[12] An indication that the pyramidalization of the gallium center is the cause of the twisting and other distortions of the fc_3Ga_2 fragment is that in the complex [**3**·2 Et_2O], which we could prepare from **2** and Et_2O, the gallium center is less pyramidalized (C-Ga-C angle sum 357°) and less twisted ($\alpha_T = 4.7°$ (average)). In addition the Ga – Ga separation is shorter (3.73 Å), so it can be assumed that in the donor-free compound **3** there is an even shorter Ga – Ga separation. Because of the poor *R* value of the structure determination the values given should be treated with caution. Whether the $Ga(C_5H_4)_3$ units of this compound are planar and mutually parallel with a Ga – Ga separation of under 3.4 Å (the Cp – Cp separation in ferrocene) should be shown by theoretical calculations (W. W. Schoeller, unpublished results).

[13] The electrochemical experiments were carried out with a Potentiostat/Galvanostat PAR-Model 273A and the corresponding software (Model 270). A three-electrode system was used of which the working electrode was a platinum disc (2 mm diameter), the counter electrode was a platinum wire, and the pseudo reference electrode was a silver wire. By each measurement with an otherwise identical sample decamethylferrocene [$FeCp_2^*$] was added as an internal standard. The measured redox potentials were later converted into ferrocene/ferrocenium ([$FeCp_2$]/[$FeCp_2$]$^+$) potentials (E([$FeCp_2^*$]) = −480 mV (in DMSO) vs. [$FeCp_2$]/[$FeCp_2$]$^+$; our own measurement). A 0.1 M solution of the electrolyte NBu_4PF_6 in DMSO was used as solvent. All the potentials given were determined by cyclic (scan rate 100 mV s^{-1}) and by square-wave voltammetry (frequency 5 Hz).

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High-Density Labeling of DNA: Preparation and Characterization of the Target Material for Single-Molecule Sequencing**

Susanne Brakmann* and Sylvia Löbermann

Single-molecule detection has emerged as the ultimate analytical tool and requires concentrations as low as femto- to zeptomolar (10^{-15} to 10^{-21}). In extreme cases the sensitivity of single-molecule techniques can even reach the yoctomole limit (10^{-24}),[1–5] and, thereby, offer insights into the chemical behavior and fate of individual molecules, as well as reflect subtle changes at the molecular level that might influence chemical reactivities.[6–10] With classic methods these effects have as yet been hidden by ensemble averaging.

Further impetus for research into this field came when it was suggested that sequencing of DNA and RNA might be accelerated by using single-molecule detection and analysis.[3, 11, 12] The sequencing attempts are based on the fact that single fluorescent molecules can be identified within milliseconds,[13] and they combine sequential enzymatic hydrolysis of individual DNA molecules with subsequent identification of released monomers by their fluorescence characteristics, either wavelengths, fluorescence lifetimes, or both.[14, 15] As natural nucleotides exhibit only marginal fluorescence at room temperature, the idea requires complete and faithful labeling of single DNA or RNA molecules with fluorescent dyes, whereby each dye distinctly codes one of the four bases (A, G, C, or T/U). Modification of DNA with fluorophores may be achieved either by enzymatic, or by postsynthetic labeling. As the chemical attachment of even a single dye molecule to oligomeric DNA does not yield 100 % modified product, synthetic introduction of dyes would result in unreliable sequencing templates. Instead, fluorophore-labeled DNA may be synthesized enzymatically using polymerase-mediated incorporation of fluorescently tagged nucleotide analogues. Nucleic acid polymerases from a variety of organisms may be used for the synthesis of complementary, fluorescently labeled DNA through nick translation, primer extension, reverse transcription, or a polymerase chain reaction (PCR). However, preliminary experiments performed by us suggested that most natural and commercially available DNA polymerases failed to accept substrates that are heavily modified with fluorescent dyes (unpublished results). Experiments by other groups,[16, 17] as well as suggestions by suppliers of dye-labeled nucleotides,[18] confirmed that a maximum 28 % of possible substitution sites could be labeled by PCR, whereas only about 18 % exchange were achieved by nick translation.[19]

Using an assay for screening polymerase activity with dye-labeled nucleotides, which was based on fluorescence correlation spectroscopy (FCS),[3] we then observed that the wild-type, as well as the exonuclease-deficient Klenow fragment, of *Escherichia coli* DNA polymerase I is capable of incorporating 55 subsequent, fluorophore-labeled deoxyribonucleotides by extension of a synthetic primer-template (100 % incorporation).[20] Although this finding was surprising, it did not contradict the previous results cited above because labeling was achieved by replication in a single round, that is, in the absence of amplification, with an artificial poly(dA) template, and the polymeric product could be detected by the highly sensitive FCS technique. FCS also provided a closer view of the enzymatic fluorophore incorporation because fluorescence intensity and diffusion time of individual molecular species were determined in parallel: Under optimal conditions, that is, in the absence of quenching phenomena and with molecular species of comparable linear shape, FCS yields data on the amount of fluorophores incorporated into a specific molecule, as well as information on the relative length of the polymeric product, which can be clearly distinguished from the length of the respective primer-template.

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[**] The authors are grateful to Dr. Dmitri Czerny of the Max Planck Institute for Biophysical Chemistry, Göttingen, for generating the electron micrograph. Our work was supported by the Max-Planck-Gesellschaft, the BMBF together with Roche Diagnostics (grant no.: 0311003), and the Deutsche Forschungsgemeinschaft (grant no.: Ei 411/1-1).

In pursuance of the labeling of long fragments for single-molecule sequencing, we applied the primer extension by Klenow polymerase to a natural DNA fragment with a length of 2.7 kilo base pairs (kb), which originated from the bacteriophage T7 genome. We started our experiments by completely substituting the dTTP[21] by its fluorescent analogue tetramethylrhodamine–4-dUTP (TMR-dUTP, Fluoro-Red; Amersham Pharmacia) and obtained a nonfluorescent reaction product with the expected electrophoretic mobility and an absorbance spectrum (in 10 mM tris(hydroxymethyl)-aminomethane–HCl (tris-HCl) buffer at pH 7.5) resembling that of natural DNA, except that its maximum was shifted from 260 nm to approximately 275 nm (Figure 1).

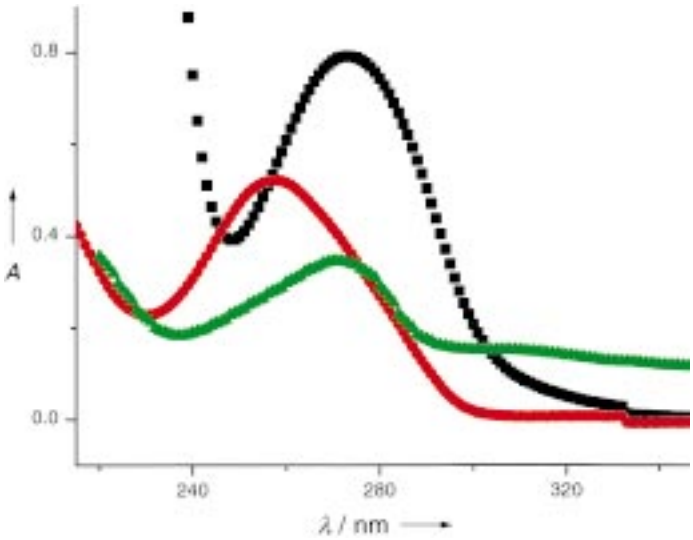

Figure 1. Comparison of the absorption spectra of natural and fluorescently labeled DNA. The spectra of nonlabeled DNA (red) and DNA labeled with TMR-dUTP (black) were determined from aqueous solutions (10 mM tris-HCl, pH 7.5); DNA labeled with TMR-dUTP and R110-dCTP (green) was dissolved in aqueous solutions of 10 mM tris-HCl buffer (pH 7.5) containing 50% dioxane.

Complete incorporation of a second fluorescence label was achieved by replacing dCTP with its analogue rhodamine-110–dCTP (R110-dCTP, Applied Biosystems), in addition to the substitution of dTTP with TMR-dUTP. Again, the primer-extension reaction of the same template yielded a product with the expected electrophoretic mobility that did not fluoresce. Optical absorbance measurements in aqueous solution (10 mM tris-HCl at pH 7.5) showed strong light scattering due to aggregation of the product molecules. However, the product was fully soluble in H_2O/dioxane (50/50) and gave an absorbance spectrum resembling that of the TMR-labeled DNA derivative with a maximum at about 275 nm (Figure 1).

DNA labeled either with TMR-dUTP or both TMR-dUTP and R110-dCTP exhibited an increasing tendency to form aggregates upon solution in aqueous solvents. This was first observed by monitoring the absorbance spectra and also became apparent with experiments to visualize the product of dye incorporation: Atomic force microscopy revealed very large aggregates (approximately 400 nm diameter and 50 nm height) of labeled DNA on the polar surface of the mica that prevented the optical differentiation of single-molecule shapes (T. Schäffer, Göttingen, personal communication). On the other hand, transmission electron microscopy (TEM) on the nonpolar graphite surface allowed the visualization of TMR-dUTP/R110-dCTP-labeled DNA (Figure 2; in cooperation with D. Czerny, Göttingen). The reaction product

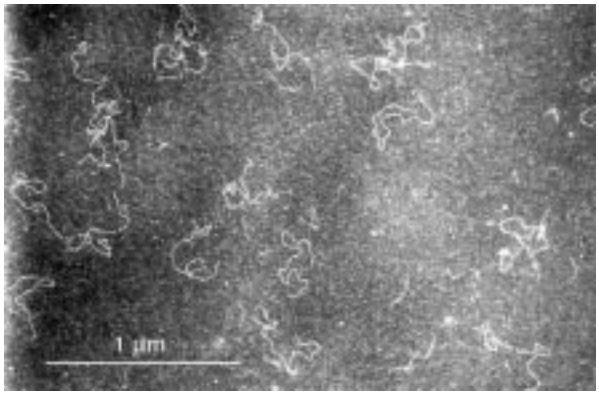

Figure 2. Transmission electron micrograph of dsDNA labeled with TMR-dUTP and R110-dCTP (graphite surface; staining with uranyl acetate). The lengths of DNA structures were determined using analySIS software (Soft Imaging System GmbH, Münster).

formed an even distribution of individual molecules, which exhibited a shape similar to that of natural double-stranded DNA, and a length of approximately 1 µm, which was expected for the 2.7 kb fragment. Although information on structural details, such as the helix characteristics, of the dye-labeled product is limited using electron microscopy, it can be concluded that full-length dsDNA represented the main product ($\geq$90%) because almost no single-stranded DNA was observed.

Furthermore, fluorescently labeled DNA did not show the cooperative melting behavior that is observed with natural DNA (D. Pörschke, Göttingen, personal communication). However, 30 min heating of a solution of dry, double-labeled DNA in 100% DMSO at 50 °C converted about 90% into single-stranded DNA (observation after medium pressure liquid chromatography (MPLC) on sepharose). We applied this finding to double-labeled DNA which was generated using a biotinylated primer, and tested whether the modified strand served as a template for further polymerase reactions: Immobilization of the biotinylated, dye-labeled dsDNA, separation of the nonlabeled strand, and subsequent PCR amplification (using natural nucleotides and proof-reading *Pfu* DNA polymerase) revealed that the labeled strand served as a template for PCR. Subcloning of the PCR product, sequence determination (*Taq* DyeDeoxy Terminator method; Applied Biosystems), and interpretation of the sequence data (approximately 10000 base pairs sequenced) revealed no alteration of the original T7 sequence. Thus, the overall error rate of label incorporation using primer-extension by exonuclease-deficient Klenow polymerase did not exceed a maximum of 10^{-4}.

The complete fluorescent labeling of the pyrimidines of one strand of dsDNA with dyes of the rhodamine type seems to

  1433-7851/01/4008-1428 $ 17.50+.50/0

induce a drastic conformational transition: Comparison of the circular dichroism spectra of the natural 2.7 kb fragment with either TMR-dUTP-modified, or TMR-dUTP- and R110-dCTP-modified DNA showed curves that represent curves that have the opposite sign to the spectrum exhibited by the natural DNA (Figure 3). This fact might indicate that the double helix, upon high-density incorporation of rhodamine dyes, undergoes a transition from the right-handed B form (natural DNA) to the left-handed Z form (labeled DNA). Inversion of the circular dichroism spectrum had originally been observed with the detection of Z-form DNA.[22]

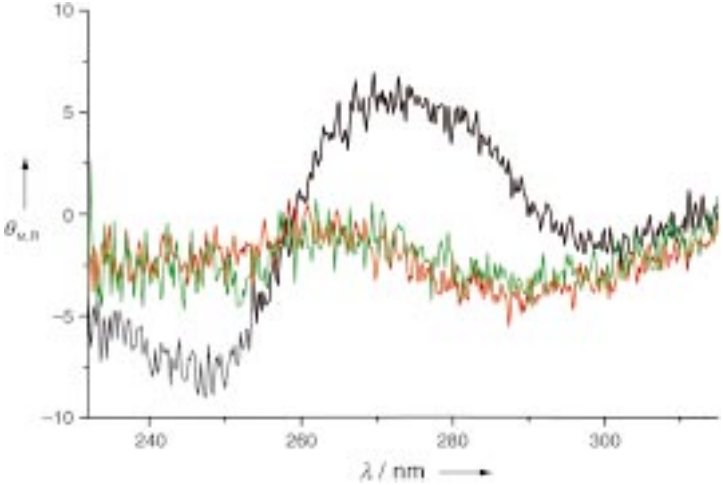

Figure 3. Comparison of the circular dichroism spectra of natural and fluorescently labeled DNA ($\theta_{M,R}$ is in deg cm^2 dmol^{-1} and is the ellipticity per mole and residue). Black: nonlabeled DNA; red: DNA labeled with TMR-dUTP; green: DNA labeled with TMR-dUTP and R110-dCTP.

Although it has not yet been proven whether the sequence of single, completely labeled DNA molecules can be resolved using successive degradation by an exonuclease, preliminary results suggest that double-labeled DNA serves as a template, for example, for exonuclease III of *Escherichia coli* (unpublished results). Regarding single-molecule sequencing, it can be concluded that distinct fluorescent tagging at each base pair is sufficient if both complementary strands of dsDNA can be labeled crosswise and degraded with time-resolved detection (two-color concept):[14] As in conventional, automated sequencing, both strands of a certain fragment could be copied into dye-labeled DNA using a set of two flanking primers and separate primer-extension reactions. This procedure does not require labeling of the second strand to start from the first labeled strand. Using both dye-labeled daughter strands in separate degradation experiments, the sequence of basepairs could, in principle, be determined provided that the exonucleolytic degradation is both highly processive and reproducible. In conclusion, the double-labeling of the A:T base pair with tetramethylrhodamine, and the tagging of G:C with rhodamine-110 might present a first solution for the complete labeling of DNA for single-molecule sequencing.

Experimental Section

A solution of dsDNA template (2.5 pmol; 2.7 kb; gene 1 fragment of bacteriophage T7 encoding T7 RNA polymerase[23]) and primer 5′-GGC GTT AGT GAT GGT GAT GGT GAT GCG CGA ACG CGA AGT-CCG ACT CTA AG-3′ (10 pmol) in 50 mM tris-HCl (80 μL, pH 7.5) was heated to 100 °C for 5 min and then cooled to 25 °C within 30 min (annealing). Primer extension and double-labeling was achieved by addition of dATP, dGTP, TMR-dUTP, and R110-dCTP (5 nmol each), as well as Klenow Exo$^-$ polymerase (50 U, New England Biolabs) in a buffer (420 μL) containing 10 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 7.5 mM dithiothreitol, and 10% DMSO. After 1 h incubation at 37 °C the reaction was stopped with 0.5 M EDTA (20 μL), and the product was purified using agarose gel electrophoresis (1.2% agarose). Labeled DNA was recovered by electroelution and ethanol precipitation, and was routinely dissolved in aqueous solutions containing 10% DMSO.

Received: November 10, 2000 [Z16071]

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