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Functional Detection of Proteins by Caged Aptamers

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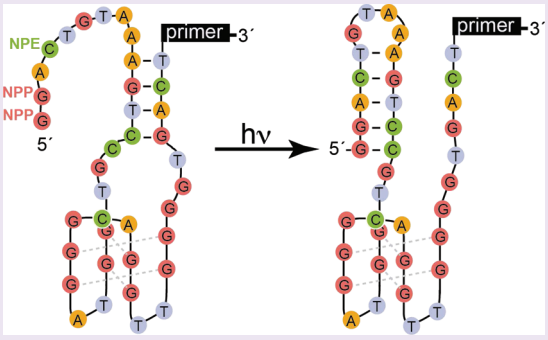
Supporting Information

ABSTRACT: While many diagnostic assay platforms enable the measurement of analytes with high sensitivity, most of them result in a disruption of the analyte's native structure and, thus, in loss of function. Consequently, the analyte can be used neither for further analytical assessment nor functional analysis. Herein we report the use of caged aptamers as templates during apta-PCR analysis of targets. Aptamers are short nucleic acids that fold into a well-defined three-dimensional structure in which they interact with target molecules with high affinity and specificity. Nucleic acid aptamers can also serve as templates for qPCR approaches and, thus, have been used as high affinity ligands to bind to target molecules and subsequently for quantification by qPCR, an assay format coined apta-PCR. Caged aptamers in turn refer to variants that bear one or more photolabile groups at strategic positions. The activity of caged aptamers can thus be turned on or off by light irradiation. The latter allows the mild elution of target-bound aptamers while the target's native structure and function remain intact. We demonstrate that this approach allows the quantitative and subsequently the functional assessment of analytes. Since caged aptamers can be generated emanating from virtually every available aptamer, the described approach can be generalized and adopted to any target−aptamer pair and, thus, have a broad applicability in proteomics and clinical diagnostics.



Immuno-polymerase chain reaction (immuno-PCR) enables the ultrasensitive detection of analytes *via* the combination of the selectivity of antibodies with the high efficiency and sensitivity of nucleic acid amplification methods.[1] Immuno-PCR utilizes a reporter antibody equipped with a short DNA strand, which is employed as a template for quantitative PCR (qPCR). Recent developments in the field have demonstrated that immuno-PCR addresses the increasing demands of diagnostic assays achieving a significant increase in sensitivity, culminating in a more than 100,000-fold enhancement of detection limits as compared to conventional ELISA.[2−6] However, immuno-PCR relies on the availability of high affinity antibodies and requires chemical conjugation of the antibody to reporter oligonucleotide tags. In the past 20 years, nucleic acid aptamers have emerged as powerful rivals to antibodies for use as diagnostic reagents. Aptamers are short nucleic acids that fold into a well-defined 3D structure in which they interact with target molecules with high affinity and selectivity.[7] Aptamers can be identified by an *in vitro* selection approach, termed SELEX (Systematic Evolution of Ligands by EXponential enrichment)[8] and have been reported to interact with a diverse set of target molecules, such as peptides, proteins, drugs, organic and inorganic molecules, or even whole cells.[9−13] Many of the aptamers reported to date reveal affinities for their target molecules comparable to, if not better than, those of respective monoclonal antibodies, with picomolar K_d values.[13,14] Furthermore, the specificity of aptamer recognition has been observed, facilitating a 10,000- to 12,000-fold[13,15] discrimination of cognate target molecules and related structures. The synthetic nature of aptamers enables their site-directed modification, which clearly sets them apart from antibodies. For example, aptamers can be generated as molecular beacon structures or equipped with functional tags for rapid adaptation to diverse assay formats without loss of aptamer activity. Consequently, due to their nucleic acid nature an approach termed aptaPCR has been developed. aptaPCR employs the dual function of aptamers acting both as a selective ligand for target molecules and as a template for qPCR. Aptamers are exceptionally well-suited for this approach, and virtually every aptamer identified to date can be used without laborious optimization procedures. Most

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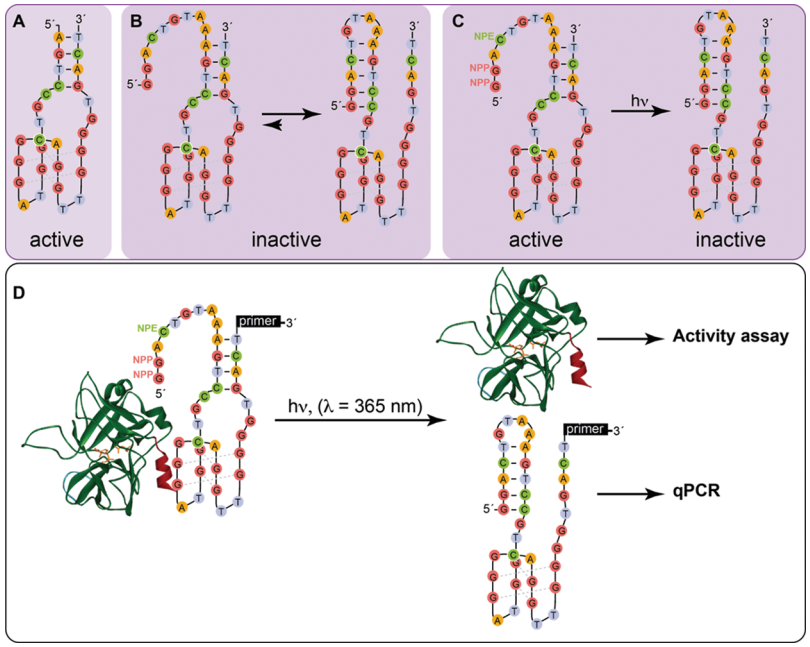

Figure 1. (A) Proposed secondary structure of the HD22 aptamer, which binds (active) thrombin *via* exosite II. (B) Extension of the aptamer with a GNRA-tetraloop sequence (GTAA) and further complementary nucleotides induces hairpin formation and, thus, unfolding of the aptamer (inactive). (C) Addition of photo-labile groups (*o*-nitrophenlypropyl, NPP, and *o*-nitrophenylethyl, NPE) at defined positions (G1, G2, and C4) prevents hairpin formation and keeps the aptamer active. Inactivation is achieved through irradiation with light (λ = 365 nm), whereby the photolabile groups are removed and hairpin formation is induced. (D) After recognition of thrombin the caged aptamer is irradiated and, thus, will be released for qPCR analysis, whereby the captured thrombin can be further assessed for specific activity.

importantly, this approach bypasses the mandatory conjugation of antibodies with DNA-reporter tags exploited in immuno-PCR, which can interfere with the recognition properties of antibodies.

Several reports have been described that use the template and recognition properties of aptamers, while different separation methods to enrich for the bound target have been employed. Most of the described assays take advantage of the unique amplification properties of aptamers, whereas the captured aptamer were amplified using qPCR or rolling circle amplification,[16−20] as well as proximity ligation assays.[21−23] We have recently employed two well-known thrombin binding aptamers and extended aptaPCR toward a sandwich-like assay format.[24] Most of the described assays for analyte detection make use of denaturing conditions to elute the bound aptamers from their target molecule, thereby potentially leading to target inactivation. Consequently, the target cannot be applied for further analyses. To ensure that the functional integrity is maintained, the implementation of mild elution methods that allow an efficient disruption of aptamer−target interactions for the liberation of the aptamer for qPCR on the one hand and the native target for further functional investigations on the other is required. One approach makes use of antisense molecules that hybridize with the aptamer, thereby disrupting its active conformation. Another alternative employs so-called caged aptamers. Caged aptamers bear photolabile groups at strategic positions, and thus their target recognition properties can be controlled by light irradiation.[25−28] In this way, we hypothesize that caged aptamers can be designed to recognize and bind a target molecule and can consequently be liberated upon irradiation. The released aptamer can subsequently be used as a template for qPCR and the remaining captured target analyte for functional analysis (Figure 1). In the present work we describe the entire route to accomplish the aforementioned strategy. Starting from the well-known aptamer HD22 that recognizes thrombin's exosite II, we illustrate the generation of a caged aptamer variant, which binds thrombin with high affinity but can be released by light irradiation. Using this molecule we established a "direct" and "sandwich"-type kind of aptaPCR assay. Furthermore, we demonstrate that after light irradiation the target protein's enzyme activity remains functional. This model system can be generalized and adopted toward the detection of a series of analytes for which quantitative, qualitative, and functional analysis in one assay format is applicable and desirable.

RESULTS AND DISCUSSION

Construction of a Light-Responsive Variant of Aptamer HD22. We previously described the synthesis and characterization of a variant of the 15mer thrombin's exosite I recognizing aptamer HD1, which can be switched off upon irradiation with light.[26] However, because of its short length it would be necessary to add primer-binding sequences to modify HD1 to render it a suitable template for qPCR. However, we recently discovered that extensions at the 5-end results in a loss of HD1 activity.[25] Consequently, we thus decided to generate variants of the aptamer HD22 whose activity can also be switched off by irradiation with light. Emanating from our previous studies we synthesized variants of HD22 that bear extensions at either its 5′- or its 3′-end, which can base pair with neighboring nucleotides of HD22 and, thus, disrupt the active aptamer structure. Herein, the aptamer was extended by the means of a GNRA-tetraloop followed by an antisense sequence of up to 9 complementary nucleotides (Table 1). This design facilitates the formation of a hairpin structure, which alters the conformation of HD22 and, thus, its recognition properties.[25,26] We evaluated the recognition behavior of the extended HD22 variants by competitive binding experiments, where we incubated radioactively labeled HD22 with a constant amount

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Table 1. Synthesized Oligonucleotides Used in This Study

NAME	MODIFICATION
3.1	5'-**HD22**-GTAAAGTCACCCC-3'
3.2	5'-**HD22**-GTAAAGTCACC-3'
3.3	5'-**HD22**-GTAAAGTCA-3'
3.4	5'-**HD22**-GTAAAGT-3'
3.5	5'-**HD22**-GTAA-3'
5.1	5'-CCACGGACTGTAA-**HD22**-3'
5.2	5'-ACGGACTGTAA-**HD22**-3'
5.3	5'-GGACTGTAA-**HD22**-3'
5.4	5'-ACTGTAA-**HD22**-3'
5.5	5'-GTAA-**HD22**-3'
5.3v	5'-AAGCAGTGGTAAGTAGGTTGATT-GGACTGTAA-**HD22**-TCTCTTCGAGCAATCCTCAC-3'
5.3v1	5'- GGACTGTAA-**HD22**-TCTCTTCGAGCAATCCTCAC-3'

of thrombin and increasing concentrations of extended HD22 variants. Those variants that still interact with thrombin will reduce the HD22 signal, whereas those that fail, most likely due to hairpin formation, will have no effect on HD22 binding. As shown in Figure 2, the extended HD22 versions differ significantly with

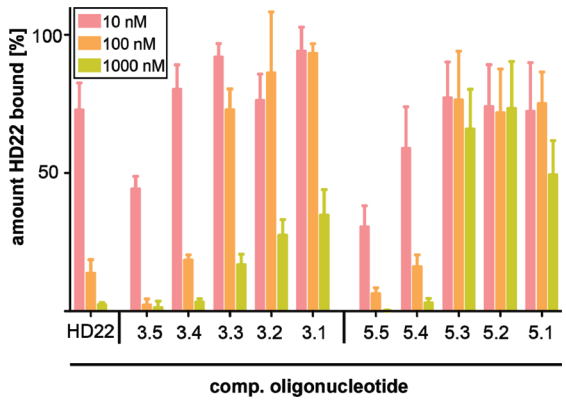

Figure 2. Competition of the HD22-thrombin interaction by HD22 variants. To evaluate the impact of the 5′- and 3′-extensions, filter retention analysis was done by incubating radioactively labeled HD22 with thrombin [10 nm] in the presence of increasing concentrations [10, 100, and 1000 nm] of each variant. After passing through nitrocellulose [0.45 μm] the retained amount of labeled HD22 was determined by phosphorimager analysis and quantified using ImageQuant software.

respect to their ability to compete with HD22 thrombin recognition. While extensions at the 3′-end have less influence on thrombin recognition, the aptamer HD22 is sensitive toward extensions at the 5′-end. Addition of 5 complementary nucleotides (variant 5.3) abolishes thrombin recognition almost completely. In contrast, a similar extension located at the 3′-end (variant 3.3) still recognizes thrombin, and even the addition of 9 complementary nucleotides at the 3′-end does not completely inactivate HD22. These data are in accordance with previous findings where both the length and position of an antisense oligonucleotide region are important factors for the perturbation of aptamer function, with the effect of the length and position being difficult to predict. The control variants, which were solely extended with the GNRA-tetraloop sequence (variants 5.5 and 3.5), still revealed high affinity binding to thrombin exemplified through a strong competition activity, which in fact seemed to be even more efficient when compared with the wild-type aptamer HD22.

Having identified the HD22 variant 5.3 as nonactive, we next started to adopt it for a photoelution apta-PCR setup. Therefore, we added primer-binding sites at either the 3′-end (5.3v1) or at both the 3′- and 5′-end (5.3v) of HD22 to enable amplification by qPCR (Table 1). These sequences were chosen to be noncomplementary, and the primer-tethered HD22 still interacted with thrombin (Supporting Information). We then synthesized the caged variants of 5.3v1 and 5.3v (Figure 1 and Table 1). To achieve this we introduced 3 photolabile groups at positions G1, G2, and C4 of the extension, functioning as a temporary block of hairpin formation, resulting in an active HD22 variant that can be inactivated upon light-irradiation. Using filter retention analysis, we analyzed whether the caged variants functioned as predicted (Figure 3). As expected, the caged variants bind to thrombin with high affinity, and interestingly, compared to the wild-type aptamer HD22, slightly increased K_D values of the modified aptamers (90 nM, 5.3v1, and 9 nM, 5.3v) were observed. Light irradiation (λ = 365 nm), however, induces a loss of the caged aptamers' recognition activity, and more importantly this can be also observed in the case of the already formed thrombin-caged aptamer complex. However, the variant with extensions on both ends (5.3v) does not react in the predicted way. It reveals recognition properties even in the irradiated version (Figure 3B), which might be explained by competitive base pairing options of the 5′-extension with the uncaged sequence (Table 1, underlined), leading to an intact HD22 structure and, thus, preserved recognition properties. As a result of these findings we focused on 5.3v1 for the further development of the photoapta-PCR assay.

Direct and Sandwich apta-PCR. In a first step, we established a direct assay format, in which thrombin was directly physically adsorbed on the surface of microtiter plate wells. Thrombin-coated wells were subsequently incubated with

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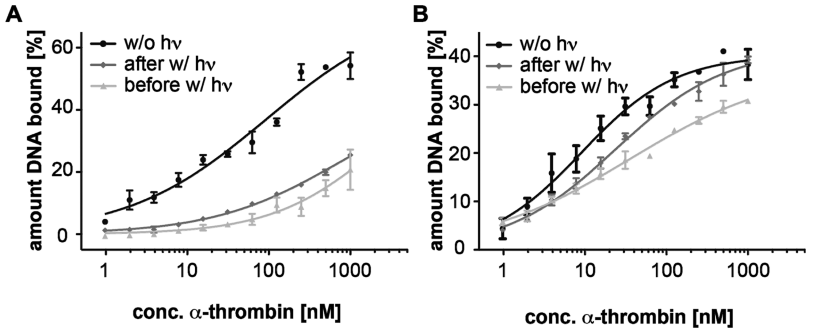

Figure 3. Light-dependent interaction of the caged aptamers 5.3v1 and 5.3v with thrombin. (A) Filter retention analysis of 5.3v1 with increasing concentrations of thrombin without (w/o *hν*) and after (after w/ *hν* and before w/ *hν*) irradiation (λ = 365 nm). Irradiation was performed either before (before w/ *hν*) or after (after w/ *hν*) incubation with thrombin. (B) Filter retention analysis of 5.3v with increasing concentrations of thrombin without irradiation (w/o *hν*) and after irradiation with light (after w/ *hν* and before w/ *hν*) (λ = 365 nm). Irradiation was performed either before (before w/ *hν*) or after (after w/ *hν*) incubation with thrombin. After passing through nitrocellulose [0.45 μM] the retained amount of labeled HD22 was determined by phosphorimager analysis and quantified using imagequant software.

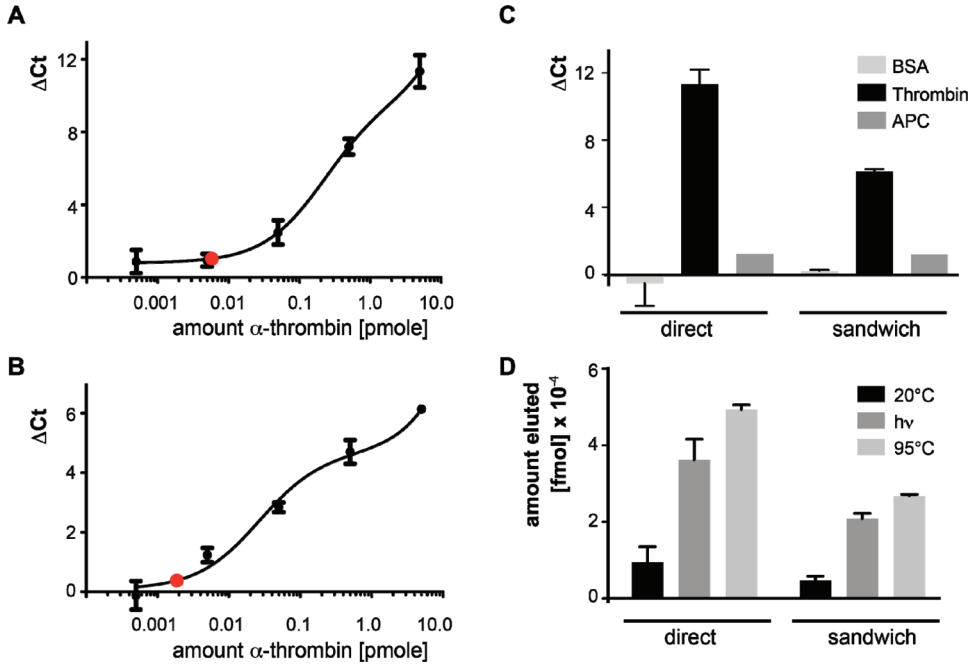

Figure 4. Photo-aptaPCR assay. Determination of the limit of detection (LoD) indicated by the red dots of the direct (A) and sandwich-type (B) photoapta-PCR assay. ΔCt values in dependence of increasing amounts of thrombin are given. (C) ΔCt values determined using 5 pmol of BSA (light gray bars), thrombin (black bars), or APC (activated protein C, dark gray bars) as analytes in the direct (left) and sandwich-type (right) photoapta-PCR assay. (D) Determination of the amount of 5.3v1 detected in dependence of different elution methods.

increasing concentrations [0.025−500 nM] of 5.3v1 and washed, and the bound 5.3v1 was then eluted by UV exposure (λ = 365 nm). The supernatant was removed and directly assayed by qPCR. The optimized concentration of caged 5.3v1 was determined to be 250 pM. At this concentration, the maximum difference between controls (absence of thrombin) and the lowest detectable concentration of thrombin was achieved, while the threshold cycle (Ct) of the control equaled that of the nontemplate control (NTC). A detection limit of 6 fmol of thrombin (120 pM) was achieved (Figure 4A).

Second, the sandwich variant of the apta-PCR was employed. This makes use of a 3′-biotinylated HD1 aptamer, immobilized on streptavidin-coated microtiter plate wells. These were exposed to increasing concentrations of thrombin [0−10 nM], and after incubation and washing, the 5.3v1 was added. The optimal concentrations of both aptamers were determined through investigating different concentrations of one aptamer while the other was kept constant. Interestingly, the optimal concentration of 5.3v1 was found to be 10-fold higher in the sandwich assay compared to the direct assay approach, which can be attributed to the fact that lower levels of thrombin had been captured by the immobilized aptamer HD1. Concentrations of 5.3v1 lower than 2.5 nM result in an increase of the Ct value comparable to those obtained from controls. After thorough washing, the bound 5.3v1 was photoeluted and directly used as a template for qPCR. The detection limit was determined to be 2 fmol (40 pM), slightly improved as compared with the direct assay format (Figure 4B). Both assays revealed specific thrombin-based detection, since control experiments using BSA or activated protein C (APC) instead of thrombin resulted in no signals (Figure 4C). To evaluate the elution efficiency of the photoelution approach, from both the direct and the sandwich

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apta-PCR, the 5.3v1 aptamer recovered from 10 pM thrombin through different elution methods was quantified *via* standard qPCR. Interesting, the results suggest than in terms of efficiency photoelution was comparable to the thermal elution, being about four times more efficient then the elution at RT (Figure 4D).

Having demonstrated the photoapta-PCR formats, we analyzed whether this approach allows the quantification of thrombin and its successive qualitative and functional assessment. To this end, the activity of thrombin in both apta-PCR assays was monitored before and after elution through cleavage analysis of PEFAFLUOR-TH (H-D-cyclohexylalanyl-alanine-arginine-7-amino-4-methylcoumarin), a fluorogenic thrombin substrate (Figure 5).[29] Using this approach we demonstrated that the photoelution approach facilitates the quantitative detection of thrombin and that photoelution of the caged aptamer does not interfere with the structural and functional integrity of thrombin, while thermal elution results in complete thrombin inactivation. The use of caged aptamers and, thus, a photoelution step for aptaPCR is very promising and avoids the disadvantages encountered through chemical or thermal release procedures. Furthermore the use of chemical release, for example, by employing chaotrope reagents such as urea, requires their subsequent removal or neutralization prior to qPCR analysis, which in turn results in dilution of the eluted aptamer and often requires precipitation of the template prior to further analysis. Thermal release generally causes protein denaturation and degradation, and thus protein fragments are frequently present in the template fraction, thus also necessitating precipitation of the eluted DNA template prior to further analysis. Photoelution, however, requires only a short UV exposure, and no further sample treatment is required. The possibility of maintaining structural and functional integrity following analysis may enable many applications in diagnostic and proteomic approaches, where small amounts of isolated proteins have to be detected and quantified and then further investigated regarding functional integrity.

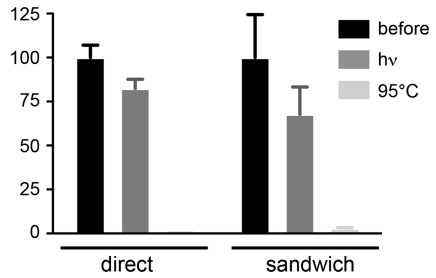

Figure 5. Thrombin activity in dependence of different elution methods. Thrombin protease activity was measured by means of the cleavage of a fluorogenic peptide substrate after thermal elution (95 °C, light gray bars) and after photoelution ($h\nu$, dark gray bars) in either the direct (left) or sandwich-type (right) assay. The values obtained prior elution were normalized to 100% (before, black bars).

Conclusions. In summary we have demonstrated a novel application of caged aptamers as light-dependent templates for apta-PCR. Caged aptamers enable the disruption of analyte and aptamer complexes by irradiation with light (λ = 365 nm). The advantages of this approach, compared to conventional apta-PCR assays, are provided through a straightforward qPCR format, where no purification steps of the eluted aptamer are necessary, and thus there is minimal loss of the reporter aptamer prior to quantification. Moreover, the elution by irradiation maintains the analyte in its native state, allowing it to be used for further testing. This has been exemplified in our study, where we investigated the functional integrity of thrombin before and after irradiation and elution of the aptamer for qPCR amplification. The approach introduced in this study can be applied to virtually every aptamer–target pair and, thus, can be generalized to measuring a diverse set of clinically relevant proteins and biomarkers. This approach may be also applicable to analyze proteomes with spatiotemporal resolution.

METHODS

Reagents. Unmodified oligonucleotides, PCR primers (forward: 5′-AAAGTCCGTGGTAGGGCA-3′; reverse: 5′-TCTCTTCGAG-CAATCCTCAC-3′), and biotinylated HD1 aptamer (5′-GGTT-GGTGTGGTTGG-biotin-3′) were synthesized and HPLC purified (Ella Biotech, Martinsried, Germany). Human α-thrombin was purchased from Cell Systems Biotechnologie Vertrieb GmbH (Troisdorf, Germany). Thrombin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Germany). Fluorogenic peptide substrate PEFA-FLUOR-TH (H-D-CHA-Ala-Arg-AMC) was purchased from Loxo (Dossenheim, Germany). Streptavidin was purchased from Applichem (Darmstadt, Germany). *E. coli* tRNA was purchased from Roche Diagnostics (Germany). Taq DNA polymerase and PCR reagents was purchased by Invitrogen (Germany). B&W buffer (binding and washing buffer) consisted of 137 mmol/L NaCl, 2.7 mmol/L KCl, 9.6 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, and 3 mM magnesium chloride at pH 7,4.

Filter Retention Analysis. The sequence of interest was radiolabeled at the 5′-end with γ^{32}-ATP using T4 polynucleotide kinase (New England Biolabs GmbH, Germany). After 1 h of incubation at 37 °C, unreacted γ^{32}-ATPs were removed using G25 microspin columns (GE healthcare, Germany) following the manufacturer's instructions, and the radioactively labeled aptamer was subsequently analyzed *via* electrophoresis on a 12% polyacrylamide denaturing gel. To determine the dissociation constant of the sequences studied, 0.4 nM radiolabeled nucleic acids were mixed with increasing concentrations (0 to 1 μM) of human α-thrombin and incubated at 37 °C for 30 min in B&W buffer in the presence of 1 mg/mL of BSA and 10 μM tRNA. After incubation the mixture was filtered thought a 0.45-μm nitrocellulose membrane (Whatman, Germany), previously activated with 0.4 M potassium hydroxide and rinsed with 600 μL of B&W buffer. Finally, the nitrocellulose membrane was washed with 800 μL of B&W buffer, dried, and exposed on a storage phosphor screen. Following overnight exposure, the screen was analyzed using a FUJIFILM FLA-3000 with AIDA Image software (Fujifilm, Germany). To characterize the affinity of the HD22 derivatives, 0.4 nM radioactively labeled HD22 was incubated with 10 nM thrombin in the presence of an increasing amount (0–1 μM) of each of the unlabeled derivatives. Following a 30 min incubation at 37 °C in B&W buffer in the presence of 1 mg/mL of BSA and 10 μM tRNA, the mixture was filtered and analysis carried out as described above. Two independent measurements were performed for each derivative.

Synthesis of the Caged Oligonucleotides. The phosphoramidite building blocks for the introduction of a caged dG^{NPP30} residue and a dC^{NPE25} residue were synthesized according to established literature procedures. Caged oligonucleotides were synthesized on an ABI-392 synthesizer using standard coupling protocols. For the cleavage, aqueous ammonia (65 °C, 4 h) was used. The resulting crude product was purified by RP-HPLC (Nucleosil 100–5 C18, 0.1 M triethylammonium acetate pH 7, acetonitrile), detritylated, and again purified by RP-HPLC (same protocol). The identity of the oligonucleotides was established by ESI-MS:

5'-AAGCAGTGGTAAGTAGGTTGATT-$G^{NPP}G^{NPP}AC^{NPE}$TGTAA-**HD22**-TCTCTTCGAGCAATCCTCAC-3':
25,681.0 Da (calcd 25,680.61)
5'-$G^{NPP}G^{NPP}AC^{NPE}$TGTAA-**HD22**-TCTCTTCGAGCAATCCTCAC-3':
15,548.9 Da (calc. 15,549.14)

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apta-PCR. *Direct apta-PCR.* A range of human α-thrombin concentrations (0 to 10 nM in 0.05 M carbonate/bicarbonate buffer pH 9.6) were incubated on the wells of a microtiter plate (50 μL/wells) for 30 min at 37 °C. Following incubation, the plate was washed three times with B&W buffer (200 μL/wells each wash), and the surface was blocked by incubating the plate for 30 min at 37 °C in the presence of Tween-20. Subsequent to a further washing step, 5.3amp1 was added to each well and incubated at 37 °C for 30 min (50 μL/well, 250 pM final concentration) in the presence of 10 μM tRNA, followed by a final wash with B&W buffer (800 μL/well). Finally, following the addition of fresh B&W buffer (50 μL/well), the reporter aptamer was eluted. Elution was carried out in three different ways: (i) incubation for 10 min at RT in B&W buffer, (ii) incubation for 5 min at 95 °C, and (iii) irradiation with UV light (λ = 365 nm) for 5 min followed by 5 min of incubation at RT. Following elution, 1 μL of the eluted supernatant was used for successive amplification using qPCR.

Sandwich apta-PCR. First, streptavidin (10 μg/mL) in carbonate/bicarbonate buffer (0.05 M, pH 9.6) was added to the microtiter plate (100 μL/well). Following an overnight incubation at +4 °C, the plate was washed with B&W (600 μL/well), and after a 1-h incubation at 37 °C in the presence of 1 mg/mL BSA in B&W buffer the 96-well plate was further rinsed with B&W buffer (600 μL/well). Finally, the buffer was removed, and the functionalized microtiter plate, stored at +4 °C, was used within 2 days. Biotinylated HD1 aptamer was dissolved in B&W buffer to a final concentration of 500 nM and added to the streptavidin-coated wells of microtiter plates (100 μL/well). Following 1 h of incubation at 37 °C, the wells were washed with B&W buffer (600 μL/well). Subsequently, a range of concentrations of human α-thrombin (0 to 10 nM) were added (50 μL/well) and incubated for 1 h at 37 °C. After a washing step, 5.3v1 (2.5 nM) was added (50 μL/well) and incubated for 1 h at 37 °C. After thorough washing (800 μL/well) the caged aptamer was eluted as described for the direct assay.

qPCR. A 1-μL sample of the elution fraction was added to 19 μL of a PCR master mix, which consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$ 100 nM forward and reverse primer, 1x final concentration SYBR Green I (Sigma-Aldrich, Germany), 10 nM FAM (Bio-Rad, Germany), and 0.25 units of Taq DNA Polymerase. The amplification was performed in an iCycler thermal cycler upgraded with the iQ5 real-time PCR detection system (Biorad, Germany) programmed according to the following protocol: 1 min at 95 °C, followed by 40 repetitions of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. The LoD was calculated from two independent experiments in which each sample was analyzed in duplicate, where the LoD was calculated as the background value plus three times the standard deviation of the background.

Measure of the Functional Integrity via Thrombin Activity. One picomole of thrombin was either captured by immobilized HD1-aptamer (sandwich assay) or physically adsorbed (direct assay) on the microtiter plate surface. Following incubation with the caged aptamer and subsequent elution, PEFAFLUOR-TH (50 μL/well, 100 μmol/L in TBS, pH 8.5) was added to each well of the microtiter plate, and the change in fluorescence (λ_{abs} = 342 nm, λ_{em} = 440 nm) after 30 min was taken as a measure of the functional active thrombin.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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