EXTENSION, IMMOBILIZATION AND CHEMICAL MODIFICATION OF DOUBLE-STRANDED DNA ON A SOLID SURFACE - TOWARD DIRECT SEQUENCING WITH MICROSCOPY -

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ABSTRACT

This paper reports a novel method to chemically modify specific nucleotides in a stretched double-stranded DNA, and to label the modified nucleotides with fluoresceinated antibodies in order to visualize them using both fluorescence microscope and AFM. We show that the modification is possible even with 48.5 kb DNA stretched to the full length and immobilized on a solid surface, where the bases are forming closed pairs and the interaction with the surface can cause steric hindrance to chemical reactions. This method is expected to be used not only for a high-resolution microscope-based direct DNA sequencing, but also for a method to align molecules and nano-particles using DNA as a template.

KEYWORDS: DNA, Sequencing, Immunofluorescence staining, Fluorescence microscopy, AFM

INTRODUCTION

Developing techniques of high-throughput DNA sequencing is one of the important topic in the field of genomic researches, because current techniques have not been enough to be applied for the medical care based on the individual genomic information due to their speed and cost. Therefore, there have been various approaches to overcome this issue, for example, direct observation of individual DNA synthesis [1], amperometric/optical detection of bases in DNA using a nano-pore [2,3], direct sequencing with a high-resolution microscope [4].

Recently, we have developed a technique to chemically modify specific nucleotides in DNA (Figure 1A) [5], so that the bases A, T, G, C can have distinct size difference, and the sequence to be determined using high-resolution microscopy. The requirements toward this novel sequencing method are, the stretched conformation of DNA for spatially resolving the bases, and high yield of modification without fragmentation of the DNA. The length of DNA to be handled should be longer than 10 kb to be competitive with existing sequencing methods. There are two ways to have stretched and modified DNA; stretch "before" modification or "after", but we found that DNA strands become fragile and break easily, so that "stretch before" scheme is employed in this paper.

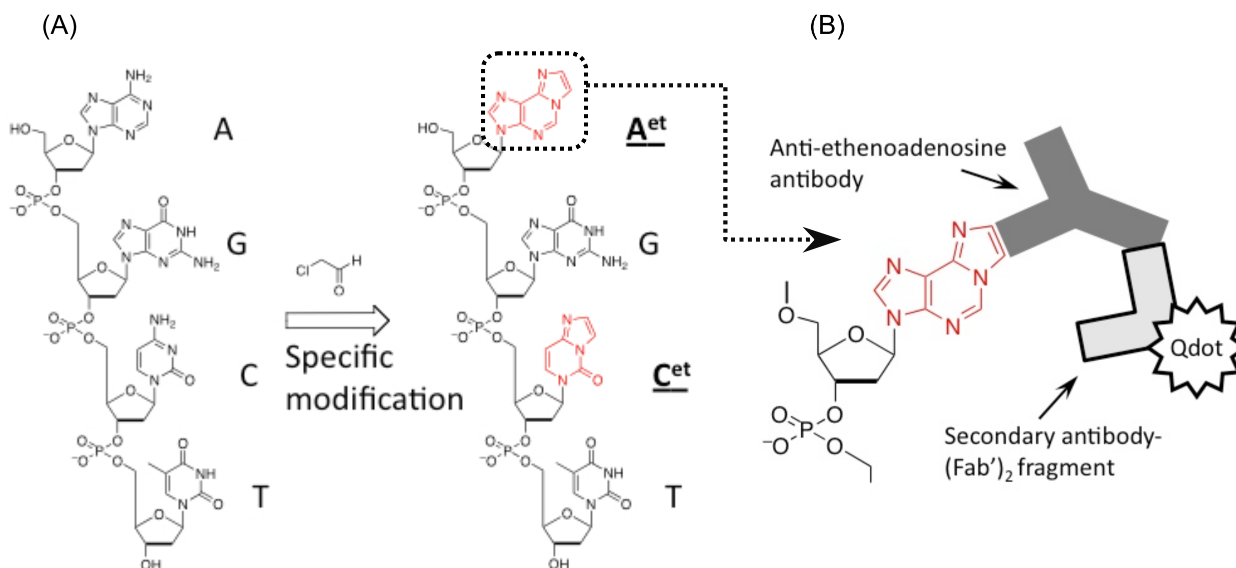

Figure 1: (A) Illustration of the modification of nucleotides (adenine and cytosine). By this modification, ethenoadenosine and ethenocytidine are produced (right) and thus, each nucleotide type can be distinguished from other nucleotide type easier by its molecular weight. (B) Schematic illustration of the visualization of the ethenoadenosine (not in scale). The position of the ethenoadenosine along the DNA strand is visualized using its specific antibody followed by Qdot-conjugated secondary antibody.

EXPERIMENTAL

λ DNA (48.5 kb, 16.5 μm) was employed as the sample and the chemical modification carried out in a reaction solution containing 1.75 M chloroacetaldehyde and 175 mM NaOAc (pH 5.5) at 37 °C. First, in order to estimate appropriate time for the reaction on a solid surface, the degree of etheno adduct was investigated by gel electrophoresis of chemically modified DNA in a microtube for 0-24 h, followed by staining with SYBR Gold. Then, DNA modification on the solid surface was carried out as follows: DNA was first stretched out and immobilized onto an amorphous carbon thin layer (formed through vapor deposition onto a cover slip) by molecular combing [6]. The amorphous carbon thin layer (ca. 10 nm) was chosen as a solid surface to simulate transmission electron microscope observation. Then the fixed DNA strands were exposed to the reaction solution. To visualize the degree of etheno adduct, monoclonal anti-ethenoadenocine antibody was added, that was fluoresceinated by a secondary antibody, i.e., anti-mouse IgG $(Fab')_2$ conjugated with Qdot (Figure 1B). Finally, the solid surface was rinsed with PBS buffer. The observations were carried out using fluorescence microscopy (in wet condition) or AFM (in dry condition).

RESULTS AND DISCUSSION

Figure 2 shows the dependence of the mobility of λ DNA on the reaction time of the chemical modification. The reaction time varied: 0-24 h. It is seen that as the reaction time become longer, the mobility gets smaller. This mobility-shift indicates increase of the molecular weight of the DNA molecule due to productions of ethenoadenosine and ethenocytidine. At 24 h reaction (lane 8), the band is slightly smear and fragmentation of the DNA seems to be occurred. Thus, we decided reaction time to be 20 h for further experiments.

Figure 3 shows example image of Qdots under a fluorescence microscope. The length of the modified DNA in the photo is about 20 μm, slightly elongated than its intrinsic length by combing, but most of them have similar lengths, showing molecular breakage did not take place during the chemical reaction. The observed fluorescence intensity is almost uniform along the DNA, indicating that the reaction took place as in the case of the solution-based experiment, even the double-stranded DNA is immobilized on the surface.

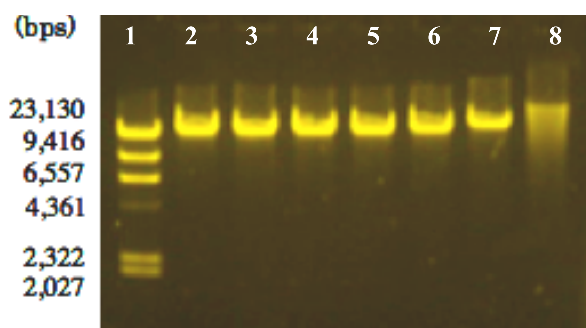

Figure 2. Dependence of the mobility of λ DNA on the reaction time of the chemical modification. Lane 1, marker, lambda/Hind III digest; 2, lambda DNA without reaction solution; 3, 0 h; 4, 0.5 h; 5, 2 h; 6, 4 h; 7, 8 h; 8, 24 h reaction.

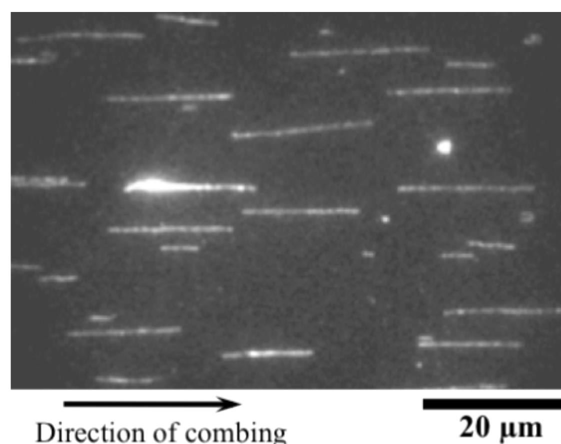

Figure 3. Example fluorescence image of the nucleotide-modified DNA molecules visualized by the fluorescence labeled antibody. λ DNA molecules (48.5 kb, 16.5 μm) are fixed with elongated conformation on the solid surface utilizing dynamic molecular combing. Then, chemically modified using chloroacetaldehyde for 20 h. After the modification, modified adenosine (i.e., ethenoadenosine) is visualized by Qdot through its specific antibody and secondary antibody.

To investigate in more detail, we conducted AFM imaging, whose typical result is shown in Figure 4. From the size analysis, the dots in the photo are identified as Qdots, which cover the entire length of DNA that extends about 20 μm, as observed in fluorescence observation of Figure 3. As seen in the uppermost (most enlarged) photo, DNA is labeled by Qdots rather uniformly, with the spacing of several ten nm. Considering the size of an antibodies-Qdot complex is about 30 nm in diameter, they are packed densely along the DNA.

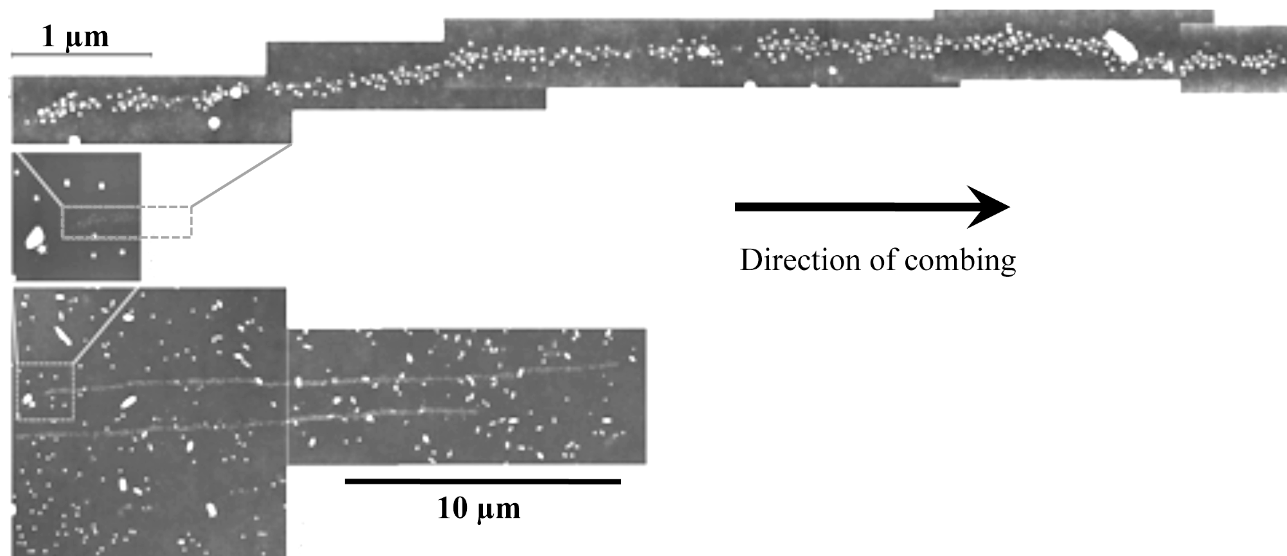

Figure 4. Example AFM image of nucleotide-modified DNA molecules with ethenoadenosine antibodies and Qdot-conjugated secondary antibodies (tapping mode). The procedure of sample preparation was the same as what brought Figure 3. The uppermost-enlarged image (obtained by scanning smaller areas) shows that an elongated string like image observed in low magnification is consisted of a large number of nano-particles.

CONCLUSION

In conclusion, we have successfully demonstrated the chemical modification and its visualization of stretched DNA immobilized on a solid surface. The method may have an application not only for direct sequencing, but also for DNA-based molecular or nano-particle constructions.

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