Fractionation of Single-stranded DNAs with/without Stable Preorganized Structures Using Capillary Sieving Electrophoresis for Aptamer Selection

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Aptamers, single-stranded DNAs/RNAs with a strong and specific interaction towards a target molecule, have wide applications in the fields of medicine and biosensors. In conventional aptamer selection methods, it is difficult to obtain “preorganized” and/or “induced-fit” type of aptamers selectively. In this study, separation and fractionation of single-stranded DNAs with/without stable preorganized structures were carried out using capillary sieving electrophoresis. The fractionated DNAs showed different mobilities and thermodynamic stabilities of their secondary structures; this outcome is deemed to be necessary for the synthesis of novel aptasensors with a desirable sensing mechanism.

Keywords Aptamer selection, capillary sieving electrophoresis, structure-induced aptamer, structure-preorganized aptamer

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Biosensing is one of the most important technologies wherein a target molecule is detected specifically using molecular recognition based on biomolecules, such as enzymes and antibodies. For example, immunoassays using various antibodies are widely employed in the antigen-based detection of pathogens, viruses, and various biomarkers. Antibodies show specific high affinities towards their target molecules; however, they are chemically unstable and expensive since they can only be obtained by in vivo synthesis. On the other hand, aptamers, which are single-stranded DNAs/RNAs with a strong and specific interaction towards their target molecules, have also attracted the attention of researchers owing to their unique characteristics. To begin with, aptamers are chemically more stable than antibodies. Next, their base and sugar structures can be easily modified for tuning their binding abilities and stabilities in vivo. They can be chemically synthesized in vitro and hence are less expensive than antibodies; this property also allows one to obtain an aptamer for a toxic compound, which is otherwise difficult to achieve with antibodies. For these reasons, aptamers are already being employed in aptamer-based biosensors (also called as aptasensors), such as fluorescence-based/electrochemical biosensors.

Aptamers are classified into two types, namely structure-induced aptamers and structure-preorganized aptamers. The underlying sensing mechanisms of aptasensors developed using structure-induced aptamers are usually based on changes in the structure of aptamers upon recognition of their target molecules. For example, Stojanovic et al. have reported an aptamer-based fluorescent sensor for cocaine. In the absence of cocaine, the fluorescence of fluorophore attached to one of the ends of the aptamer can be observed as a quencher attached to the other end which remains far apart from the fluorophore. However, in the presence of cocaine, the fluorophore and the quencher come close to each other due to structural changes in the aptamer resulting from binding with cocaine, thus leading to a decrease in the fluorescence intensity. In other words, this structural change in the cocaine aptamer is induced by its specific complexation with cocaine. The structure-preorganized aptamers, however, have rigid and stable structures that recognize the target molecules and exhibit almost no structural changes in their preorganized structures upon complexation with their targets. Taking advantage of these characteristics, they are used in biosensors that employ a different biosensing mechanism. For example, Lu et al. have reported catalytic DNA biosensors for metal ions using structure-preorganized aptamers. In this biosensor, the aptamer was used not only as a recognition molecule, but also as a scaffold for the catalytic reaction with the metal ion, suggesting a wider usability of the structure-preorganized aptamers. Therefore, it is necessary to separate structure-induced and structure-preorganized aptamers for developing specific aptasensors. However, the conventional aptamer preparation techniques, including systematic evolution of ligands by exponential enrichment, SELEX, usually yield a mixture of structure-preorganized and structure-induced aptamers that are indistinguishable in a DNA library with random sequences. It is well-known that the combination of CE and SELEX is one of the most effective selection methods to obtain aptamers specifically binding with target molecules, but it is still extremely difficult to decisively obtain structure-preorganized or structure-induced aptamers for aptasensors with a desirable sensing mechanism.

In this study, we focused on three-dimensional structural differences between structure-preorganized and structure-induced aptamers, and proposed a capillary sieving electrophoresis (CSE)-based application using polymer solutions for preparing novel libraries consisting of DNAs with/without
stable preorganized structures (Fig. 1). It is well-known that CSE is a powerful separation method based on the molecular sieving effect of polymer solutions filled in a capillary. CSE has many advantages including high resolution, requiring small quantities of samples, and the rapid separation of especially large biomolecules, such as proteins and nucleic acids. Therefore, we envisaged that the application of CSE would enable the separation of DNAs with/without a stable preorganized structure, based on the differences in their tertiary structures.

In previous studies, it was reported that the DNAs with different tertiary structures showed different apparent migration velocities for almost the same length in native polyacrylamide gel electrophoresis (native-PAGE). In other words, these results indicated that DNAs with different tertiary structures can be separated by a molecular sieving effect. Thus, DNAs with different migration times detected as peaks in CSE are expected to have different three-dimensional structures. To confirm this expectation, DNAs located in front of and behind the peak in CSE were fractionated by changing the cathodic-side vial. They were then amplified by a polymerase chain reaction (PCR). The DNAs in the PCR product were sequenced by next-generation sequencing (NGS), and the sequencing data were analyzed by AptamCORE, which is an application used for aptamer enrichment analysis. Finally, the stabilities of the DNA secondary structures, based on their sequences, were evaluated by mfold, which is a web application that can estimate the secondary structures, based on their sequences, were evaluated by mfold, which is a web application that can estimate the secondary structure/s of DNAs and RNAs.

We used single-stranded DNAs with 23 consecutive random nucleotides (N23, wherein N = A, T, G, or C) between the 21 sequences of the forward primers (5′-GAC TAC CCG GGT ATC TAA TCC-3′) and 17 sequences of the reverse primers (5′-CTG CCG CCA CCC GTA GG-3′) used for the NGS analysis; these DNAs were obtained from Tsukuba Oligo Service Co., Ltd. (Ibaraki, Japan). In the background solution (BGS), hydroxypropyl cellulose (HPC, Mw ~80000) in a 10 mM Tris-HCl buffer (pH 8.0) was added as a sieving matrix. The proposed separation was carried out using the CE 7100 system (Agilent Technology, Santa Clara, CA, USA) equipped with a fused-silica capillary (100 μm i.d., 360 μm o.d., total/effective length 33/24.5 cm). The inner surface of the capillary was modified by poly(vinyl alcohol) to suppress both electroosmotic flow (EOF) and non-specific adsorption. To begin with, a 10 μM DNA library solution was introduced into a capillary filled with a 10% HPC solution by applying an injection voltage of −5 kV for 5 s; it was then electrophoresed at −10 kV. After the absorbance detection of DNAs at a wavelength of 260 nm, the portions in front of and behind the observed peak of DNA library (indicated by (i) and (ii) in Fig. 2A) were fractionated using small vials filled with 20 μL of the BGS. The fractionated solutions were mixed with forward/reverse primers and a polymerase solution (KAPA HiFi HotStart ReadyMix purchased from NIPPON Genetics Co., Ltd., Japan), and were then amplified using PCR. The PCR was conducted in a thermal cycler (Blue-Ray Biotech, Taiwan), and consisted of 25 cycles of thermal denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 10 s. As shown in Fig. 2B, the results of the PCR amplification were confirmed by gel electrophoresis, wherein 5 μL of the PCR product solutions were analyzed using the precast agarose gels (3%(w/v)). The remaining PCR products were cleaned up using a PCR clean-up kit (Takara Bio, Japan). After cleaning-up, the obtained solutions were incubated at 95°C for 5 min, followed by 0°C for 10 min, for denaturation and stabilization of the single-stranded DNAs. The samples were analyzed again under almost similar CSE conditions, except for the applied injection voltage of −10 kV for 20 s. The observed electropherograms were compared to those of the DNA library before fractionation.

In the CSE analyses of fractionated, PCR-amplified DNAs, the detection time of the fractionated DNAs almost corresponded to that of the DNA library (Fig. 2), except for a slight decrease in the migration time due to the extended injection time. This indicates that the DNAs showing different mobilities under the CSE analysis conditions were successfully separated and fractionated from the DNA library based on the molecular sieving effect.

Moreover, the DNAs in each fraction were sequenced by Miseq (Illumina, San Diego, CA, USA) and analyzed by AptamCORE. Each of the 100 randomly selected sequences...
was used as an input in the mfold software, which can estimate any change in the Gibbs’s free energy (ΔG) from primary sequences to the plausible secondary structures of DNAs. The evaluated ΔG values are summarized using a box plot depicted in Fig. 3. The obtained values of the lower, median, and upper quartiles as well as those of the means and standard deviations are summarized in Table 1. The differences in the mean and standard deviations of the ΔG values were found to be 1.17 and 0.62 kcal mol⁻¹, respectively. The evaluated ρ value (1.3 × 10⁻¹⁰) was less than 0.01, indicating a statistically significant difference between groups (i) and (ii). It also means that more DNAs in fraction (i) had highly stable (= rigid) secondary structures than those in fraction (ii). Additionally, many of the DNAs randomly selected from fraction (i) were estimated to form some rigid structures.

**Table 1 Values in the distribution of changes in Gibbs’s free energy calculated from the obtained fractions (i) and (ii)**

| Values in the box plots | Fraction (i) | Fraction (ii) |
|-------------------------|--------------|---------------|
| Lower quartile (kcal mol⁻¹) | -2.51 | -0.64 |
| Median quartile (kcal mol⁻¹) | -1.32 | -0.32 |
| Upper quartile (kcal mol⁻¹) | -0.62 | 0.02 |
| Mean (kcal mol⁻¹) | -1.70 | -0.53 |
| Standard deviation (kcal mol⁻¹) | 1.39 | 0.77 |

The values of the lower, median, and upper quartile, and mean of fraction(i) are smaller than those of fraction (ii). The standard deviation of fraction (i) is larger than that of fraction (ii), probably because fraction (i) contained DNAs with various secondary structures.
secondary structures, like hairpins (Fig. 3B, (i)), whereas those from fraction (ii) were estimated to form long and flexible structures with few hairpins (Fig. 3B, (ii)). Consequently, these results clearly showed the successful separation of DNAs with/without stable preorganized structures by CSE, indicating the preparation of subset DNA libraries with/without stable preorganized structures.

In conclusion, the separation of DNAs with/without stable preorganized structures was successfully demonstrated using CSE to fractionate a random DNA library. The NGS analyses of the obtained DNAs in the different fractions clearly showed different thermostable abilities of the secondary structures, indicating the successful preparation of DNA libraries with/without stable preorganized structures. In the future, structure-preorganized and structure-induced aptamers can be obtained selectively from the prepared DNA libraries with/without stable preorganized structures, which would contribute to the development of desirable aptamers as molecular recognition elements for various aptasensors.

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