Reversible, Meniscus-Free Molecular Combing of Long-Chain DNA

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We introduce a method for reversibly orienting long-chain DNA on solid hydrophilic substrates without a fluid meniscus. End-tethered λ-DNA mushrooms are elongated by a hydrodynamic flow in the presence of trivalent cations, resulting in electrostatic adsorption of the extended DNA to the surface. By complexation of the cations the part of the DNA which is unspecifically bound to the surface desorbs quantitatively, and the mushroom conformation is restored. With the use of multiple deposition−combing steps, combined with a final desorption step, tethering densities higher than attainable with single deposition steps can be obtained.

Introduction

With the use of the stretching force exerted by the flow in a receding fluid meniscus, long-chain DNA can be deposited with high orientation on a solid substrate.[1] This technique, called "molecular combing" by their inventors, has proven useful for the identification of restriction sites in genomic DNA, as immobilized restriction products can be measured and quantified with fluorescence microscopy.[2] The original method of combing DNA to hydrophobic polymer substrates appears to be initiated by selective attachment of the hydrophobic, single-stranded ends of the DNA molecule to the substrate via van der Waals attraction,[3] allowing the fluid flow in the meniscus to apply forces large enough to overstretch DNA. After the meniscus has receded, the DNA chain adheres to the surface, possibly by capillary and van der Waals forces, strongly enough that DNA cannot be released by rehydrating the sample. Furthermore, the strong affinity of both exposed single-stranded ends to the surface may lead to the formation of U-shaped conformations. Methods for reversible combing of DNA in a meniscus have been developed, using the lipid CTAB to induce electrostatic attachment.[4] In some applications, however, the drying of DNA in a meniscus is not wanted.

In this publication we present a method for reversible combing of end-tethered DNA onto hydrophilic surfaces without the need of a meniscus. This method, which we call "soft combing", is based on the condensation of DNA in the presence of trivalent cations.[5,6] By stretching end-tethered DNA in a flow field, the trivalent cations in the solvent induce DNA to adsorb to the substrate rather than to condense into the compact tori observed in condensation in bulk solution. By complexation of the cations by poly(acrylic acid)[7] DNA is desorbed quantitatively from the surface with intact tethering, which renders the combing reversible. We demonstrate that by using multiple tethering−elongation steps followed by complexation-induced desorption, tethering density can be significantly enhanced (see Figure 1).

Experimental Section

Sample Preparation. Plasmid λ-DNA (48 502 base pairs, New England Biolabs) was linearized by heating in TRIS−EDTA (TE) buffer at pH 8.0 to 75 °C for 15 min and hybridized at the 3′-end with oligomers labeled with four biotin functionalities (IBA, Göttingen). Functionalized λ-DNA was purified by precipitation with PEG (molecular weight 8000 g/mol). For fluorescence microscopy, DNA was labeled with YOYO-1 (Molecular Probes) at 50 °C for 1 h with a density of one YOYO-1 per four base pairs. Borosilicate glass coverslips were cleaned by a 1:1 v/v mixture of H_2SO_4 and H_2O_2, silanized with aminopropyl-triethoxysilane (Sigma), and coated with 8% glutardialdehyde (Polyscience) and streptavidin (Roche) in saline phosphate buffer (PBS) at pH 7 at a concentration of 100 μg/mL. The slides were then incubated with bovine serum albumin (BSA; New England Biolabs) at a concentration of 100 μg/mL in PBS at pH 7. This step suppresses irreversible, nonspecific adsorption of DNA to the surface, while it leaves the streptavidin binding sites accessible. Drops of biotin-labeled DNA at a concentration of 2 ng/μL in a TRIS−borate−EDTA (TBE) buffer with 0.5 M NaCl, pH 9 were incubated on the streptavidin-coated slides for 20 min. Unbound DNA was removed by thorough, gentle washing with TBE and TE buffer.

^{32}P-labeled λ-DNA was prepared by transfer of the 5′-γ^{32}P from labeled ATP (Hartmann Analytic) to the hydroxylated 5′-end of the biotin-labeled λ-DNA using T4 kinase (Roche), following the protocol from the supplier. T4 kinase and remaining ATP were removed by centrifugation through a Sephadex G25 column (Amersham). The DNA was then diluted in TBE and 0.5 M NaCl.

Combing Procedure. Tethered DNA molecules were combed in the flow induced by adding a drop of buffer containing the trivalent cations spermidine and La^{3+} with a pipet. For the spermidine combing we used a solution of 20 mM spermidine (Sigma) and 25 mM NaCl in TE buffer. For the La^{3+} combing we used solutions of $LaCl_3$ (Sigma) in water at concentrations between 0.01 and 4 mM.

For the desorption experiments we exchanged the cation-containing buffers with 10 mM poly(acrylic acid) (Sigma, molecular weight 135 000 g/mol) in 0.5× TE at pH 9.

Sample Characterization. The fluorescence from YOYO-1 bound to DNA was imaged with a confocal microscope equipped with a 100× oil immersion objective with numerical aperture 1.45, Nipkow

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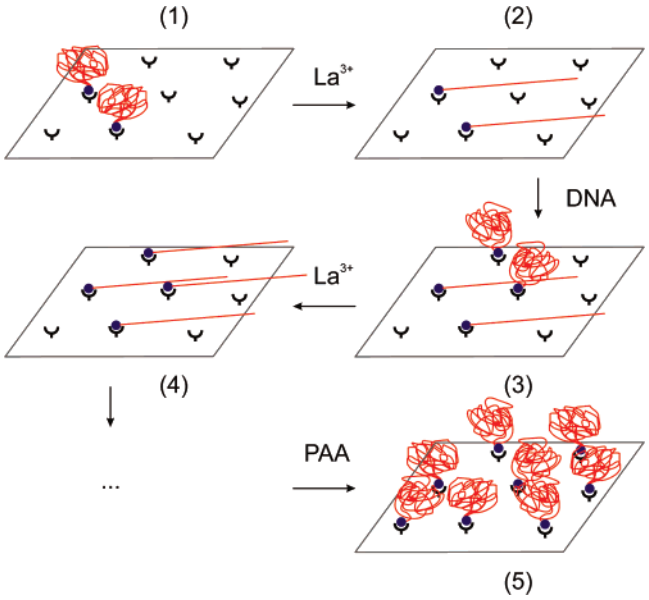

Figure 1. Schematic diagram of soft combing: DNA end-tethered to a solid substrate (1) is flushed with a buffer containing La^{3+}, resulting in an array of combed DNA attached to the surface by electrostatic attraction. Incubation with end-labeled DNA leads to arrays of DNA end-tethered at the interstitials between the combed molecules (3). These may be deposited by renewed flushing with La^{3+} (4). Removal of the trivalent cations by poly(acrylic acid) (PAA) releases the unspecifically bound parts of the DNA chain and results in an array of end-tethered DNA at enhanced density (5).

disk, and a cooled CCD camera (Hamamatsu Orca II). By recording two-dimensional intensity distributions at different heights above the surface we determined the location of the substrate surface to within 650 nm. The frame located closest to the surface was used to measure the tethering density by counting bright spots (representing individual coiled DNA molecules) or lines (representing combed DNA).

The concentration of ^{32}P-labeled λ-DNA was measured with a liquid scintillation counter (LS 6500, Beckman) by transferring the DNA-covered coverslips into the scintillation liquid (Aquassure (New England Nuclear) in xylene). Calibration curves were measured on nontethered, biotin-labeled λ-DNA diluted into scintillation liquid at amounts between 2.8 and 280 amol. The detection limit was about 0.3 amol. DNA quantities from tethered samples on coverslips were above 6 amol.

Results and Discussion

λ-DNA which is end-tethered by biotin to streptavidin-coated substrates forms, after thorough washing, low-density arrays in which the molecules are in a mushroom conformation, with a radius of gyration $R_g = 0.77\,\mu m$[8] (see Figure 2a). Typical tethering densities obtained by counting DNA coils are $\sigma \approx 0.08\ \mu m^{-2}$ for an incubation time of 20 min. Although this density is considerably lower than the estimate for close-packing, $\sigma^* = (\pi R_g^2)^{-1} = 0.54\,\mu m^{-2}$ of coiled λ-DNA labeled with 0.2 YOYO-1/base pair and $L = 19.8\ \mu m$,[8] it is low enough that individual tethered DNA coils can be resolved.

When a solution of spermidine or La^{3+} is added to such arrays with a pipet, the DNA molecules are elongated by the flow and highly oriented. Confocal fluorescence microscopy shows that the elongated molecules are immobilized onto the substrate within less than 500 ms (see Figure 2b).

While the orientation is due to the viscous drag exerted onto the molecules by the fluid flow, the immobilization arises from the electrostatic attraction between the negatively charged DNA and the BSA/streptavidin surface induced by the trivalent cations. This behavior is quite in contrast to the effect of trivalent cations in bulk solutions which leads to condensed toroidal DNA aggregates.[5] Confocal fluorescence microscopy (CFM) on DNA mushroom arrays combed with spermidine show, in addition to the straight segments, globular structures which are mostly in close proximity of straight segments (see Figure 2b). These structures are likely to be DNA segments condensed during the hydrodynamic elongation of the molecule, but before adsorption to the surface. Mushroom arrays combed with La^{3+} showed significantly less such globular structures. For relatively high-density mushroom arrays such as in Figure 2 one can see strong variations in brightness across the image (see Figure 2b) which are due to bundling of neighboring end-tethered DNA chains. We found that after removal of the trivalent cations by washing with TBE the DNA molecules remain immobilized for at least 24 h if stored at 4 °C.

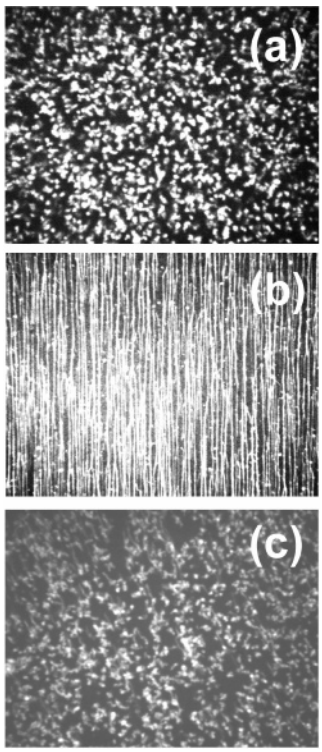

Figure 2. Confocal fluorescence micrographs of YOYO-1-labeled λ-DNA end-tethered to a streptavidin-coated glass slide before (a) and after addition of a solution of 20 mM spermidine (b). Individual bright spots in (a) are single end-tethered λ-DNA molecules. After incubation with poly(acrylic acid) (PAA) for 15 min the DNA desorbs from the surface (c). Brightness variations in (b) are due to the formation of bundles. The reduced fluorescence intensity in (c) is due to photobleaching of the YOYO-1; the slightly reduced number of DNA molecules in (c) is due to the PAA treatment. Field of view: 90 μm $\times$ 70 μm.

Removing the cations by washing the sample with a solution of poly(acrylic acid) (PAA) leads to rapid desorption of the DNA molecules from the surface (see Figure 2c), while it leaves the tethering largely intact. Upon addition of PAA we consistently observe a slight reduction of the tethering density by typically about 10% whose origin is not clear. Desorption was found to be complete within typically 15 min. Subsequent washing with TBE did not decrease the number of visible DNA molecules, showing that desorbed DNA is still attached to the surface.

In order to investigate whether the desorption with PAA restores end-tethering, we have subjected a combed and desorbed DNA

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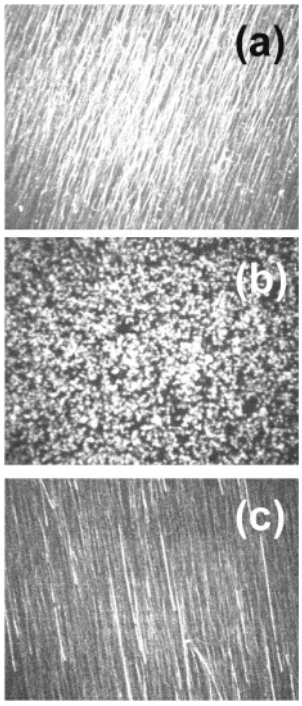

Figure 3. (a) Confocal fluorescence micrographs of end-tethered λ-DNA combed with 4 mM $LaCl_3$; (b) is the same field of view as in (a) after complexation of the La^{3+} ions with poly(acrylic acid) (PAA). Panel (c) shows the sample after a second adsorption−combing step, with the flow direction making an angle to the one used to produce the combed structure (a). The absence of kinked DNA chains in (c) shows that the desorption with PAA restores end-tethering in (b). The reduced fluorescence intensity in (c) is due to photobleaching of the YOYO-1; the slightly reduced number of DNA molecules in (c) is due to the PAA treatment. Field of view: 90 μm × 70 μm.

carpet to a second elongation−adsorption step, at an angle with the first combing direction. Incompletely detached DNA molecules would then appear as kinks or loops. We find that the resulting structure contains only straight and nearly straight molecules (see Figure 3), which shows that the PAA-induced desorption quantitatively restores end-tethering.

Due to its large aspect ratio ($L/d \approx 9900$ with diameter $d =$ 2 nm), YOYO-1-labeled λ-DNA tethered to the surface with a density σ occupies an area fraction $\varphi_{comb} = Ld\sigma$ in the combed state which is much smaller than the area fraction $\varphi_{coil} = \pi R_g^2\sigma$ in the coiled state. Combed DNA arrays should thus provide ample space for further end-tethering of DNA coils, and repeated elongation−adsorption steps should allow one to increase the tethering density to a value $\sigma^{**} = (Ld)^{-1} \approx 25\ \mu m^{-2}$, much larger than the close-packing density $\sigma^* = (\pi R_g^2)^{-1} \approx 0.54\ \mu m^{-2}$ of coiled molecules.

Indeed, after removal of the spermidine by washing with TBE, biotin-labeled DNA is found to attach to the interstitials between the combed molecules (see Figure 4). By counting the end-tethered DNA molecules before each combing step, we find that the incremental amount of end-tethered DNA of $(0.13 \pm 0.01)\ \mu m^{-2}$ is nearly constant from one step to the other for three deposition−combing steps, indicating that the interaction between the DNA adsorbed to the surface and the end-tethered DNA mushrooms is negligible.

Already after two deposition−combing steps the final surface density of DNA after PAA treatment becomes so high that individual molecules can no longer be discriminated with CFM, in the combed as well as in the mushroom state. Nevertheless, by addition of the density increments measured after each deposition step we estimate a density of about $(0.39 \pm 0.03)\ \mu m^{-2}$ after three deposition−combing steps.

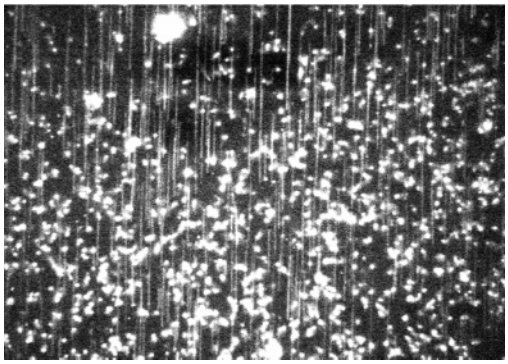

Figure 4. DNA array formed by deposition of biotin-labeled λ-DNA (bright spots) to a streptavidin surface covered with end-tethered λ-DNA which had been combed with 2 M La^{3+} (bright lines). Field of view: 90 μm × 70 μm.

The tethering density of ^{32}P-labeled λ-DNA measured after a single deposition step is $\sigma = (0.05 \pm 0.02)\ \mu m^{-2}$, considerably smaller than the value $\sigma \approx 0.13\ \mu m^{-2}$ obtained from counting molecules in CFM images. We attribute this to partial condensation of biotin-labeled DNA before deposition due to the presence of spermidine in the kinase buffer used for ^{32}P labeling. In the following, we will thus correct the densities measured by ^{32}P scintillation for the loss incurred during preparation by the factor $\sigma_{CFM}/\sigma^{32P} = 0.13/0.05 = 2.6$. Two subsequent steps consisting of deposition and washing with TBE, but without combing, roughly doubles the tethering density to a value $\sigma = (0.26 \pm 0.03)\ \mu m^{-2}$. This shows that after the first deposition step the biotin binding capacity of the streptavidin is still intact and that the coils do not significantly overlap. This value agrees well with the CFM result. DNA attached to the surface by two subsequent deposition steps of 20 min, each followed by removal of nonbound DNA and combing by La^{3+} results in a tethering density $\sigma = (0.55 \pm 0.05)\ \mu m^{-2}$. This value is about twice as high as would be expected from the constant incremental density $(0.13 \pm 0.01)\ \mu m^{-2}$ measured with CFM. We attribute the high value of σ to unspecifically bound DNA molecules which are not removed by washing, indicating a progressive degradation of the binding capacity of the substrate which may be due to the removal of BSA during successive combing−release steps.

Increasing the incubation time to several hours resulted in DNA carpets too dense to be quantified by direct counting. However, the relative increase of tethering density after one deposition−combing step can be estimated from the average image gray levels. By this method, we obtained an increase of the tethering density by about $(9 \pm 3)\%$ after one deposition−combing step with La^{3+} and subsequent washing with PAA and TBE. However, scintillation measurements using ^{32}P-labeled DNA on dense DNA arrays showed very large variations from sample to sample making it impossible to discern a quantitative increase of tethering density with increasing number of deposition−combing steps. The reason for these large variations is unclear.

Conclusions

Our results show that the combination of hydrodynamic extension of end-tethered DNA with the electrostatic attraction between substrate and DNA molecules induced by trivalent cations can be used to deposit long-chain DNA with controlled orientation onto a solid substrate with moderate stretching force. This method, which we term "soft combing", does, in contrast

to conventional molecular combing, not require a receding meniscus (entailing large forces and drying of the DNA) and allows us to use hydrophilic substrates such as glass. Our results show that with multiple deposition−combing steps, combined with a final desorption step, tethering densities can be achieved which are higher than those obtained in single deposition steps. Although in the present study the final tethering densities achieved with multiple deposition−combing steps are only slightly larger than the overlap density of coils, $\sigma^* = 0.54\ \mu m^{-2}$ (possibly due to unbinding of end-tethered DNA induced by the complexing agent PAA and due to degradation of the DNA binding capacity of the substrate), we expect that with optimized complexation agents for desorption and better-defined flow geometries for deposition and combing, such as stagnation flow in a closed sample cell, high-density DNA arrays near the theoretical limit which for λ-DNA is $\sigma^{**} \approx 25\ \mu m^{-2}$ can be obtained with the method presented here. Our results could bear some relevance for the preparation of monodisperse brushes of long-chain polyelectrolytes, e.g., for biosensor applications.

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