Real-time Detection of Nucleotide Incorporation During Complementary DNA Strand Synthesis

Alexander Krieg, Stephan Laib, Thomas Ruckstuhl, and Stefan Seeger*[a]

Real-time observation of DNA strand synthesis by using a supercritical angle fluorescence detection apparatus for surface-selective fluorescence detection is described. DNA template molecules were immobilized on a glass surface and the synthesis of the complementary strand was observed after addition of enzyme, dTTP, dATP, dGTP, and fluorescently labeled dCTP (d, deoxy; TP, triphosphate; T, A, G, and C, nucleobases). The fluorescence increase during the Klenow-fragment-catalyzed polymerization depends on the number of labeled dCTP nucleotides incorporated. The efficiency of this reaction is of the same order of magnitude as that of a bimolecular hybridization reaction.

KEYWORDS:

biosensors · DNA labeling · fluorescent probes · polymerase · sequencing

Introduction

The synthesis of complementary DNA strands in vitro is a central step in all existing DNA-sequencing procedures.[1–3] A new DNA-sequencing concept based on real-time single-molecule detection of nucleotide incorporation has recently been described.[4] In this technique, a template molecule is fixed at a surface, and DNA polymerase catalysis is used for specific incorporation of fluorescently labeled nucleotides. These nucleotides are detected selectively during the incorporation processes. Nucleotides that have already been incorporated into molecules are inactivated by bleaching or by other photochemical processes. However, base-specific, real-time observation of nucleotide incorporation has not yet been described because of the high concentrations of labeled nucleotides required in all fluorescence-based methods, which result in strong background signals. This does not allow specific and selective detection of the incorporated nucleotides. In contrast, DNA synthesis monitoring by surface plasmon resonance enables the synthesis of the complementary strand to be detected but lacks the potential to observe base-specific nucleotide incorporation.[5–7]

Here we report the real-time monitoring of DNA strand synthesis of an ensemble of surface-fixed single-stranded DNA (ssDNA) species[8] by use of the supercritical angle fluorescence (SAF) detection technique.[9–11] In order to prevent signal perturbation due to a high concentration of fluorescent dyes in solution, the detection volume has to be restricted to the coverslip/analyte interface as far as possible. In the detection of SAF, only molecules with a surface distance well below 100 nm contribute to the fluorescence signal.

In brief, the coverslip/analyte interface is illuminated orthogonally through the glass with a focused beam from a HeNe laser (Figure 1). To detect surface binding (incorporation) in the presence of high concentrations of fluorescently labeled nucleotides in solution, it is necessary to confine the detection region strictly to the surface. This is accomplished by collecting the fluorescence emitted into the glass above the critical angle of refraction ($\alpha_c = 61^\circ$). Only molecules at a distance of less than 100 nm from the surface efficiently emit fluorescence above 61°, which minimizes background from bulk fluorescence. The SAF emission is made parallel by a parabolic glass element and the fluorescence photons are counted with a single-photon

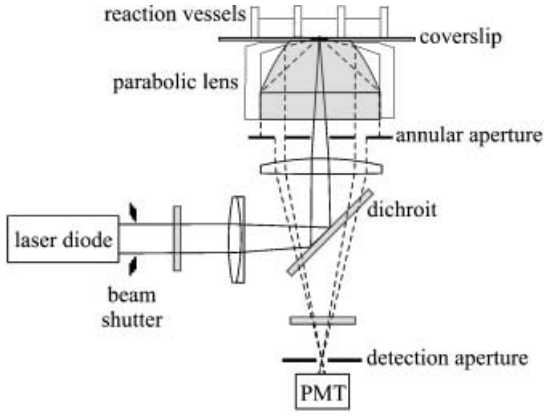

Figure 1. *Setup of the supercritical angle fluorescence (SAF) detection system. PMT, photomultiplier tube.*

[a] *Prof. Dr. S. Seeger, A. Krieg, S. Laib, Dr. T. Ruckstuhl*
Physikalisch-Chemisches Institut
Universität Zürich
Winterthurerstrasse 190
8057 Zürich (Switzerland)
Fax: (+41) 1-6356813
E-mail: sseeger@pci.unizh.ch

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counting photomultiplier tube. As we describe herein, this setup allows the direct observation of incorporation of deoxynucleotide triphosphates (dNTPs). This approach is also a method for direct real-time observation of polymerase activity.[12–14]

Results and Discussion

In the experiment described below, we observed the detection of Cy5-labeled dCTP incorporation in the complementary DNA strand. Strands with one or three guanine bases were chosen, together with a reference containing no guanine bases in the strand (for sequences, see Table 1).

The guanine bases used for the incorporation of three dyes were separated from each other by nine thymine bases to reduce steric hindrance of the polymerase by the dye chromophores attached to the base units and to prevent quenching of the dyes. In addition, in order to avoid surface effects, such as repulsion forces between enzyme and surface, we introduced a C-6 spacer (approximately 0.6 nm, for chemical composition, see Table 1) between the DNA strand and the end-labeled amino group necessary for the coupling and the DNA-strand synthesis. To increase the distance from the surface further, the last Cy5-dCTP incorporation chosen took place eleven bases before the end of the sequence. In addition, to minimize effects due to the surface environment, the primer elongation started at the free unbound end of the strand.

Investigations of different polymerase enzymes have been performed elsewhere.[15, 16] In our examination, the highest incorporation efficiency was shown with the Klenow exonuclease-free fragment and the Cy5-dCTP dye (data not shown); this enzyme/labeled dNTP combination was therefore used for the experiment described herein. The dNTP mix was added to the surface-bound ssDNA with unlabeled dATP, dGTP, dTTP, and labeled dCTP. The enzyme was added to the reaction mixture by pipette, and one data point was measured every 90 seconds.

We did not observe any increase in fluorescence during complementary DNA strand synthesis with Seq0dCTP-ssDNA (Figure 2). However, we did observe increases in fluorescence with incorporation in the cases of Seq1dCTP and Seq3dCTP. The average increase in fluorescence for the incorporation of one Cy5-labeled dCTP unit is about 59000 counts, and for three labeled dCTPs about 250000 counts, once the reaction is complete. Figure 3 shows the dependence of the fluorescence signal on the number of incorporated Cy5-dCTP units, which is linear within the range of experimental accuracy.

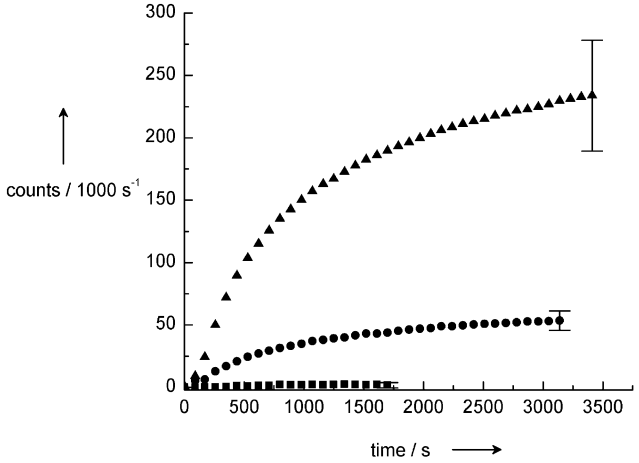

Figure 2. *Fluorescence signal against time during DNA strand synthesis. The background signal was subtracted from the data and the start time was set to zero. Triangles: Seq3dCTP; circles: Seq1dCTP; squares: Seq0dCTP.*

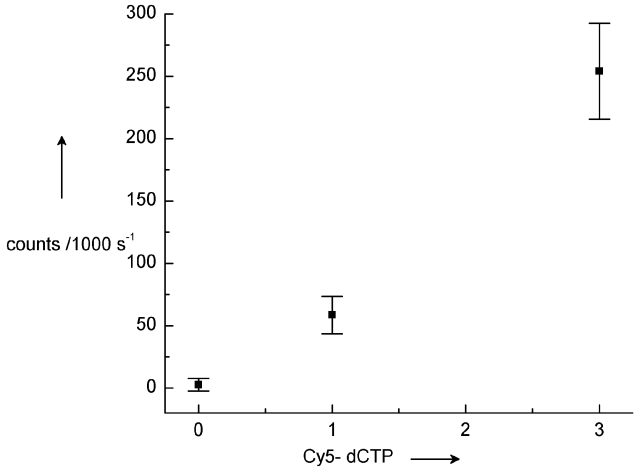

Figure 3. *Fluorescence signal against number of incorporated Cy5-labeled dCTP units (signal intensity at endpoint).*

The hybridization of Seq1dCTP-ssDNA with a corresponding Cy5-labeled primer was also carried out and monitored by use of the same setup.[17] By comparing the result of the polymerase-induced strand synthesis with the data for the hybridization reaction, both of which result in single-dye-labeled double-stranded DNA, the synthesis efficiency in toto (that is, how many strands are labeled overall) was estimated.

Table 1. *Sequences and chemical structures of the oligonucleotides and linker used.*

Name	Sequence (5′ – 3′)
Seq0dCTP	TTT TTT TTT TTT TTT TTT TTT TTT TTT TTA TCA TCT CTT ATT ACC TCT AA
Seq1dCTP	TTT TTT TTT TTT TTT TTT TTT TTT TGT TTA TCA TCT CTT ATT ACC TCT AA
Seq3dCTP	TTT TTT TTT TGT TTT TTT TTG TTT TTT TTT GTT TAT CAT CTC TTA TTA CCT CTA A
PrimerSeqdCTP, PrimerSeqdCTP-Cy5	TTA GAG GTA ATA AGA GAT GAT
Primer-Mismatch-Cy5	ATT GCG TCG CTT TTT GCT GTC C
Chemical composition of the linker	-O-P(O_2)-O-(CH_2)$_6$-NH_2

Figure 4 shows the kinetic data for the hybridization reaction and the strand synthesis. The concentrations were 10^{-7} M Cy5-monolabeled primer and Cy5-dCTP, respectively. The annealing of a noncorresponding primer sequence could not be detected (circle base line).

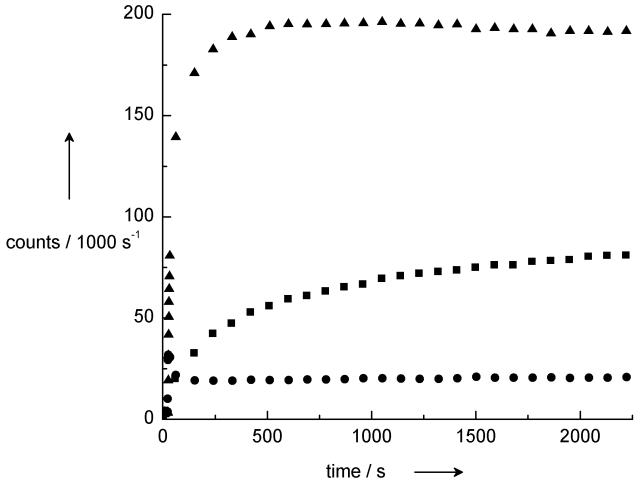

Figure 4. *Fluorescence signals of the hybridization reaction (triangles) and DNA strand synthesis (squares); circles: mismatching primer control (primer-mismatch-Cy5). The start time was set to zero.*

The fluorescence increase due to the single incorporation event is of the same order of magnitude as that due to the annealing reaction. The difference between the fluorescence signals (a factor of about three) is probably due to the lower efficiency of the polymerization process in relation to the bimolecular hybridization reaction. The results also show that the strand synthesis reaction is slower than the annealing process.

Conclusion

The obtained data clearly show the detection of the incorporation of dye-labeled dNTPs into the strand in real time and hence the potential to detect and compare the incorporation efficiencies of labeled dNTPs and different polymerases. This work is an important step towards direct sequencing by polymerase-catalyzed strand synthesis with fluorescently tagged nucleotides. To achieve this goal, the next step should be to focus on one single DNA strand to detect nucleotide incorporation at this level.

The variation in photon counts detected in different experiments is due to changing of the slides and probably has optical causes. However, this problem has no relevance for observation of polymerase activity at the single-molecule level; that is, for DNA sequencing, which is also carried out at the single-molecule level.

Investigation into highly efficient polymerases and dyes that allow complete strand synthesis is necessary to establish a single-molecule DNA-sequencing technique based on monitoring of the incorporation of labeled dNTPs, in contrast to the digestion method that uses exonuclease.[18–20]

Methods

Annealing: Annealing of ssDNA with the corresponding primer (ssDNA and primers were purchased from Microsynth, Switzerland; for sequences, see Table 1) was performed by addition of ssDNA (1 μL, 10^{-4} M) and primer solution (4 μL, 10^{-4} M) to hybridization buffer (15 μL, Roche, Germany, hybridization buffer for PCR/DIG ELISA), and the annealing took place over 2 h (hybridization solution).

Fixation of aminated ssDNA to glass slides: Glass slides (Menzel–Glaser, Germany) were used. They were cleaned in an ultrasonic bath with ethanol solution (70%, 30 min) followed by further cleaning with an NaOH/ethanol solution (25 g NaOH dissolved in 250 mL 60% ethanol solution; 2 h) and by washing three times with distilled water. Slides were then coated by treatment with a poly-L-lysine solution (0.01% w/v, Sigma Diagnostic, USA) in phosphate-buffered saline (PBS buffer, pH 7.48, Fluka, Switzerland) for 1 h, followed by drying (1 h at 45 °C).

The poly-L-lysine-coated glass slides were treated with a solution of glutaraldehyde (20 mL, 2.5%, Fluka, Switzerland) in Na_2HPO_4 buffer (0.05 M, adjusted to pH 7.0) for at least 1 h. After exhaustive washing with distilled water, the hybridization solutions, dissolved in Na_2HPO_4 buffer (0.05 M, pH 7.0, Fluka, Switzerland), were added and the slides were left for 12 h. After incubation, the glass slides were washed once with sodium dodecylsulfate (0.1%) and twice with distilled water, and were then incubated for 5 min with sodium borohydride (50 mg $NaBH_4$, Fluka, Switzerland) solution in PBS (15 mL)/ethanol (5 mL, 100%) and rinsed with water.[21]

After this step, the glass slides were glued to a mask with wells and measurements were made on the SAF detection system. Cy5-dCTP (3.3×10^{-7} M, Amersham Biosciences Pa55021), dATP, dGTP, and dTTP (3.3×10^{-6} M, MBI Fermentas, Germany) were dissolved in Klenow reaction buffer 10× (150 μL, 0.5 M tris(hydroxymethyl)aminomethane-HCl, pH 7.5, 0.1 M $MgCl_2$, 0.1 mM dithiothreitol, 0.5 mg mL^{-1} bovine serum albumin). To start the reaction, one unit (unit defined by supplier) of exonuclease-free Klenow fragment (Amersham Biosciences 70057Y) was dissolved in Klenow reaction buffer (10 μL) and the solution was added to the mixture in the well.

Hybridization: Fixation of the Seq1dCTP ssDNA to the surface and preparation of the slide was performed as described above. Hybridization buffer (10 μL, Roche, hybridization buffer for PCR/DIG ELISA) was added to the well and the measurement was started. Cy5-labeled hybridization probe (10^{-7} M, 150 μL, Microsynth, Switzerland; for sequences, see Table 1) dissolved in hybridization buffer was then added.

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