Preparation of Oligonucleotide Arrays with High-Density DNA Deposition and High Hybridization Efficiency[†]

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Received June 5, 2004

In DNA microarray produced by DNA-deposition technology, DNA-immobilization and -hybridization yields on a solid support are most important factors for its accuracy and sensitivity. We have developed a dendrimeric support using silylated aldehyde slides and polyamidoamine (PAMAM) dendrimers. An oligonucleotide array was prepared through a crosslinking between the dendrimeric support and an oligonucleotide. Both DNA-immobilization and -hybridization yields on the solid support increased by the modification with the dendrimers. The increase of the immobilization and hybridization efficiency seems to result from a three-dimensional arrangement of the attached oligonucleotide. Therefore, our dendrimeric support may provide a simple and efficient solution to the preparation of DNA microarrays with high-density DNA-deposition and high hybridization efficiency.

Key Words : Dendrimer, DNA chip, Hybridization, Immobilization, Oligonucleotide

Introduction

Microarray-based hybridization analysis is the most widely used technology with diverse applications. This technique evolved from key insight that a nucleic acid molecule could be used to detect other nucleic acid molecule attached to a solid support, through hybridization. With the development of array technology, a number of solid supports and various attaching methods have been published.[1-7] Glass slides are generally used as standard supports for making microarray. The deposition of pre-fabricated nucleic acids and direct *in situ* synthesis on the solid surface are prevailing protocols to produce DNA array. Despite the extremely high information content of the direct *in situ* synthesis,[8] the deposition of nucleic acids has its own advantages, including easy construction, a high capacity for long-length DNA, and excellent flexibility[9] Many researchers have focused on the production of better solid supports to improve the deposition protocol. Modified glass slides are developed as the best supports because they can be easily produced, and they usually provide high sensitivity.[1,9-11] Currently, glass slides that contain high-density amine or aldehyde groups on their surface are widely used for the immobilization of nucleic acids. Amine or aldehyde groups react with other functional groups on nucleic acids to form covalent bonds. These amine- or aldehyde-modified glass supports can accommodate short-length oligonucleotides, as well as long-length cDNAs.

However, the array technology using the modified glass support is still hampered by both the low-surface density of attached nucleic acids and the inefficient hybridization between target and probe nucleic acids. Many researchers developed various solid supports to overcome these limitations: i) Several techniques using polyacrylamide or agarose gels were applied to develop three-dimensional supports, which consequently provided a great capacity of immobilization.[3,12,13] ii) Various spacers were introduced between solid supports and attached nucleic acids to improve the yield of hybridization.[14,15] However, most of the methods are complex and should be used only under specific conditions. Recently, some researchers[16,17] used polyamidoamine (PAMAM) dendrimers, which are a new class of polymers containing radially branched amidoamine groups beginning with either a nucleophilic or an electrophilic core,[18,19] in modifying silylated amine or epoxy slides, as solid supports, in order to increase the surface density of nucleic acids attached the supports. They showed that hybridization signals were increased by the modification with the dendrimers. However, it was not clear yet whether the increase of hybridization signals resulted from the increase of immobilization of oligonucleotides or from the improvement of hybridization yield. In this study, we modified silylated aldehyde slides with PAMAM dendrimers because Schiff base formation between glass-aldehyde and dendrimer amine groups is followed by the treatment with sodium borate that reduces a background signal.[20] Here, we report, using the modified aldehyde slides with PAMAM dendrimers, that the dendrimeric surface improved both the immobilization of oligonucleotides and the hybridization yield.

Experimental Section

Chemicals and oligonucleotides. All chemicals were purchased from Sigma-Aldrich Korea (Seoul, Korea), unless stated otherwise. Silylated aldehyde-coated slides (CSS Silylated Slides®) were purchased from Cel Associates (Huston, Texas, USA). Oligonucleotides used for immobilization and hybridization were purchased from Genotech (Daejeon, Korea).

Preparation of dendrimeric glass support. The aldehyde slides were pre-cleaned by washing with water and methanol. The pre-cleaned aldehyde slides were immersed in methanol containing 0.5% PAMAM dendrimer generation 3 (G3) or generation 4 (G4) for 12-16 h. The slides were washed three times with methanol and dried for 30 min under a vacuum. The unreacted aldehyde groups were reduced to non-reactive primary alcohols by a NaBH₄ solution (0.05 g NaBH₄ dissolved in 30 mL of 100% methanol) for 5 min and the slides were washed as above.[21] For linker formation, the slides were incubated with 0.2% 1,4-phenylene diisothiocyanate (PDC) in 10% pyridine/ dimethyl formamide for 3 h under Ar and washed with methanol ten times. The PDC-treated slides were kept in a vacuum desiccator before the immobilization of oligonucleotides.
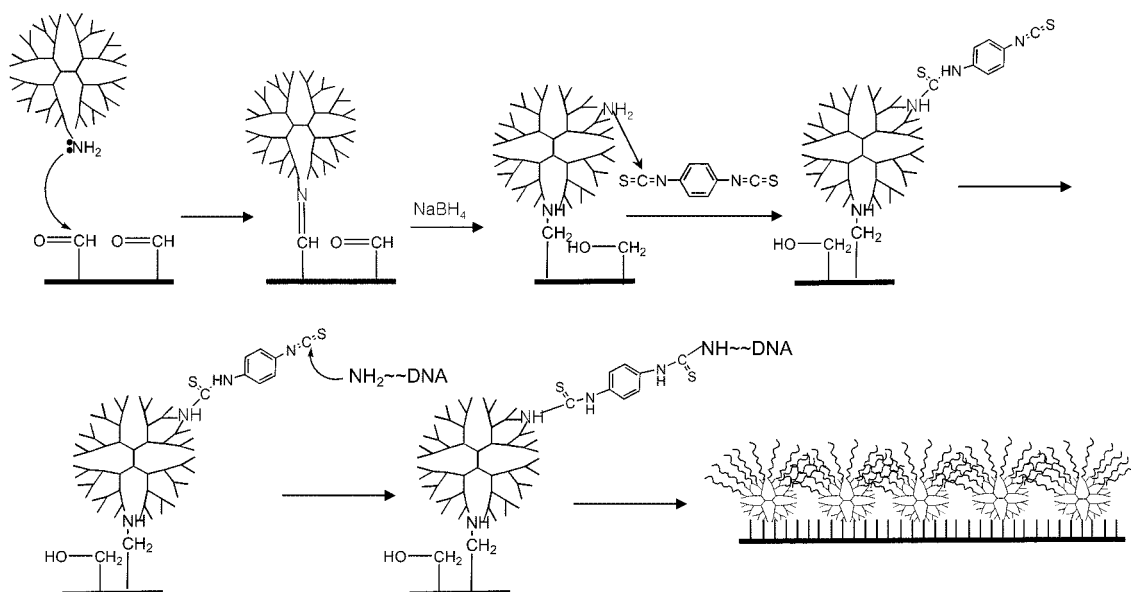
Immobilization of oligonucleotides. For the immobilization of olignucleotides, a 3'-amine modified oligonucleotide (5'-CCGACCGGAATAAAT-NH₂-3') was spotted on slides manually (500 nL/spot). The oligonucleotide was labeled with [γ-³²P]ATP and T4 polynucleotide kinase at the 5'-end, as described,[22] when required. The labeled oligonucleotide was purified through a G50 column before use. The oligonucleotide-spotted slides were dried at room temperature for about 16 h. The slides were washed with water, 3 M NH₄OH, and finally a Wash Buffer I (1× SSPE, 0.2% SDS) to clearly remove unbound oligonucleotides. For quanti-

fication of immobilization, the labeled oligonucleotide was used and the amount of the immobilized oligonucleotide was quantitated by analyzing the slide with a Molecular Dynamics PhosphoroImager.

Hybridization. For hybridization, the complementary oligonucleotide (5'-ATTTATTCCGGTCGG-3') was labeled with [γ-³²P]ATP and T4 polynucleotide kinase at the 5'-end. The oligonucleotide-deposited slides were pre-hybridized in a Hybridization Buffer (5× SSPE, 0.2% SDS) for 2 h followed by the addition of the ³²P-labeled complementary oligonucleotide to a final concentration of 2 pmol/mL. Hybridization was performed at 42 °C for 16 h, as described.[23] Unhybridized probes were washed away with a Wash Buffer I (1× SSPE, 0.2% SDS) followed by a Wash Buffer II (0.1× SSPE, 0.2% SDS) at 40°C for 30 min. The hybridization signals were quantitated by analyzing the slide with a Molecular Dynamics PhosphoroImager.

Results and Discussion

Dendrimer monolayer immobilized on several supports, such as Au electrodes and silicon wafers, are known to be highly stable and nearly closed-packed.[24,25] The terminal groups of the immobilized dendrimers can be post-functionalized easily without their loss from the surface.[24] A computer based simulation of the dendrimer monolayer formation has revealed that the high-generation dendrimers tend to flatten and spread on the surface, while the low-generation dendrimers, such as generation 1 (G1) or G2, retain their bulk-phase conformation, but are less immobilized.[23] Therefore, we assumed that G3 or G4 dendrimers retain the three-dimensional identity of dendrimers with high immobilization when forming the monolayer on the solid support. For this reason, in this study, we used PAMAM G3 and G4 dendrimers to generate dendrimer surfaces. Figure 1 shows the reactions that we carried out for the generation of


Figure 1. Modification of solid support with dendrimers and immobilization of oligonucleotides on the modified support.

dendrimeric solid supports. The dendrimer layer was formed on the glass surface via the Schiff base chemistry between glass-aldehyde and dendrimer amine groups. The coupling of a dendrimer-amine group with 1.4-phenylene diisothiocyanate (PDC) led to the formation of a phenylthiocyanate group on each terminus of the dendrimer. This phenylthiocyanate group functions as a reactive group for the bond formation with the 3'-amine group of oligonucleotides.[14] It also increases the diameter of the dendrimer. which provides an additional space between adjacent oligonucleotides when they are coupled.

A 17-mer oligonucleotide labeled with ^{32}P at the 5'-end was deposited on the PDC-treated dendrimeric surface, and the surface density of the immobilized oligonucleotide was quantitated (Fig. 2). The amount of the immobilized oligonucleotide on the dendrimeric support increased linearly with the concentration of the input-oligonucleotide. The signal of the immobilized oligonucleotide on the dendrimeric solid supports was 1.5- to 4.5-fold higher than that of the aldehyde support (Fig. 2B). This finding indicated that the modification with dendrimers increased the immobilization of the oligonucleotide on the solid support. The diameters of PAMAM G3 and G4 dendrimers are ~40 Å and ~50 Å. and the number of their terminal amine groups are 32 and 64, respectively.[24] The proposed model of molecular ordering assumes that they have highly deformed ellipsoidal shapes in condensed monolayers.[24,25] The coupling with PDC at the terminal end increases the diameter by ~17 Å. Therefore, the dendrimeric monolayer can provide active thiocyanate groups with the surface density of ~0.06 nmole/cm². This surface density gives an average distance of ~18 Å between adjacent thiocyanate groups, which is nearly same as the diameter of the DNA helix.[15,26-28] On the other hand. two-dimensional solid supports have the surface density of terminal functional groups of ~0.3 nmol/cm² with the distance of ~10 Å between adjacent functional groups.[14] However. the dendrimeric support carried more oligonucleotides than did the aldehyde support (Fig. 2), despite the lower availability of functional groups for the bond formation with oligonucleotides. In this respect, an appropriate three-dimensional spacing between terminal functional groups on the solid support may be essential for providing a high yield of immobilization of oligonucleotides.

The hybridization efficiency of the oligonucleotide on the dendrimeric support was determined by using the complementary oligonucleotide. In this experiment, we spotted an unlabeled oligonucleotide on the dendrimeric support and performed the hybridization reaction with the complementary oligonucleotide labeled at the 5' end with ^{32}P (Fig. 2). The hybridization signals of the dendrimeric support were 4.5- to 8-fold higher than those of the aldehyde support depending on the concentration of the input-oligonucleotide. In most cases. the increase of both the immobilization and the hybridization signals was a little higher when it was modified with G4 dendrimers than with G3 dendrimers. Since the increase of the immobilization contributed to the
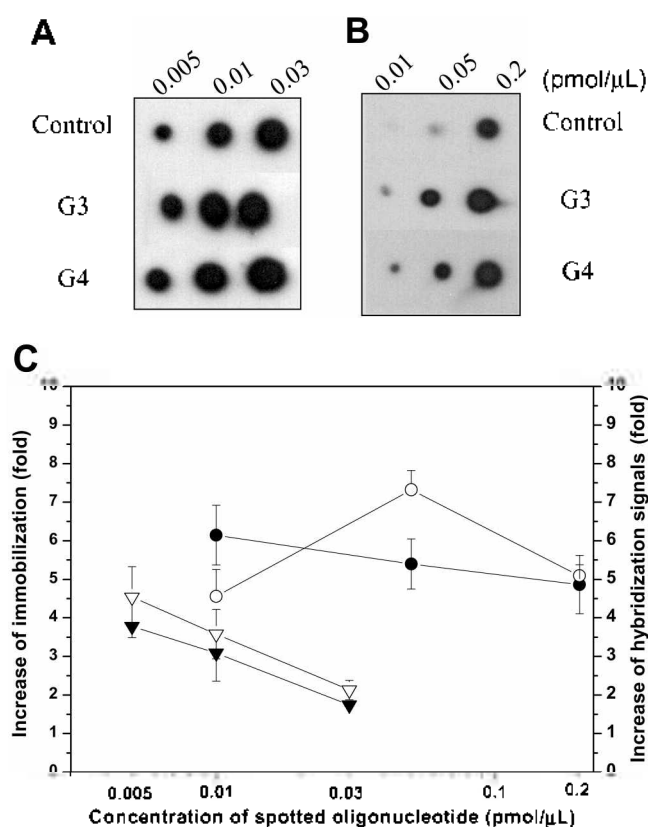
Figure 2. Immobilization of oligonucleotides and hybridization to the immobilized oligonucleotides on the dendrimeric support. (A) The ^{32}P-labeled oligonucleotides were spotted on the indicated supports and the amounts of the immobilized oligonucleotides were quantitated by analyzing the slides with a Molecular Dynamics PhosphoroImager. (B) The oligonucleotides immobilized on the dendrimeric support were hybridized with the ^{32}P-labeled complementary oligonucleotides. The hybridization signals were quantitated by analyzing the slides with a Molecular Dynamics PhosphoroImager. Control, unmodified aldehyde slide; G3, modified support with dendrimer G3; G4, modified support with dendrimer G4. The concentration of the spotted oligonucleotides was indicated above the figure. (C) The increase of the immobilization or the hybridization signals was represented as a ratio of the value on the dendrimeric slide to the unmodified aldehyde slide. Triangles and circles show immobilization and hybridization, respectively. Slides modified with G3 and G4 dendrimers are represented with filled and open symbols, respectively. All quantitations are means of at least four different determinations.

increase of the hybridization signals. the actual increase of the hybridization efficiency by the dendrimeric support would be 2- to 3-fold. Among factors affecting the hybridization efficiency of nucleic acids. steric hindrance is the most important factor.[15] As described above, the space between immobilized oligonucleotides in the dendrimeric solid support is expected to be wide enough to accommodate the incoming oligonucleotide probe. Therefore, this property probably contributed to the improvement of the hybridization yield.

In many cases of DNA microarray using modified glass slides. the improvement of the surface density of immobi-

lized nucleic acids usually accompanies the increase of non-specific binding of the probe to the activated surface.[14] The high background signal by the non-specific binding could significantly reduce the sensitivity of array analysis. However, we did not observe any nonspecific binding of the probe to our dendrimeric surface (data not shown). This finding indicates that the dendrimeric surface does not have nonspecific binding affinity to oligonucleotides. The absence of the nonspecific binding affinity suggests that all amino terminal groups of the dendrimer were converted to thiocyanate groups by the reaction with PDC. Otherwise the unreacted amino groups with positive charges could interact electrostatically with negative-charged oligonucleotides.[28] Alternatively, the treatment of the surface with sodium borohydride to reduce unreacted glass aldehyde groups after the cross-linking reaction between glass aldehyde and dendrimer amine groups might contribute to the absence of the nonspecific binding affinity presumably by reduction of trace impurities of molecules, which otherwise would lead to nonspecific binding of oligonucleotides.[20]

In summary, we have developed a three-dimensional support using silylated aldehyde slides and PAMAM dendrimers. An oligonucleotide array was prepared through a covalent crosslinking of oligonucleotides on the dendrimeric support. Both the immobilization and hybridization yields were increased by the modification with the dendrimers. We reasoned that the increase of the immobilization and hybridization efficiency would result from a three-dimensional arrangement of the attached oligonucleotide. With this feature, the dendrimeric solid support may provide a simple and efficient solution to the preparation of DNA microarrays with high-density DNA deposition and high hybridization efficiency.

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