Photoassisted Recovery of DNA Molecules for On-chip Directed Evolution

Shingo Ueno1,2,3, Aiko Ono2, Ryo Kobayashi2, Yoko Tanaka2, Shusuke Sato2, Manish Biyani2,3 Naoto Nemoto1,3 and Takanori Ichiki2,3

1Department of Functional Materials Science, Faculty of Engineering, Saitama University, Saitama 338-8570, Japan
2Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, Tokyo 113-8656, Japan
3CREST, Japan Science and Technology Agency, Tokyo 102-0075, Japan

ueshin@bionano.t.u-tokyo.ac.jp

Directed evolution is a powerful approach to creating functional biomolecules, and a combinative use of microarray chip technology is expected to have a great potential for realizing a sophisticated directed evolution with higher speed and quantitative performance than ever before. Although “recovery” of evaluated and selected molecules followed by amplification is an essential step in the performance of directed evolution, conventional DNA or protein microarray chips are limited only to analytical use. In this study, we have developed a novel technology for DNA microarray that is applicable to an artificial Darwinian selection on a chip. DNA molecules were microarrayed on a gold surface via an oligonucleotide linker that contains a nitrobenzyl group as a photocleavable moiety and were recovered from the surface by spot-selective photoirradiation at few micrometer precision. The recovered DNA molecules were confirmed by polymerase chain reaction. Thus, in this study, we have realized a new function of biomolecular microarray technology that is useful for the establishment of on-chip directed molecular evolution.

Keywords: directed evolution, in vitro selection, DNA chip, protein chip, photo cleavage, high-throughput screening

1. Introduction

Since the 1990s, directed evolution has been applied in the laboratory evolution of functional biomolecules [1]. A rational design of novel biomolecules (e.g., enzymes, peptides, functional DNAs/RNAs) has not been established because the relationship between the structure and function of biomolecules is still inadequately understood. In contrast, directed evolution does not require a complete solution to this difficult problem. Indeed, directed evolution has proven to be much more effective for generating many functional proteins including enzymes than using a rational design.

Conventional directed evolution is performed using organisms such as bacteria, yeasts and phages [2-4]. Recently, however, an organism-free method, namely, in vitro evolution has emerged, and its use in the generation of functional peptides, enzymes, DNA/RNA aptamers and DNA/RNA enzymes has been reported [5-7]. In vitro evolution has some advantages over organism-dependent methods; in particular, screening conditions are not necessarily limited to physiological conditions, and even severe conditions for organisms to survive are applicable. Although in vitro evolution methods have already been put into practical use, there remains much room for improving their screening process. Screening is usually performed in a single tube as a batch system without evaluating the function or activity of each molecule and is not quantitative enough for the unequivocal evolution of desired molecules.

Therefore, the development of quantitative
screening technology with a high throughput is required for performing a sophisticated evolution, and we believe that the use of microarray chip technology will fulfill this requirement. Microarray technology offers some significant advantages over the batch screening method. Microarray technology is suitable for high-throughput screening using automatic robotic and image analysis. Moreover, a quantitative evaluation of each molecule can be performed at once using microarrays, which is difficult in batch screening owing to the fact that molecules compete with each other according to specific features of the screen [8]. Figure 1 shows the concept of on-chip directed evolution.

To realize our concept of on-chip directed evolution, we need to prepare several element technologies. Recently, we have developed an in vitro evolution method termed cDNA display [9-10], which can evolve functional protein/peptide molecules using puromycin technology [11]. Moreover, immobilization of proteins and peptides on a microchip using the cDNA display [12] and other puromycin technologies [13-14] has already been achieved. The distribution of a large mutant library onto a microchip surface with the format of a single mutant per spot is required for carrying out the directed evolution, and we previously developed a method that satisfies this requirement [15]. In the method, 10^8 species of mRNA mutants could be patterned on a chip. However, we still do not have the technology for recovering genetic information on the immobilized proteins or peptides, which is essential for performing directed evolution. In this study, we developed a technology for recovering genetic molecules, which were immobilized on a chip, using a photocleavable molecule and photoirradiation.

2. Method

2.1. DNA and RNA preparation

Photocleavable oligonucleotide linker (PC linker): 5'-thiol-(CH2)6-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-(NB)-CCCGCCGCCCCCCTT CCT-3' was custom-synthesized by Nihon Bio-Service (Saitama, Japan) using general phosphoramidite chemistry using the reagents “5'-Thiol-Modifier C6 S-S” and “PC spacer” from Glen Research (VA, USA), where NB is a 2-nitrobenzyl group (see Fig. 3). The Cy5-modified mRNA encoding the GFP gene (839 mer) was prepared by in vitro transcription using RiboMAX-T7 (Promega, WI, USA) and Cy5-UTP (GE Healthcare, Little Chalfont, UK) from 869 bp of DNA containing a T7 promoter sequence [12].

2.2. Immobilization of PC linker on gold surface

A gold surface (200 nm thick) was prepared by evaporation on a glass substrate, which was preliminarily coated with a 10-nm-thick Cr layer. The piranha-cleaved gold surface was soaked with 1 µM PC linker in 1x phosphate-buffered saline (PBS) for 180-240 min, and then immersed in 1 mM 6-mercaptotetchedanol (MCH) for 60 min at...
room temperature. The surface was rinsed in 1x PBS and water and then spin-dried.

2.3. Preparation of APTES-modified glass and examination of cDNA adsorption

3-aminopropyltriethoxysilane (APTES)-modified glass was prepared as follows: a piranha-cleaned glass slide was soaked with 0.1 M sodium hydroxide for 1 min and blow-dried with nitrogen gas. The glass slide was soaked with 1 % APTES (Tokyo Chemical Industry, Tokyo, Japan) in 0.1 % acetic acid for 60 min at 90°C, followed by washing with ethanol and water, and blow drying with nitrogen gas.

DNA adsorption onto the APTES-modified glass was evaluated as follows: the Cy5-modified mRNA was reverse-transcribed with Cy3-dCTP using SuperScript III and the primer 5’-TTTCCCCGCCGCCCCCCTCTCGTCCCGCCTGATGAT-3’, followed by purification using a QIAquick PCR purification column (Qiagen, Hilden, Germany). The reverse-transcribed product was dissolved in a series of buffers with different pHs at a concentration of 10 nM. Each cDNA solution (3 µl) was dropped on the APTES-modified glass and incubated for 5 min. After incubation, the dropped solutions were removed, and adsorbed cDNA spots were analyzed by the fluorescence of the Cy3-modified cDNA using the fluorescent image scanner. The buffer solutions used in this examination were 29 mM glycine buffer for pH 2, 104 mM glycine buffer for pH 3, 123 mM acetic acid buffer for pH 4, 30 mM acetic acid buffer for pH 5, 50 mM MES buffer for pH 6, 23 mM MES buffer for pH 7, 25 mM Tris buffer for pH 7.5, 36 mM Tris buffer for pH 8, 175 mM Tris buffer for pH 9, 11 mM carbonate buffer for pH 10, 7.5 mM carbonate buffer for pH 11 and 1x PBS for pH 7.4. These buffers have the same ionic strength of 0.02 M without 1x PBS.

2.4. Micropatterning of mRNA and cDNA

mRNA was micropatterned by microintaglio printing as previously reported [12]. Briefly, a polydimethylsiloxane (PDMS) micromold plate with fine holes of 60 or 4 µm in diameter and depth was fabricated using an SU-8 mold. After sandwiching a droplet of 500 pM Cy5-modified mRNA between the PC linker-modified substrate and the PDMS micromold plate, the PC-linker and mRNA were hybridized by heating to 70°C and then gradually cooling to room temperature. The surface was washed successively with 3x PBS containing 0.02% Tween 20, 3x PBS and 1x PBS. The micropatterned mRNA was examined using the fluorescence of the Cy5-modified mRNA using a fluorescent image scanner (Typhoon, GE healthcare). The immobilized mRNA was reverse-transcribed as follows: 50 µl of a DTT-free reaction solution for SuperScript III (Invitrogen, Carlsbad, CA, USA) with Cy3-dCTP (GE Healthcare) was dropped on the surface and covered with a cover glass. The sample was incubated for 60 min at 45°C in a sealed chamber. The surface was washed successively with 3x PBS containing 0.02% Tween 20, 3x PBS and 1x PBS. The reverse-transcribed mRNA was examined using the fluorescence of the synthesized Cy3-modified cDNA using the above-mentioned fluorescent image scanner and a laser-coupled scanning confocal microscope (Digital Eclipse C1; Nikon, Tokyo, Japan).

2.5. Photocleavage and recovery of cDNA

The cDNA-immobilized surface was immersed in 1x PBS and sealed with the APTES-modified glass and silicone grease (high-vacuum grease; Dow Corning, Midland, MI, USA). cDNA-immobilized spots on the surface were observed by the fluorescence of the Cy3-modified cDNA, and a 377 nm laser (0.5-1.0 µJ/µm² for the spots of 60 µm diameter, 130-630 µJ/µm² for the spots of 4 µm diameter) was irradiated on any spots to cleave the nitrobenzyl group in the PC-linker using a laser-coupled scanning confocal microscope.

After irradiation, cleaved cDNA fragments, which were adsorbed onto the APTES substrate, were recovered by dissolving them in 30 µl of 11 mM sodium carbonate buffer (pH 10), which was dropped on the APTES surface. The recovered cDNA molecules were amplified by polymerase chain reaction (PCR) using Ex Taq polymerase (Takara Bio, Osaka, Japan) and the primers 5’-GATCCCGCGAATTATAGACTCATATAGGGGAAATTATTACAAATTTACATTACCCCA-3’ and 5’-TTTGTAGAGCTCACTCCATGCA-3’. The amplified products were analyzed by nondenaturing polyacrylamide gel electrophoresis (PAGE) with SybrGold (Invitrogen) staining.

3. Results and discussion

3.1. Procedure of photoassisted DNA recovery from chips

Figure 2 shows a schematic representation of our recovery technology. cDNA molecules (genetic molecules) are immobilized on a gold surface via a photocleavable oligonucleotide linker (i.e., PC linker, see Fig. 3) with a micrometer-scale spot
pattern. The cDNA molecules are detached from the surface by photolysis and recovered via adsorption onto the APTES-modified glass.

3.2. Preparation of APTES-modified glass and examination of cDNA adsorption.

For efficient recovery of the photocleaved cDNA with a low background, an APTES-modified glass was used as an adsorption substrate of the cDNA [16]. Prior to the photorecovery experiment, adsorption of DNAs onto the APTES substrate was investigated in a series of buffers with different pHs. Figure 4 shows the result of adsorption of the fluorescence-modified cDNA in the buffer solutions with different pHs, which were dropped on the APTES surface. The cDNA was efficiently adsorbed at pHs below 10 and hardly adsorbed at pHs 10 and 11. Therefore, we used 1x PBS (pH 7.4), which is commonly used in biological experiments, as the adsorption solution and 11 mM sodium carbonate buffer (pH 10) as the desorption solution.

3.3. Preparation of the photorecoverable DNA microarray

Biomolecules are known to be damaged by short-wavelength UV light (200-320 nm) radiation. Because a nitrobenzyl group can be cleaved using relatively long-wavelength UV light (320-400 nm), it is used in the field of biotechnology for the photopurification [17] or photopatterning [18] of biomolecules. Therefore, we synthesized the PC linker that possessed the nitrobenzyl group as a photocleavable moiety (Fig. 3). A self-assembled monolayer (SAM) of the PC linker on a gold surface was prepared as described in the method section. Microintaglio printing of the mRNA on the gold surface using the PDMS micromold plate with a fine-hole pattern 60 µm in diameter and depth was performed. Subsequently, reverse-transcription was performed on the same surface. Figure 5A shows a fluorescent image of Cy5-modified mRNA and Cy3-modified cDNA spots, which were patterned by microintaglio printing and reverse-transcription, respectively. Thus, fine patterns of mRNA and cDNA were successfully obtained.

Figure 3. Structure of photocleavable oligonucleotide linker (PC linker). The PC linker was composed of 18 mer single-strand DNA for hybridization with mRNA, the 2-nitrobenzyl group for photocleavage, 30 mer poly-deoxythimidine (T30) and hexane as a spacer and thiol for immobilization on the gold surface. The 2-nitrobenzyl group is easily cleaved by UV irradiation.
3.4. Photorecovery and amplification of cDNA

Spot-selective photocleavage and adsorption of cDNA onto the APTES substrate were investigated using the gold surface, on which 60-µm-diameter cDNA spots were patterned. After irradiation on three spots of cDNA at 377 nm wavelength using the laser-coupled scanning confocal microscope, the fluorescence intensity of irradiated spots decreased and three corresponding fluorescent spots were observed on the APTES surface (Fig. 5B). It was observed that 30-40% of the immobilized cDNA molecules were cleaved by these irradiations. This observation indicates that spot-selective photocleavage and adsorption of the cDNA onto the APTES substrate were successfully achieved. Finally, we performed the recovery and amplification of the spot-selectively photocleaved cDNA molecules using the gold surface on which 4-µm-diameter cDNA spots were patterned as shown in Fig. 6. After the photoirradiation and adsorption of cDNA onto the APTES surface, 30 µl of 11 mM sodium carbonate buffer (pH 10) was
dropped on the APTES surface of corresponding photoirradiated and nonirradiated areas, followed by recovery of the solutions. Two recovered solutions were subjected to PCR amplification. As a result, the sample recovered from the photoirradiated area was highly amplified compared with the sample recovered from the nonirradiated area (Fig. 6B). This result indicates that the spot-selective recovery and amplification of the cDNA molecules were successfully achieved in micrometer-scale spots. However, a slight nonselective recovery of cDNA molecules from the nonirradiated area was also observed (Fig. 6B). This may result from insufficient removal of nonspecifically adsorbed cDNA molecules or nonselective detachment of cDNA molecules due to the relatively weak bond between thiol and gold [19]. The nonselective recovery of genetic molecules from a chip causes a noise of selected genetic information and disturbs an efficient selection of functional biomolecules. Therefore, it is necessary to reduce the nonselective recovery of the cDNA molecules as much as possible.

4. Conclusions

We studied the photoassisted and spot-selective recovery of DNA molecules from microarrays to develop a novel platform for decoding genetic information for on-chip molecular directed evolution. The microspot array (4 or 60 μm in diameter) of DNA molecules was fabricated using microintaglio printing. The DNA molecules were immobilized on a gold surface via a photocleavable oligonucleotide linker, which contains a nitrobenzyl group as a photocleavable moiety. These DNAs were detached from the gold surface by spot-selective photoirradiation and recovered via adsorption onto the amino-modified substrate. Finally, the adsorbed DNAs were desorbed using alkaline buffer pH 10 and amplified by PCR successfully. The achievement in this study will enable the directed evolution of biomolecules on microchips.

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References