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DOI: 10.1002/cbic.200700296

Controlling DNA Polymerization with a Switchable Aptamer

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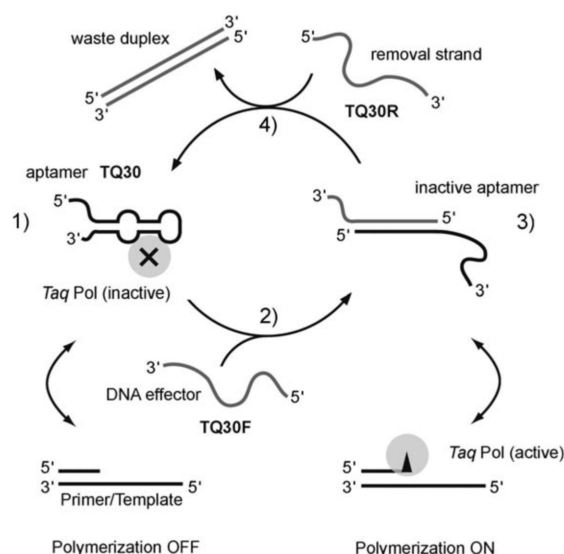
In recent years, the unique molecular recognition properties of DNA and RNA molecules have been used to realize impressively complex supramolecular structures.[1] Through utilization of the mechanical properties of DNA molecules and specific biochemical effects such as strand displacement by branch migration, DNA could even be used to produce a number of switchable, machine-like molecular devices.[2] Among these were devices with the ability to stretch, rotate, and even translocate.[3] In an attempt to add more function to DNA-based molecular devices, several groups have recently also incorporated DNA aptamers into such structures.[4,5] Aptamers are functional nucleic acids that have been selected for their high binding affinity and specificity to certain molecular targets such as proteins or small molecules. In the selection process, DNA or RNA sequences with good binding capabilities are selected from an initial random pool of oligonucleotides through several rounds of binding assays of increasing stringency (systematic evolution of ligands by exponential enrichment, SELEX).[6] Through the use of such an aptamer, the operation principles previously developed for DNA nanomechanical devices were applied to construct a simple molecular device that was able repeatedly to bind and release the protein thrombin.[4] To this end, the known sequence for a thrombin-binding aptamer[7] was modified with a random 12-nucleotide (nt) sequence—a "toehold"—at which a DNA strand partly complementary to the aptamer sequence could attach. This "effector" or "fuel" strand could then displace the protein from the aptamer by binding to the aptamer sequence. A second toehold on the effector strand was used to remove the effector strand from the aptamer by a "removal" strand. In this process, the aptamer's binding capabilities were restored and the protein was bound again.

In this work the operation principle of the thrombin-binding aptamer device has been applied to a DNA aptamer capable of binding to DNA polymerase from *Thermus aquaticus* (Taq polymerase).[8] The result is a simple molecular device that allows us to control the enzymatic activity of Taq polymerase. In contrast to ref. [4], we show here that a switchable aptamer can actually be used to switch the biological activity of an enzyme reversibly. Device operation has been characterized by gel electrophoresis and fluorescence correlation spectroscopy.

The general concept of our aptamer-based device is shown in Scheme 1. Two DNA effector strands—fuel strand TQ30F and removal strand TQ30R—are used to switch the aptamer TQ30 between its active, folded form and an inactive duplex form. Removal of TQ30F by TQ30R results in a waste duplex. As toehold on the aptamer TQ30, the 5′ constant flanking region, which was expected not to bind strongly to the protein, is used. In the folded state, the aptamer can bind to Taq polymerase and effectively inhibit its enzymatic function. Taq polymerase is turned OFF. On the other hand, Taq polymerase does not bind strongly to the duplex form of the aptamer and polymerization is switched ON. Like all known DNA polymerases, Taq polymerase only extends partly double-stranded DNA to a full-length complementary dsDNA. More precisely, activated nucleotides are added to the 3′-termini of double-stranded regions according to the sequence given by the 5′-overhang of the template strand. Taq polymerase neither initiates new strands nor can it add nucleotides to the 5′-termini of the strands. The inactive aptamer duplex contains ssDNA and dsDNA sections, but no ssDNA 5′-overhangs. Thus, Taq polymerase is expected not to interfere with the switching strands TQ30, TQ30, and TQ30R. To monitor whether the enzymatic function of Taq polymerase is indeed switched ON or OFF, we added a monitoring strand consisting of a 17 nt primer (PRIM) hybridized to a 78 nt oligonucleotide (TMPL) that serves as an elongation template for Taq polymerase.



Scheme 1. Operation cycle of the device. 1) In its folded form, aptamer TQ30 can bind to Taq polymerase. In this state, DNA polymerization is effectively switched OFF. 2) Fuel strand TQ30F can bind to the 5′-toehold of the aptamer, and the aptamer is forced into a duplex conformation (3). In this form, Taq polymerase is not bound, and DNA polymerization can proceed. 4) The fuel strand can be displaced from the aptamer by a removal strand TQ30R, returning the device to its initial state.

Our system is designed for isothermal room temperature operation. Important timescales of the reaction cycle are set by the time required for hybridization and branch migration reactions, but also by the speed of DNA polymerization. As experiments with Taq polymerase are usually performed in a PCR setting at elevated temperatures, we first had to characterize the polymerization properties of Taq polymerase at room tempera-

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ture. The undisturbed progress of primer extension of the monitor strand was characterized by denaturating PAGE. Full-length extended primer strands (TMPL*) appear as discrete bands, as the two complementary 78 nt strands (TMPL, TMPL*) clearly differ in terms of their gel migration properties and appear as two distinguishable bands. For unextended monitor strands the TMPL* band does not exist. Thus, the extension progress of the monitor strand—and therefore the ON or OFF state of Taq polymerase—can be deduced from the appearance of the TMPL* band. Figure 1 A displays the progress of primer extension between 0 and 120 min. Each lane represents a sample taken after another 10 min of extension. While the TMPL band intensity stays nearly constant, the TMPL* band intensity increases with time. The overall performance (amount of polymerized dNTPs per unit time) of Taq polymerase at 75 °C is 0.33 nmol $unit^{-1}$ min^{-1}). To determine the room temperature performance of Taq polymerase, intensity values of TMPL*—normalized with respect to TMPL band intensity—in this gel were plotted against time (Figure 1 B). Intensity analysis yields roughly 0.4 % TaqPol activity at room temperature, compared with its maximum at 75 °C (details are given in the Supporting Information).

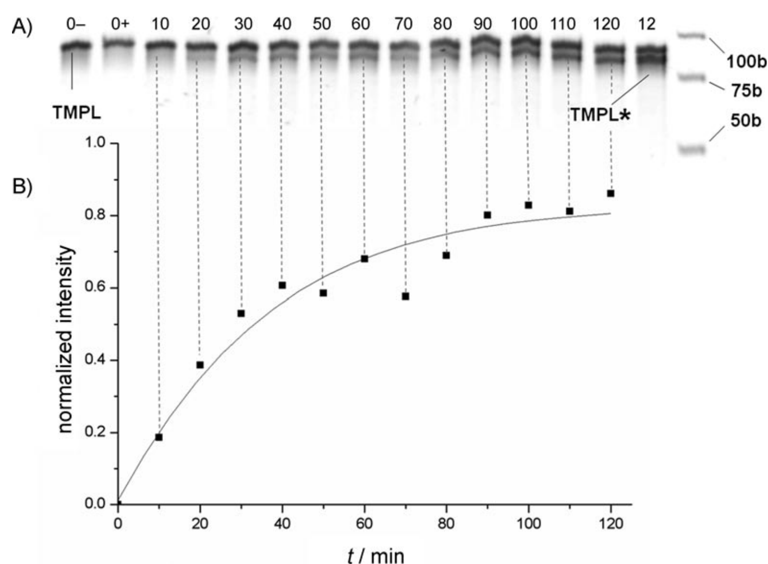

Figure 1. Primer extension analysis. A) Denaturating PAGE of the monitor strand extension progress. From left to right: Immediately before (0−) and after (0+) addition of dNTPs. Lanes 3–14: Primer extension progress after 10, 20, 30,… 120 min. Lane 15: Primer extension progress after 12 h. Lane 16: Low MW weight marker. B) Intensity of TMPL* band (fully extended primer) versus time.

The room temperature activity of Taq Pol having been determined, the switching cycle of our device was analyzed. Switching analysis (Figure 2) was performed by adding the system components (Taq polymerase, dNTPs, monitor and switching strands) one by one, documenting the system state by taking a sample before each step, and leaving it on the workbench for 12 h. Firstly, Taq polymerase and the monitoring strand were added. Prior to loading dNTPs there was no detectable polymerase activity (lane 1). After addition of dNTPs the primer was extended to a full-length strand, and the TMPL* band appeared (lane 2). When the aptamer TQ30 was added quickly after the addition of dNTPs (ca. 2–3 min), no TMPL* band became visible. This shows that polymerization is completely suppressed in the presence of TQ30: the polymerase is turned OFF (lane 3). After addition of the fuel strand TQ30F, however, a TMPL* band appeared (lane 4); this demonstrated that Taq polymerase was now switched ON. TQ30F had hybridized to the aptamer and by this had forced it into its inactive duplex form. If the release strand TQ30R was added before the polymerase had produced a detectable amount of full-length extended primers (15 min), no TMPL* band became visible (lane 5). TQ30R displaced TQ30F from its binding to the aptamer, which folded back into its polymerase-inhibiting conformation. Taq polymerase was effectively turned OFF again, and a TQ30F–TQ30R waste duplex was formed.

Some of the bands in lane 4 of the PAGE gel shown in Figure 2 neither represent one of the switching strands (TQ30, TQ30F, TQ30R) nor do they represent one of the monitoring strands (primer, TMPL, TMPL*). They did not appear with Taq polymerase active in the presence of monitor strands or of free primer strands only—as primer is always added in excess (lane 2 in Figure 2). These multiple bands are a result of interaction between Taq polymerase and the switching strands: more exactly, misextension, DNase-activity, or possibly pausing of the Taq polymerase (for details see analysis in the Supporting Information). Misperformance is dependent on the probability of Taq polymerase interacting with a switching strand in relation to the probability of encountering a monitor strand. Because of its small dissociation constant (K_D~40 pM)[8] the aptamer concen-

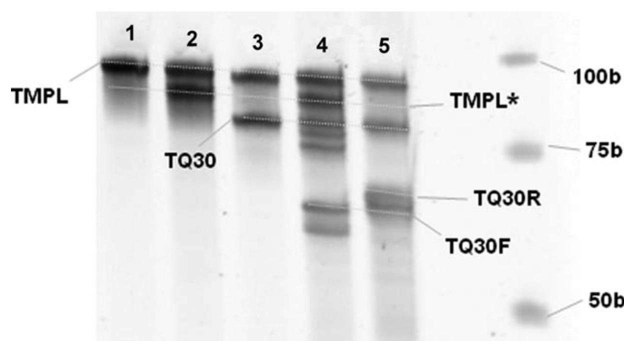

Figure 2. Switching analysis. Denaturating PAGE. From left to right: Control bands of the monitor strand without (lane 1) and with (lane 2) added dNTPs. Addition of TQ30 turns Taq polymerase OFF. No TMPL* band appears (lane 3). Upon addition of TQ30F the TMPL* becomes visible again (lane 4). An inactive aptamer–fuel duplex has formed, releasing Taq polymerase and turning its activity ON. Multiple bands in lane 4 can be explained by misextension, DNase-activity, or pausing of Taq polymerase (see fidelity analysis in the Supporting Information). Lane 5 demonstrates that no TMPL* band is visible when TQ30R is added to the TQ30–TQ30F duplex before the polymerase has had time to synthesize a considerable amount of product. TQ30R releases TQ30, which subsequently inhibits TaqPol. The rightmost lane contains a MW ladder.

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tration could be easily reduced by a factor of ten without switching on the Taq polymerase. Reducing the switching strand concentrations by this factor while keeping the monitor strand concentration constant would reduce misperformance drastically. In this study, monitor and switching strand concentrations have been chosen to be of the same order (1 μM) to allow them both to be detectable in the same gel.

To substantiate the binding and unbinding of the aptamer TQ30 to Taq polymerase and to address questions of reaction kinetics we performed a series of fluorescence correlation spectroscopy (FCS) measurements. In FCS, the diffusion coefficient of a fluorescently labeled molecule is extracted from the statistical properties of the fluorescence intensity fluctuations.[9] As the diffusion properties of a macromolecule vary with its shape and size, FCS is perfectly suited for investigation of biomolecular binding events such as aptamer–ligand interactions.[10] Figure 3 shows diffusion coefficients of fluorescently labeled TQ30 before and after the addition of Taq polymerase derived by standard means from FCS data. Each data point is obtained from a fit to an experimentally determined fluorescence autorrelation function (Supporting Information). There is a drastic change in the diffusion coefficient of TQ30 after addition of the polymerase. Diffusion coefficients calculated immediately after addition and 15 min later do not significantly differ from each other; this demonstrates that binding to TQ30 is completed quickly after the addition of the polymerase. Our data also indicate the stability and the typical variation of diffusion coefficients determined by FCS, enabling differentiation between only slightly differing diffusion coefficients.

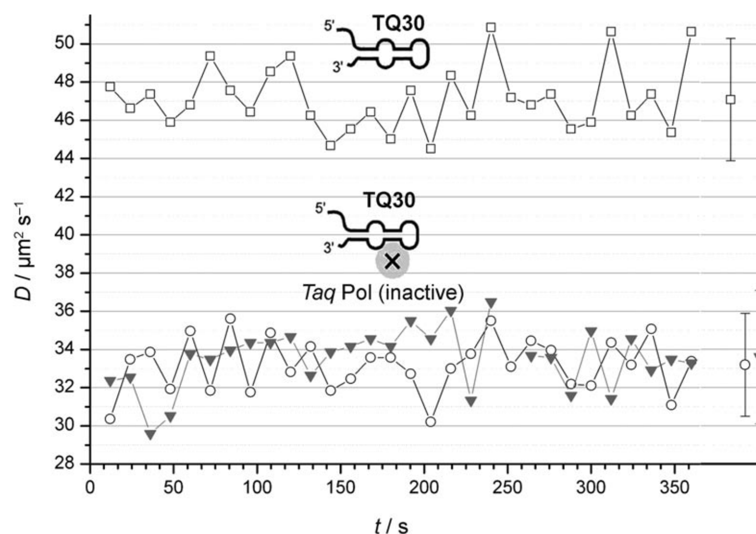

Figure 3. FCS analysis of binding. Diffusion constants as measured in 12 s FCS assays for a total time of 360 s each. A reference record displays diffusion times of TQ30 alone (□). The curve recorded immediately upon addition of Taq polymerase shows a drastic change in diffusion time (▼) that does not undergo any significant change in comparison with the curve 15 min later (○).

In Figure 4 a complete switching cycle is demonstrated. Three reference records give a set of diffusion coefficients for the free TQ30, for the inactive aptamer–fuel TQ30–TQ30F duplex conformation, and for TQ30 in its aptamer conformation when it is bound to Taq polymerase. Starting in the last state, upon addition of TQ30F the diffusion coefficient gradually approaches the value for the inactive aptamer–fuel TQ30–TQ30F duplex conformation, which suggests the displacement of Taq polymerase by TQ30F, setting the polymerase free and thereby turning its activity ON. Adding TQ30R in turn results in a reduction in the diffusion coefficient. The value for the diffusion coefficient returns to that of Taq polymerase bound aptamer within one hour. Thus, upon addition of the effector strands TQ30F and TQ30R, the aptamer TQ30 can indeed be reversibly switched between a folded form capable of binding to Taq polymerase and an inactive duplex form. Generally, displacement reactions in FCS measurements are slower, due to the fact that the concentrations are reduced by a factor of 100 in relation to those used for PAGE analysis.

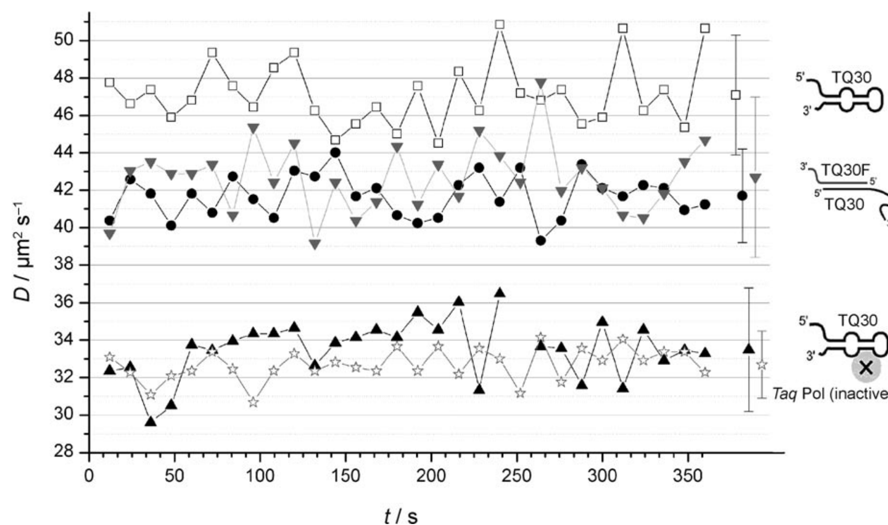

Figure 4. FCS analysis of a switching cycle. Diffusion constants as measured in 12 s FCS assays for a total time of 360 s each. Reference records show diffusion times for TQ30 without Taq polymerase (□), the inactive aptamer–fuel duplex (●), and aptamer bound Taq polymerase (▲). Upon addition of TQ30F after 80 min (▼) the diffusion time has converged to that of the inactive aptamer–fuel duplex, which suggests that the fuel strand TQ30F has displaced Taq polymerase from TQ30, forming the duplex conformation. Addition of the release strand TQ30R results in a diffusion time akin to that of the aptamer-bound Taq polymerase reference curve after around 70 min (☆), indicating the release of the aptamer by the TQ30R release strand and repeated formation of the aptamer–Taq polymerase complex.

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We have shown that the previously introduced concept of a switchable aptamer device[4] capable of binding and releasing a protein can be also applied to a Taq polymerase inhibiting aptamer, TQ30. Whereas for the thrombin binding aptamer a "toehold" section had to be attached to the aptamer sequence, here we have simply utilized the constant flanking regions of the aptamer sequence used in the SELEX process. Thanks to the larger size and the stronger secondary structure of the TQ30 aptamer, the switching processes appear to proceed considerably more slowly than in the case of the small thrombin aptamer.

In contrast to earlier experiments, the switchable aptamer was used here to turn a biochemical reaction ON or OFF selectively, and the progress of the reaction was used to monitor the state of the system. Controllable switching of biochemical reactions such as DNA polymerization is an important capability for the construction of artificial biochemical networks, and might find application in the context of advanced biosensors.[11]

Experimental Section

Oligonucleotides: All oligonucleotides were purchased from biomers.net (Ulm, Germany). Their sequences are given in Table 1.

TQ30 was also ordered with a Cy5-modification at its 5′-end. Amplification strands (TMPL, Primer) were chosen compliant to switching strands (TQ30, TQ30F, TQ30R) in such a way as to have complementary regions of minimum length to exclude unwanted hybridization between switching and amplification strands. Fuel (TQ30F) and release (TQ30R) strands were designed with self-complementary regions of minimum length to prevent stable secondary structures of these strands, which may inhibit the desired fast hybridization between complementary strands. TQ30R was tested not to inhibit Taq polymerase. Concentrations of nucleic acids were determined by measuring UV absorption at 260 nm with a NanoDrop system (NanoDrop Technologies, USA). Extinction coefficients were calculated with inclusion of nearest-neighbor interactions. The fuel strand TQ30F uses the 5′-flanking region of TQ30 as a "toehold" and is also partly complementary to the binding region of TQ30 (Table 1). It contains an additional 15 nt toehold at which the removal strand TQ30R can attach and displace TQ30F from TQ30. As previously described,[4] the fuel strand is not chosen completely complementary to the aptamer in order to avoid binding of the removal strand to the aptamer target. The toehold was chosen at the 5′-end of the aptamer to ensure that the duplex formed by TQ30F and TQ30 cannot serve as substrate for a DNA polymerase (Figure 1). Aptamer and switching strands were checked for secondary structures with the aid of the program RNA Structure[12] (Supporting Information) in order to ensure that these did not also form a substrate for Taq polymerase.

Polymerase: *Thermus aquaticus* DNA polymerase (Taq polymerase) was purchased from NEB (New England Biolabs). Taq polymerase ($M_W = 90\,000$ g mol^{-1}) stock solution concentration (5000 units per mL) is equivalent to 0.05 g L^{-1} (550 nM) according to NEB information.

General reaction conditions: Generally, all reactions (primer extension analysis, misperformance analysis, and Taq polymerase switching) were carried out in a standard PCR buffer [KCl (50 mM), $MgCl_2$ (4.0 mM), Tris-HCl (10 mM), pH 8.3] at 25 °C. The Taq polymerase concentration was 10% (*v/v*) of the stock solution (~55 nM); those of dNTPs were 375 μM (each dNTP). To ensure temperature stability, reactions were performed in an Eppendorf thermomixer at 25 °C.

Primer extension analysis: A sample (600 μL) was prepared as described under the general reaction conditions, containing primer extension template (1 μM; i.e., previously hybridized primer and TMPL strands) with a slight primer excess. Before addition of dNTPs a reference sample (30 μL (0−) was taken. Primer extension samples (30 μL) were taken every ten minutes after addition of dNTPs, with the first taken just after addition of dNTPs (0+) and the last after 120 min. A 12 h primer extension sample was taken as well (12 h). Primer extension was stopped by inhibiting Taq polymerase by addition of EDTA (9% (*v/v*), 0.5 M).

Misperformance analysis: Samples (50 μL) variously containing TQ30F, TQ30R, hybridized TQ30F–TQ30R, and hybridized TQ30–TQ30F (each 1 μM) were mixed as described under the general reaction conditions. To ensure that the Taq polymerase was not inhibited by unhybridized TQ30, the TQ30–TQ30F sample was prepared with a slight TQ30F excess. Samples were analyzed after 12 h of incubation.

Taq polymerase switching analysis: A sample (1200 μL) was prepared as described under the general reaction conditions. One after another, all relevant DNA strands were added and the state of the system was documented by saving a sample (60 μL) before each step. All samples were left on the workbench before analysis by denaturating 20% PAGE.

PAGE: All gels shown are 20% denaturating polyacrylamide gels (containing 8 M urea) that were run under standard conditions in 1×TBE at 19 V cm^{-1} for 4–5 h at 65 °C. Gels were stained with SYBR gold. A formamide loading buffer (95%) was used.

FCS: FCS experiments were performed with a ConfoCor2 system (Zeiss, Germany) with use of a standard PCR buffer consisting of KCl (50 mM), $MgCl_2$ (4.0 mM), and Tris-HCl (pH 8.3, 10 mM) at 25 °C. The Taq polymerase concentration was 10% (*v/v*) of the stock solution (~55 nM). The TQ30 concentration was 10 nM. TQ30F was added in a 25% excess (12.5 nM) relative to TQ30; TQ30R was

Table 1. Sequences for the DNA strands used in the experiments. Lower case letters denote the constant flanking regions used during SELEX in ref. [8]. The complementary regions of TQ30 and TQ30F are underlined.

Oligonucleotide[a]	Sequence
TQ30	ttc tcg gtt ggt ctc tgg cgg agc AAG ACC AGA CAA TGT ACA GTA TTG GCC TGA tct tgt gta tga ttc gct ttt ccc
TQ30F	AAT ACT GTA CAT TGT CTG GTC TTG CTC CGC CAG AGA CCA ACC GAG AAG GAC TAC TAC TAC TA
TQ30R	TAG TAG TAG TAG TCC TTC TCG GTT GGT CTC TGG CGG AGC AAG ACC AGA CAA TGT ACA GTA TT
TMPL	TGA AGT GAA GTG AAG TGA AGT GAA GTG AAG TGA AGT GAA GTG AAG TGA AGT GAA GTG AAG TGA AGT GAA GTG AAG TGG ATA GAT TCT T
Primer	AAT CTA TCC ACT TC

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added in a 25% excess relative to TQ30F. For each sample, 30 autocorrelation functions were calculated each from a series of 12 s fluorescence signal data acquisition measurements. Plots show fitted diffusion times for each of the 12 s acquisition measurements.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (Emmy Noether grant SI 761/2-3), Nanosystems Initiative Munich (NIM) and the Human Frontier Science Program (HFSP) is gratefully acknowledged. We would also like to express our thanks for cooperation within CeNS, especially the ongoing support of Jörg. P. Kotthaus and Joachim O. Rädler.

Keywords: aptamers · molecular devices · oligonucleotides · switches · Taq polymerase

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Received: May 29, 2007
Published online on August 6, 2007

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