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Using DNA-Binding Proteins as an Analytical Tool

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Abstract: We propose that DNA-binding proteins can be used as highly efficient and versatile tools in analyses of DNA, RNA, and proteins. This work reports assays applying specific affinity probes: hybridization probes for analyses of DNA and RNA, and aptamer probes for analyses of proteins. Both types of probes are single-stranded DNA. In affinity analyses, in general, the probe (P) binds to a target molecule (T), and the amounts of the probe−target complex (P·T) and unbound P are determined. Distinguishing between P and P·T can be achieved by electrophoretic separation. If the electrophoretic mobilities of P and P·T are close in gel-free media, which is always the case for hybridization analyses, separation typically requires the use of a sieving matrix. Here we utilized a single-stranded DNA binding protein (SSB) to facilitate highly efficient gel-free separation of P and P·T in capillary electrophoresis (CE) for three types of targets: DNA, RNA, and proteins. When present in the CE run buffer, SSB binds differently to P and P·T. Due to this selective binding, SSB induces difference in electrophoretic mobilities of P and P·T in an SSB concentration-dependent fashion. The difference in the electrophoretic mobilities allows for affinity analyses of DNA, RNA, and proteins in gel-free CE. The large number of well-characterized DNA- and RNA-binding proteins and the diversity of their properties will allow researchers to design a comprehensive tool set for quantitative analyses of DNA, RNA, and proteins. Such analyses will facilitate identification of genomic DNA in ultra-small samples without error-prone and time-consuming PCR. They can also be used for monitoring gene expression at both mRNA and protein levels.

Introduction

A large number of proteins involved in DNA replication, DNA damage control, DNA repair, and gene expression are capable of binding DNA and RNA with different affinities and sequence specificities.[1−3] This ability of DNA- and RNA-binding proteins has a yet-to-be realized potential in analytical sciences. We suggest that they can be used as highly efficient and versatile tools in analyses of DNA, RNA, and proteins. In this proof-of-principle work, we utilized a single-stranded DNA binding protein (SSB) to facilitate affinity analyses of DNA, RNA, and proteins in gel-free electrophoresis.

This work deals with affinity assays using specific affinity probes: hybridization probes for analyses of DNA and RNA,[4−6] and aptamer probes for analyses of proteins.[7,8] Both types of probes are single-stranded DNA (ssDNA). In such analyses, in general, the probe (P) binds to a target molecule (T) and the amounts of the probe−target complex (P·T) and unbound P are determined. Distinguishing between P and P·T requires a physical-chemical property (e.g. optical spectrum, polarization, electrophoretic mobility, etc.) that is different for P than for P·T. Finding such a property and optimizing its use is one of the major challenges in designing affinity analyses.

This study was inspired by the insight that any DNA-binding protein that binds differently to P and P·T can induce the required change in their physical-chemical properties. P is a ssDNA; thus, we decided to examine SSB (source *Escherichia coli*) that binds ssDNA and ssRNA of eight bases or more in length but does not bind double-stranded DNA (dsDNA), dsRNA, or double stranded DNA−RNA hybrids.[9,10] The affinity of ssDNA to SSB is about 10 times higher than that of ssRNA.[11] The dissociation constant of the SSB−ssDNA complex is equal to 0.3 μM under conditions used in this work.[12] Our hypothesis was that SSB could induce and control the difference in electrophoretic mobilities of P and P·T in gel-free electrophoresis (electrophoretic mobility is linearly dependent on the "charge-to-size" ratio of the molecule). We examined this

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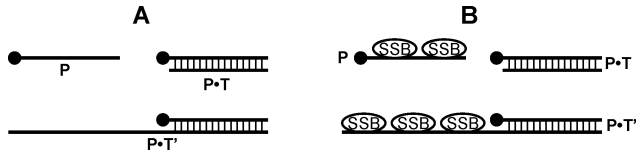

Figure 1. Panel A schematically depicts the hybridization probe (P), the complex of the probe with the complementary target (P·T), and the complex of the probe with the elongated target (P·T′). Panel B schematically illustrates binding of SSB to the single-stranded P and single-stranded overhang of P·T′ and the lack of such binding to double-stranded P·T.

hypothesis for three types of target molecules: DNA, RNA, and proteins. Capillary electrophoresis (CE) was used to monitor apparent electrophoretic mobilities of P and P·T. SSB was added to the CE run buffer to maintain equilibrium interaction of SSB with P and P·T. The use of mediators capable of shifting analyte mobility in CE is well documented.[13−15] This work demonstrates for the first time that a mediator, such as a DNA-binding protein, can facilitate highly efficient separation of P and P·T in affinity analyses of T.

Results and Discussion

DNA and RNA as Targets. All hybridization experiments employed the same probe (P), a fluorescently labeled 15-base-long ssDNA. Two types of ssDNA and ssRNA targets were studied: (i) complementary targets (T) which had the same length as P and (ii) elongated targets (T′) which were longer than P and included the sequence complementary to P at their 5′ ends (see the Experimental Section for sequences of P, T, and T′). P and the hybridization complexes, P·T and P·T′, are schematically depicted in Figure 1A. Every nucleotide base in DNA or RNA bears a single negative charge. Therefore, the "charge-to-size" ratio of both DNA and RNA is highly negative and does not depend on their lengths or hybridization. As a result, single-stranded P, double-stranded P·T, and P·T′, which has a single-stranded overhang, have similar highly negative electrophoretic mobilities in gel-free electrophoresis. An SSB molecule comprises of 178 amino acids and bears only a small negative charge of −4 to −8 for pH ranging from 9.0 to 10.0. Thus, the electrophoretic mobility of SSB is much less negative than those of P, P·T, and P·T′. SSB binds to single-stranded P and single-stranded overhang of P·T′ but does not bind to double-stranded P·T (Figure 1B). Upon binding to P and P·T′, SSB should make their electrophoretic mobility less negative while the mobility of P·T should not be affected as it does not bind SSB. P·T′ contains a double-stranded region that cannot bind SSB. Therefore, P·T′ binds fewer SSB molecules per base of DNA or RNA than P. As a result, P·T′ should have a more negative electrophoretic mobility than P. Moreover, due to the lower affinity of SSB to RNA with respect to that of the affinity of SSB to DNA, P·T′ with an RNA target will bind fewer SSB molecules than P·T′ with a DNA target. Thus, P·T′ with RNA as a target should have more negative electrophoretic mobility than P·T′ with DNA as a target. The following experiments confirmed all these predictions.

First, we examined how SSB influences the electrophoretic mobilities of P and P·T. One hundred picoliters of the mixture of P (40 nM) and P·T (60 nM) was injected into the capillary and subjected to electrophoresis in a gel-free run buffer supplemented with different concentrations of SSB. In the absence of SSB in the run buffer, P could not be separated from P·T, confirming that P and P·T had similar mobilities in the gel-free electrophoresis (Figure 2, lower trace). As expected, the presence of SSB in the run buffer induced the mobility shift of P. When the concentration of SSB increased, the electrophoretic mobility of P increased while that of P·T remained virtually the same (Figure 2, middle and top traces). The optimum difference in electrophoretic mobilities of P and P·T was achieved when the concentration of SSB was in the range of the dissociation constant of the complex between P and SSB, $K_d \approx 0.3\ \mu$M.[11] The results were identical for RNA and DNA as a target. Thus, we demonstrated that SSB induces and effectively controls the difference in electrophoretic mobilities of P and P·T for DNA and RNA targets.

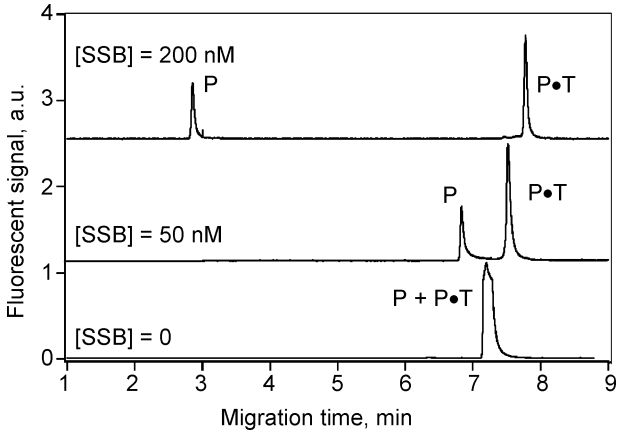

Figure 2. SSB-mediated DNA hybridization analysis in gel-free capillary electrophoresis. P is a fluorescently labeled ssDNA probe. P·T is dsDNA hybrid of P with a complementary target DNA. The amounts of P and P·T were 4×10^{-18} mol and 6×10^{-18} mol, respectively. The run buffer was 25 mM Borax at pH 9.4 supplemented with different concentrations of SSB (shown in the graph).

Second, we examined whether SSB can facilitate the separation of P·T′ from P and P·T. One hundred picoliters of the mixture of P (40 nM) and P·T (40 nM) and P·T′ (10 nM) was injected into the capillary and subjected to electrophoresis in a gel-free run buffer supplemented with 200 nM SSB. P, P·T, and P·T′ were baseline separated for both DNA and RNA as a target (Figure 3). As we predicted, P·T′ had more negative electrophoretic mobility with RNA as a target (Figure 3, insert) than with DNA as a target (Figure 3, main panel). Thus, we demonstrated that SSB induces and effectively controls the difference in electrophoretic mobilities of P, P·T, and P·T′ for DNA and RNA targets.

SSB provides a unique means of gel-free CE analysis of short and long DNA and RNA targets. Advanced CE instrumentation used in this work allows the quantitation of fewer than 1000 molecules,[16] which is comparable with the sensitivity of quantitative PCR.[17] In addition, the accuracy of CE greatly exceeds that of PCR. Thus, SSB-mediated CE analyses will facilitate highly sensitive and accurate quantitation of genomic DNA and messenger RNA without time-consuming and error-prone PCR and RT-PCR.

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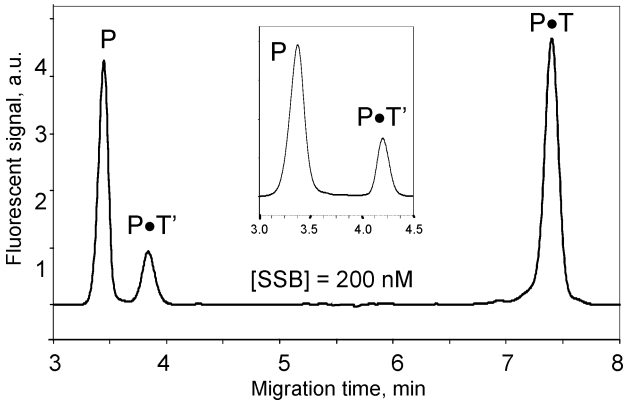

Figure 3. SSB-mediated hybridization analysis of two target oligonucleotides: T and T′. P and T are similar to those in Figure 1. T′ is an elongated target with 22 additional bases at one end of the target. The amounts of P, P·T, and P·T′ were 4×10^{-18} mol, 4×10^{-18} mol, and 1×10^{-18} mol, respectively. The run buffer was 25 mM Borax with 200 nM SSB at pH 9.4. The main panel and the insert show the results for DNA and RNA targets, respectively.

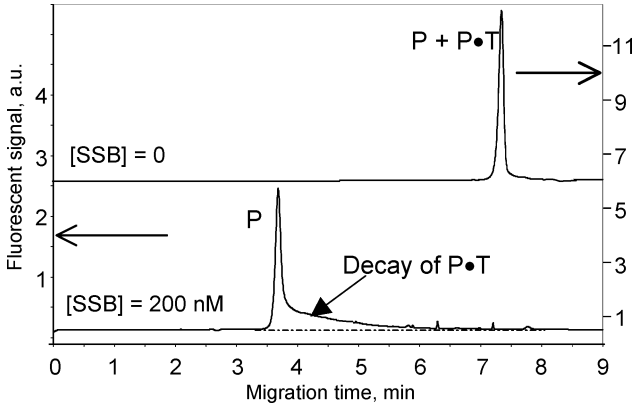

Figure 4. SSB-mediated NECEEM affinity analysis of thrombin (P) with its aptamer (P). The amounts of P and P·T were 5×10^{-18} mol and 5×10^{-18} mol, respectively. The run buffers were 25 mM Borax at pH 9.2 without SSB (top trace) and with 200 nM SSB (bottom trace).

SSB is a representative of a very large family of DNA- and RNA-binding proteins. Among them, there are proteins that bind DNA and RNA sequence-nonspecifically. Such proteins will facilitate universal hybridization analyses similar to those demonstrated here with SSB. Moreover, sequence-specific DNA- and RNA-binding proteins can be used to add sequence selectivity to the analyses when required. The diversity of properties of DNA- and RNA-binding proteins will allow researchers to design a comprehensive tool set for quantitative analyses of DNA and RNA.

Protein as a Target. Finally, we examined our hypothesis for a protein as a target (T) and an ssDNA aptamer as a probe (P). Aptamers are DNA or RNA molecules that are selected from random libraries of DNA or RNA oligonucleotides in an artificial evolution process. They are able to bind target molecules with very high selectivity and affinity and are often viewed as artificial antibodies.[18−20] When the target protein has electrophoretic mobilities close to that of its oligonucleotide aptamer, separation of P from P·T becomes more difficult. This may happen if the target protein has a large number of anionic amino acids or if protein migration is retarded by its adhesion to capillary walls. Our hypothesis suggests that SSB can induce the mobility shift of the aptamer, P, without affecting the mobility of the aptamer−protein complex, P·T, and facilitate the separation of P and P·T, which otherwise would be difficult to achieve.

In this series of experiments, we used thrombin as T and its fluorescently labeled aptamer as P.[21,22] One hundred picoliters of an equilibrium mixture containing P (50 nM), T (1 μM), and P·T (50 nM) was injected into the capillary and subjected to nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM).[23] In the absence of SSB in the run buffer, P could not be separated from P·T as was confirmed by observing a single electrophoretic peak (Figure 4, upper trace). Two effects could contribute to our inability to resolve P and P·T in the SSB-free buffer. First, at pH 9.2, a thrombin molecule has a large negative charge of −14. Together with the relatively small size of the molecule, this defines its negative electrophoretic mobility that may be close to that of the aptamer. Thrombin's adhesion to capillary walls could also contribute to the retardation of its electrophoretic migration. When SSB was present in the run buffer, it bound P, which resulted in the shift of peak P and appearance of an exponential trace, corresponding to the decay of P·T during NECEEM (Figure 4, lower trace). P·T decays completely during the separation; thus, no peak corresponding to intact P·T was observed. However, the areas under the peak and the exponential curve correspond to concentrations of P and P·T in the equilibrium mixture, and the unknown concentration of T in the sample can be accurately determined.[22] These experiments proved that SSB could also be a very efficient mediator of electrophoretic mobilities of P and P·T in aptamer-based affinity analyses of proteins.

Concluding Remarks

In conclusion, we demonstrated that SSB could be used to separate an ssDNA probe from the probe−target complex in free-solution electrophoresis. This approach will be utilized to design highly efficient electrophoretic affinity analyses of DNA, RNA, and proteins. Such analyses will allow for the accurate quantitation of genomic DNA in ultra-small samples without error-prone PCR amplification. They will also facilitate extremely sensitive monitoring of gene expression at both mRNA and protein levels. SSB is a representative of a very large family of DNA- and RNA-binding proteins. We foresee that many proteins of this family will find applications in analytical sciences.

Experimental Section

Chemicals and Materials. Single-stranded DNA binding protein from *E. coli*, human thrombin, RNA oligonucleotides, and buffer components were obtained from Sigma-Aldrich (Oakville, ON). Normal and modified DNA oligonucleotides were kindly donated by Dr. Yingfu Li (McMaster University, Hamilton, ON). Fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). All solutions were made using the Milli-Q-quality deionized water and filtered through a 0.22 μm filter (Millipore, Nepean, ON).

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Probes. Two ssDNA probes were used. First, a fluorescein-labeled 15-mer oligonucleotide, 5′-GCGGAGCGTGGCAGG, was utilized as a hybridization probe for analyses of DNA and RNA targets. Second, a fluorescein-labeled 15-mer oligonucleotide GGTTGGTGTGGTTGG was used as an aptamer probe for the analysis of thrombin.[21]

Targets. Two types of DNA and RNA targets were examined. The first type of targets included 15-mer DNA and RNA oligonucleotides with sequences complementary to that of the hybridization probe. The second type of targets included 37-mer DNA and RNA oligonucleotides, which included the 15-mer sequence complementary to the hybridization probe at their 5′ end. The remaining 22-mer region had the following sequence: 5′-TCACTGTGGTTGGTGTGGTTGG for DNA and 5′-UCACUGUGGUUGGUGUGGUUGG for RNA. Thrombin was used as a protein target for its aptamer probe.

Probe−Target Complexes. The hybridization probe was annealed with its targets in a Mastercycler themocycler (Westbury, NY) at 95 °C for 2 min in a CE run buffer (see below). After annealing the complexes were cooled to room temperature. The aptamer probe was reacted with thrombin at room temperature for 30 min in 20 mM Tris-HCl buffer at pH 8.3 supplemented with 5 mM KCl and 1 mM $MgCl_2$. To prepare mixtures containing free probe and probe−target complexes, the targets were annealed or reacted with the excess of the probe.

Capillary Electrophoresis. Capillary electrophoresis analyses were performed using a laboratory-built CE instrument with fluorescence detection described in detail elsewhere.[24] A 488-nm line of an Ar-ion laser was utilized to excite fluorescence of labeled probes. Uncoated fused silica capillaries of 40 cm × 20 μm i.d. × 150 μm o.d. were used. Electrophoresis was carried out with a positive electrode at the injection end biased at +16 kV, resulting in the electric field of 400 V/cm across the 40-cm long capillary. The run buffer was 25.0 mM sodium tetraborate at pH 9.2 for hybridization analyses of DNA and RNA, and at pH 9.4 for affinity analyses of thrombin. The run buffer was supplemented with SSB in concentrations ranging from 0 to 200 nM. The samples were injected into the capillary by a pressure pulse of 1 s × 9.1 kPa; the length and the volume of corresponding sample plug were 0.3 mm and 100 pL as was calculated using the Poiseulle equation. The capillary was rinsed with the run buffer solution for 2 min prior to each run. At the end of each run, the capillary was rinsed with 100 mM NaOH for 2 min, followed by a rinse with deionized water for 2 min.

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