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Three-Dimensional Surface Patterning by DNA-Modifying Enzymes

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ABSTRACT Self-assembled patterned multilayers may be fabricated using DNA monolayers and the orchestrated reactions of DNA-modifying enzymes. To demonstrate this approach, DNA monolayers were formed on silicon and cleaved quantitatively with a restriction enzyme. Subsequently, fluorescently labeled nucleotides were covalently incorporated to the cleaved DNA. Nucleotide addition was shown to be highly selective according to the sequence at the cleavage site, and no nonspecific adsorption to the surface was observed. The dual action of the DNA-modifying enzymes was quantitative and could be utilized in the fabrication of multilayered structures. Other DNA-modifying enzymes can be exploited in this manner to enrich the repertoire of self-assembly supramolecular structure fabrication.

KEYWORDS: DNA monolayer • surface patterning • multilayers • supramolecular structures • DNA-modifying enzymes

INTRODUCTION

The fabrication of molecular-based nanodevices is expanding into three dimensions in order to extend their complexity and versatility (1−3). As with two-dimensional patterning, it is a great challenge to form predesigned self-assembled multilayers toward the construction of supramolecular architectures. The incorporation of biomolecules in nanodevices has become a widespread phenomenon based on their self-assembly capabilities (4−6), and their potential to form complex three-dimensional structures has already been demonstrated (7−9). These capabilities have been exploited by utilizing DNA as a structural scaffold for the formation of RNA and protein arrays (10−15) in addition to patterning of inorganic particles (16, 17).

The attachment of DNA and proteins to surfaces has been studied extensively in recent years because of the prevailing role of DNA and protein microarrays in biological and medicinal research (18, 19). In these systems, the DNA and proteins serve as sensors to reveal the presence of analytes through their specific interactions. A few studies demonstrated the use of DNA and proteins as building blocks in electronic devices (20−22). However, the potential to utilize biological molecules as a chemical toolbox to pattern surfaces has only begun to be realized. For example, a restriction enzyme has been shown to cleave its DNA substrate in a sequence-specific manner when the latter was immobilized to the surface (23), while adsorbing such an enzyme on an atomic force microscopy (AFM) tip retained its activity, allowing it to form a pattern within a DNA monolayer (24). DNA adsorbed on a surface or on gold nanoparticles (25−27) has been shown to act as a template for the synthesis and amplification of single-stranded DNA in the form of rolling circle replication by a DNA polymerase. Finally, DNA brushes composed of 2000-base-pair (bp)-long DNA fragments have been self-assembled on surfaces and shown to support gene expression by the dual action of an RNA polymerase and the protein translation machinery (28).

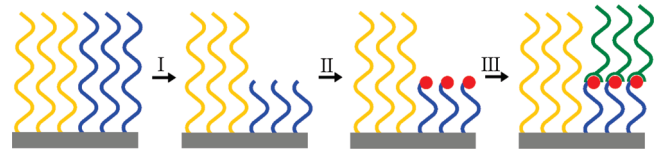

FIGURE 1. New approach toward multilayer biopatterning. A surface is patterned with two types of DNA, one containing (blue) and the other lacking (yellow) a recognition site for a restriction enzyme. A restriction enzyme cleaves only one of the DNAs (step I, blue), leaving the other intact (step I, yellow). A DNA polymerase incorporates modified nucleotides (red circles) only at the end of the cleaved DNA (step II). These modified nucleotides serve as docking sites for additional DNA molecules (step III, green) or other organic and inorganic materials in subsequent steps.

Here we demonstrate the consecutive action of two DNA-modifying enzymes, a restriction enzyme and a DNA polymerase, to form patterns within a DNA monolayer that could be sequence-specifically extended to multilayered structures by the selective addition of modified nucleotides. This tool can also be used to quantitatively characterize label- free DNA monolayers postadsorption. Figure 1 depicts this new approach toward biopatterning that can yield multilayers of biomolecules and biofunctionalized inorganic particles produced with full control and characterization capabilities. Subsequent to DNA monolayer formation, a restriction enzyme cleaves the DNA in a single position along the DNA, according to the position of its recognition sequence (Figure 1, step I). The selectivity of this reaction allows DNA fragments with no recognition sequence to remain intact on the surface. These intact DNA fragments may contain other

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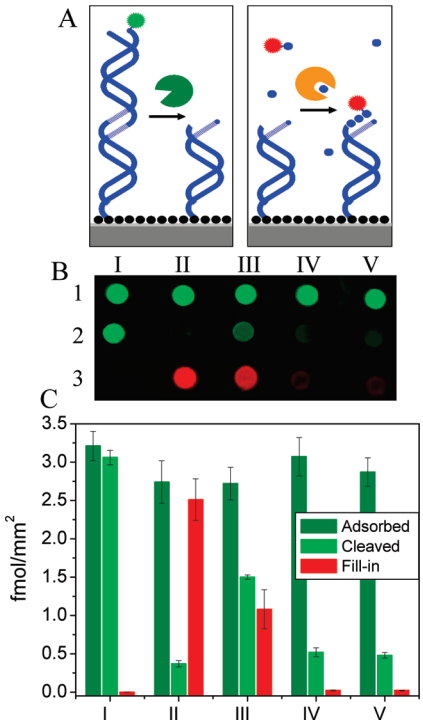

FIGURE 2. Imaging and quantifying enzymatic modifications of DNA immobilized on a surface. (A) Depiction of a restriction enzyme (dark green) cleaving the DNA only at the recognition sequence (light blue), leaving a short DNA with no fluorescence label (light green). A DNA polymerase (orange) fills in the DNA gap at the cleavage site with natural (blue) and fluorescently modified nucleotides (red). (B) Fluorescently labeled DNA on a glass surface before cleavage (green) and after the fill-in reaction (red). Row 1: Fluorescently labeled DNA before cleavage. Row 2: Addition of no enzyme (I) and the restriction enzyme *Bam*HI at high (II, IV, and V) and low (III) concentrations. Row 3: Addition of a DNA polymerase I Klenow fragment (II, III and V) with fluorescently labeled dCTP and natural dATP, dTTP, and dGTP (II–IV). (C) Quantification of the fluorescent spots in part B.

restriction enzyme recognition sequences and thus be modified in a sequence-specific manner in later steps.

Following DNA cleavage, an additional DNA-modifying enzyme is added (Figure 1, step II), which reacts only with the DNA that had been cut by the restriction enzyme. As previously shown (29), upon the addition of a sequence-selective DNA polymerase, nucleotides can be incorporated to the end of the cleaved DNA. Using unnatural nucleotides, the DNA monolayer could be modified chemically and could react in subsequent steps to form an additional monolayer of DNA, proteins, or nanoparticles (Figure 1, step III). Besides a DNA polymerization reaction, other DNA-modifying enzymes can be utilized, such as DNA ligase, which had been previously shown to perform efficient sequence-selective covalent attachment of long DNA fragments to DNA immobilized on the surface (30), or a DNA polynucleotide kinase, which could phosphorylate the 5′ end of surface-anchored DNA (31).

EXPERIMENTAL METHODS AND MATERIALS

Silicon Slide Preparation. An oxide-coated silicon (100) substrate (600 nm, 1–100 Ω) was cut into 1 × 1.5 cm slides. The slides were cleaned with a Piranha solution [a 1:1 mixture of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2)] by dipping them for 10 min in the cleaning solution, followed by double rinsing in deionized (DI) water and blow-drying with nitrogen. Next, they were etched in 1:1 argon and oxygen plasma with a radio-frequency power of 100 W and a base pressure of 1000 mTorr for 10 min. This process results in a SiO_2 surface with an excess of oxygen molecules. In the hydroxylation process, the samples were immersed in a 6:1:1 solution of DI water, hydrochloric acid (HCl), and H_2O_2 at 85 °C for 10 min. This process leaves –OH groups on the surface. The samples were immersed in a (mercaptopropyl)trimethoxysilane (MPTMS) solution [5 mM MPTMS in 99% bicyclohexyl] for 2 h, followed by double rinsing in DI water and blow-drying with nitrogen, creating a modified surface with thiol [–SH] functional groups on top. Monolayers were characterized by contact-angle and ellipsometric measurements.

DNA Preparation. All oligonucleotides were purchased from MWG. 150bp-long DNA fragments were produced by a polymerase chain reaction (PCR) using pUC18 linear plasmid as the template. PCR fragments containing the *Bam*HI recognition sequence were prepared with the reverse primer CGAATTC-GAGCTCGGTACCCGGG and the forward primer GCGAAAGGGG-GATGTGCTGCAAG. For fragments lacking the *Bam*HI recognition sequence, the reverse and forward primers were CTA-GAGTCGACCTGCAGGCATGC and GGGCCTCTTCGCTATTACGC-CAGC. The reverse and forward primers were purchased with a 5′-thiol modifier and a FAM modification, respectively. The PCR products were purified by Wizard SV PCR purification columns (Promega).

Binding Thiolated DNA to the MPTMS-Modified Silicon. A 5′-thiolated dsDNA was reduced by a 1 h incubation with 10 mM tris(2-carboxyethyl)phosphine hydrochloride, followed by purification on a Micro Bio-Spins 6 (Bio-Rad) to remove the reducing agent and thiol-protecting group. Thiolated DNA (1 μM) was dissolved in a 50 mM sodium carbonate buffer (pH 8) and incubated with activated slides for 12 h, followed by rinsing with a 100 mM sodium phosphate buffer (pH 7.5).

DNA Restriction on the Surface. MPTMS-modified surfaces bound with DNA were placed on a board cooled to 4 °C. A total of 2 μL of a reaction solution containing 0.5 fmol/μL *Bam*HI endonuclease (Fermentas) in a suitable buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.9) were placed on top of the DNA spots. The reactions were sealed with a lid made of 50-μm-thick parafilm, with round 6-mm-diameter holes attached to a Piranha-cleaned glass. The cleavage reaction was initiated by transferring the surface to a 37 °C heating block. The reaction was stopped by returning the chamber back to the 4 °C board, followed by opening of the well and washing of the silicon-coated DNA for 15 min with a 100 mM sodium phosphate buffer (pH 7.5, 5 mM $MgCl_2$). Fluorescene on the surface was imaged using a FLA-5100 scanner (FUJI).

Nucleotide Incorporation on the Surface. The cleaved DNA-modified surface was incubated with 50 μM d(G/A/T)TPs, 0.1 μM 5-(propargylamino)-dCTP-ATTO-647N (Jena), and 0.1–1 U of DNA polymerase I (Klenow fragment, NEB) for 1–90 min in 25 °C. The reaction was stopped by intensive washing with a 100 mM sodium phosphate buffer (pH 7.5). The fluorescence from the surface was imaged using a FLA-5100 scanner (FUJI).

Surface Patterning. Selective surface patterning was performed using a modification of the magnetolithography method (32). Thiolated DNA (1 μM) containing the *Bam*HI cleavage site was incubated with 1 mg/mL, 10-nm-diameter magnetoparticles (Fe_3O_4) in a 50 mM sodium carbonate buffer (pH 8) for 1 h. The solution was added to an MPTMS-modified surface placed on a magnetic mask. After 2 h, a solution containing thiolated DNA lacking the *Bam*HI cleavage site was added to the surface, which was farther incubated for 10 h. The surface was then gently rinsed with a sodium phosphate buffer (pH 7.5).

RESULTS AND DISCUSSION

To demonstrate the applicability of the multilayer biopatterning approach, we formed DNA monolayers on a silicon surface and followed their cleavage upon the addition of a restriction enzyme. The DNA monolayers were constituted of 150bp-long double-stranded DNA (dsDNA) fragments prepared by PCR such that one of their ends was modified with a fluorescent probe and the other with a thiol modification (Figure 2A and see the Experimental Methods and Materials section). Upon adsorption on a MPTMS-modified silicone surface, disulfide bonds were formed between the thiol on the surface and the thiol at the end of the DNA. Low-resolution scanning of the fluorescent signal revealed that fairly homogeneous DNA monolayers were formed (Figure 2B), as confirmed by high-resolution AFM imaging (data not shown). The density of the adsorbed DNA was found to be 8 fmol/mm^2, as measured in a separate radio-labeled monolayer following our published protocol (31). The fluorescent signal disappeared completely when the enzyme *Bam*HI was added to the DNA monolayer (Figure 2B, row 2). The *Bam*HI cut the adsorbed DNA at its recognition site located 25bp from the surface, thereby releasing the DNA harboring the fluorescent label to the solution (Figure 2A). The decrease in the signal was observed only when *Bam*HI was added (Figure 2B; compare columns I and II) and was proportional to the amount of enzyme, as is evident by only a partial reduction in the signal observed when the DNA monolayer was exposed to a lower concentration of the enzyme for the same time (Figure 2B, column III). Hence, DNA cleavage on the surface was controllable and specific.

The cleaved DNA monolayer was susceptible to further enzymatic manipulations, by the addition of DNA polymerase I to the monolayer (see the Experimental Methods and Materials section) together with the fluorescently labeled dCTP nucleotide and the three other nonlabeled nucleotides. DNA polymerase binds to a primer/template structure with a protruding 5′ DNA end, such as that created by the former action of the restriction enzyme (depicted in Figure 2A). Once bound, DNA polymerase I catalyzes the incorporation of nucleotides to the 3′ DNA end in a template-dependent manner, resulting in a full double-stranded structure. Thus, by monitoring the fluorescence at different emission wavelengths, it is possible to follow not just the reduction in emission from the originally adsorbed DNA but also an increase in the fluorescence due to the incorporation of fluorescently labeled nucleotides at the *Bam*HI cleavage site (Figure 2B, green and red spots, respectively). Indeed, the effect was specific to the location of the originally adsorbed DNA and only at the spots that had been reacted with *Bam*HI (Figure 2; compare columns II and III to column I). The appearance of the "red" fluorescent signal was shown to specifically correlate with the incorporation of the nucleotide to the DNA by two control reactions in which either the fluorescent nucleotide was present but no DNA polymerase was added (Figure 2, column IV) or both the enzyme and fluorescent nucleotide were added but the other three nucleotides were missing (Figure 2, column V).

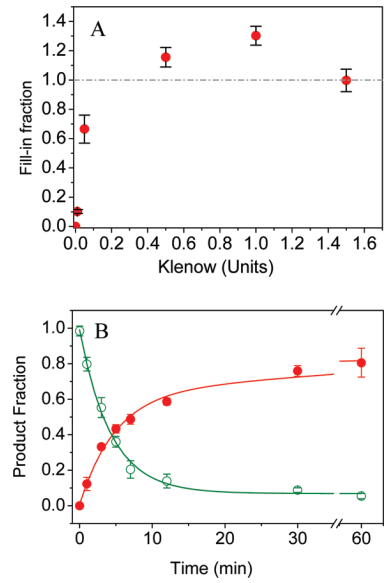

FIGURE 3. Characterization of the DNA polymerase I reaction on a DNA-modified surface. (A) Reaction efficiency as a function of the DNA polymerase I concentration. (B) Time dependence on the fluorescent nucleotide incorporation with 0.2 u/μL DNA polymerase I (red) compared to the disappearance of the original fluorescent signal after *Bam*HI cleavage (green).

In order to demonstrate that the DNA polymerase I reaction can be used quantitatively, we monitored the dependence of the DNA polymerase labeling reaction on the concentration of DNA polymerase I. As can be seen in Figure 3A, the reaction reached a saturation value as the concentration of DNA polymerase I was increased, suggesting that the entire DNA that had been cleaved by the restriction enzyme was labeled and no further reaction had taken place. Because at high concentrations a value slightly greater than unity has been obtained, which may indicate some nonspecific reactions taking place on the surface, we chose to continue working with a lower concentration of DNA polymerase I. At this concentration (0.2 u/μL) and after 1 h of incubation, the entire DNA reacted, and no further fluorescent accumulation occurred (Figure 3B).

To verify that the DNA polymerase I reaction reports an accurate value of the number of DNA molecules that had been cleaved, we compared the time course of nucleotide incorporation and fluorescent appearance to that of *Bam*HI DNA cleavage and the disappearance of the original DNA label. As can be seen in Figure 3B, the amount of DNA labeled with DNA polymerase I was always lower by a few percent than the amount revealed by DNA cleavage and loss of the signal. We attribute the higher values of loss of the signal to the fact that about 14% of the original DNA label is lost during washes of the surfaces.

In order to demonstrate the utilization of our approach in biopatterning, we combined the specificity of the DNA restriction reaction with the selectivity of the DNA polymerization reaction on one surface patterned by the magnetolithography method (32). Figure 4A depicts a fluorescently labeled DNA monolayer composed of DNA fragments with and without a *Bam*HI recognition site. The DNA harboring the *Bam*HI site was first covered by magnetic nanoparticles,

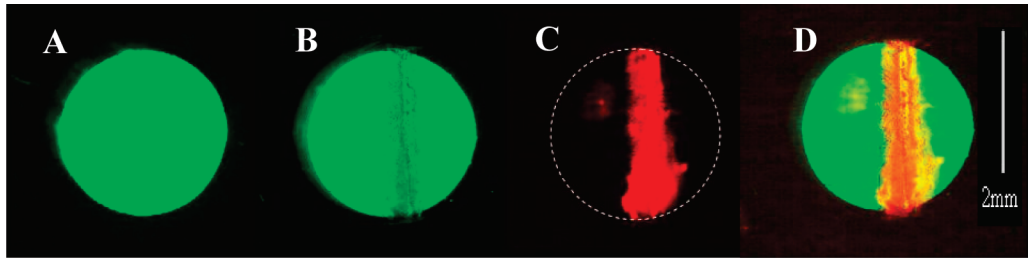

FIGURE 4. Surface patterning by the successive action of two DNA-modifying enzymes. (A) Surface patterned with magnetolithography of two types of DNA, both fluorescently labeled but with only one containing the *Bam*HI recognition site. (B) Addition of *Bam*HI to the entire surface causing removal of the fluorescent signal only from the patterned DNA with the recognition site. (C) Addition of a DNA polymerase I Klenow fragment resulting in the incorporation of fluorescent nucleotides only at the patterned stripe. (D) Image of the original uncut DNA (green) overlaid on the pattern of incorporated nucleotides (red).

directed to their position on the surface using a permanent magnetic field and a paramagnetic patterned mask that modified the external magnetic field. As a result of this process, a line covered with magnetic nanoparticles was formed.

DNA with no *Bam*HI site was then added to the surface, resulting in its adsorption to the surface in those regions that had not been blocked by magnetic particles. The magnetic nanoparticles were then removed, and the DNA harboring the *Bam*HI was adsorbed in the area formally covered by the nanoparticles. Because both DNA types were equally fluorescently labeled, a complete circle was formed (Figure 4A). The addition of the *Bam*HI restriction enzyme to the entire surface resulted in a reduction in the fluorescent signal only in the region where the quantum dots containing the DNA with the *Bam*HI site had been applied (Figure 4B). The subsequent addition of DNA polymerase I and fluorescently labeled nucleotides resulted in their incorporation to the *Bam*HI cleavage site, according to the pattern that had been initially formed by the mask (Figure 4C). Scanning of the surface with two different wavelengths revealed the two patterns, that of the intact DNA and that of the cleaved and extended nucleotides (green and red, Figure 4D).

Although we have demonstrated the selective incorporation of a fluorescently labeled nucleotide to a DNA monolayer, other commercially available modified nucleotides could be incorporated into a DNA chain. A straightforward example is the incorporation of biotinylated nucleotides that would direct the binding of avidin specifically to those DNA molecules that had incorporated the nucleotides. Finally, besides nucleotide incorporation, DNA fragments can be directly added to the restriction site via enzymatic ligation (30), further expanding the repertoire of reactions.

In conclusion, we have demonstrated the ability to pattern surfaces that had been modified with an initial DNA monolayer. This DNA monolayer can be modified with enzymes to create multilayers. By patterning of a surface with more than one type of DNA, patterned multilayers could be created (Figure 1). The large collection of restriction enzymes and their specific recognition sequences, together with the wide variety of DNA-modifying enzymes and chemically modified nucleotides and nanoparticles, make this self-assembly approach highly versatile, relying on the natural selectivity of DNA-dependent enzymes. The resolution that can be achieved by enzymatic manipulations is at the molecular nanoscale and exceeds the resolution of the micrometer-scale DNA patterning using such methods as magnetolithography (32) or photolithography (28).

As a quantitative and characterization tool to DNA manipulations on surfaces, the constructive nucleotide incorporation approach is the preferred methodology, for two main reasons: First, the appearance of a signal is more accurate and sensitive than its disappearance. Second, this methodology allows the quantitative evaluation of DNA on a surface postmanipulation such that the original DNA monolayer can be formed and analyzed with no structural perturbation and the label is only introduced in a subsequent step.

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