Molecular Devices

Individual Molecules of Dye-Labeled DNA Act as a Reversible Two-Color Switch upon Application of an Electric Field**

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Over the past few years, molecular switches have been heavily researched in the quest for molecular electronic devices. Some switching systems operate by a conformational change in the molecule which is induced by either an electric field,[1,2] an STM (scanning tunneling microscope) tip,[3] an electrochemical reaction,[4] or light.[5] Alternatively, molecular switches can be operated nonconformationally by redox reactions[6] or a chemical binding event.[7] Biomolecules have recently been adapted for new purposes; for example, DNA has been used as part of a conducting wire,[8] a molecular machine,[9–12] a crystal template,[13] a scaffold for nanoscale construction,[14] and even as a component of a "computing machine".[15,16] Herein we show that the fluorescence of individual dye-labeled DNA molecules can be reversibly switched from green to red and vice versa upon application of an electric field.

The use of nucleic acids as molecular switches is a relatively new concept and, so far, has had limited success. The use of an electric field to modulate the fluorescence of labeled DNA adsorbed onto an electrode[17] and the use of a magnetic field to control the hybridization state of DNA[18] are known. Conformational switching of DNA by the variation of buffer conditions,[11,19] the binding of adenosine to an aptamer sequence on DNA,[20] or the binding of single-stranded DNA (ssDNA) to a DNA quadruplex[9] have also recently been reported. The main limitation with the current mechanisms for DNA conformational switches is that they require bimolecular hybridization or a change of buffer, thus switching is slow. Furthermore, in some cases switching is not reversible[20] or the process creates undesirable "waste" DNA.[9,10] Our DNA switch overcomes these problems by using an electric field to alter the DNA–dye interaction which renders the switching process rapid (<100 ms) and reversible with no by-products.

Here a controllable electric field and single-molecule fluorescence detection were used to probe the switching of the fluorescent states of individual DNA molecules that were labeled with two different fluorophores at the same end of the DNA duplex. We previously utilized the very high electric-field gradients generated at the tip of a nanopipette to controllably concentrate and deliver DNA.[21–23] The pipettes have an inner diameter of 100 nm and the voltage drop occurs in the last few microns of the tip owing to its tapered shape. By application of a potential of 1 V between the electrode in the bath and the electrode in the pipette, a very high electric field of $\approx 8000\ \mathrm{V\,cm^{-1}}$ is generated at the tip of the pipette.[22]

The fluorescence of both fluorophores was detected simultaneously with either two-color direct excitation of both fluorophores or single-color excitation of the donor fluorophore only. In the latter experiments, the excited-state energy is transferred nonradiatively from a donor to an acceptor fluorophore by FRET (fluorescence resonance energy transfer). The efficiency of transfer, E, depends on the relative orientation of the fluorophores and the distance that separates them, R, according to Equation (1). R_0 is the Förster distance and this was calculated to be 53 Å for the dye-pair used in these FRET experiments. This inverse-sixth-power separation dependency of energy transfer allows FRET to be used as a sensitive probe of intramolecular distances over the 10–100 Å range.

$$E = R^6/(R^6 + {R_0}^6) \quad (1)$$

In FRET, the principle property of interest is not the intensity of fluorescence, but rather the proximity ratio, P. The FRET proximity ratio of each DNA molecule was calculated according to Equation (2), in which I_A and I_D are the intensities of the acceptor and donor fluorescence, respectively. This ratio is strongly dependent on the distance between the fluorophores, whereas it is independent of diffusional motion.[24] If the excitation power is kept low, photodriven processes such as photobleaching can also be neglected and the proximity ratio becomes independent of the laser intensity.[25]

$$P = I_A/(I_A + I_D) \quad (2)$$

We studied a 40-base double-stranded DNA (dsDNA) labeled with a donor fluorophore, Rhodamine Green, and an acceptor fluorophore, Alexa 647. Both fluorophores were attached to the bases by C6 linkers that do not constrain the motion of the fluorophore. DNA 1 is a dsDNA with the donor and acceptor fluorophores directly opposite each other on separate strands; it is able to undergo FRET owing to the close proximity of the strands (Figure 1). DNA 2 has the donor and acceptor fluorophores at opposite ends of the duplex and is unable to undergo FRET as their separation is larger than the Förster distance for the dye-pair used (53 Å; Figure 1). A single-molecule study of DNA 1 in solution unexpectedly revealed two peaks in the FRET histogram (Figure 2a), one at $P = 0.05$ and the other at $P = 0.95$, both of which had approximately equal amplitudes. The average single-molecule proximity ratio ($P = 0.40$) gave a value that was almost identical to that of the bulk value ($P = 0.44$). Experiments in which the laser power was varied by two orders of magnitude and in which a radical scavenger (ascorbic acid, 200 μM) was introduced did not lead to any changes in the single-molecule FRET histogram (data not shown). This indicates the absence of photobleaching in this

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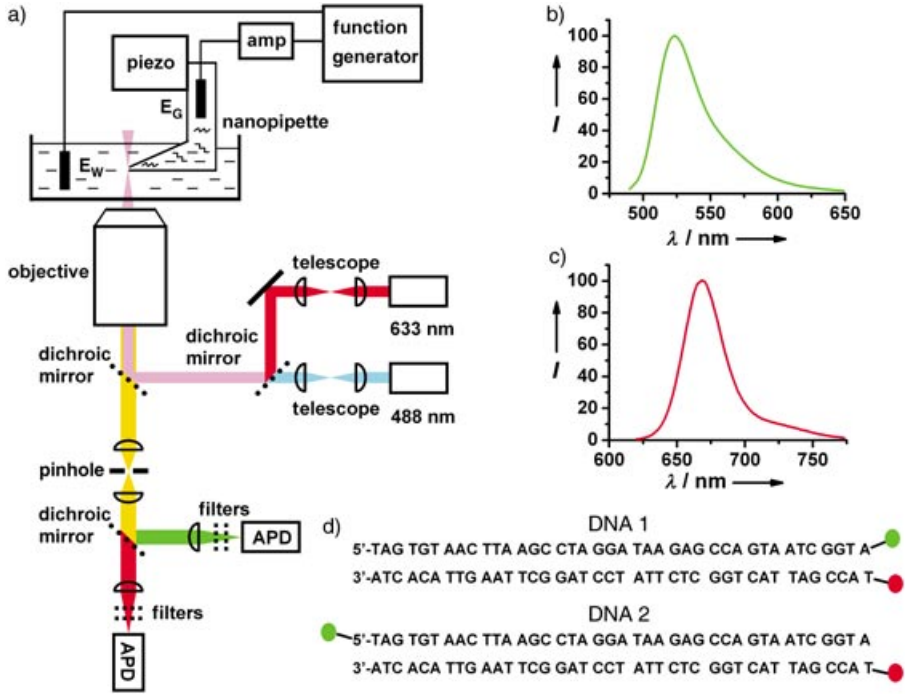

Figure 1. a) A schematic of the experimental setup used (E_G = ground electrode, E_W = working electrode, APD = Avalanche Photo Diode detector); b) and c) the fluorescence emission spectra of the fluorophores Rhodamine Green (donor) and Alexa 647 (acceptor), respectively; d) the two DNA samples labeled with the donor (green circle) and the acceptor (red circle) dyes.

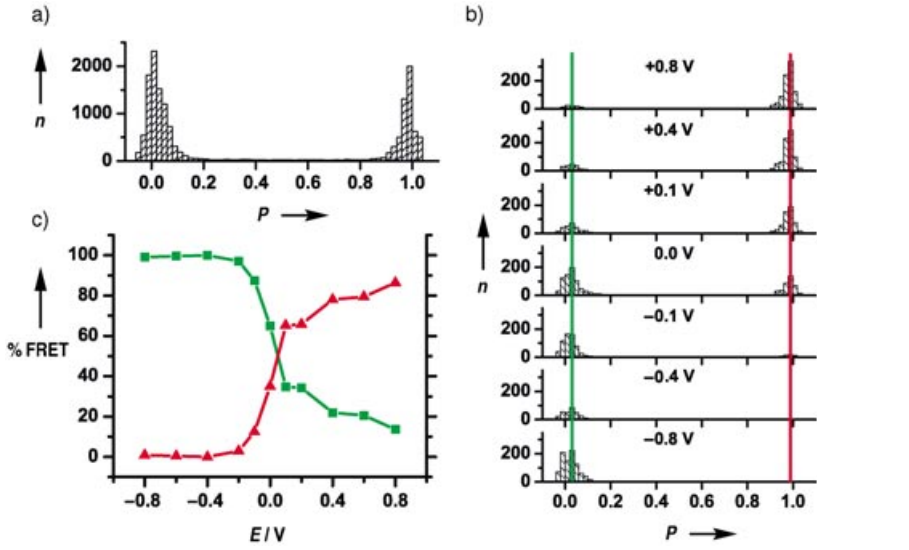

Figure 2. a) Single-molecule FRET histogram of DNA 1 (50 pM) in solution; b) single-molecule FRET histogram of DNA 1 (1 nM) in a nanopipette upon excitation at $\lambda = 488$ nm (on the tip); P = FRET proximity ratio, n = number of molecules. c) Variation of the percentages of the low-FRET peak (green; donor emission) and the high-FRET (red; acceptor emission) with the applied dc potential, E.

experiment. A temperature-dependent study over the range 12–38 °C also revealed no variation in the two FRET peaks (data not shown). These results indicated that two equally occupied conformational states are present in solution: one low-FRET state that is green-fluorescent and one high-FRET state that is red-fluorescent.

The possibility of altering the populations of these two fluorescent states through the generation of an electric field in the nanopipette was then explored. Indeed, the amplitudes of the two FRET peaks were dramatically altered by the application of different potentials Figure 2b. No additional states were observed. The pipette in these experiments was earthed, and a potential was applied to the electrode in the bath. At a negative potential below −0.2 V only the low-FRET peak was observed, whereas a positive potential above 0.4 V favored almost entirely the high-FRET peak. This implies that the low-FRET peak is not due to the presence of DNA labeled only with the donor, but that it is a true FRET state because its population can be altered by an applied voltage. The absolute values in Figure 2b cannot be compared because of the slight differences in the alignment of the pipette, thus the percentage of each FRET population at a given voltage is displayed in Figure 2c. This shows a clear switching between the two states with the applied voltage. To determine if the change was reversible, the laser was then focused outside the pipette at approximately 20 μm from the tip, and a positive voltage was applied so that the DNA flowed out of the pipette. The DNA is in a high-FRET state in the pipette tip, however, the measured single-molecule FRET histogram and the mean proximity ratio value were identical to those in free solution which indicates no irreversible processes (e.g. redox reactions) had taken place in the tip. Switching was determined to be rapid (< 100 ms) from the application of a square wave (± 0.8 V, 0.25 Hz) and from measurement of the time taken for the FRET to change from low to high and vice versa.

Fluorophore excimers,[26,27] exciplexes,[28] and excitonic interactions[29,30] have previously been observed in DNA or RNA. The main characteristic of an excitonic interaction is a change in the UV/Vis absorption spectrum, whereas the main characteristic of an excimer or exciplex is a change in the fluorescence spectrum. No changes in the UV/Vis absorption or fluorescence spectra were

observed for DNA 1 compared to the control sample, DNA 2, which indicates that neither a fluorophore exciplex nor an exciton interaction between the two fluorophores is likely to be present in solution. No perturbation of the electronic state of the Alexa 647 dye attached to the DNA could be detected in the absorption or fluorescence spectra relative to the free dye. The melting temperatures for DNA 1 and 2 were $68 \pm 0.2\,^{\circ}C$ and $67 \pm 0.3\,^{\circ}C$, respectively. This also suggests that there is no significant dye–dye interaction in solution.

Each fluorophore was then excited directly by using a two-color setup. When a negative potential was applied to DNA 1 in a nanopipette and upon excitation at $\lambda = 488$ nm, fluorescence from Rhodamine Green was observed. However, emission from Alexa 647 was not detected even upon direct excitation at $\lambda = 633$ nm. The same behavior was observed with DNA 2. In contrast, it was possible to excite directly the Alexa 647 moieties in both DNA 1 and 2 upon application of a positive potential. An experiment was then performed to determine if dsDNA was required to observe this effect. A 5′-Alexa 647-labeled ss40-mer and a Rhodamine Green-labeled noncomplementary ss65-mer (used as a control to check the presence of DNA at the tip) were excited at $\lambda = 488$ and 633 nm in the nanopipette. A square-wave waveform was applied, and the fluorescence was monitored. Many bursts of Alexa 647 emission were observed during the positive half-cycle because of the electrophoretic flow to the pipette tip, and fewer bursts were detected during the negative half-cycle. However, when the fluorescence counts per burst were individually analyzed, no differences were observed in the fluorescence amplitude histogram (Figure 3a). Double-stranded DNA would therefore seem to be a prerequisite for the switching process. These experiments suggest that dye–dye interactions are not the reason for the observed switching, but rather switching occurs upon a change in the interaction between the Alexa 647 moiety and the duplex DNA upon application of an electric field.

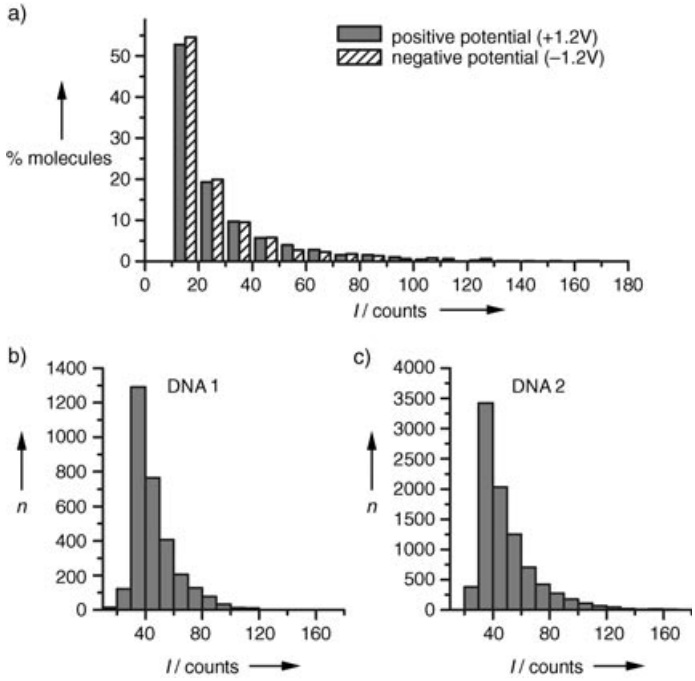

Figure 3. a) Fluorescence amplitude histogram of 5′-Alexa 647-labeled ss40-mer DNA; excited at $\lambda = 633$ nm with a ± 1.2-V square-wave (0.125 Hz) applied. b) and c) Donor fluorescence amplitude histograms of DNA 1 (only the low-FRET population is selected, that is, $P < 0.5$; mean fluorescence burst amplitude = 46) and DNA 2 (mean fluorescence burst amplitude = 50), respectively, in free solution.

Quenching of the donor was ruled out by analysis of the individual fluorescence counts from the donor per burst of DNA 1 (upon selection of only the low-FRET bursts) and DNA 2 in free solution. No differences in the fluorescence amplitude histogram were seen (Figure 3, b and c), and mean amplitudes of 46 and 50 counts were obtained for DNA 1 and DNA 2, respectively, which indicate that the donor is not quenched.

We postulate that there are two states for the Alexa 647 dye: 1) Under a positive potential, the dye is free to move such that the Rhodamine Green donor can transfer energy to Alexa 647 through FRET with nearly 100% efficiency owing to the proximity of the fluorophores. 2) Under a negative potential, the Alexa 647 dye interacts with the duplex DNA, possibly by base-stacking on the end of the DNA duplex, whereupon it is quenched by the DNA. Base-stacking of the Alexa dye that results in restricted movement of the dye is suggested by the high bulk fluorescence anisotropy value (0.204 ± 0.004). Rhodamine Green has a low bulk fluorescence anisotropy value (0.090 ± 0.008) which implies that it is relatively mobile. The fluorophore Cy3, a cyanine dye like Alexa 647, is known to stack onto the end of DNA helices in a similar manner to that of an additional base pair.[31] Base-stacking has been observed for other dyes, with millisecond-range rates of conversion between the stacked and unstacked states observed at room temperature.[32,33] In this case, the Alexa dye is closer to the Rhodamine Green moiety and is not free to rotate. Only Rhodamine Green emission is observed in this state for two possible reasons: First, the weak coupling assumed in the Förster transfer process breaks down, and an excitonic state is formed whose fluorescence originates entirely from the Rhodamine fluorophore. This excitonic state was not observed in solution so the interactions, separation, or orientation of the fluorophore must be altered in the presence of the high electric field. Alternatively, weak coupling between the dyes may still occur, but Rhodamine Green cannot transfer energy to the Alexa dye owing to the relative orientation of the two dyes, and thus Rhodamine Green fluorescence is observed.

In conclusion, we have shown that duplex DNA with donor and acceptor fluorophores at the same end can be reversibly switched between a donor-emitting green state and an acceptor-emitting red state on the application of an electric field. The electric field appears to alter the conformation of the acceptor dye only which results in a significant change in its fluorescence quantum yield presumably owing to interactions with the DNA strand. This is important for applications as a two-state switch in single-molecule optoelectronic devices and indicates that the conformations of other

biological molecules as well as voltage-gated ion channels may be switched by the application of an electric field.

Experimental Section

HPLC-purified 40-base oligonucleotide 5′-TAG TGT AAC TTA AGC CTA GGA TAA GAG CCA GTA ATC GGT A-3′ (MWG–Biotech, Ebersberg, Germany) was labeled at the 3′ terminus with the fluorophore Rhodamine Green; a 5′-labeled and unlabeled versions were also purchased. Its 40-base complementary oligonucleotide, modified at the 5′ terminus with a C6 linker chain (IBA, Göttingen, Germany), was desalted (NAP 5 column; Amersham, U.K.) and labeled by using an Alexa Fluor 647 Oligonucleotide Amine Labeling Kit (Molecular Probes, Eugene, USA). Dimethylsulfoxide was removed from the labeled DNA solution by using a NAP 5 column. The labeled oligonucleotide was separated from excess dye by precipitation in ethanol and further purified by HPLC to remove unlabelled DNA and any remaining dye. Acetonitrile (from the HPLC fractions) was removed with a NAP 10 column (Amersham, U.K.). All oligonucleotides were prepared in a buffer solution containing Tris-HCl (10 mM; Amersham, U.K.), EDTA (1 mM; Amersham, U.K.), and NaCl (100 mM; Acros Organics, Fairlawn, USA) at pH 7.4. The concentration of the dye-labeled DNA was determined by the absorbance at $\lambda = 260$ nm; the absorbances at $\lambda =$ 505 (Rhodamine Green) or 650 nm (Alexa 647) were used as internal references. Double-stranded DNA (dsDNA) samples were prepared by mixing two complementary single-stranded oligonucleotides, heating the mixture to 90 °C and then slowly cooling this to room temperature. Melting temperatures of 68 °C and 67 °C (in NaCl–Tris–EDTA buffer) for DNA 1 and DNA 2, respectively, were observed by the evolution of UV/Vis spectra with temperature.

The nanopipettes were made by using a laser-based pipette puller (Model P-2000, Sutter Instrument Co.). A two-line program was used to pull borosilicate glass capillaries (0.58- and 1-mm inside and outside diameters, respectively) with the following parameters: Heat = 350, Fil = 3, Vel = 30, Del = 220, Pull = ; Heat = 330, Fil = 2, Vel = 27, Del = 180, Pull = 250. For the pipette experiments, a solution of DNA (1 nM) was back-filled into the bent nanopipette by a microfiller (Microfil 34, World Precision Instruments, Sarasota, USA). A coverglass-bottomed dish (Willco Wells GWST-1000) that contained buffer solution (2–3 mL) was used as the bath. The pipette tip was placed 5 to 10 μm above the surface of the dish. Two Ag/AgCl electrodes, one placed in the bath and the other placed inside the pipette, served as the working and reference electrodes, respectively. The ion current flowing through the pipette was the same in the presence and absence of DNA because the ion current is dominated by the flow of sodium and chloride ions. Furthermore, no reduction in the ion current could be detected with DNA present in the pipette (from partial blocking). The same buffer solutions (Tris-HCl, 10 mM; EDTA, 1 mM; NaCl, 100 mM) were used both in the pipette and in the bath. EDTA served to remove multivalent cations in the solution. For experiments in solution, DNA was diluted to 50 pM in the NaCl–Tris–EDTA buffer solution (pH 7.4) and Tween 20 (0.01 %) was added to prevent surface adhesion of the DNA molecules.

The two-color setup and apparatus for the experiments performed in the pipette were described previously.[34] The potential waveforms applied to the electrodes were created by using a function generator (Model DS345, Stanford Research Systems, Sunnyvale, USA). This function generator was also used to provide a trigger for the MCS (Multi Chanel Scalar) cards. Solution experiments were performed on the same setup, except that the laser beams were focused 5 μm into a 1-mL aliquot of the solution of the sample supported in a Lab-TeK chambered coverglass (Scientific Laboratory Suppliers Ltd, U.K.). All experiments were performed at room temperature with a 1-ms bin time(data-acquisition time) used on both MCS cards. A threshold of 35 counts per millisecond bin for the sum of the donor and acceptor fluorescence signals was used to differentiate between the signal bursts of the background and the single-molecules. A background of 1–2 counts per millisecond, obtained from independent measurements of buffer solution that did not contain labeled DNA, was subtracted from each burst. The emission spectra of the two fluorophores are very well separated (see Figure 1 which reduces the cross-talk to a negligible level.[34]

Steady-state fluorescence measurements were recorded on an Aminco-Bowman Series 2 fluorimeter, which was equipped with a water bath set to 20 °C. A 50-nM concentration of DNA was used. Fluorescence anisotropies, r, for Rhodamine Green and Alexa 647 were calculated from the polarization of the emission components $I_{\parallel\parallel}$, $I_{\parallel\perp}$, $I_{\perp\parallel}$, and $I_{\perp\perp}$ ($\parallel$ and $\perp$ denote the parallel and perpendicular orientations, respectively, of the excitation and emission polarisers) according to Equation (3), with $G = I_{\perp\parallel}/I_{\perp\perp}$.

$$r = (I_{\parallel\parallel} - GI_{\parallel\perp})/(I_{\parallel\parallel} + 2GI_{\parallel\perp}) \quad (3)$$

For the fluorescence anisotropy of Rhodamine Green, excitation was carried out at $\lambda = 505$ nm and the emission was monitored at $\lambda =$ 530 nm. For Alexa 647, excitation was carried out at $\lambda = 649$ nm and the emission was monitored at $\lambda = 666$ nm.

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[1] Z. J. Donhauser, B. A. Mantooth, K. F. Kelly, L. A. Bumm, J. D. Monnell, J. J. Stapleton, D. W. Price, A. M. Rawlett, D. L. Allara, J. M. Tour, P. S. Weiss, *Science* **2001**, *292*, 2303.
[2] V. Bermudez, N. Capron, T. Gase, F. G. Gatti, F. Kajzar, D. A. Leigh, F. Zerbetto, S. W. Zhang, *Nature* **2000**, *406*, 608.
[3] F. Moresco, G. Meyer, K. H. Rieder, H. Tang, A. Gourdon, C. Joachim, *Phys. Rev. Lett.* **2001**, *86*, 672.
[4] R. A. Bissell, E. Cordova, A. E. Kaifer, J. F. Stoddart, *Nature* **1994**, *369*, 133.
[5] L. Giordano, T. M. Jovin, M. Irie, E. A. Jares-Erijman, *J. Am. Chem. Soc.* **2002**, *124*, 7481.
[6] D. I. Gittins, D. Bethell, D. J. Schiffrin, R. J. Nichols, *Nature* **2000**, *408*, 67.
[7] B. S. T. Kasibhatla, A. P. Labonte, F. Zahid, R. G. Reifenberger, S. Datta, C. P. Kubiak, *J. Phys. Chem. B* **2003**, *107*, 12378.
[8] E. Braun, Y. Eichen, U. Sivan, G. Ben-Yoseph, *Nature* **1998**, *391*, 775.
[9] P. Alberti, J. L. Mergny, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1569.
[10] B. Yurke, A. J. Turberfield, A. P. Mills, F. C. Simmel, J. L. Neumann, *Nature* **2000**, *406*, 605.
[11] C. D. Mao, W. Q. Sun, Z. Y. Shen, N. C. Seeman, *Nature* **1999**, *397*, 144.
[12] H. Yan, X. P. Zhang, Z. Y. Shen, N. C. Seeman, *Nature* **2002**, *415*, 62.
[13] E. Winfree, F. R. Liu, L. A. Wenzler, N. C. Seeman, *Nature* **1998**, *394*, 539.
[14] J. H. Chen, N. C. Seeman, *Nature* **1991**, *350*, 631.
[15] Y. Benenson, R. Adar, T. Paz-Elizur, Z. Livneh, E. Shapiro, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 2191.
[16] M. N. Stojanovic, D. Stefanovic, *Nat. Biotechnol.* **2003**, *21*, 1069.
[17] M. Ueda, M. Takai, K. Taniguchi, Y. Takamura, Y. Horiike, Y. Baba, *Appl. Phys. Lett.* **2003**, *83*, 5086.
[18] K. Hamad-Schifferli, J. J. Schwartz, A. T. Santos, S. G. Zhang, J. M. Jacobson, *Nature* **2002**, *415*, 152.
[19] D. S. Liu, S. Balasubramanian, *Angew. Chem.* **2003**, *115*, 5912; *Angew. Chem. Int. Ed.* **2003**, *42*, 5734.

[20] R. P. Fahlman, D. Sen, *J. Am. Chem. Soc.* **2002**, *124*, 4610.
[21] L. M. Ying, A. Bruckbauer, A. M. Rothery, Y. E. Korchev, D. Klenerman, *Anal. Chem.* **2002**, *74*, 1380.
[22] L. M. Ying, S. S. White, A. Bruckbauer, L. Meadows, Y. E. Korchev, D. Klenerman, *Biophys. J.* **2004**, *86*, 1018.
[23] A. Bruckbauer, L. M. Ying, A. M. Rothery, D. J. Zhou, A. I. Shevchuk, C. Abell, Y. E. Korchev, D. Klenerman, *J. Am. Chem. Soc.* **2002**, *124*, 8810.
[24] A. A. Deniz, M. Dahan, J. R. Grunwell, T. J. Ha, A. E. Faulhaber, D. S. Chemla, S. Weiss, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 3670.
[25] M. I. Wallace, L. M. Ying, S. Balasubramanian, D. Klenerman, *J. Phys. Chem. B* **2000**, *104*, 11551.
[26] M. Masuko, H. Ohtani, K. Ebata, A. Shimadzu, *Nucleic Acids Res.* **1998**, *26*, 5409.
[27] E. Kostenko, M. Dobrikov, D. Pyshnyi, V. Petyuk, N. Komarova, V. Vlassov, M. Zenkova, *Nucleic Acids Res.* **2001**, *29*, 3611.
[28] S. G. Kruglik, P. Mojzes, Y. Mizutani, T. Kitagawa, P. Y. Turpin, *J. Phys. Chem. B* **2001**, *105*, 5018.
[29] S. Bernacchi, Y. Mely, *Nucleic Acids Res.* **2001**, *29*, e62.
[30] S. Bernacchi, E. Piemont, N. Potier, A. van Dorsselaer, Y. Mely, *Biophys. J.* **2003**, *84*, 643.
[31] D. G. Norman, R. J. Grainger, D. Uhrin, D. M. J. Lilley, *Biochemistry* **2000**, *39*, 6317.
[32] C. Eggeling, J. R. Fries, L. Brand, R. Gunther, C. A. M. Seidel, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 1556.
[33] L. Edman, U. Mets, R. Rigler, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 6710.
[34] H. T. Li, L. M. Ying, J. J. Green, S. Balasubramanian, D. Klenerman, *Anal. Chem.* **2003**, *75*, 1664.

