Optimization of on-chip elongation for fabricating double-stranded DNA microarrays

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Abstract

The sequence-specific recognitions between DNA and proteins are playing important roles in many biological functions. The double-stranded DNA microarrays (dsDNA microarrays) can be used to study the sequence-specific recognitions between DNAs and proteins in highly parallel way. In this paper, two different elongation processes in forming dsDNA from the immobilized oligonucleotides have been compared in order to optimize the fabrication of dsDNA microarrays: (1) elongation from the hairpins formed by the self-hybridized oligonucleotides spotted on a glass; (2) elongation from the complementary primers hybridized on the spotted oligonucleotides. The results suggested that the dsDNA probes density produced by the hybridized-primer extension was about four times lower than those by the self-hybridized hairpins. Meanwhile, in order to reduce the cost of dsDNA microarrays, we have replaced the Klenow DNA polymerase with Taq DNA polymerase, and optimized the reaction conditions of on-chip elongation. Our experiments showed that the elongation temperature of 50°C and the Mg²⁺ concentration of 2.5 mM are the optimized conditions in elongation with Taq DNA polymerase. A dsDNA microarray has been successfully constructed with the above method to detect NF-κB protein.

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1. Introduction

The sequence-specific interactions between DNA and proteins play important roles in regulating cellular processes such as transcription [1], recombination [2], restriction [3], replication [4] and DNA–drugs interaction [5,6]. Several methods have been developed to analyze the DNA–protein sequence-specific interactions, which mainly are gel-shift assays [7,8] and DNasel footprinting assays [9]. However, they are laborious, time-consuming, and incapable of high-parallel analysis.

DNA microarray has provided a platform for high-throughput detecting different biological molecules and analyzing the interaction between them, which has been successfully used for mRNA expression analysis [10], mutation analysis [11], deletion strain analysis [12], and now used in analyzing DNA-binding proteins [13,16]. The present studies of double-stranded DNA (dsDNA) microarrays suggested that these kinds of microarrays are well suited for the analysis of DNA–protein interactions, particularly for the discovery of the sequence recognition by transcription factors. However, only limited publications on dsDNA microarrays in scientific journals, which might be due to several technical and economic problems. Until now, most dsDNA microarrays were fabricated through making the ssDNA microarrays firstly and then converted the ssDNAs into dsDNAs by adding another complimentary sequences on the ssDNA microarrays. The ssDNA could be fabricated either by on-chip synthesis [13] or by the spotting methods [14–18]. The former method could make high-density microarray, while the spotting method has been widely used due to easy making and economic reasons. The conversion of ssDNA to dsDNA could be conducted by two processes. One was to convert an ssDNA microarray into a dsDNA microarray directly by hybridizing with a mixture of complementary oligonucleotides [17,18]. Another approach was to anneal one common primer onto ss-
DNA probes firstly and extend the primer along the different ssDNA probes by enzymatic reactions [13–16]. However, the former method was suffered from its high fabrication cost due to its necessity in synthesizing a number of the different complementary oligonucleotides and its difficulty in fabricating dsDNA microarrays with the sequence-similar probes, such as the probes for single-nucleotide variations [17,18]. Most published dsDNA microarray relied on the surface photo-addressable synthesis of oligonucleotides that currently is expensive and proprietary. The efficiency of the single-step synthesis of oligonucleotides was thought to be only 92–96% [19,20], that is, only 4–20% of the sequences on a chip could be desired length and sequence for a 40mer oligonucleotides [21]. Furthermore, oligonucleotide microarrays constructed in this fashion are easy to be contaminated with truncated molecules [18,22]. The presence of competing truncated molecules and single-stranded oligonucleotides might interfere and mislead binding experiments [21]. Finally, considering the possible instability of dsDNA towards binding or washing reactions, the usefulness of this kind of dsDNA microarrays might be diminished.

In order to develop dsDNA microarray techniques for practical applications, we have presented several new methods to fabricate dsDNA microarrays economically for detecting DNA–protein interactions [14–16]. A series of oligonucleotides containing a certain consensus of a DNA-binding protein and a hairpin in the 3′ end were chemically synthesized on microscope glasses with Klenow DNA polymerase. (a) Method I: elongation of self-hybridized hairpin of the spotted oligonucleotides on microscope glasses with Klenow DNA polymerase. (b) Method II: elongation of primer hybridized on spotted oligonucleotides with Taq DNA polymerase. In this paper, a comparative study on the elongation efficiency from both self-hybridized hairpin and the complementary primers hybridized on spotted oligonucleotides will be discussed. In most previous studies, Klenow DNA polymerase was usually used in elongation process for making dsDNA probes, which are almost too much at the temperature as low as 37 °C. The elongation reaction at the temperature would be expected to produce branched or ‘christmas tree’ structures. In order to produce the consistent dsDNA probes and reduce the cost of the enzyme, we replaced Klenow DNA polymerase with Taq DNA polymerase, and optimized the reaction conditions of on-chip elongation. We used Taq DNA polymerase to produce dsDNA microarrays, which can specifically bind to Cy3 labeled NF-kB protein.

2. Materials and methods

2.1. Preparation of glutaraldehyde-derived glass slides

The aminosilane-derived glass slides were cleaned with distilled water and incubated in 5% glutaraldehyde in 0.1 M PBS, pH 7.4, for 2 h. Then the slides were thoroughly washed twice with methanol, acetone and deionized distilled water, and dried.

2.2. Preparation of probes and primer

Three kinds of special single-stranded oligonucleotides were chemically synthesized from Shengyou Inc. (Shanghai, China) for manufacturing dsDNA microarrays (summarized in Table 1). Probe I was a constant oligonucleotide consisting of a 10-base consensus of protein dNF-kB p50 homodimer (5′...GGGACTTTC...3′) at the middle, 12-base proximal flanking sequence linked with −NH2 at the 3′ end and a 14-base sequence with a hairpin allowed being self-hybridization.

Probe II was a target oligonucleotide containing a 5-base proximal flanking sequence labeled with −NH2 at the 3′ end, a 16-base sequence allowed to annealed with its complementary oligonucleotides (primer), which contained the NF-kB protein DNA-binding site, and a 13-base distal flanking sequence contained 3-base A.

2.3. Fabrication of dsDNA microarrays

To fabricate dsDNA microarrays with the Method I (shown in Fig. 1a), the probe I dissolved in sodium carbonate buffer (0.1 M, pH 9.0) was spotted in the concentration of 80 μM onto glutaraldehyde-derived glass slides with a pin-based robot PixSys5500 (Cartesian Technology Inc.). The glasses were incubated in 37 °C for 1 h and treated with NaBH4 (0.28% (w/v) NaBH4/76% (v/v) PBS/24% (v/v) alcohol) for 1 h. Then rinsed with 2 × SSC/0.5% SDS and deionized distilled water, and dried. As shown in Table 1, the probe I could be hybridized with itself in the 3′ end hairpin. The glasses were incubated in boiling water for 2 min and incubated with hybridization buffer for 1 h at 50 °C then the 25 μl Klenow DNA polymerase (Amersham Pharmacia Biotech, UK) elongation system, which included 1 x nick translation buffer, 4 μM dATP, 4 μM dCTP, 4 μM dGTP, 0.02 μM Cy3-dUTP (Amersham Pharmacia Biotech, UK), 3U Klenow DNA polymerase I was added onto the microarrays, 37 °C for 1 h. Subsequently the microarrays were rinsed with 2 x SSC/0.5% SDS and deionized distilled water, and dried.

Fig. 1. Two methods used to manufacture dsDNA microarrays. (a) Method I: elongation of self-hybridized hairpin of the spotted oligonucleotides on microscope glasses with Klenow DNA polymerase. (b) Method II: elongation of their complementary primers hybridized on spotted oligonucleotides on microscope glass with Klenow DNA polymerase.
Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Length (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe I</td>
<td>5′-NH2TTTAGTGGAGGGGACCTTTGCCGGGACGCTTAAAGCTT...3′</td>
<td>41</td>
</tr>
<tr>
<td>Probe II</td>
<td>5′-TAATTTATATCCCGGGAAGCCCTCTTGT...NH2...3′</td>
<td>34</td>
</tr>
</tbody>
</table>

To fabricate dsDNA microarrays with Method II (shown in Fig. 1b), the probe II was printed onto glutaraldehyde-derived glass slides and hybridized with the primer. To convert the ssDNA microarrays into dsDNA microarrays, the glasses were incubated in boiling water for 2 min and added with primer in the concentration of 100 nM in hybridization buffer and then put into a humid incubation chamber in 55°C for 1 h, rinsed with 2× SSC/0.5% SDS and deionized distilled water, and dried. The same 25 μl Klenow DNA polymerase (Amersham Pharmacia Biotech, UK) elongation system as in Method I was added onto the microarrays, 37°C for 1 h. Subsequently the microarrays were rinsed with 2× SSC/0.5% SDS and deionized distilled water, and dried.

2.4 Optimization of the reaction conditions of the elongation by using Taq DNA polymerase

In Method I, we replaced Klenow DNA polymerase with Taq DNA polymerase and optimized the reaction conditions of on-chip elongation.

To optimize the temperature and Mg2+ concentration for the Taq DNA polymerase reaction system, experiments were taken in seven different temperatures (37, 45, 50, 55, 60, 65, and 72°C) under 2.0 mM Mg2+, respectively. Subsequently, six different concentrations of Mg2+ (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM) were used. The Taq DNA polymerase reaction system included 1× reaction buffer, 20 μM dATP, 20 μM dCTP, 20 μM dGTP, 0.02 μM Cy3-dUTP (Amersham Pharmacia Biotech, UK). The microarrays were then rinsed with 2× SSC/0.5% SDS and deionized distilled water, and dried.

2.5 The binding reaction of dsDNA microarrays created with Cy3 labeled rhNF-kB protein

The 45 ml rhNF-kB protein (human recombinant p50 expressed in bacteria from a full-length DNA encoding 453 amino acids, Promega) was centrifuged at 5000 rpm for 5 min with BioRad Biospin P6 column. The liquid was spun off and the protein was washed with phosphate-buffer saline (PBS) solution (141 mM NaCl, 7.2 mM Na2HPO4, 2.8 mM NaH2PO4, pH 7.4) for two times. Then dissolved the protein in 0.1 M carbonate buffer (0.1 M, pH 9.0) at the concentration of 1 mg/ml. Added 25 μl carbonate buffer (0.1 M, pH 9.0) to a tube with FluoroLinkTM Cy3 monofunctional dye (Amersham Pharmacia Biotech, Piscataway, NJ) and mixed well. Transferred 1.5 μl Cy3 dye into protein solution and kept in reaction for about 30 min avoiding light and mixed round every 10 min. Spun off carbonate buffer and dissociated Cy3 dye at 3000 rPm with BioRad Biospin p6 column. The protein above the column was rinsed with PBS for two times and dissolved in 100 μl PBS solution, and divided into 10 small packs, kept at −40°C for future use.

The three kinds of dsDNA microarrays were firstly hydrated in 10% BSA/PBS (0.01 M, pH 7.4) at 37°C for at least 1 h, then washed with 0.01 M, pH 7.4 PBS/0.1% Tween-20 for two times, 5 min/time, and then rinsed with 0.01 M PBS (pH 7.4) for 5 min, and dried. Dissolved 1 μl Cy3 labeled protein into binding buffer (10 nM HEPES, pH 7.9, 50 mM KCl, 0.0039 in EDTA, 2.5 mM DTT, 10% glycerol, 0.05% NP-40, 1–5% BSA) at the final concentration of 6.39 ng/μl. The protein was added to microarrays before mixing well. The microarrays were kept at the condition of avoiding light and at room temperature for 1 h. After the binding reaction, the microarrays were rinsed with 0.01 M, pH 7.4, PBS/0.1% Tween-20 for two times, 5 min/time, and then with 0.01 M, pH 7.4 PBS for 20 min, and dried.

All the slides were scanned by using ScanMicroarray® Lite (Packard Bioship Technologies) in the Cy3 channel at 85% laser power, 80% PMT gain, 5 μm resolution.

3. Results and discussion

Two methods were used to fabricate dsDNA microarrays in the present study. To compare these two different methods, we chemically synthesized two single-stranded oligonucleotides (probe I and probe II) and printed them onto glutaraldehyde-derived glass slides to fabricate ssDNA microarrays, respectively. The images and average fluorescence intensity of dsDNA microarrays manufactured with these methods were shown in Fig. 2. According to the fluorescence intensity in Fig. 2, the dsDNA probes density produced by Method II (Fig. 2B) was about four times lower than that by Method I (Fig. 2A and C). The results also indicated that the image of dsDNA microarray created with Method I was better than Method II in the signal consistency. The image created by using Taq DNA polymerase was also better than that created by Method II in both fluorescence intensity and signal consistency. As the study reported previously, in the case of the lowest probe density situation, the hybridization efficiency could be as high as 100% for the corresponding complementary sequences, whereas in the case of high probe density situation, the efficiencies could be dropped to 10%
In our experiments, the high-density of ssDNA probes on solid-phase has been achieved, because we spotted as high as 80 μM amino-labeled probes on slides. The hybridization efficiency might be low under such a high-density of the immobilized probes in Method II. Therefore, the lower fluorescence intensity of the dsDNA microarray by annealing with its complementary extension primer was obtained. On the other hand, hairpin structure probe could overcome the problem of the low hybridization efficiency by its self-hybridized process, which could obtain primer extension without the hybridization to the solution-phase targets. In this case, all the immobilized probes could be converted into dsDNA probes.

To determine the proper on-chip reaction temperature of Taq DNA polymerase, seven different temperatures under 2.0 mM Mg2+ concentration were performed in Method I. As shown in Fig. 3, the images of dsDNA microarrays, which extended at the temperature of 50 °C were most satisfied. The standard errors of the average fluorescent intensities (S.E., here T represented different temperature) of the images at the temperatures of 37, 45, 50, 55, 60, 65, and 72 °C were 2000, 3200, 3210, 8000, 8200, 8400 and 14,400, respectively. The fluorescence consistencies of the different temperature could be calculated from the ratios of S.E.37/S.E. T. So the signal consistencies of the images at seven different temperatures were calculated as 1.0, 0.625, 0.623, 0.25, 0.244, 0.238, and 0.139 for the temperatures of 37, 45, 50, 55, 60, 65, and 72 °C, respectively (as shown in Fig. 4). Therefore, the signal consistence in the images became worse when the elongation temperature increased. The low signal consistency of the image under the high-temperature was likely due to the reduced annealing efficiency of the hairpins. On the other hand, the fluorescence intensity was enhanced with the increasing of temperature, since the higher temperature was more suitable for Taq DNA polymerase. So the results suggested that dsDNA microarrays extended by Taq DNA polymerase could be carried out at the temperature of 50 °C.

In Taq DNA polymerase reaction system, the Mg2+ concentration usually plays an important role. We investigated the most favorable Mg2+ concentration in the reaction of Taq DNA polymerase elongation. Six Mg2+ concentrations were tested, and the reaction temperature of 50 °C was chosen. Results shown in Fig. 5 demonstrated that the variation of Mg2+ concentration affected the elongation reaction of Taq DNA polymerase. According to the present experiment results, the optimal Mg2+ concentration of 2.5 mM was selected, which was higher than the Mg2+ concentration in solution PCR reaction system (1.5–2.0 mM). It might be due to the surface extension on solid-phase.

To demonstrate the usage of microarrays made in this experiment, dsDNA microarrays created by using Method I, Method II and Method I elongated with Taq DNA polymerase were used to interact with Cy3 labeled rNF-kB protein. Fig. 6 shows the binding efficiency of NF-kB to the dsDNA probe. We can see that the NF-kB binding signal of
the microarray made by Method II is much lower than that by Method I. Furthermore, the results also show that the microarrays created with Taq DNA polymerase under the conditions of 50 °C and 2.5 mM MgCl2 had the same feature in the interaction between dsDNA and DNA-binding protein as the one with Klenow DNA polymerase.

**4. Conclusion**

Investigation of the DNA–protein interactions is important in the biological and medical research areas. The dsDNA microarray, as a novel type microarray, will become a powerful tool. It can be used in a way of high-parallel analysis in not only the interactions of DNA–protein, but also the concentrations of the DNA-binding proteins. In this study, we successfully replaced the Klenow DNA polymerase with Taq DNA polymerase in the method to elongate the hairpin into a complete dsDNA microarray on a microscope glass. We also optimized the reaction conditions of the Taq DNA polymerase elongation system and used this kind of microarrays to bind with Cy3 labeled rhNF-kB p50 monodimer. The optimization conditions for fabricating microarrays by Taq DNA polymerase were obtained, which are the extending temperature of 50 °C and MgCl2 concentration of 2.5 mM. Our researches provided a practical, reliable, and economical method for fabricating the dsDNA microarray and promote the studies of DNA-binding proteins and its interaction with the corresponding DNAs.

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**References**


