotors (11–13), and even peptide engineering (14–16). These reports involved the use of enzymes that naturally act on DNA. However, we are interested in regulating enzymes that do not naturally act on DNA, and, at the same time,

we want to take advantage of the special reactivity of azobenzene to photon energy. Therefore, we will focus our molecular design on using azobenzene to regulate the binding of DNA aptamers that have enzyme inhibitory capabilities.

The rational design of nucleic acid (DNA) aptamers for this purpose is, therefore, a key part of our developmental strategy. As a previously uncharacterized class of binding elements, nucleic acid aptamers selected by the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) method have shown some outstanding features (17–20). For instance, DNA-aptamers possess a relatively small molecular weight coupled with high binding affinity. Structurally, DNA-aptamers are easily modifiable with robust structural restoration under ambient conditions (20). For these reasons, many nucleic acid sequences have been selected for various types of targets, and some of them have shown considerable clinical potential. Among them, thrombin-binding aptamers (TBAs) are some of the most popular DNA sequences (21, 22). Of the 2 known TBAs, 1 is 15 bases long (15Apt) and binds to exosite 1 of thrombin. Because it occupies the fibrinogen-binding pocket, only 15Apt can inhibit thrombin-mediated coagulation (23). Thrombin is important in the regulation of homeostasis and is associated with many types of cancer. Therefore, engineering probes that target thrombin and, moreover, whose function can be precisely regulated by photonic energy, can advance anticoagulation therapy for many diseases (24, 25).

Photoregulation of azobenzene conformation and DNA duplex structures using azobenzene is a well-known and studied phenomenon. We also believe that it is a key component to the functionalization of probes engineered to target thrombin. This conclusion is based on the work that has been done on synthesizing azobenzene phosphoramidite monomers and the fact that the NMR structures of both *cis*- and *trans*- states have been described (26–27). Most recently, a full thermodynamic description based on nearest-neighbor models of DNA duplex formation has been presented and successfully used to predict melting temperatures of azobenzene-incorporated DNA duplexes (29). Finally, because researchers have shown its ability to regulate enzymes (30, 31), we chose azobenzene as the fundamental component of our thrombin-binding aptamer, which we term photocontrollable inhibitor, or PCI.

Our PCI design has 3 functional domains: inhibitory, regulatory, and linking (Fig. 1). The inhibitory domain is a 15Apt sequence. As noted above, 15Apt binds to exosite 1 of thrombin. Because it occupies the fibrinogen-binding pocket, only 15Apt can inhibit thrombin-mediated coagulation (23). Next, the regulatory domain is composed of the complementary sequence to 15Apt, but with azobenzene molecules inserted into the phosphate backbone of the

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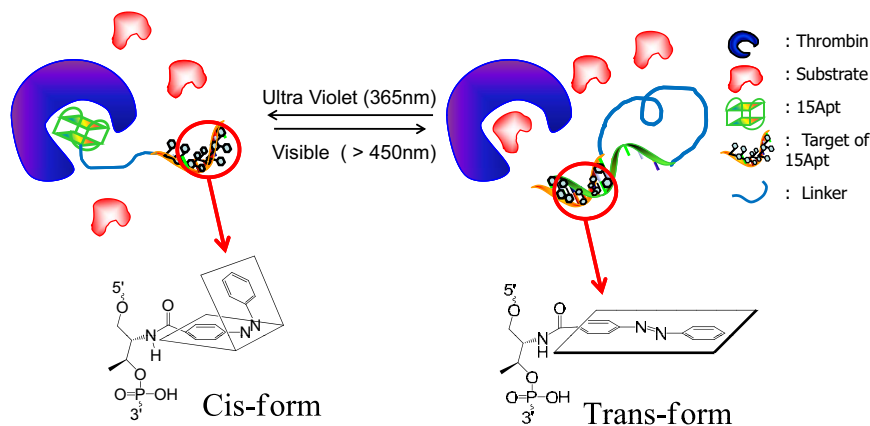


Fig. 1. Xcomp/Yazo probes. The working principle is that dissociation and association of the 2 domains report high and quenched fluorescence signal, respectively. We assign test probes the following nomenclature. Xcomp equals the number of complementary sequences, and Yazo equals the number of incorporated azobenzene molecules. The *trans*- and *cis*- conformation is reversibly regulated by different input of electromagnetic radiation of the energy. Thus, when probes are treated with visible light, the regulatory domain is hybridized, and the probe is released from its target, resulting in activation of the enzyme (thrombin). When treated with UV light, probes form the open conformation, and the inhibitory domain can bind to the target, causing low enzymatic activity. The use of Xcomp/Yazo creates combinations that afford the opportunity to optimize probe design in accordance with thermal stability, thrombin affinity, and the results of clotting assays.

DNA sequence using an automated DNA synthesizer (31). Finally, the linking domain ties the inhibitory and regulatory domains together. Importantly, because the regulatory domain is composed of photochromic molecules, the affinity of the inhibitory domain can be altered by different light treatments, as discussed above. Briefly, UV light induces *trans*-to-*cis* isomerization, resulting in a low binding affinity of the regulatory domain to 15Apt. This alteration frees 15Apt for binding to exosite 1 of thrombin. On the other hand, visible light reverses the conformation of the regulatory domain, allowing it to hybridize 15Apt. This results in the low affinity of 15Apt for thrombin, thus enabling thrombin to hydrolyze fibrinogen for coagulation. Or, stated another way, the inhibition of thrombin is disabled because the probe hybridizes with the cDNA in the *trans*-azobenzene conformation so that the aptamer cannot bind its target thrombin.

To optimize the PCI, we designed and characterized a series of candidate molecules with different lengths of cDNA and different amounts of azobenzene in the inhibitory domain. The inhibitory potency of these probes in both the ground and excited states was measured by using prothrombin time (PT) measurement in human plasma. The results demonstrate the real-time ability of the *cis*-form of these probes to switch on thrombin's activity under visible illumination, or, conversely, switch off thrombin's activity under UV illumination. Moreover, by using a microscopic fluorescence-based PT assay, a second key part of our investigation sought to confirm the feasibility of sequential site-specific activation of coagulation by spatial control of thrombin inhibition.

Results and Discussion

Optimization of Probe Designs. To design effective photon-controllable inhibitors, the complementary sequences to 15Apt (regulatory domain) were modified with azobenzenes, and the inhibitory and regulatory domains were linked by using a polyethylene glycol (PEG) linker composed of 5 units of hexa-ethylene glycols conjugated by using phosphoramidite chemistry. Several factors influenced our decision to incorporate azobenzene into the complementary sequences of 15Apt rather than the 15Apt sequence itself. First, the complementary sequence of 15Apt can function as an excellent antidote for 15Apt, as a result of its high binding affinity via Watson–Crick base pairing (32–33). In other words, had we decided to incorporate azobenzene moieties to 15Apt, we would have lost the complementary antidote effect.

Second, even minor modifications to 15Apt can result in significant disruption of interacting configurations (34), which are generally unpredictable without computational simulation or experimental investigation. Thus, modifying the complementary 15Apt sequence with azobenzene is a more feasible approach. Five units of hexa-ethylene glycol (≈ 10 nm) were used to ensure close proximity of the inhibitory and regulatory domains (35). This complex then resulted in a unimolecular conjugate. In fact, it is the unimolecular nature of our design that allows the molecule to switch between active and inactive inhibitory states without the addition of another strand of DNA. In this way, reaction was allowed to occur between the regulatory and inhibitory domains in a manner that maximized the efficiency of dissociation and association between them according to the number and position of azobenzene insertions, which is explained later in this article. This was expected because small alterations are enough to disrupt intramolecular interactions, whereas more significant alterations are required to break intermolecular interactions.

Optimization of probe design also depends on maximizing the efficacy of photoisomerization. This involves adjusting both the number and position of azobenzene molecules and base pairs in the regulatory domain. Previous reports have shown that the insertion of azobenzene molecules (*trans* conformation) to the DNA chain can destabilize or stabilize duplexes of DNAs depending on their positions. Thus, the most common method of regulating DNA duplex conformations is to alternate every 2 bases with a single azobenzene phosphoramidite. Although this strategy works well at high temperatures, a maximum of 7 azobenzene molecule insertions did not result in a kinetically favorable duplex transition within the 15-bp stem under the reaction conditions necessary to perform the PT assay (37 °C and physiological salt). Therefore, we investigated the feasibility of alternating azobenzene moieties between every other nucleotide. Using this protocol, we could potentially have a probe with 15 or 16 azobenzene incorporations within the regulatory domain.

These conditions combined with the potential of azobenzenes to destabilize our probe design required us to test a series of molecular probes having different numbers of azobenzene and base pairings [supporting information (SI) Table S1]. Each probe contained a FRET pair (fluorescein and dabcy) as a signaling element to monitor the hybridization and dehybridization between the regulatory and inhibitory domains (36). The working principle is that

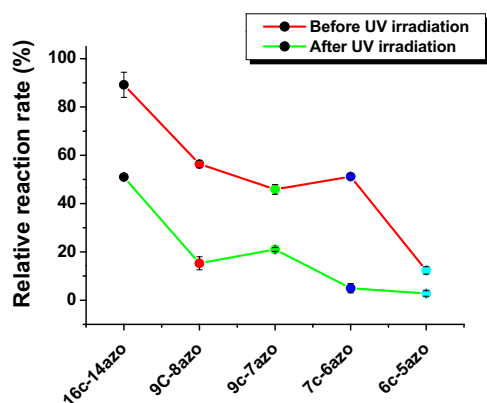


Fig. 2. Photo-regulating inhibitory function of probes.

dissociation and association of the 2 domains report high and quenched fluorescence signal, respectively. Our protocol can best be understood if we assign probes the following nomenclature. Let Xcomp equal the number of complementary sequences, and let Yazo equal the number of incorporated azobenzene molecules. A series of combinations, such as 8comp-4azo or 10comp-4azo, then arise that enable us to measure, compare, and characterize these probe combinations by (i) melting temperature, (ii) binding affinity to thrombin, and (iii) clotting assay. Depending on the number of azobenzene incorporations, these molecular probes had significantly different properties, as summarized in Table S2. Specifically, in this experiment, we investigated what prototype probe design would work best at room temperature (RT) and whether different ranges of photoalteration could be achieved by adjusting the number of base pairing.

First, we will consider the property of thermal stability. We found that probes with $\leq 40\%$ azobenzenes per nucleotide, e.g., 10c-4azo, 8c-4azo, 7c-3azo, and 6c-3azo, showed higher melting temperatures than non-azobenzene-incorporated probes. It is a well-known phenomenon that the insertion of azobenzenes between at least 2 nucleotides can enhance duplex stability by enhanced π - π stacking interaction. On the other hand, the continued insertion of azobenzenes causes significantly reduced stability. The reduced thermal stability is clearly shown by comparing the T_m s of probes with the same number of base pairs, but different percentages of azobenzene incorporations, e.g., 7c-6azo < 7c-4azo < 7c-3azo. In this example, a negative correlation is established because thermal stability decreases with the increase of azobenzene molecules, even though the number of base pairs stays the same. Next, we ran a fluorescent assay to determine the maximum number of azobenzene incorporations consistent with duplex stability under either UV or visible (Vis) light. Results showed that molecular probes containing a low percentage of azobenzenes did not respond differentially to either UV or Vis light, indicating that the duplex had not dissociated. On the other hand, probes possessing the maximum number of azobenzenes per regulatory domains (16c-15azo, 14c-13azo, 12c-11azo, 10c-9azo, 9c-8azo, 7c-6azo, and 6c-5azo) showed dramatic decreases in duplex stability after illumination with UV light (Fig. 2). Taking these findings together, we concluded that probe optimization depended on incorporation of at least 1 azobenzene between every 2 nucleic acids in the regulatory domain and that the prototype probes demonstrate different ranges of photoalteration based on the number of base pairings.

Probes Can Act as a Photoswitching Anticoagulant. Although the above results showed that the duplex structure of our probe design thus far can be regulated with UV and Vis light under physiological conditions (i.e., RT), complete optimization still required an additional step. Specifically, to demonstrate that these probes are also

capable of regulating human blood clotting reaction, the PT of each probe was measured in the *cis*- or *trans*-conformations (37). PT measures how long it takes for blood to clot. As such, PT testing is used frequently as a standard and accurate method to assess bleeding problems and the efficiency of anticoagulants. In our experiment, the results obtained from the PT tests were plotted against probe concentration and fitted to a sigmoid curve. Then, the concentration most effective in doubling the plasma clotting time, the IC_{200} , was calculated for each probe. By the theory underlying our probe engineering, it was expected that probes in the *trans* state would show shorter PTs and higher IC_{200} , whereas probes in the *cis* state would have relatively longer PTs with lower IC_{200} under identical sample preparation and experimental conditions. Typical results are shown in Fig. 3 A and B. In accordance with the reasoning indicated above, the PTs of *trans* probe samples are much shorter than those of *cis* probe samples. Also, a general trend of increased clotting time correlated with increased number of base pairs as can be seen by comparing probes 6c-5-azo with 10c-9-azo (Fig. 3 A and D). However, probes 12c-11-azo through 16c-15-azo showed only negligible difference in PT (Fig. 3 C and D).

The difference in probes is more obvious when we compare the concentration most effective in doubling the plasma clotting time, or the IC_{200} value (Table 1). That is, to determine the optimal probe design, we looked for probes showing the highest differential IC_{200} , thereby showing the largest difference in anticoagulation response upon treatment with UV or Vis light. We expected the IC_{200} values of both states of the 6c-5-azo probe samples to be the lowest. We also expected that IC_{200} values for the *cis* forms would increase with an increasing number of base pairs. We found that probes with >10 bp were too stable to have a detectable difference in IC_{200} between the *trans* and *cis* states. The difference between $IC_{200-cis}$ and $IC_{200-trans}$ was maximized with the 10c-9-azo and 9c-8-azo probe (Table 1). On the other hand, probes with >10 bp showed abrupt increase of $IC_{200-cis}$ (from 9.5 to 16 μ M), which is highly unfavorable, even though these probes formed highly stable duplexes when Vis light was treated. This tendency causes nondetectable difference in IC_{200} between the *trans* and *cis* states of those probes, but it does not refer to the huge *cis*-to-*trans* or *trans*-to-*cis* transition. Thus, we concluded that the 10c-9-azo and 9c-8-azo probes were the optimal probe designs because they showed the highest differential IC_{200} , thereby showing the largest difference in anticoagulation response upon treatment with UV or Vis light. In subsequent experiments on real-time and spatial (submillimeter) control of clotting activity, we chose probe 9c-8-azo over 10c-9-azo because probe 9c-8-azo had a lower melting temperature and faster overall kinetics than the 10c-9-azo probe (51 °C and 60 °C, respectively).

Dynamic Photoconversion to Restore Coagulation Process. In most cases, it is difficult to reverse an enzyme's activity once a drug molecule has bound, but this conversion can be useful in many respects, especially in clinical applications. For instance, immediate deactivation of drugs can limit side effects in medical treatment and potentially save lives. In some cases, there are antidotes that disrupt inhibitor-enzyme interactions, but there is still no general way of finding molecules that can perform this function. In this sense, aptamers can be advantageous compared with other molecule- or antibody-based enzyme inhibitors because an aptamer's cDNA sequences naturally function as effective antidotes. This can occur because the cDNA sequence of the aptamer can replace the protein to form a DNA duplex, thus disabling the aptamer's inhibition function with target proteins. However, this example also illustrates a key potential drawback of this approach in that a large quantity of antidote sequences must be administered to overcome the slow reaction time of diffusion and hybridization in the case of in vivo applications. Another attempt used caged thrombin aptamers to deactivate thrombin. However, because this mechanism is the opposite of restoring the protein's function, it does not fit the

light treatment can activate the clotting reaction, resulting in selectively blocking the blood vessels, whereas nontargeted sites (no Vis light) remain unaffected. We expect that this approach will be highly beneficial in future biomedical and pharmaceutical applications. For example, as a class of cancer treatment regulating angiogenesis has been highlighted as a means of minimizing nutrition delivery to the tumor site. Based on the working principle of our PCI probes, this nutritional supply line to the tumor site could be cut off in a similar manner.

Materials and Methods

Chemicals and Reagents. DNA synthesis reagents were purchased from Glen Research. A physiological buffer contained 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 5% (vol/vol) glycerol for the buffer experiment. Human α -thrombin was obtained from Haematologic Technologies. Fibrinogen was obtained from Sigma-Aldrich. Universal coagulation reference plasma (UCRP) and thromboplastin-DL for human sample testing were purchased from Pacific Hemostasis.

Preparation of Probes. We designed, prepared, and characterized a series of molecular probes, which are shown in Table S1. The probes were synthesized by using an ABI 3400 DNA/RNA synthesizer (Applied Biosystems) at 1- μ mol scale with the standard synthesis protocol. Details of the synthesis procedures are given in SI Text.

Real-Time Monitoring of Clotting Reaction. To monitor the clotting time alteration in response to different light treatments, we designed a simple buffer experiment that contained only thrombin, one of the nucleic acid inhibitors, and fibrinogen substrate in physiological buffer. The underlying principle of the experiment is based on the mixture of sample that becomes nonfluidic and tends to scatter light by fibrin aggregation, the product of thrombin's catalytic reaction. As a result, the clotting process can be measured by the decrease of light penetration through the sample cell. To compare the efficacy of inhibition, each probe was pretreated with UV (365 nm) or Vis (white light). The mixture containing 1 μ L of 10 μ M thrombin and 1 μ L of 100 μ M concentrations of each inhibitor in 200 μ L of physiological buffer was then incubated for 10 min under either UV (365 nm) or Vis (500 nm) light in the spectrometer. In sequence, 4 μ L of 20 mg/mL fibrinogen was quickly added and mixed while the UV spectrometer was monitoring either the UV or the Vis wavelength as a function of time. Reaction mixtures containing only thrombin and fibrinogen with or without

15Apt were always tested together with other samples as internal standards. All clotting times were normalized based on the internal standard and compared with it.

Human Plasma Tests. To evaluate the feasibility of the inhibitor as a potential anticoagulant reagent, we determined PT for each ligand using human plasma samples. Details are given in SI Text.

Monitoring Site-Specific Activation of Enzymatic Reaction in Microfluidic Channel. To demonstrate site-specific activation of thrombin's activity, we used a microfluidic channel to create a 2-dimensional model of the clotting process using fluorescent fibrinogen to visualize fibrin networks. Fibrinogen was labeled with Alexa Fluor 488. Fibrinogen (10 mg) was dissolved in 1 mL of 0.1 M sodium bicarbonate buffer. Then, 0.5 mg of Alexa Fluor 488 in dimethyl sulfoxide (DMSO) was added to the protein, followed by stirring for 1 h. The conjugated protein was separated from unreacted Alexa Fluor 488 by using a Nap-25 column (GE Healthcare Bio-Sciences). The purified protein had approximately a single fluorophore on each protein.

Microfluidic channels were prepared by the following procedure. Microscope slides and 18-mm-square no. 1 coverglasses (Fisher) were rinsed with deionized H₂O and dried under nitrogen. Strips of double-sided tape (3M) were placed 4 mm apart on a microscope slide, and the coverglass was placed on top. Devices were filled by capillary action. Solution exchange was performed by simultaneously pipetting solution at one end and withdrawing fluid from the other end with P8 filter paper (Fisher). An Olympus FV500-IX81 confocal microscope was used to both illuminate and image specific regions of the channel using a 10 \times objective and the 494-nm laser line. The length scale of the 10 \times field of view was calibrated by using a micrometer, and the image was 1.286 \times 1.286 mm. A human plasma sample (10 μ L) was prepared for aPTT measurement containing 10 μ M concentrations of *cis*-probes and Alexa 488-labeled fibrinogen, and the mixture was loaded into the channel and incubated at RT. The aPTT is a performance indicator for measuring the efficacy of both the "intrinsic" pathway (now referred to as the contact activation pathway) and the common coagulation pathway. The fluorescent time-lapse images were obtained every 15 s until the reaction was completed.

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