Surface-Initiated Growth of Poly d(A-T) by *Taq* DNA Polymerase

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In this paper, we report surface-initiated d(A-T) polymerization by *Taq* DNA polymerase as a method for constructing DNA-tethered surfaces using an enzyme. The enzymatic polymerization was conducted successfully via two steps: tethering of oligo d(A-T)s onto the surface presenting carboxylic acids by amide coupling and surface-initiated polymerization using *Taq* DNA polymerase. In this enzymatic polymerization process, the design and construction of carboxylic acid-presenting surfaces were found to be an important factor: DNA growth did not occur on the gold surface coated only with the self-assembled monolayer (SAM) of 16-mercaptohexadecanoic acid (MHDA), but effectively proceeded on the surfaces presenting mixed SAMs of MHDA and 1-pentadecanethiol. The coupling of oligo d(A-T)s and the subsequent DNA polymerization reaction were characterized by polarized infrared external reflectance spectroscopy, ellipsometry, X-ray photoelectron spectroscopy, and atomic force microscopy.

Introduction

Due to the tremendous growth in biosensing and microarray technologies based on nucleic acids, surface-tethering of nucleic acids has been the target of interest in wide areas from fundamental research fields to industrial areas.[1] Not only conventional techniques such as in situ solid-phase synthesis[2,3] and robotic deposition[4] but also alternative methods such as the formation of self-assembled monolayers (SAMs) presenting DNAs[5−8] and the covalent attachment of DNAs onto surfaces using organic reactions[9−13] were widely used for the construction of solid surfaces presenting surface-tethered nucleic acids.

Because enzymes have generally been used for tailoring DNAs in genetic engineering as well as in the body,[14] there were many efforts for the enzymatic treatments of surface-tethered DNAs via various processes such as primer extension by DNA polymerase, digestion by DNase, and ligation by ligase.[15−24] However, enzyme-catalyzed DNA synthesis on surfaces has not intensively been investigated because the enzymatic reaction process needs essentially a primer and a template, and the process would not be efficient when it is compared with conventional methods. From the recognition of such substantial limitation, we investigated a possibility that enzymatic, self-priming-based DNA synthesis in solution could be applicable to enzymatic, surface-initiated DNA synthesis.

In this paper, we demonstrated the surface-initiated d(A-T) polymerization by *Taq* DNA polymerase as a method for DNA growth from surfaces using an enzyme, one of the examples of enzymatic, surface-initiated polymerization.[25] *Taq* DNA polymerase has been known to catalyze the polymerization of dATP and dTTP into poly d(A-T) without requiring any added primer/template.[26] The possible mechanism of this activity has been investigated in depth by Yoshikura.[26,27] The primer/template-independent polymerization appears to proceed via two reactions: the slow process of the formation of a "precursor

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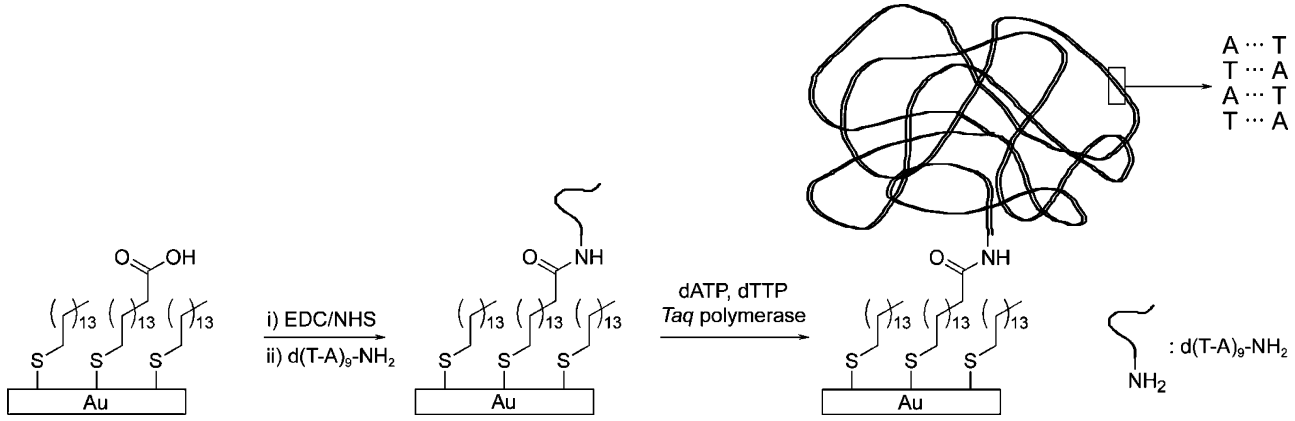

Figure 1. Schematic representation for surface-initiated poly d(A-T) growth by *Taq* DNA polymerase.

oligonucleotide" without primer/template and the rapid process of polymerization of poly d(A-T).[27] When dATP and dTTP are supplied to the *Taq* DNA polymerase reaction mixture, a short precursor oligonucleotide is formed in the slow process. The molecular nature of the short precursor oligonucleotide is a 16−19 nucleotide-long alternating dA/dT oligomer. The precursor oligonucleotide then serves as a primer/template via the formation of a hairpin structure for the rapid process of the elongation of the oligo d(A-T) by self-priming. Providing a short oligo d(A-T) such as $d(A\text{-}T)_9$ could skip the first slow process of forming the precursor oligonucleotide, so that the rate of the polymerization process of poly d(A-T) is greatly accelerated.[27] The reaction behaviors of *Taq* DNA polymerase are suitable for the application to the surface-initiated poly d(A-T) growth via a two-step procedure, tethering of an oligo d(A-T) onto surfaces and the subsequent *Taq* DNA polymerase-catalyzed polymerization (Figure 1). The oligo d(A-T) referred to the precursor oligonucleotide on surfaces would act as an initiation site for the formation of poly d(A-T) and the surface reaction would be more favored than the polymerization in solution because of the fast reaction rate of the elongation process.

This enzymatic process would provide an attractive possibility for surface-tethering of DNAs, and the surfaces formed can be used as a platform for various applications. The surface-initiated growth of DNA polymers from the surface may benefit over the simple attachment of DNA polymers onto the surface in its efficiency and manipulability. In addition, a programmable enzymatic manipulation by the initial attachment of oligo DNAs and the following polymerization using *Taq* DNA polymerase introduce an additional level of the control (surface-initiation and enzymatic polymerization). DNA-tethered surfaces would have many applications in materials science, such as DNA microarrays, nanobiosensors, and the assembly of nanostructures. DNA polymers on the surface could provide (sizable amounts of) sites for further modifications such as biotinylation, and the sequence specificity of the nature of DNA polymers on the surface could be used for the directed assembly of nano-building blocks by strong interactions between complementary DNA polymers than building blocks.

Experimental Section

Materials. Si⟨100⟩ wafers were obtained from Prolog Semicor, Ltd., Ukraine. Absolute ethanol (EtOH, 99.8+%, Merck), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 98+%, Aldrich), *N*-hydroxysuccinimide (NHS, 97%, Aldrich), 16-mercaptohexadecanoic acid (MHDA, 90%, Aldrich), 1-pentadecanethiol (PDT, 98%, Aldrich), precursor oligonucleotide (3′-d(T-A)$_9$−(CH$_2$)$_6$−NH$_2$-5′, d(T-A)$_9$−NH$_2$, Bioneer Corp., Korea), *Taq* DNA polymerase (Koma Biotechnology, Korea), dATP (Takara Bio Inc., Japan), dTTP (Takara Bio Inc., Japan), sulfuric acid (H_2SO_4, 95.0+%, Junsei), acetic acid (99.0+%, Junsei), and hydrogen peroxide (H_2O_2, 30−35%, Junsei) were used as received. Ultrapure water (18.3 MΩ/cm) from the Human Ultra Pure System (Human Corp., Korea) was used.

Preparation of Self-Assembled Monolayers. The gold substrates were prepared by thermal evaporation of 5 nm of titanium and 100 nm of gold onto silicon wafers. Prior to use, gold substrates were cleaned for 1 min in piranha solution (3:7 by volume of 30% H_2O_2 and H_2SO_4, *Caution: piranha solution reacts violently with most organic materials and must be handled with extreme care*), rinsed with H_2O and ethanol, and dried under a stream of argon.

The SAMs of MHDA were prepared by immersing the gold substrates in a 1 mM solution of ethanol/water/acetic acid (85/10/5, v/v/v) overnight according to the procedure reported previously.[28] The mixed SAMs of MHDA and PDT (1:2 or 1:4) were prepared by immersing the gold substrates in solutions of ethanol/acetic acid (95/5, v/v) containing a mixture of two thiol compounds (1 mM of MHDA and 2 mM of PDT for 1:2 mixed SAMs and 1 mM of MHDA and 4 mM of PDT for 1:4 mixed SAMs) overnight. After the formation of SAMs, the substrates were rinsed with ethanol several times and then dried under a stream of argon.

EDC/NHS Activation and Coupling of Precursor Oligonucleotides. A gold substrate presenting carboxylic acids was immersed in an aqueous solution of EDC (75 mM) and NHS (15 mM) for 1 h at room temperature, washed with water and ethanol, and dried in a stream of argon. The NHS-activated surface was immersed in a 0.1 M sodium phosphate buffer solution (pH 8.0) of d(T-A)$_9$−NH$_2$ (30 μM) for 7 h at room temperature. After the reaction, the substrate was rinsed with 0.1 M sodium phosphate buffer, water, and ethanol and dried in a stream of argon.

Surface-Initiated Synthesis of Poly d(A-T) by *Taq* DNA Polymerase. The reaction mixture contained 100 units of *Taq* DNA polymerase and 200 μM each of dATP and dTTP in 1 mL of reaction buffer (2 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4 at 25 °C)). For the polymerization reaction, a gold substrate presenting the precursor oligonucleotide was immersed in the solution without stirring at 65 °C. After 3 h of reaction, additional 20 μL of 10 mM dATP and 20 μL of 10 mM dTTP were supplemented to the reaction mixture. After another 3 h of reaction at 65 °C, the reaction mixture was cooled to room temperature and then the gold substrate was rinsed with 0.1 M sodium phosphate buffer and water and dried in a stream of argon.

Instrumentation. Polarized infrared external reflectance spectroscopy (PIERS) spectra were obtained in a single reflection mode using a dry N_2-purged Thermo Nicolet Nexus FT-IR spectrophotometer equipped with the smart SAGA (smart apertured grazing angle) accessory. The *p*-polarized light was

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Table 1. Thickness at Each Reaction Step

	thickness (Å)			
	SAM	after NHS activation	after precursor coupling	after polymerization
SAM of MHDA	16.2 ± 1.1	20.6 ± 1.2	21.3 ± 1.0	20.1 ± 1.6
1:2 mixed SAM	14.6 ± 1.2	17.0 ± 1.4	22.3 ± 1.4	93.5 ± 17.9
1:4 mixed SAM	13.1 ± 1.2	14.5 ± 1.1	17.0 ± 1.1	100.4 ± 9.1

incident at 80° relative to the surface normal of the substrate and a narrow band mercury−cadmium−telluride (MCT) detector cooled with liquid nitrogen was used to detect the reflected light. We averaged 2000 scans to yield the spectrum at a resolution of 2 cm^{-1}, and all spectra were reported in the absorption mode relative to a clean gold surface. The thicknesses of the monolayer films were measured with a Gaertner L116s ellipsometer (Gaertner Scientific Corp., IL) equipped with a He−Ne laser (632.8 nm) at a 70° angle of incidence. A refractive index of 1.46 was used for all films. The X-ray photoelectron spectroscopy (XPS) study was performed with a VG-Scientific ESCALAB 250 spectrometer (U.K.) with monochromatized Al Kα X-ray source. Emitted photoelectrons were detected by a multichannel detector at a takeoff angle of 90° relative to the surface. During the measurements, the base pressure was 10^{-9}−10^{-10} Torr. Survey spectra were obtained at a resolution of 1 eV from 3 scans and high-resolution spectra were acquired at a resolution of 0.05 eV from 5 to 20 scans. Atomic force microscopy (AFM) imaging was performed in the Tapping Mode on a Nanoscope IIIa multimode scanning probe microscope (Veeco, USA) with Tapping Mode etched silicon probes (TESP).

Results and Discussion

Formation of SAMs and Coupling of Precursor Oligonucleotide. Three types of SAMs, SAM of MHDA and 1:2 and 1:4 mixed SAMs of MHDA and PDT, were prepared as the platforms for surface-initiated, enzymatic poly d(A-T) growth. The thicknesses of the SAMs were measured to be 16.2 Å (for the SAM of MHDA), 14.6 Å (for the 1:2 mixed SAM), and 13.1 Å (for the 1:4 mixed SAM), respectively (Table 1). Characteristic peaks in the IR spectra confirmed the formation of SAMs presenting COOH groups with a highly ordered structure (Figure 2a). The CH_2 stretching vibrations of the alkyl chain are very sensitive to the lateral packing density and to the presence of gauche defects, which makes these vibration modes ideally suited as probes to determine the crystallinity of SAMs. In the IR spectra of the three types of SAMs, the CH_2 stretching vibration peaks were observed around at 2850 cm^{-1} (symmetric CH_2 stretching) and 2919 cm^{-1} (asymmetric CH_2 stretching). The peak positions of the CH_2 stretching vibration are well in agreement with the peak positions of the CH_2 stretching vibration of the highly crystalline, well-ordered SAM.[29] In the cases of the mixed SAMs, the bands at 2964 and 2881 cm^{-1} from the asymmetric C−H stretching and the symmetric C−H stretching of the terminal methyl group of PDT additionally appeared.[30] We also observed the peaks from the COOH group at 1719 cm^{-1} (C═O stretching of acyclic dimers) and 1742 cm^{-1} (C═O stretching of monomers) in the IR spectrum of the SAM of MHDA and at only 1718 cm^{-1} in the cases of the mixed SAMs.[30]

After the treatment of the SAMs with EDC and NHS, the carboxylic acids at surfaces were completely converted into NHS esters. Representatively, in the case of the 1:2 mixed SAMs, the C═O band of carboxylic acid at 1718 cm^{-1} completely disappeared and three absorption peaks from the symmetric (1797 cm^{-1}) and asymmetric (1755 cm^{-1}) C═O stretch of NHS and the C═O stretch of ester bond (1828 cm^{-1}) newly appeared after the reaction (Figure 2b).[31,32] The successful reactions were further confirmed by the increases in the film thicknesses by 4.4 Å (for the SAM of MHDA), 2.4 Å (for the 1:2 mixed SAM), and 1.4 Å (for the 1:4 mixed SAM), respectively (Table 1).

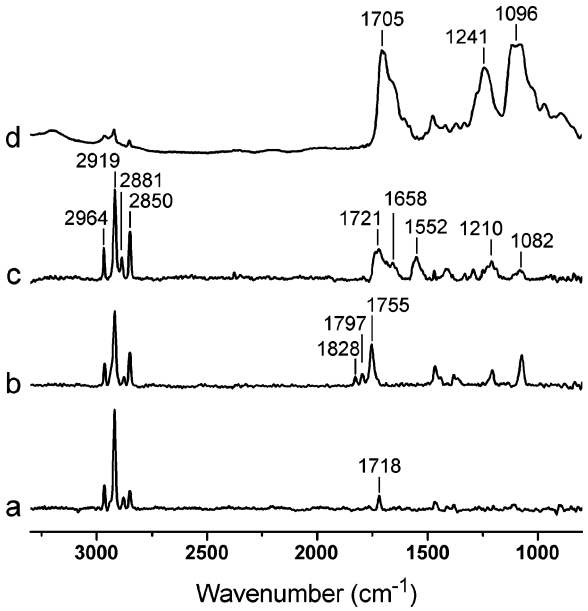

Figure 2. IR spectra of (a) the 1:2 mixed SAM of MHDA and PDT and the mixed SAM (b) after the EDC/NHS activation, (c) after the coupling of d(T-A)$_9$−NH_2, and (d) after the formation of poly d(A-T) film by *Taq* DNA polymerase.

We conducted the amide coupling between d(T-A)$_9$−NH_2 and the terminal NHS ester groups at surfaces. The NHS-activated SAMs were reacted with d(T-A)$_9$−NH_2 (30 μM) in 0.1 M sodium phosphate buffer (pH 8.0) for 7 h at room temperature. Characteristic amide peaks after the coupling appeared around 1658 cm^{-1} (amide I) and 1552 cm^{-1} (amide II) (Figure 2c).[30] Three main characteristic peaks, the absorptions at 1721 cm^{-1} due to the stretching vibrations of double bonds in base plane, at 1210 cm^{-1} due to the phosphate groups, and at 1082 cm^{-1} due to the deoxyriboses of oligonucleotides also proved the successful anchoring of d(T-A)$_9$−NH_2.[33] The coupling was further confirmed by XPS studies (Figure 3). After the coupling, the N 1s peak from amide bonds and DNA bases was observed at 400.6 eV. In addition to the N 1s peak, the appearance of the P 2p peak at 134.3 eV confirmed the successful attachment of d(T-A)$_9$−NH_2 onto surfaces because phosphorus (P) is the unique element of phosphate backbone of nucleic acids. Ellipsometric measurements showed that the increase in the film thickness was 5.1 Å (for the SAM of MHDA), 7.7 Å (for the 1:2 mixed SAM), and 3.9 Å (for the 1:4 mixed SAM), respectively (Table 1). Because we did not observe NHS groups at surfaces after 7 h of reaction of the amide coupling, we proceeded to the DNA polymerization step without any further treatment, such as the hydrolysis of NHS esters.

Synthesis of Poly d(A-T) by *Taq* DNA Polymerase. Poly d(A-T) was synthesized using *Taq* DNA polymerase from the surface-tethered precursor oligonucleotides prepared by the direct coupling in the previous step. We adopted the polymerization conditions, 200 μM of dATP, 200 μM of dTTP, 2 mM of Mg^{2+}, and 65 °C as a reaction

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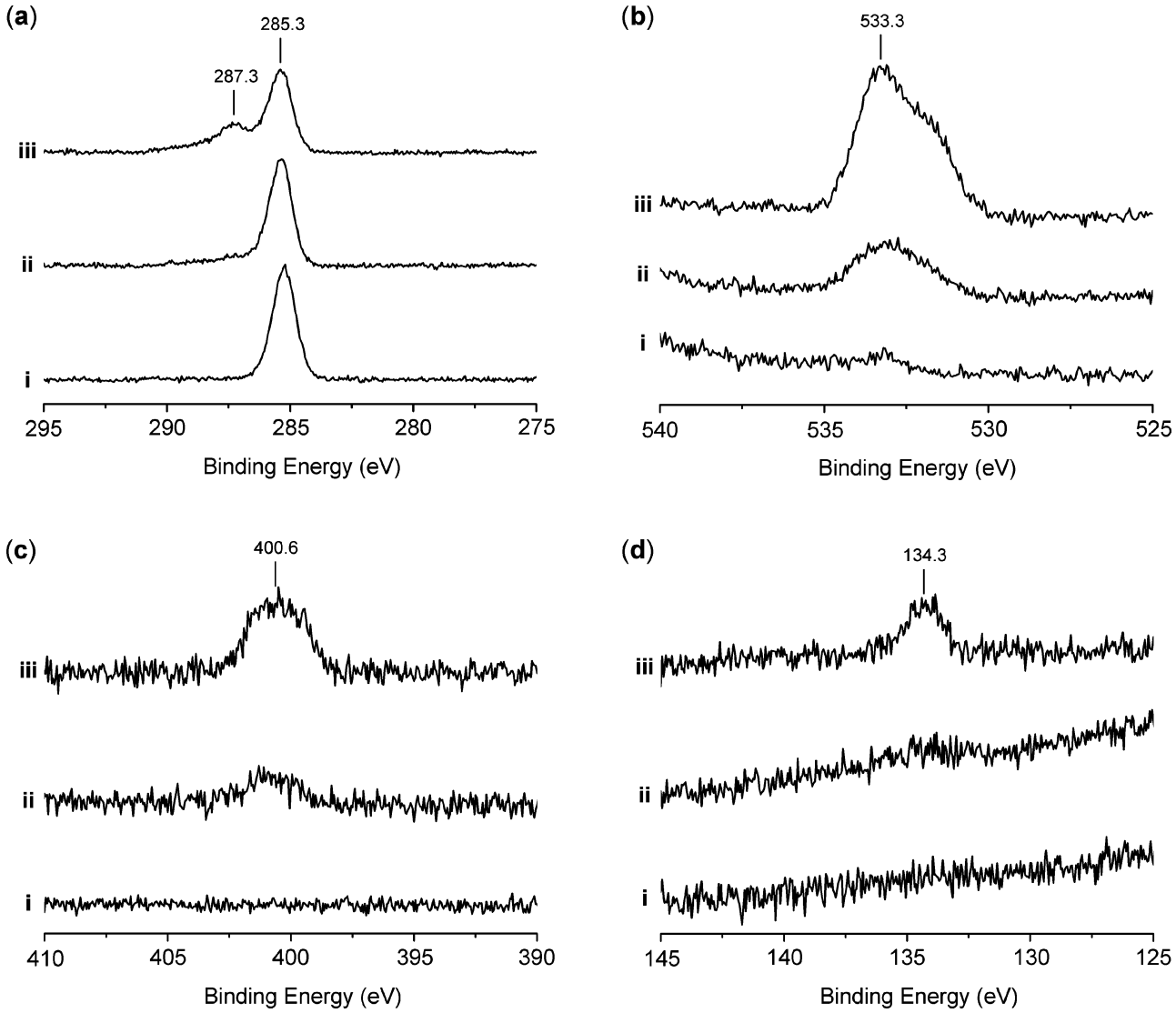

Figure 3. High-resolution XPS spectra of (a) C 1s, (b) O 1s, (c) N 1s, and (d) P 2p regions acquired from (i) the surface having intact 1:2 mixed SAM of MHDA and PDT; (ii) the surface after the coupling with d(T-A)$_9$−NH$_2$; (iii) the surface after the polymerization by *Taq* DNA polymerase.

temperature, known to be suitable for synthesizing poly d(A-T) by *Taq* DNA polymerase in the solution.[26,27] After the polymerization, the thicknesses of the polymer films grown from the 1:2 and 1:4 mixed SAMs were 93.5 and 100.4 Å, respectively. In the case of the SAM of MHDA, however, no increase of thickness was observed from the ellipsometric measurement (Table 1). The result showed that poly d(A-T) was synthesized from the oligonucleotides coupled only with the 1:2 and 1:4 mixed SAMs, not with the SAM of MHDA. In the IR spectra after the polymerization from the 1:2 and 1:4 mixed SAMs, the relative intensity of peaks from the SAMs drastically decreased and the peaks at 1705, 1241, and 1096 cm^{-1} due to poly d(A-T) were observed predominantly with strong absorption bands (Figure 2d). The polymerization was further confirmed by XPS studies (Figure 3). After the polymerization, peak intensities of elements, C, N, O, and P from poly d(A-T), greatly increased and the analysis of a carbon (C 1s) region revealed that there was an additional peak at 287.3 eV along with a main peak at 285.3 eV. Although the precise analysis of the carbon peak was difficult because of the complex structure of DNAs, the characteristic peak of poly d(A-T) film at 287.3 eV was thought to be due to carbons having neighboring oxygen in deoxyriboses and bases. We further analyzed the XPS data acquired after the polymerization, and the N:P ratio was calculated to 3.57:1. The formed DNA polymer is of poly d(A-T) structure, the alternating form of adenine (A) containing five nitrogen atoms and thymine (T) containing two nitrogen atoms and the expected N:P ratio is 3.5:1. The experimental XPS result was in agreement with the expected value. The morphologies of the poly d(A-T) films were characterized by AFM in the tapping mode (Figure 4). The root-mean-square (RMS) roughness of the poly d(A-T) films was about 50.9 Å for the 93.5-Å-thick film on the 1:2 mixed SAM and about 44.5 Å for the 100.4-Å-thick film on the 1:4 mixed SAM. At these surfaces, the poly d(A-T) films showed the domains of spherical shapes having the maximum height of about 400 Å, and the domains were more uniform in the case of the 1:4 mixed SAM than in the case of the 1:2 mixed SAM.

No formation of poly d(A-T) films on the single-component SAM could be explained by steric crowding at surfaces, which is characteristic of interfacial reactions.[34] Mrksich et al. studied enzymatic glucosylation of *N*-acetylglucosamine immobilized in mixed SAMs on gold.[35] They found that the extent of reaction dropped dramati-

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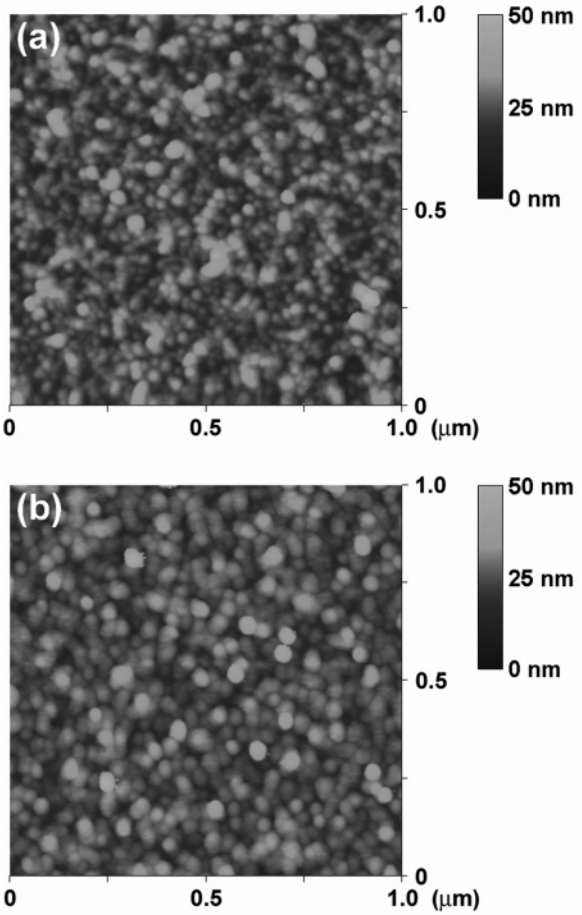

Figure 4. AFM images of the poly d(A-T) films polymerized from (a) the 1:2 mixed SAM and (b) the 1:4 mixed SAM.

cally if the surface density of *N*-acetylglucosamine was higher than a certain threshold. The low yields at high coverages were thought to be due to the steric crowding at the surface, which inhibited the enzymatic reaction. In our system, because of the high density of precursor oligonucleotides on the SAM of MHDA, *Taq* DNA polymerase might not be capable of conducting the efficient polymerization reaction from the precursor oligonuclotides. Another possibility could be drawn from the observation that only a 5.1-Å increase of thickness was achieved on the SAM of MHDA after the precursor oligonucleotides coupling, which is less than the thickness increase for the 1:2 mixed SAM (7.7 Å). Deng et al. reported that DNA molecules could be attached onto carboxylate-terminated alkanethiol SAMs on gold surfaces via the EDC/NHS cross-linking reaction.[36] The DNA attachment was attributed to the formation of amide bonds between the carboxylate groups and the amine groups on the DNA bases. In our system, particularly adenine base has a primary amine group, which could react with the NHS-activated surface. If the adenine bases in the precursor oligonucleotide were anchored covalently onto the surface, the oligo d(T-A) would not be capable of acting as an initiation site. Although the primary amines at adenines have much lower reactivity than the primary amine at 5′-end of the precursor oligonucleotide,[12] there are more chances for the covalent anchoring of adenines because of the number of carboxylate groups in the SAM of MHDA.

Conclusions

We demonstrated the surface-initiated d(A-T) polymerization by *Taq* DNA polymerase as a method for the construction of DNA-tethered surfaces using an enzyme. The polymerization was conducted successfully via two steps: tethering of long oligo d(A-T)s onto surfaces and polymerization using *Taq* DNA polymerase. The molecule-level design of surfaces presenting carboxylic acids was found to be an important factor for the successful enzymatic process: enzymatic polymerization did not occur on the SAM of MHDA but proceeded only on the mixed SAMs with PDT. We believe that the enzymatic process described herein would provide an attractive approach to the surface tethering of DNAs and could be applied effectively to surface-initiated synthesis of DNAs containing various sequences, such as poly d(A-U) and poly d(G-C), which are achievable via the self-priming process.[37] Moreover, the elongation process also would be realized generally by various enzymes, including the intact *Thermus*-derived DNA polymerases such as *Taq* DNA polymerase (the enzyme used in this study), Δ*Tth* DNA polymerase, *E. coli* DNA polymerase I, and T4 DNA polymerase.[27]

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