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Differential Regulation of Protein Subdomain Activity with Caged Bivalent Ligands

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The functional characterisation of biomolecules requires the development of precise tools that act either as inhibitors or activators of signalling pathways. The most common tools for target validation are, among others, so-called loss-of-function strategies that include siRNA, antisense and genetic knock-out approaches.[1] These act on the genetic or mRNA level and cause the disappearance of the protein of interest from the proteome of the cell or organism under investigation. Because every protein is embedded in a complex network of biomolecules the loss of one member often causes secondary effects that contribute to altered phenotypes but are not necessarily related to the protein of interest.[2] Similarly, off-target effects caused by the unintended down-regulation of other than the targeted mRNA might lead to misinterpretation of functional data.[3] Alternative approaches thus make use of small molecules and macromolecular tools that act directly at the protein and protein subdomain levels to investigate the function of the protein of interest.[4]

Macromolecules that bind and inhibit proteins include peptide aptamers, antibodies and nucleic acid aptamers.[5] In the last case, we have recently developed aptamers that bear photolabile groups at defined positions, so-called caged aptamers, whose activity with regard to protein function inhibition can be exogenously regulated with UV-A light,[6] either by sterically blocking the interaction site[7a] or by induction[7b] of the formation or depletion[7c] of the active conformation. These kinds of inhibitors pave the way for the regulation of protein function with spatiotemporal resolution. Furthermore, we have recently constructed the bivalent aptamer **HD1–22** (Figure 1), which simultaneously targets and accordingly inhibits the regulatory exosites I and II of thrombin.[8] The serine protease thrombin is a key player in the blood clotting cascade. It converts fibrinogen into fibrin, activates platelets and augments the clotting cascade by upstream activation of clotting factors. Both exosites of thrombin are required for the activation of coagulation cofactors and platelets. Thrombin's exosite I recruits fibrinogen, which is cleaved to fibrin by the active site, leading to clot formation. Exosite II is the domain to which heparin can bind—mediating the binding of antithrombin (AT), which then blocks the thrombin active site.[9] Here we report that derivatives of

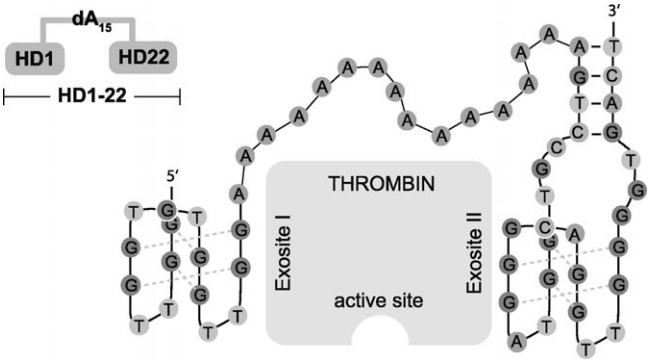
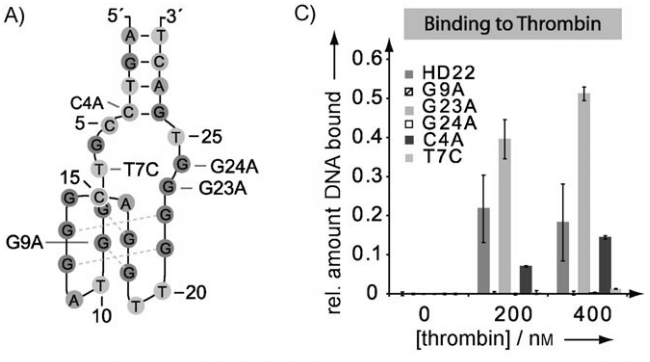


Figure 1. HD1–22 is a bivalent aptamer consisting of the two parts **HD1** and **HD22**, which are connected through a poly-dA linker. Each part targets a different regulatory site on the serine protease thrombin.



B)

HD22	5′- AGTCCGTGGTAGGGCAGGTTGGGGTGACT -3′
C4A	5′- AGT**A**CGTGGTAGGGCAGGTTGGGGTGACT -3′
T7C	5′- AGTCCG**C**GGTAGGGCAGGTTGGGGTGACT -3′
G9A	5′- AGTCCGTG**A**TAGGGCAGGTTGGGGTGACT -3′
G23A	5′- AGTCCGTGGTAGGGCAGGTTGG**A**GTGACT -3′
G24A	5′- AGTCCGTGGTAGGGCAGGTTGGG**A**TGACT -3′

Figure 2. A), B) Cartoon representation of the mutants of the aptamer **HD22**, together with their sequences. C) Result of filter retention studies with these mutants and thrombin. In the case of the mutants **G9A**, **G24A** and **T7C** the bars coincide with the base line. For further results of filter binding studies with the mutants **G23A** and **C4A** see the Supporting Information.

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HD1–**22** bearing cages in different positions offer potential for the exogenous and differential control of protein subdomain activity.

The bivalent aptamer **HD1**–**22** is made up of the aptamer module **HD1**, a 15-nucleotide G-quadruplex-forming nucleic acid that binds to the exosite I,[10] and a second aptamer module, termed **HD22**, also forming a G-quadruplex motif that has been shown to bind to thrombin's exosite II.[11] The two modules are connected through a dA_{15}-linker. Although photoreactive variants of the aptamer **HD1** have been reported previously,[7] no such variations of the **HD22** aptamer are known. Thus, we first determined nucleotide positions of the aptamer **HD22** that are sensitive to mutations, by defining several mutants of the aptamer **HD22** and analysing them for thrombin-binding activity by filter retention analysis. As shown in Figure 2, mutation at positions T7, G9 and G24 results in complete loss of binding capacity. These findings are in accordance with previously reported data that show the importance of the formation of the G-quadruplex and the presence of the hinge regions (C5–T7 and G23–T25), most probably involved in high-affinity binding.[11]

The mutation at position C4 resulted in a weakly binding aptamer (Figure 2C and Figure S1 in the Supporting Information), whereas mutation at position G23 had almost no effect on high-affinity binding of the aptamer [K_D value of (6.2 ± 1.4) nM for **G23A**, relative to (11.9 ± 1.1) nM for **HD22**;[8] Figures 2C and S1]. From these data we chose positions G9 and G24 to be modified with photolabile groups, thus allowing access to light-sensitive **HD22** variants. To test this, we synthesized the **HD22** derivatives **G9**^{cage} and **G24**^{cage} bearing the photolabile *o*-nitrophenylpropyl (NPP) group at the exocyclic position O^6 in the indicated guanine nucleobases (Figure 3A and B; for the mechanism of the uncaging reaction see refs. [6f] and [12]).

Filter retention analyses were performed to elucidate the light-dependent activities of the two modified aptamers. As shown in Figure 3C and D, the interacting activities of both caged aptamers can clearly be regulated by light. The nonirradiated variants showed almost no or rather low binding to thrombin, whereas upon irradiation the binding activities of the aptamers could be restored.

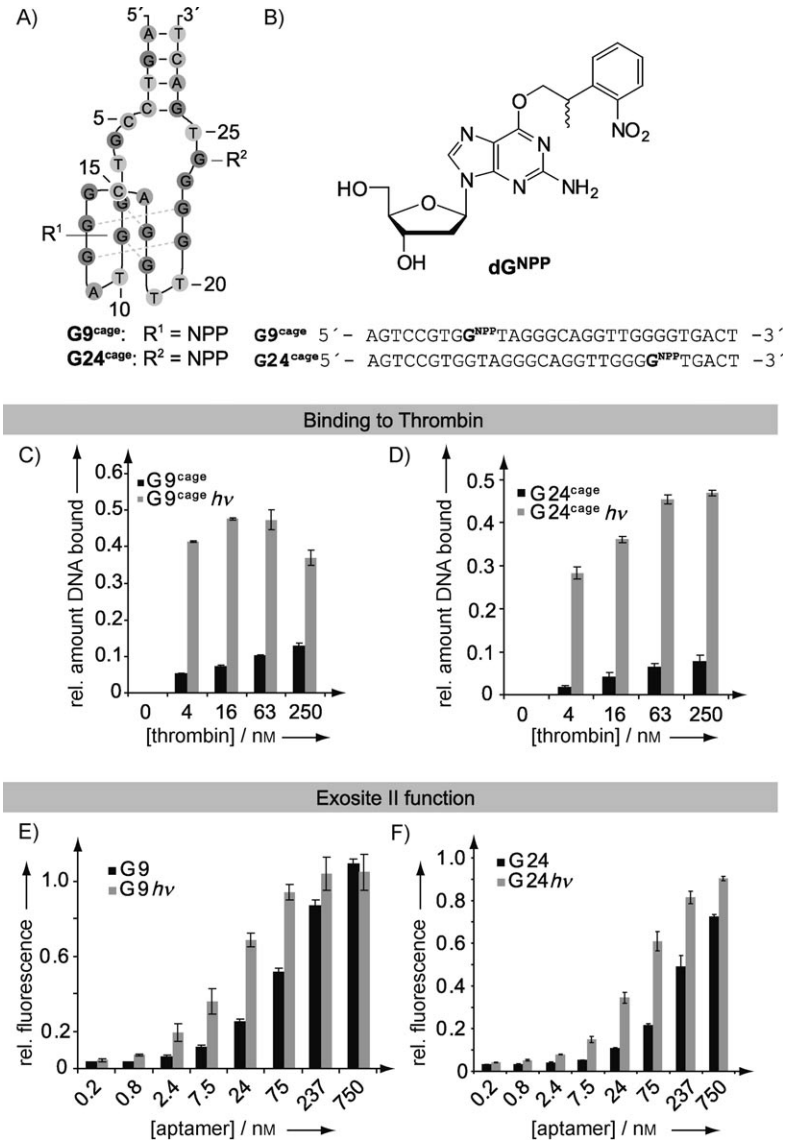
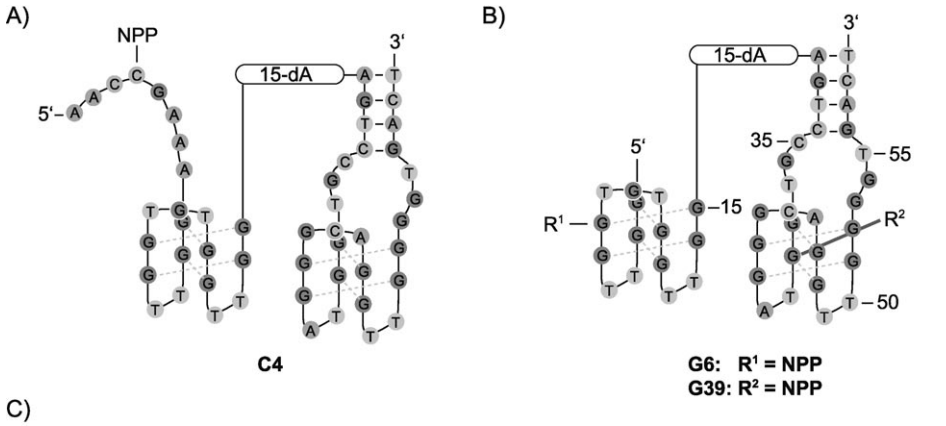


Figure 3. A), B) Representation of the caged derivatives **G9**^{cage} and **G24**^{cage} bearing the caged nucleoside **dG**^{NPP} at the indicated positions. C), D) Results of filter retention studies with these caged aptamers and thrombin before and after irradiation. E), F) Results of studies assessing the exosite II function of thrombin with increasing concentrations of **G9**^{cage} (E) or **G24**^{cage} (F) before (black bars) and after irradiation (grey bars). Normalized values were obtained by using **HD22** as reference.

We next elucidated whether **G9**^{cage} and **G24**^{cage} allow light-control of thrombin's exosite II function, by employing a fluorogenic peptide cleavage assay.[8,13] In brief, heparin binds to the exosite II of thrombin and accelerates AT-mediated inhibition of thrombin. Because **HD22** interacts with exosite II it competes with heparin binding and counteracts the heparin effect. Thrombin was incubated in the presence of heparin and with increasing concentrations of **G9**^{cage} and **G24**^{cage} without or with UV-A irradiation, and the kinetics of AT-mediated thrombin inhibition were monitored through the hydrolysis rates of a simultaneously added fluorogenic peptide. As shown in Figure 3E and F, both aptamers obviously reveal light-dependent competition with heparin binding, resulting in increased rates of thrombin activity after irradiation. However, high concentrations of the non-irradiated caged aptamers also induce the preservation of thrombin activity.

On the basis of these findings we defined caged bivalent anti-thrombin aptamers. These aptamers were designed to be light-dependent with regard either to the exosite I or to the exosite II activity. In this way it should be possible to generate ligands that target distinct protein subdomains, thereby inhibiting the associated signalling cascade and protein activity without interfering with the other domain and vice versa. The light-dependent release of the photolabile protecting group



C)

HD1-22	5′-GGTTG G TGTGGTTGG-15dA-AGTCCGTG G TAGGGCAGGTTGGGGTGACT-3′
C4	5′-AACC^{NPE}GAAAGGTTG G TGTGGTTGG-15dA-AGTCCGTG G TAGGGCAGGTTGGGGTGACT-3′
G6	5′-GGTTGG^{NPP}TGTGGTTGG-15dA-AGTCCGTG G TAGGGCAGGTTGGGGTGACT-3′
G39	5′-GGTTG G TGTGGTTGG-15dA-AGTCCGTGG^{NPP}TAGGGCAGGTTGGGGTGACT-3′

Figure 4. The caged derivatives **C4**, **G6** and **G39** are designed in such a way that the **HD1** part can be turned OFF or ON, or the **HD22** part can be turned ON by UV-A irradiation.

subsequently offers exogenous control over the second protein subdomain, thus enabling protein functions to be switched off precisely at a desired time point. To test this hypothesis we designed the **HD1**–**22** derivatives **C4**, **G6** and **G39**, each bearing one photolabile protecting group at the indicated position (Figure 4). The derivative **G39** is equivalent to the previously discussed **G9^{cage}**, which showed a slightly better response to light irradiation than **G24^{cage}**, while the derivatives **G6** and **C4** are analogous to compounds used in fprevious studies in which we showed that the activity of **HD1** can be turned ON[7b] or OFF,[7c] respectively, with light.

Functional analyses were performed by conducting fibrinogen-based clotting assays to assess the function of exosite I on the one hand, whereas on the other hand exosite II-related assays, as described above, were conducted to address inhibition of thrombin's exosite II. As shown in Figure 5 A, the caged bivalent aptamer **G6** clearly shows light-dependent inhibition of thrombin-mediated clot formation. The position chosen for caging effectively turns the exosite I inhibiting property off until uncaging, a result similar those that we had previous observed with **HD1** alone.[7c] On the other hand, irradiation of **G6** had only little effect on the interference of exosite II function (Figure 5 B).

In the derivative **G39**, however, the exosite II function was light-inducible (Figure 5 D)—similarly to what we observed with the **HD22** derivative **G9^{cage}**. However, irradiation also showed an effect on the exosite I function of **G39** (Figure 5 C). To explain this it is necessary to consider the dissociation constants of the reference compounds: **HD1** is a weaker binder [(123.1 ± 7.2) nM] than **HD22** [(11.9 ± 1.1) nM] and the fusion aptamer **HD1**–**22** binds most strongly [(4.9 ± 1.6) nM], as reported previously.[8] Thus, turning on of a second interaction domain in the aptamer also leads to a higher affinity—hence the apparent increase in efficiency of **G39** upon irradiation. In fact, the same is visible for **G6** (Figure 5 B), but to a smaller extent because of the smaller differences in affinity between **HD22** and **HD1**–**22**. In further support of this point, we have included dose dependence studies with the reference compounds in the Supporting Information.

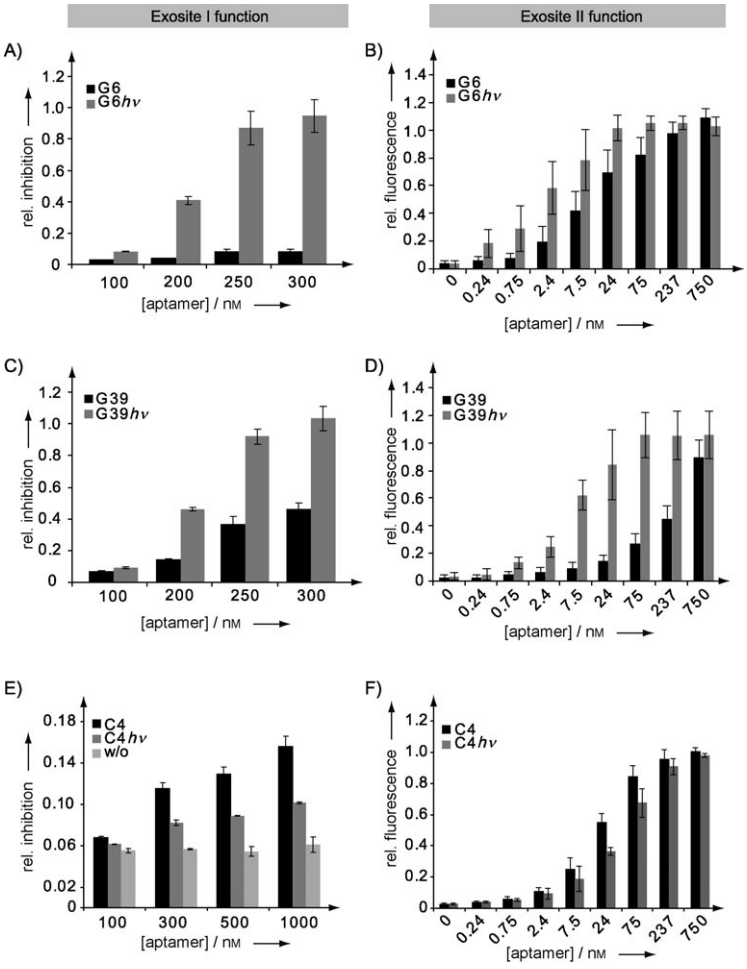


Figure 5. Results of studies assessing the exosite I (A, C, E) and exosites II B, D, F) functions of thrombin in the presence of increasing concentrations of the caged bivalent aptamers before (black bars) and after (grey bars) irradiation. Normalization was done by using the respective saturation values of **HD1**–**22** (A–D, F) or **HD1** (E) as reference.

These data indicate that both aptamers exhibit reciprocal light-dependent activities with regard to exosite I and exosite II of thrombin. The bivalent aptamer **C4** bears a 5′-extension designed to allow light-dependent deactivation of exosite I binding through inhibition of module **HD1** of the bivalent aptamer. As would be expected, this aptamer interferes with exosite II in an almost light-independent fashion. Arguably there is a small drop in activity, which would be expected because of the slight loss in affinity observed upon deactivation of the **HD1** module. Inhibition of thrombin-dependent clot formation, however, was shown to be clearly light-dependent, although the **C4** molecule is less active than the parent bivalent aptamer **HD1**–**22**. These observations are consistent with previously described results obtained with the **HD1** module in combination with a similar 5′-extension.[7c] In addition, the extended **HD1** aptamer proved to be less efficient in inhibiting thrombin-dependent blood clotting than the aptamer **HD1**.

In conclusion, we have demonstrated that the combination of the caging technology with bivalent aptamers enables access to tailored molecules with superior activities, now exogenously controllable in a reciprocal manner. To the best of our knowledge, this is the first study to show that it is possible to modulate individual domain activity in aptamers—and therefore also domain activity in proteins—with light. In particular, in the caged aptamer **G6**, we present a molecule that, before light activation, even enhances the activity of thrombin because it inhibits its natural inactivation by antithrombin with the **HD22** part, even in the absence of heparin.[13] After irradiation, the activity is completely reversed: the **HD1** part is activated, and its function now dominates, whereas the **HD22** part now synergistically enhances the antithrombin activity. Because aptamers are available by in vitro selection, and because sophisticated selection schemes allow different protein sites to be addressed, our approach should be easily adaptable to other aptamers and target proteins. It can thus be regarded as a general strategy for precise analysis of biomolecular function, not only limited to spatiotemporal control but also refined by individual protein subdomain activities.

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