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Design Variations for an Aptamer-Based DNA Nanodevice

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The performance of DNA nanodevices based on a thrombin-binding DNA aptamer is strongly dependent on chemical modifications and extensions of the protein-binding core sequence. We here give an overview of the influence of fluorescent labeling and extension of the aptamer device sequence on its binding properties. Sequence extension on the 5′ end as well as labeling on the 5′ or 3′ end does not significantly reduce the binding capabilities. Sequence extension on the 3′ end completely suppresses binding. This knowledge can be utilized for the construction of a doubly labeled aptamer device which can be operated as a switchable molecular beacon.

Keywords: DNA Nanodevices, Molecular Machines, Aptamers, Molecular Beacons, Biosensors.

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1. INTRODUCTION

DNA nanodevices are artificial supramolecular structures composed of DNA which can be controllably switched between different conformations. Switching can be accomplished by changes in buffer composition[1–3] or by the introduction of specific DNA operator strands.[4–10] In the latter case the conformational changes are actively driven by the free energy released from the hybridization between complementary DNA strands. Recently it was shown that a similar operation principle as employed for DNA nanodevices can also be utilized for the construction of a switchable DNA aptamer.[11] Aptamers are DNA or RNA structures which are evolved from a random pool of nucleic acids to bind strongly and specifically to a target molecule.[12–14] The interaction of aptamers with their target molecules is comparable to protein antibody-antigen interactions which makes them most interesting structures for biomedical applications, e.g. in biosensing,[15–22] screening,[23, 24] or as enzyme inhibitors.[25, 26] Using the concept of DNA strand displacement by branch migration, a DNA aptamer structure can be easily switched between a binding and a non-binding form.[11] The result is a simple DNA nanodevice which can repeatedly bind and release a specific molecule. To apply the branch migration principle to aptamers, DNA address tags which serve as binding sites for DNA operator strands have to be attached to the aptamer structure without significantly deteriorating the binding properties of the aptamer. Furthermore, for the characterization of the devices, the structures have to be fluorescently tagged. In previous studies on aptamer-based fluorescent sensors, the influence of the fluorescent label on the binding properties of aptamers has already been investigated.[18–22] From selection experiments it is well known that only slight alterations of aptamer sequences can significantly deteriorate the performance of the aptamers. We here specifically investigate the influence of fluorescent labeling positions and sequence extensions on the binding properties of a switchable DNA nanodevice based on a thrombin-binding aptamer. The device is labeled at the 5′ and the 3′ end as well as on both ends. It is found that labeling does not have a significant impact on the binding properties of the aptamer. We present evidence that labeling at the 3′ end slightly decreases the affinity of the device for thrombin, whereas extension and labeling at the 5′ end does not seem to hinder binding to thrombin. By contrast, with an extension of the base sequence on the 3′ end the device loses its binding properties altogether. This result is particularly important for the development of surface-bound devices and for the incorporation of aptamers into DNA supramolecular structures.

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2. OVERVIEW

The operation principle of the device is schematically shown in Figure 1(a). On the left hand side, the protein thrombin is bound to the folded DNA device A based on the well-known anti-thrombin aptamer.[25] Upon addition of a DNA strand F which can attach to the "toehold" section of the device (blue), thrombin is displaced from the device. In the released state (right hand side), the aptamer is forced into a double-helical conformation (A-F) which cannot bind the protein. Assisted by a second toehold section (green), the strand F can be removed from the device again by branch migration, a principle which has been previously employed for other DNA nanomechanical devices. The free aptamer device can refold and bind to thrombin again and a "waste" product F-R is released. The operation of the device depends critically on the blue toehold section which significantly increases the speed of the protein release. Also, for characterization purposes and in biosensing applications, fluorescent labeling is usually necessary. In the present study, we investigate several variants of the aptamer device which differ in labeling position or in an extension of the core aptamer sequence by single-stranded arms. The various devices are depicted in Figure 1(b). The core device (A-C) consists of just the original 15 base long thrombin-binding aptamer sequence.[25] Devices A-5, A-3 and A-5-3 are extended with a random 12 base toehold section on the 5′ end and labeled at the 5′ position, the 3′ position or on both positions, respectively. The 3′ labels have been attached through an additional thymine spacer base. A-0 is the device with a 12 base toehold without any label. In many applications for a switchable aptamer device it would be important to immobilize the device and still have a free operator (toehold) section for the release process. In this case, the core aptamer would have to be extended on both 5′ and 3′ ends as in device A-E. We investigated two devices (A-E_1 and A-E_2) with extensions of 20 and 28 bases on each end, respectively.

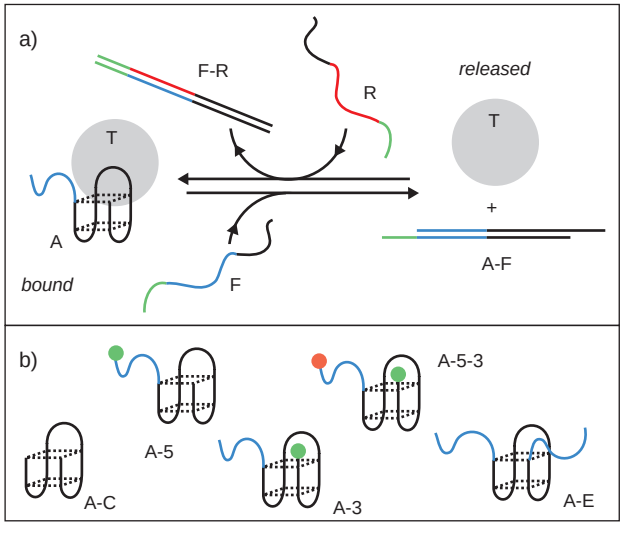

Fig. 1. (a) Operation principle of a thrombin-binding DNA device. On the left hand side the aptamer device A is folded into a chair-like conformation which results from two stacked G quartets. In this conformation it binds to the protein thrombin (T). The addition of F destroys this conformation by duplex formation with A and releases the protein (right hand side). This process can be reversed by the addition of the strand R which displaces F from A and therefore allows A to assume its thrombin-binding conformation again. (b) Variants of the aptamer devices investigated in this study. Devices are labeled or extended at the 5′ or 3′ ends as indicated.

3. METHODS

Experimental. The DNA sequences of the devices in Figure 1(b) and of the fuel and removal strands F and R are given in Table I. The random sequence extensions were designed using the *DNASequenceGenerator*[27] and checked for secondary structure and interaction with the aptamer core sequence with the program *RNA structure*.[28] All oligonucleotides were synthesized, labeled and purified by biomers.net, Ulm, Germany. Other chemicals were obtained from Sigma-Aldrich, Germany, unless stated otherwise, and used without further purification.

The buffer used for all experiments was a modified physiological buffer consisting of 20 mM Tris HCl, 150 mM NaCl, 10 mM $MgCl_2$ and 10 mM KCl at pH 8.5. Polyacrylamide gel electrophoresis was performed on a 12 % native gel in TBE buffer (89 mM Tris HCl, 89 mM boric acid) with 10 mM KCl and run for 1 h with a field of 10 V/cm at 24 °C. Gels were stained using the nucleic acid stain SYBR gold (Molecular Probes). The binding affinity of the aptamer device variants to thrombin was assayed in gel band shift experiments in which the concentration ratio between device strands and thrombin was varied. Before the addition of thrombin, the devices were heated to 65 °C and slowly cooled down to room temperature. The concentration of aptamer devices was between 0.5 μM and 1 μM in each lane and constant within each titration experiment.

Table I. Sequences of the DNA strands used. The aptamer core sequence is shown in bold letters. OG488 stands for the fluorescent label Oregon Green 488 (Molecular Probes, Oregon), Cy3 is the fluorescent dye indocarbocyanine.

A-C	5′-**GGTTGGTGTGGTTGG**-3′
A-0	5′-TAAGTTCATCTC**GGTTGGTGTGGTTGG**-3′
A-5	5′-OG488-TAAGTTCATCTC**GGTTGGTGTGGTTGG**-3′
A-3	5′-TAAGTTCATCTC**GGTTGGTGTGGTTGG**T-OG488-3′
A-5-3	5′-Cy3-TAAGTTCATCTC**GGTTGGTGTGGTTGG** T-OG488-3′
A-E1	5′-CCTCATCAACAGACAGCGTG**GGTTGGT GTGGTTGG**CAGATATGCGTCAGGA CATG-3′
A-E2	5′- CCTCATCAACAGACAGCGTGCCATACGC**GGTTGGT GTGGTTGG**AATCCTAG CAGATATGCGTCAGG ACATG-3′
F	5′-CACACCAACCGAGATGAACTTACGGCGTTG-3′
R	5′-CAACGCCGTAAGTTCATCTCGGTTGGTGTG-3′

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The doubly labeled device A-5-3 can also function as a switchable, thrombin-binding molecular beacon and was further characterized in fluorescence resonance energy transfer (FRET) experiments. In FRET, an excited fluorescent "donor" dye can transfer its energy to an "acceptor" chromophore in its neighborhood whose absorption overlaps with the emission of the donor. The distance over which this process is effective, the Förster radius, is typically on the order of a few nanometers.[29, 30] In the case of the OG488-Cy3 pair it is ≈6 nm. FRET experiments were carried out on a Fluorolog-3 fluorescence spectrometer (Jobin Yvon Horiba, France). The excitation monochromator was set to $\lambda = 488$ nm with a slit width of 5 nm. Emission was detected $\lambda = 521$ nm through a slit with width 5 nm. The temperature for the FRET experiments was 37 °C regulated with a Peltier-thermostated sample holder. The concentration of device strands was 100 nM in all cases, strands F and R were added stoichiometrically. Thrombin was added at 2.5-fold excess over the device strands.

Gel Analysis. Depending on their quality, some of the gels were amenable to a more quantitative analysis. Band intensities were quantified with an image analysis program. After subtraction of background signal, the amounts of DNA in the upper and lower bands were determined by integration of the band intensities. As the intensity of the lower band usually saturated at a finite value even at the highest thrombin concentrations, we assumed that a certain amount of the devices was misfolded and therefore unable to bind to the protein at all. This was corrected for by subtraction. The ratio of the concentration of unbound device to the total concentration $[A]/[A]_0$ was then determined as the ratio of the intensity of the corrected lower band to the corrected total intensity.

4. RESULTS AND DISCUSSION

Polyacrylamide gels resulting from three representative gel titration experiments are displayed in Figure 2. In each gel, the added thrombin amount increases from left to right (see figure caption). The binding of the devices to the protein results in the appearance of a high molecular weight band, accompanied by the disappearance of the lower band from the unbound device. It is found that the devices A-E1 and A-E2 do not seem to bind thrombin at all. Devices A-5 and A-0 bind similarly well to thrombin as the core sequence A-C. In a direct comparison of A-5 and A-3, device A-5 binds more strongly to thrombin than the device labeled on the 3′ end (see Fig. 3). For some gels, the binding affinity could be further quantified by the analysis of the band intensities (compare Methods). The result of this procedure is shown for three of the devices in Figure 4, in which the ratio between bound and total device $Y = 1 - [A]/[A]_0$ is plotted against the initial thrombin concentration $[T]_0$.

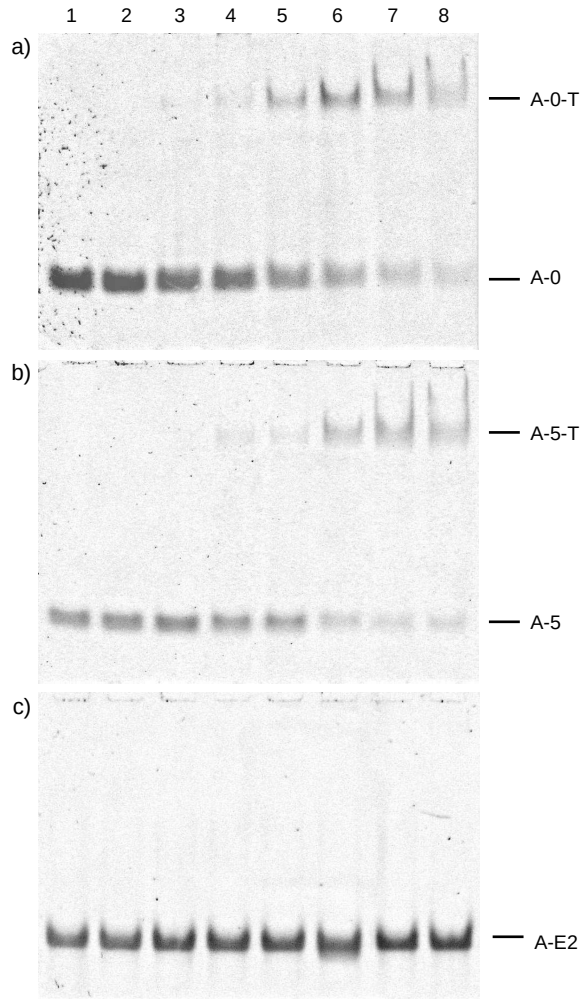

Fig. 2. Gel band shift experiments with the aptamer devices: The first lane contains no thrombin. The ratio between initial concentrations of thrombin and aptamer devices is 0.5:1,1:1,1.5:1,2:1,3.5:1,7:1, and 10:1 from lanes 2 to 8. Gel (a) contains the unlabeled device A-0, (b) the 5′ labeled device, and gel (c) contains the extended device A-E_2. Binding to thrombin can be judged from the appearance of a high molecular weight band (marked with A-0-T and A-5-T, respectively) in the gels. A-0 and A-5 bind comparatively well to the protein, whereas A-E_2 does not seem to bind to thrombin at all.

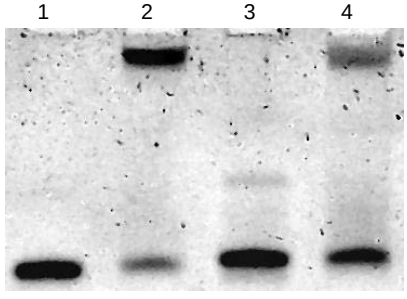

Fig. 3. Direct comparison of A-5 and A-3. Lane 1: 1 μM A-5, no thrombin. Lane 2: 1 μM A-5, thrombin in fivefold excess. Lane 3: 1 μM A-3. Lane 4: 1 μM A-3, thrombin in fivefold excess. A-5 binds more strongly to the protein than A-3, as can be judged from the stronger high molecular weight band for the bound complex.

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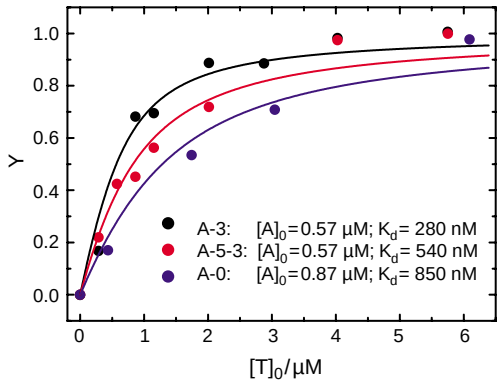

Fig. 4. Plot of the binding ratio Y against the total thrombin concentration for the devices A-3, A-5-3 and A-0. Data points are plotted as dots and fits to the data are continuous lines.

Fits of a theoretical binding curve to the data were used to determine the binding affinities of the various aptamer devices to thrombin. From the definition of the dissociation constant

$$K_d = \frac{[A][T]}{[C]} \quad (1)$$

where $[C]$ denotes the concentration of the aptamer-thrombin complex, one obtains the theoretical binding curve

$$Y = \frac{1}{2[A]_0}\left\{[A]_0 + [T]_0 + K_d - \sqrt{([A]_0 + [T]_0 + K_d)^2 - 4[A]_0[T]_0}\right\} \quad (2)$$

This function can be fit to the data using K_d as the single fit parameter. A fit to the data obtained for the 3′ labeled device A-3 yields a K_d of 280 nM, whereas the device A-5-3 has an apparent dissociation constant of 540 nM. The fit to the data obtained for the unlabeled A-0 yields an unexpected higher K_d of 850 nM. Whereas these values are in the same range as those obtained for the original aptamer sequence by other groups,[21] their exact quantitative value is probably not reliable. Our results varied between 200 nM and 2.5 μM with no clear dependence on the labeling position. In particular, we were unable to quantify the conclusion drawn from Figure 3 as the K_d obtained for A-5 was unreasonably high. The quantitative analysis of the gels was compromised by several factors: The smearing of the higher molecular weight band for the device-protein complex made a determination of the intensity difficult. It is also unclear, whether dye labeling of the DNAstrands is efficient—and linear in the concentration—for the DNA-protein complex. We also suspect that some of the original complexes decay in the gel due to heating or other effects. In a different set of experiments using fluorescence correlation spectroscopy, we obtained a much lower K_d for A-5 of 10 nM which seems to be a more reasonable value.[31]

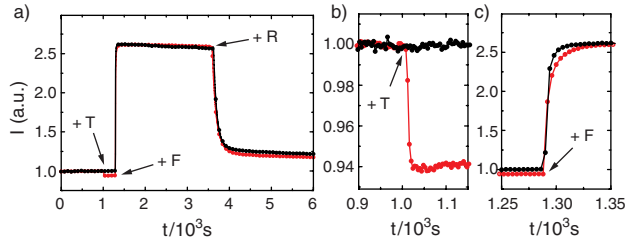

Fig. 5. FRET characterization of the beacon-like aptamer device A-5-3. (a) The device is opened and closed by the addition of operator strands F and R. In the case of the red trace, the protein thrombin (T) is added at the point indicated in the graph. Conformational transitions result in changes in the energy transfer efficiency between the donor and acceptor dyes attached to A-5-3 which can be monitored by the fluorescence intensity. The binding to thrombin stabilizes the chair-conformation of A-5-3 leading to a slight decrease in fluorescence intensity. This "sensor action" is shown more clearly in (b). The effect of dilution alone would be a fluorescence change less than 0.5 %. The maximum fluorescence is measured for the completely stretched device (after the addition of F) and is independent of the presence of thrombin. After removal of F by R, the fluorescence of A-5-3 is lower in the presence of thrombin, as it again binds to the protein. (b) Similarly to aptamer beacon sensors, A-5-3 responds to the presence of thrombin. (c) The opening kinetics of the device is slowed down by the presence of thrombin.

We have previously demonstrated that A-3 and A-5 can be used for the characterization of the behavior of the aptamer devices in fluorescence resonance energy transfer experiments.[11] We here show that this can also be done in a molecular beacon configuration as employed in A-5-3. In Figure 5(a), fluorescence traces recorded for A-5-3 in the presence and in the absence of thrombin are presented for one operation cycle. With the addition of thrombin, the fluorescence signal decreases slightly (see Fig. 5(b)). The binding of thrombin stabilizes the chair-like conformation of the aptamer core of the device which changes the mean distance between the fluorescent labels at the ends of the device strand. The effect is not as dramatic as in aptamer-based fluorescent sensors as the device is not specifically constructed for sensing purposes. With the addition of fuel strand F, the device A-5-3 is forced into a stretched duplex conformation (compare Fig. 1(a)). This is accompanied by a sharp increase in the fluorescence signal as the FRET acceptor Cy3 is now far apart from the donor OG488. The same fluorescence level is attained in the absence as in the presence of thrombin. However, the transition from the low fluorescence to the high fluorescence state is less rapid in the presence of thrombin as the bound protein slows down the formation of the stretched duplex (Fig. 5(c)). Addition of the removal strand R removes F from the duplex A-5-3-F and A-5-3 returns into the folded state. In the presence of thrombin, the fluorescence of A-5-3 again is lower than in the absence of thrombin, indicating the repeated binding of the protein to the device.

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5. CONCLUSIONS

We have investigated a variety of DNA nanodevices based on a thrombin-binding aptamer structure. The devices differ in fluorescent labeling and extensions of the aptamer core sequence on the 5′ and 3′ end. In gel titration experiments it is found that long sequence extensions on both the 5′ and 3′ ends destroy the protein binding capability of the device. Extension at the 5′ end alone as well as fluorescent labeling does not reduce the binding affinity of the devices significantly. For the devices which retain their binding ability, apparent dissociation constants derived from the titration experiments are on the order of a few hundred nM. These values are probably systematically overestimated. The fact that the aptamer device can be labeled at both 5′ and 3′ end is utilized for the construction of a switchable aptamer beacon which can sense the presence of thrombin and also repeatedly bind and release it. Our findings have important consequences for the immobilization of our aptamer-based devices or their incorporation into larger DNA nanostructures. For example, aptamers could be added as appendages to lattices made from DNA double crossover molecules or similar 2D structures.[32–35] Such structures could serve to spatially arrange the molecules recognized by the aptamers in a complex pattern. In the case of the thrombin aptamer, any incorporation will have to be made over the 5′ end of the sequence. In particular, it will not be possible to attach the device over a spacer on the 3′ end and simultaneously add an operator sequence for switching on the 5′ end.

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