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A novel approach to Au nanoparticle-based identification of DNA nanoarrays

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TABLE OF CONTENTS

1. ABSTRACT

The combination of electron beam lithography and gold nanoparticle-based detection method is subject to a novel high-resolution approach to detecting DNA nanoarrays. In this work, gold nanoparticle-based detection of DNA hybridization on nanostructured arrays is presented. The nanostructured arrays were created by electron beam lithography of a self-assembled monolayer. Amine groups, which are active moieties and are used for attachment of DNA, were introduced to the nanostructures, and the amine-modified structures were characterized by scanning Maxwell-stress microscopy (SMM) for seeing the modification process. The DNA probe covalently immobilized within the nanostructures was hybridized with a biotinylated target DNA. Streptavidin-gold conjugate was then bound to the biotin, thereby assembling inside the nanostructured arrays. The sequence-specific hybridization was imaged by atomic force microscopy (AFM). On the other hand, the activity of the DNA molecules within the nanostructured arrays was verified by fluorescence microscopy using streptavidin-Cy 5 conjugate instead of streptavidin-gold nanoparticles conjugate. On the basis of fluorescent detection, an alternative method has been developed for detection of DNA nanostructures, which will benefit the development of DNA chips.

2. INTRODUCTION

Improvements to DNA chip technology enable one to quickly and inexpensively detect the presence of a whole array of genetically based diseases. In addition, the technology can also enable one to cost-effectively conduct widespread disease screening, and to monitor the effectiveness of patient therapies (1-4). The next generation of DNA chips is expected to have smaller component size down to the nanometer scale, resulting in high-density DNA arrays having controlled homogeneous spots, high detection sensitivity and massive parallelization with low amount of samples.

The conventional label for DNA chip detection is the fluorescent dye, whose use is well established in the labs as well as in industries. However, there are a number of demerits concerning the fluorescence detection methods. First, photobleaching phenomenon exists in the fluorescence dye methods; secondly, the detection of fluorescence dyes basically involves the use of expensive equipments; thirdly, the use of fluorescence markers is hazardous; fourthly, because of the resolution limitations inherent in optical detection, it is barely feasible to optically visualize fluorescent DNA nanostructures down to 50 nanometers. Gold nanoparticle is one of the promising

candidates. The signals of gold nanoparticles are stable for extended periods of time, conferring the ability for individual particle measurement by AFM. Consequently, labeling with gold nanoparticles can overcome the problems of labeling using fluorescent dyes and achieve high-resolution detection of DNA nanostructures. In one word, traditional fluorescence detection methods in chip technology suffer from photobleaching, use of expensive equipments and hazardous markers, and limitations of inherent detection. New method using gold nanoparticle labeling is easy of use capable of attaining reasonable high-resolution and high-throughput. Recently, DNA array detection methods using gold nanoparticles rather than fluorescence dyes as labels have been explored (5). Thus far, a scanometric DNA array (6-7), optical readout (8), electrical detection (9-10), colorimetric detection (11-12), electrochemical stripping detection (14-15), and scanning electrochemical microscopy (SECM) imaging (16) based on gold nanoparticle labeling have been developed as alternatives in chip technology. Thus, the unusual properties of gold nanoparticles make them unique labels for DNA chip detection. However, most of the methods related to array detection utilizing gold nanoparticle labeling are limited to micron size.

Dip-pen nanolithography-based methodology was used to generate arrays of gold nanostructures functionalized with oligonucleotides (17-18), but the technique suffers from a low throughput. Electron beam (EB) lithography of self-assembled monolayer (SAM) is capable of achieving a high-resolution down to several nanometers with a high throughput, allowing fabrication of single molecule arrays. More recently, DNA nanostructures were produced by EB lithography of octadecyltrimethoxysilane (ODS) SAM, in which nanoscale patterns of the ODS SAM on a silicon surface were fabricated by EB lithography, followed by immobilization of oligonucleotides onto these nanopatterns and subsequent hybridization of fluorescently labeled target to the immobilized DNA (19-20). To investigate the technique of generating DNA nanostructures detectable by nanoparticles, we show here an electron beam lithography-based methodology for producing DNA nanostructured arrays. Nanostructured arrays were fabricated on a silicon surface by electron beam lithography of ODS-SAM. Pre-synthesized probe oligonucleotides were covalently and selectively immobilized onto the nanostructured regions, and the surface hybridization process was carried out using complementary target oligonucleotides labeled with biotin, streptavidin-Cy 5 or streptavidin-gold nanoparticles conjugate was applied to the substrates and bound to the biotin inside nanostructures. Finally the binding event was illustrated by two methods: fluorescent dyes or Au nanoparticles labeling. This approach allows gold nanoparticle-based detection of the DNA nanostructured arrays on the ODS-SAM, which will provide a novel technique for high-resolution detection of DNA nanoarrays.

3. MATERIALS AND METHODS

3.1. Materials

All oligonucleotides were purchased from Sigma Genosys Japan (Hokkaido, Japan). Two types of oligonucleotides were employed in this experiment: those for immobilization onto the surface, and those for hybridization to the surface. Complementary oligonucleotides to be immobilized onto the surface were amine-modified at the 5′-end, those employed for hybridization to the surface were biotin-labeled at the 5′-end. Each of these oligonucleotides was 21-mers in length. The two 21-mer sequences used were: H_2N-5′-CCACGGACTACTTCAAAACTA-3′ (complementary probe)
biotin-5′-TAGTTTTGAAGTAGTCCGTGG-3′ (target)

All chemicals and solvents were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) except the following: 3-aminopropyltriethoxysilane (APTES, Acros, USA), octadecyltrimethoxysilane (Acros, USA), Tween-20 (BBInternational, UK), and 20-nm streptavidin-gold conjugate (BBInternational, UK). Wafers used for the experiments were n-type Si (100) wafers. Ultra pure water was obtained from a Millipore system.

3.2. Formation of 10 nm SiO_2 layer on silicon surface

All wafers were subjected to thorough cleaning prior to use. The n-type Si (100) wafers were cut into small square pieces (1.5 cm × 1.5 cm). After immersing in a solution of HCl : H_2O_2 : H_2O (1 : 1 : 7) at 80 °C for 5 min, the wafers were rinsed with a solution of HF : NH_4F : H_2O (1 : 4 : 180) to remove the native oxide layer. After each step, the wafers were washed with MilliQ water and blow-dried. A layer of silicon dioxide, 10 nm thick, was grown in an oxidation furnace at 950 °C for 10 min.

3.3. Deposition of ODS-SAM on silicon surface

ODS-SAM was deposited to a SiO_2 layer at 110 °C for 3 h in N_2 by placing the wafers face down inside a Teflon chamber to be exposed to the ODS vapor. The chamber was filled with a solution of 300 μL ODS. The surface was washed with chloroform for 10 min by ultrasonication. The thickness of the ODS monolayer measured by ellipsometer measurements is approximately 2.0 nm.

3.4. Fabrication of nanostructured arrays

EB lithography of the ODS-SAM was performed with a Hitachi S-4200 scanning electron microscope (SEM) specially equipped for the EB draw system (Tokyo Technology Inc.) to produce nanostructures by exposing the SAM to an electron beam at a dose of 960 $\mu C/cm^2$ at 20 keV. The patterned monolayer was developed in buffered hydrofluoric acid (BHF; HF : NH_4F : H_2O=1 : 4 : 180) for 60 s, leaving exposed SiO_2 nanoholes in the monolayer.

3.5. DNA immobilization on nanostructured arrays

Nanostructured substrates were immersed into a solution of NH_4OH : H_2O_2 : H_2O (1 : 1 : 4) for 5 min, washed with MilliQ water and blow-dried. Then the substrates were immersed into a solution consisting of 2 % APTES in 95 % ethanol / H_2O for 1 h, washed with ethanol three times for 5 min by ultrasonication. Next, the substrates were immersed into a solution of 1 % glutaraldehyde in water for 1 h, then rinsed three times with water. 21-mer 5′ amine-modified oligonucleotide probes were diluted with 3 × SSC to a final concentration of 20 μM. Small droplets of the solution were deposited

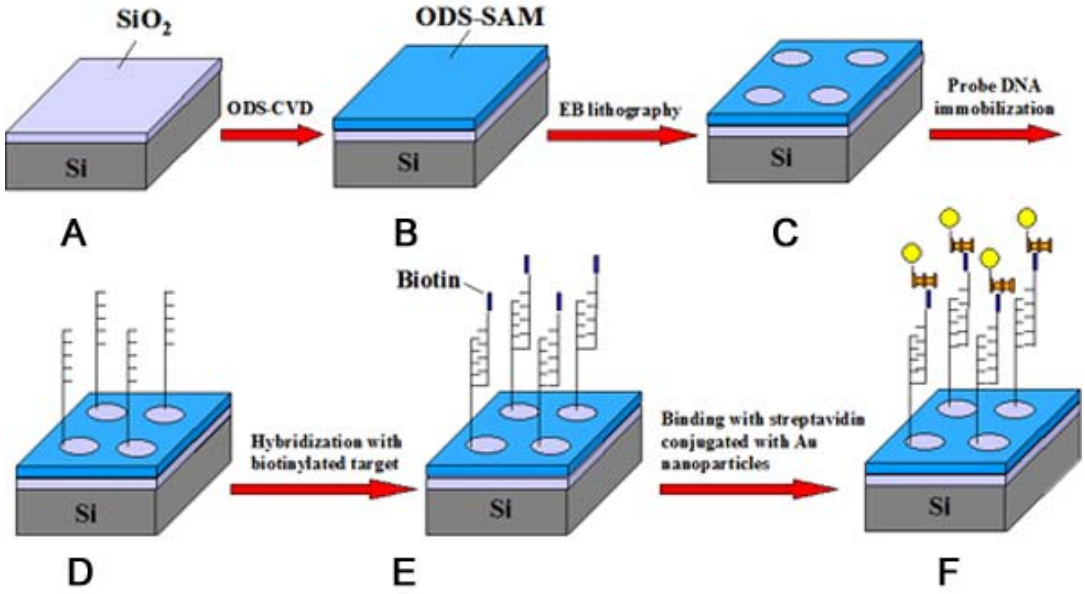

Figure 1. Schematic representation of gold nanoparticle-based detection of DNA hybridization on a nanoarray.

manually on the surface to cover the nanostructured arrays. The substrates were incubated at 38 °C for 2 h in a humidified hybridization chamber to prevent drying of the droplets, then washed three time with washing buffer (PBS, 0.1 % Tween-20), and rinsed with MilliQ water, blow-dried.

3.6. Hybridization inside nanostructured arrays

Nanostructured arrays immobilized with probe DNA were incubated with 2.5 μM of target DNA labeled with biotin in 2 × SSC at room temperature for 4 h in a humid chamber. The hybridized surfaces were then washed once for 5 min in 2 × SSC, 0.2 % SDS, once for 5 min in 2 × SSC, and once for 5 min in 0.2 × SSC to remove any unhybridized target.

3.7. Atomic Force Microscopy

Hybridized substrates were incubated for 5 min with streptavidin-gold nanoparticle (20-nm) conjugate diluted 50 × in PBS buffer containing 1 % BSA and 0.1 % Tween-20. The substrates were then washed five times with the same wash buffer. AFM experiments were carried out on a Molecular Force Probe 3D (MFD-3D) AFM (Asylum Research, Santa Barbara, CA). The silicon microcantilevers (AC160TS-24) were purchased from Olympus and used with a force constant of ~40 N/m. Topographic images were taken in tapping mode in air, collected at a scan rate of 1 Hz, and analyzed with IGOR Pro software.

4. RESULTS AND DISCUSSION

A schematic diagram describing the preparation of DNA nanostructured arrays by EB lithography of ODS-SAM based on gold nanoparticle detection is shown in Figure 1. A layer of silicon dioxide is grown on a silicon surface (Figure 1a). ODS-SAM is deposited on the silicon surface by the chemical vapor deposition (CVD) method (Figure 1b). EB lithography is employed to produce nanostructures (Figure 1c). Probe oligonucleotides are preferentially immobilized to the nanostructured arrays by covalent binding (Figure 1d). The hybridization of the biotinylated target with the probe introduces biotin to the nanostructured arrays (Figure 1e), subsequently leading to the formation of biotin with streptavidin-gold nanoparticle conjugate (Figure 1f). Consequently, the nanostructures covered with such a conjugate can be observed with AFM.

To covalently immobilize amine-modified oligonucleotides onto the nanostructured arrays, the regions within the nanostructures must be activated. Many crosslink methods are applicable, of which APTES is one. This technique is employed to produce amine groups within the nanostructures and involves immersing the surface with 2 % APTES in the presence of ethanol and water. APTES, however, is a small molecule, leading to difficulties in characterizing its presence by conventional AFM. Prior to introduction of amine groups, the arrays were etched back to the silicon dioxide surface to form periodic holes by developing the substrates in buffered hydrofluoric acid, whereas the areas outside the holes were still coated with ODS-SAM. A contact-mode AFM image, in which an image is taken successfully for identifying soft structures since it introduces high lateral forces as well as compressive forces to the sample surface with the consequence of plastic deformations, when the tip and sample remain in close contact as the scanning proceeds, indicating the arrays of holes is presented in Figure 2a. In this figure, the holes are periodic and homogeneous.

Scanning Maxwell-stress microscopy (SMM), which is capable of obtaining images of surface potential, is an effective tool for visualizing features composed of positively charged amine groups (21). The SMM images were taken on arrays with dots of 1 μm diameter spaced 1 μm apart from each other. Figure 2b shows an SMM image of an array of dots composed of the amine deposits. The

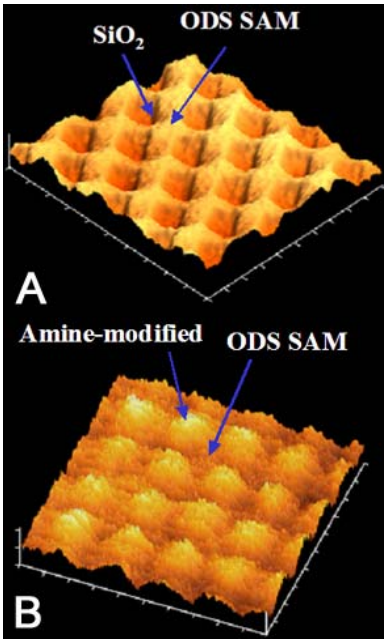

Figure 2. A. A contact-mode AFM image of an EB-patterned ODS-SAM surface on a silicon dioxide layer (Size: 1μm, Interval: 1μm). B. A scanning Maxwell-stress microscopy (SMM) image of an amine-modified ODS-SAM array.

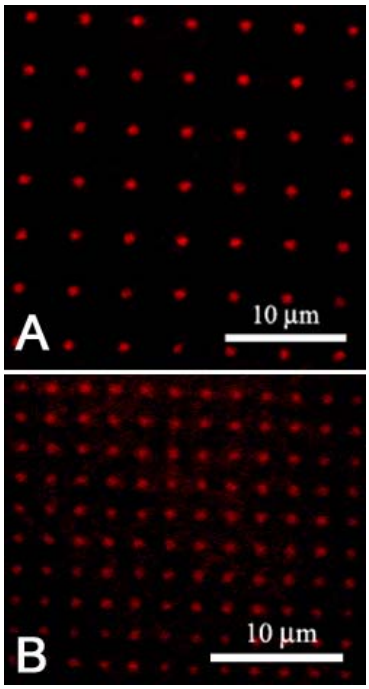

Figure 3. Fluorescence images of the attachment of the streptavidin-Cy 5 conjugate to the spots where the biotinylated target has been hybridized to the complementary probe. (a) Dots of 500 nm diameter with spacing of 5 μm in between. (b) Dots of 250 nm diameter with spacing of 2.5 μm in between.

bright peaks correspond to the amine-modified moieties, whereas the bottom is covered with ODS-SAM. The higher surface potential in the arrays verifies that the APTES is selectively deposited and only produces amine groups inside the arrays.

Not only can fluorescent detection reach a high sensitivity, but it can also verify the biological activity of the oligonucleotides that are immobilized in the nanostructured arrays. In the proposed protocol, an indirect method was employed in which the biotinylated target was hybridized to the probe, and gold nanoparticles were conjugated to the nanostrucutured arrays via affinity biotin-avidin binding. It is well known that the binding between biotin and avidin is rapid and of high affinity. Thus, the activity of the patterned DNA molecules needed to be investigated. To this end, 1 μM of a streptavidin-Cy 5 conjugate in PBS was used for binding to the biotin in the nanostructured arrays. The DNA nanostructures were then characterized by epifluorescence microscopy. Fluorescence images were collected by a SIT camera (C2400-08, Hamamatsu Photonics, Japan) coupled to an image intensifier (VS4-1845, Video Scope International, USA) under red (632.8 nm) laser excitations. In this case, only fluorescence corresponding to the complementary target within the nanostructures was visualized. As shown in Figure 3a and 3b, DNA nanostructures with dot arrays (500-nm dots, 5-μm pitch and 250-nm dots, 2.5-μm pitch) were strongly observed. The brightness of each spot is strong, suggesting that hybridization of the target took place at all of the spots covered with the same probe, and binding between biotin and avidin happened at the same time. However, as described previously, ODS-SAM is a hydrophobic surface, leading to protein adsorption on the surface (22). A slightly non-specific binding is visible in Figure 3b since the amount of the DNA molecules in 250-nm nanostructures is small and streptavidin-Cy 5 conjugate somewhat adsorbs on ODS-SAM. These results indicate that the attached oligonucleotides retain their activity, and therefore hybridize to the complementary target labeled with the biotin bound to the streptavidin-Cy 5 conjugate. The fluorescent detection approach also confirms that the proposed method can be used to detect the assembly of streptavidin-gold nanoparticle conjugate.

Recently, colorimetric silver detection of DNA microarrays (13) and silver enhanced imaging of DNA microarrays with scanning electrochemical microscopy (16) have been developed. However, both detection methods are not applicable to nanostructured arrays due to limited resolution. AFM imaging, which is capable of high resolution, has proven to be highly selective and sensitive in visualizing the hybridization of single-stranded DNA with nanostructures (23). Therefore, AFM-based approaches to detecting DNA hybridization within the nanostructures are promising. Prior to the immobilization of probe DNA, the nanostructured arrays were fabricated on ODS-SAM by EB lithography. The nanoholes in ODS-SAM were characterized by tapping-mode AFM, which allows high resolution topographic imaging of sample surfaces that are easily damaged, loosely hold to their substrate, or difficult to image by other AFM techniques.

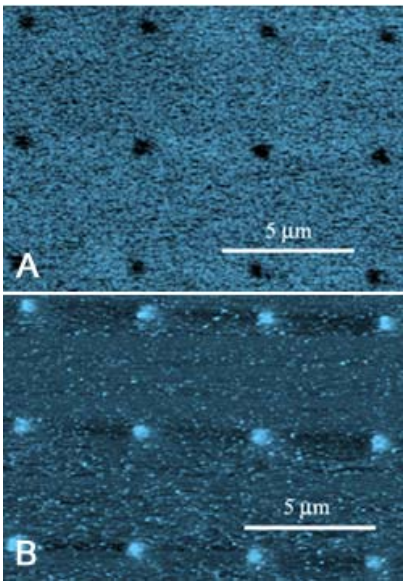

Figure 4. Nanometer-sized characterization by tapping-mode AFM in air. (a) An AFM topographic image of the nanoholes on ODS-SAM created by EB lithography (500-nm holes with 5-µm distance in between). (b) An AFM topographic image of the attachment of the streptavidin-gold nanoparticle conjugate to the spots where the biotinylated target has been hybridized to the complementary probe (500-nm features with 5-µm distance in between).

Figure 4a shows submicro-sized arrays indicating 500-nm-size dots separate from each other by a distance of 5 µm distance, demonstrating that the nanoholes corresponding to the dark regions are regular and periodic, as expected. As mentioned previously, the thickness of ODS-SAM is approximately 2.0 nm and the measured depth of each dot is approximately 3 nm, indicating that SiO_2 surfaces inside the dots are exposed (19). Subsequently, streptavidin-gold nanoparticle conjugate instead of streptavidin-Cy 5 conjugate was utilized for binding with biotinylated oligonucleotides that were hybridized to the nanostructured arrays. Figure 4b shows an image of the nanostructured arrays of 500-nm-size and 5-µm-pitch assembled with gold nanoparticles. The bright features correspond to the DNA nanostructures. A little nonspecific binding of gold nanoparticles on ODS-SAM is observed mainly because streptavidin is adsorbed on the hydrophobic surface, thereby resulting in adsorption of gold nanoparticles. The measured height of each spot is approximately 17 nm, which is in good agreement with the diameter of the gold nanoparticles used (20-nm). The results of Figure 4b show that the DNA nanostrucured arrays combined with the formation of the biotin / avidin-gold nanoparticle conjugate can be examined with AFM.

5. CONCLUSIONS

This work demonstrates a new method for imaging DNA hybridization in nanostructured arrays that is based on gold nanoparticle labeling by AFM. Gold nanoparticles point to a preferable label, which can be used to amplify the signals inside nanostructures. By conjugating the streptavidin-gold nanoparticles with the nanostructures that are immobilized with the complementary probe, the hybridization of the biotinylated target with the probe can be effectively visualized. The DNA molecules within the nanostructured arrays maintain their activity, as demonstrated by hybridization using streptavidin-Cy 5 conjugate imaged by fluorescence microscopy. Both detection methods indicate that the hybridization occurs between a pair of complementary duplexes. Compared to fluorescent detection, the nanoparticle-based method is not capable of high-resolution detection of DNA nanostructures on ODS-SAM template mainly because of the adsorption of protein on the hydrophobic surface. The proposed approach is preliminary, but may lead to the development of an effective way of generating DNA nanoarrays if a hydrophilic template is chosen. Further research focused on detection of smaller features based on gold nanoparticles on a hydrophilic SAM is underway. Such nanostructured DNA arrays fabricated by EB lithography of self-assembled monolayer based on gold nanoparticle detection are expected to make possible the easy and simple readout.

6. ACKNOWLEDGMENTS

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