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Light-controlled thrombin catalysis and clot formation using a photoswitchable G-quadruplex DNA aptamer†

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The reversible photocontrol of an enzyme governing blood coagulation is demonstrated. The thrombin binding aptamer (TBA), was rendered photochromic by modification with two anthracene groups. Light-triggered anthracene photodimerisation distorts its structure, inhibiting binding of the enzyme thrombin, which in turn triggers catalysis and the resulting clotting process.

External control of biomolecule function is important for regulating biological processes that are relevant to various therapeutic and diagnostic applications. Photoregulation is a popular method of control due to the high level of specificity gained without the use of additional chemical reagents,[1] with photoswitchable groups allowing properties to be controlled in a reversible manner. As part of ongoing interest in this area, there are now a number of examples of different photoactive groups used for photocontrollable catalysis.[1a,2,3] Here, through the use of a photoswitchable DNA aptamer that displays anthracene photochromism, we demonstrate a new way of using light to reversibly control a catalytic process.

Human α-thrombin, an enzyme from the family of serine proteases, is a key biological protein involved in the process of blood coagulation at the site of a blood vessel injury.[4] It has two anion binding exosites, one of which (exosite I) binds fibrinogen. During the formation of a blood clot, thrombin catalyses the production of insoluble fibrin polymers from soluble fibrinogen monomers. DNA aptamers, short single stranded oligonucleotides generated synthetically to bind proteins and small molecules, are widely used for a range of applications linked to diagnostics and therapy.[5] The thrombin binding aptamer or **TBA** (Fig. 1) is a 15-mer that adopts an anti-parallel G-quadruplex (G4) tertiary structure in the presence of K^+ ions and binds to thrombin at exosite I,

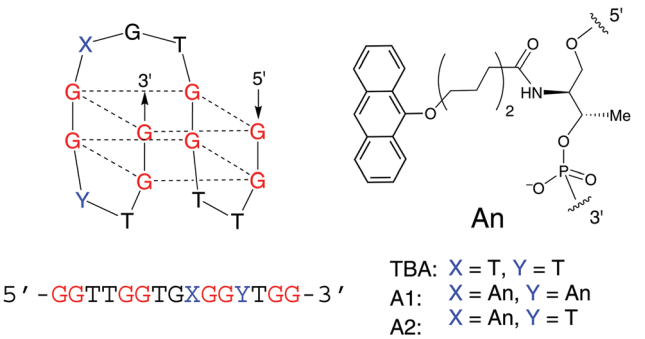

Fig. 1 Structures of **TBA** and modified aptamers **A1** and **A2** depicting the G4 quadruplex structure held together by H-bonds at guanine residues (in red) and sequence variations (in blue) at positions X (in top loop) and Y (in bottom loops); An denotes the anthracene connected to a D-threoninol linker.

the fibrinogen binding site.[6,7] The resulting complex competitively inhibits the binding of the enzyme to fibrinogen, thereby inhibiting the process of blood coagulation. This has led to the consideration of **TBA** and related compounds as treatments for medical conditions linked to blood clotting disorders.[5d] However the use of anticoagulants, including traditional ones (*e.g.* heparin or warfarin), can often result in other healthcare risks.[8] For example, the reduction in systemic clotting capability could mean that wound healing is compromised at injury sites away from where anti-coagulation is required. A dynamic system in which anticoagulation could be deactivated locally using an orthogonal and external source, and more quickly than the body's clearance systems, could provide an effective solution to such an issue.

Previous examples exploring this idea using **TBA** derivatives[2d,f,7b] have included azobenzene[2d] and nitrophenyl[2f] systems in which the application of light was found to alter clotting times. Anthracene photochromism is known to be particularly effective in photo-regulatory processes within supramolecular systems as its reversible photodimerisation reaction can induce significant structural changes.[9] Here we show how it can be successfully applied for the first time to a catalytic system by enabling thrombin-mediated clotting to be controlled using light (Fig. 2).

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† Electronic supplementary information (ESI) available: DNA synthesis & characterization details, photoirradiation & thermal reversion studies, CD studies, EMSA studies and Claus assay conditions. See DOI: 10.1039/c9cc01540j

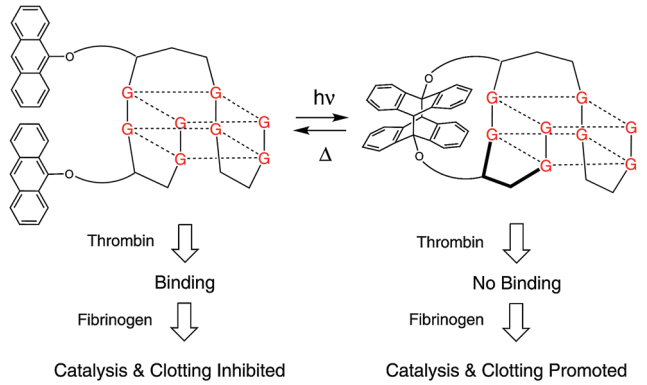

Fig. 2 Diagrammatic representation of anthracene photochromism in aptamer **A1** involving a [4π + 4π] photocycloaddition reaction, forming a head-to-tail isomer. Distortion of the functionalised bottom loop, depicted in bold, restricts thrombin binding, promoting catalysis and fibrin clot formation.

As part of our interest in photoregulated biological systems,[10] we have previously demonstrated that DNA duplex formation can be reversibly controlled by anthracene photodimerisation within one DNA strand.[10a] We therefore decided on a similar synthetic approach for this work by attaching anthracene to a D-threoninol linker group *via* its C-9 position (Fig. 1), to enable its incorporation into the **TBA** sequence using phosphoramidite chemistry. However important considerations for the successful design of such a photoswitchable system were firstly the position of the two anthracene groups within the **TBA** sequence and secondly, which bases they would replace. It was decided to retain those guanines partaking in G-quadruplex formation as this motif gives the required tertiary structure for strong thrombin binding. Furthermore, it was reasoned that replacing thymines in both TT loops would adversely affect thrombin binding in its unphotodimerised form as this bottom face binds to the protein.[6] Therefore, one anthracene moiety was introduced into one TT loop at the T12 position, with the other replacing a thymine at T9 in the top TGT loop, giving the sequence **A1**. It was anticipated that this strand would retain the ability to bind thrombin while at the same time having two anthracene groups in close enough proximity (*i.e.* separated by two bases) to undergo intramolecular photodimerisation. In addition to **A1**, the sequence **A2**, containing only one anthracene in the top loop at T9, was synthesised as a control strand. Each strand was purified by reversed-phase HPLC and characterised by ESMS (see ESI†).

To induce the desired anthracene photodimerisation, samples of **A1** at 2 μM in aqueous buffer at pH 7.4 (10 mM KCl, 20 mM Tris HCl, 1 mM $MgCl_2$, 120 mM NaCl, 2 mM $CaCl_2$) were subjected to photoirradiation (using a 365 nm bandpass filter) over a period 4 hours. Over this time, the characteristic anthracene absorbance band centred at *ca.* 370 nm was found to decrease by 73% (Fig. 3a), indicating successful photodimer formation.‡ In contrast, the corresponding spectrum of sequence **A2**, containing only one anthracene unit, hardly changed over the same time period (Fig. 3b), confirming that the light-induced reaction was predominantly an intramolecular process. Upon heating the irradiated solution of **A1** at 80 °C, the anthracene signal gradually

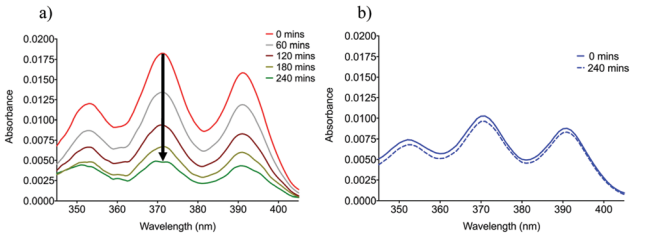

Fig. 3 Overlaid absorbance spectra (340–410 nm region) of a solution of (a) aptamer **A1** and (b) aptamer **A2**, showing the effect of photoirradiation over a period of four hours (2 μM, pH 7.4).

reappeared (67% reversion after 12 hours, see ESI†), with the timescale and conditions for the reversion consistent with the photocycloadduct from the forward reaction being predominantly the head-to-tail isomer (Fig. 2).[9b,10a]

Circular dichroism (CD) spectra were recorded to assess the extent to which the G-quadruplex tertiary structure of **TBA** was affected by anthracene modification and photodimer formation. Although **A1** gave a much weaker spectrum than that of unmodified **TBA** or the control **A2**, both derivatives gave characteristic signals of a G4 structure,[6,7] with positive peaks at 292 nm and 245 nm and a negative peak at 261 nm for **A1** (Fig. 4). The photoirradiated sample of **A1** gave an even weaker signal and slightly shifted peak maxima, but with a pattern that also suggests a G4-type tertiary structure.

In order to probe the tertiary structures further, melting temperature (T_m) values were determined using VT CD spectroscopy. In each case the data showed a single co-operative unfolding transition (see ESI†), with the T_m value of 50 °C for **TBA** higher than that of **A1** (42 °C) but lower than that of **A2** (56 °C). This indicates that while the presence of one anthracene in the lower TGT loop is stabilising, an additional anthracene in the top loop is destabilising. However, while the photoirradiated sample of **A2** gave only a small change in T_m value (55 °C), that for the **A1** sample rose to 52 °C after photoirradiation. This increase in thermal stability can be explained by the unfolding of the tertiary structure being hampered by the presence of two intramolecular covalent crosslinks provided by the anthracene photodimer.

Studies on the binding of thrombin to **TBA** and its modified derivatives were undertaken using electromobility shift assays (EMSAs). The open form of aptamer **A1** was found to bind thrombin, as shown by the appearance of DNA bands at the top

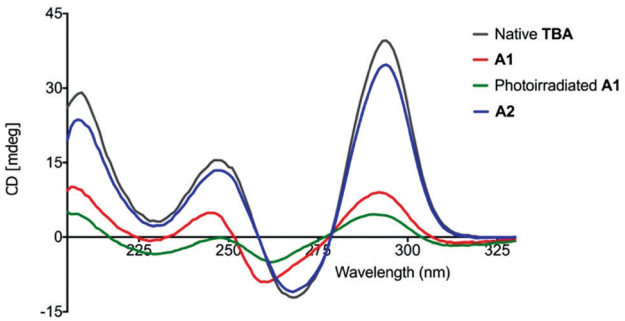

Fig. 4 CD spectra of native **TBA**, aptamer **A1** both before and after 4 h photoirradiation and aptamer **A2** (2 μM, pH 7.4).

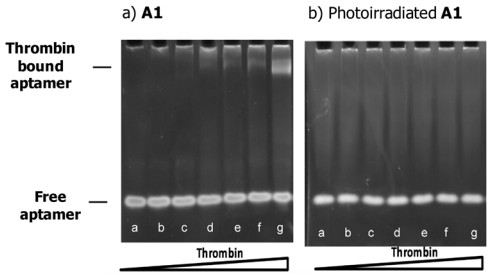

Fig. 5 EMSA gels (stained for DNA) of aptamer **A1** in the presence of increasing amounts of thrombin. The images show **A1** (a) before & (b) after 4 h photoirradiation. Both gels contain 1 μM of the aptamer & lanes a–g contain thrombin concentrations of: (a) 0 μM, (b) 0.2 μM, (c) 0.5 μM, (d) 1 μM, (e) 1.5 μM, (f) 2 μM, (g) 2.5 μM.

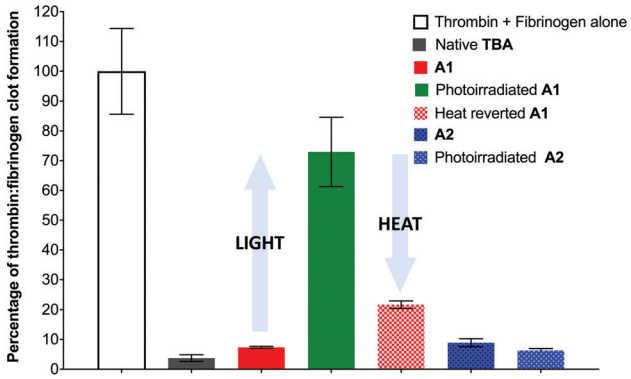

Fig. 6 Clauss assay data after 40 minutes of addition of thrombin (1 μM) to fibrinogen for no aptamer, native **TBA**, aptamer **A1** and aptamer **A2**, presented as the difference in percentage UV transmission at 450 nm and normalised to data obtained with no aptamer. Aptamer concentration = 1 μM. Each test was done in triplicate; error bars represent standard deviation.

of the gel as a function of increasing concentration of the enzyme (Fig. 5a). The location of these DNA bands matched those visualised through protein stains, with the results similar to those for both unfunctionalised **TBA** and **A2** (see ESI†). These results show that modification with either one or two anthracene groups does not prevent binding to thrombin. However in contrast, the photoirradiated sample of **A1** did not show any new DNA bands on the EMSA under the same conditions (Fig. 5b).§ These results provide good evidence for photo-switched binding arising through photodimerisation of the two spatially separated anthracene units (Fig. 2), which induces sufficient distortion of the G4 tertiary structure within the modified aptamer to severely restrict its binding properties. In support of these interpretations, the heat-reverted sample of **A1** was found to bind thrombin again, confirming that the anthracene photochromism was responsible for this behaviour (see ESI†). Furthermore, photoirradiation of a solution of the control sample **A2** had no detrimental effect on its ability to successfully bind thrombin due to its inability to form intramolecular photoadducts (*vide supra*).

With photocontrolled binding of thrombin established, the ability of light to control its catalytic activity was then investigated using the Clauss assay (see ESI†), which measures changes in UV transmission resulting from the polymerisation of fibrinogen to the insoluble clotting product fibrin. The assay was first carried out in the absence (as a control) and in the presence of the unmodified aptamer **TBA**. As expected **TBA** inhibited thrombin's catalytic activity under these conditions, with fibrin formation after an incubation time of 40 minutes reduced by 96% compared to the control (Fig. 6). Experiments were then repeated using **A1** and **A2**, which revealed both to be almost as effective as **TBA**, with amounts of clotting product at 7% and 9% respectively of the control. These results support the EMSA data, which demonstrate effective binding of these functionalised aptamers to thrombin. However, repeating the assay in the presence of a solution of photo-irradiated **A1** indicated a significant increase in activity, with the clotting product level raised to 73%. In contrast, photoirradiation of **A2** had no effect on catalytic function. Finally, also in good agreement with the UV/vis data and EMSA binding studies, much of the original inhibitory activity of **A1** could be restored after heat treatment, with the level of clotting product lowered again to 22%.

In conclusion, this work demonstrates the first application of anthracene photochromism to the control of a catalytic process in the form of thrombin catalysed fibrin clot formation. While being relevant to the development of smart anticoagulation therapies, such an approach also has the potential to be applied more widely to other photopharmacological and optogenetic processes.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

‡ The **A1** photoproduct had an identical retention time by HPLC to its open form, which precluded its isolation from the photoirradiated sample.
§ Gel data indicate a thrombin binding order of **TBA** > **A2** > **A1**, with dissociation constant (K_d) ratios estimated from relative band intensities as: $K_d^{A1}/K_d^{TBA} = 8$ and $K_d^{A2}/K_d^{TBA} = 2$. No detectable binding was found for the photoirradiated **A1** sample under these conditions.

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