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Evaluation of the formation of a junctional DNA nanostructure through annealing curve analysis

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ABSTRACT

During the self-assembly of different numbers of oligonucleotides comprising junctional DNA nanostructures, a change in environmental variables (e.g., temperature or salt concentration) has a substantial influence on the final products. Further, distinctive annealing temperatures of oligonucleotides are observed depending on the state of hybridization. Here, we present an evaluation of the annealing characteristics of oligonucleotides for the formation of a simple junctional DNA nanostructure using an annealing curve analysis. This method may be useful for analyzing the formation of complex junctional DNA nanostructures.

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1. Introduction

For several decades, DNA has gained much attention in a variety of bioengineering fields owing to its intrinsic biocompatibility and ease of precise manipulation [1–3]. In addition, single DNA strands recognize their complements and precisely pair with one another. Explicit selectivity of both strands makes DNA a promising nanobuilding block for the achievement of novel polymeric architectures. One example of DNA blocks is a Holliday junction, which possesses a tripodal spot formed by a simple combination of three different oligonucleotides [4–7]. A plethora of DNA-based nanostructures have been created and exploited for use in cellular delivery [8], cancer therapy [9], cell-free protein production [10], and energy storage devices [11]. Despite the extensive development of DNA constructs in several research fields, an accurate study of either the mechanism or conditions influencing the formation of simple DNA nanostructures has not yet been conducted [12]. Numerous environmental factors affect the yields of conventional DNA hybridization. These include temperature variation [13], salt concentration [14] and pH [15]. Therefore, consideration of several environmental parameters deserves in-depth studies of their influence on the creation of junctional DNA nanostructures. In this study, environmental factors (e.g., salt concentration, temperature decrease rate) were evaluated in terms of influence on the formation of junctional DNA nanostructures. As a study model, an X-shaped DNA (X-DNA), which serves as a simple example of a junctional DNA nanostructure, was used [16].

For the studies of external conditions, several temperatures and salt concentrations were chosen as representatives and were analyzed for their effects on X-DNA formation. The complementary hybridization of each oligonucleotide was measured successively while decreasing temperature and monitored via the hybridization mechanism of X-DNA nanostructures. Hybridization was assessed by monitoring the green I fluorescence intensity change during temperature variation using RT-PCR (real time-polymerase chain reaction). When bound to double-stranded DNA, SYBR® Green I is known to enhance the fluorescence emission by 1000-fold compared to that of free molecules in solution [17]. The increased fluorescence intensity of SYBR® green I by DNA hybridization can be quantified over the entire process, and the results can then be used to produce an annealing curve.

2. Materials and methods

2.1. Model junctional DNA nanostructure (X-DNA) preparation

All oligonucleotides were purchased from Integrated DNA Technology (IDT). The sequence information of the oligonucleotides is described in Table S1. SYBR® Green I nucleic acid staining dye was purchased from Molecular Probes.

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2.2. Gel electrophoresis

Hybridization of X-DNA was indicated with gel electrophoresis. To indicate a size increase in hybridized strands, samples were assayed from single strands to complete X-DNA. To obtain appropriate resolution of samples, 3% (w/v) agarose gel was used. Each sample loading amount was fixed at 0.5 μg, and equimolar strands were used.

2.3. Annealing curve measurement

The amount of base pairing in several combinations of single oligonucleotides was measured by monitoring the intensity of SYBR® Green I. Equimolar single strands were mixed thoroughly with 10,000X-diluted SYBR® Green I. Salt concentration was adjusted with NaCl according to the required experimental conditions. Final solution volume was 12 μl, and the concentration of oligonucleotides was fixed at 0.07 μM. The SYBR® Green I intensity change and differentiated plot were measured using a real-time quantitative RT-PCR program in a LightCycler 480 (Roche Applied Science). The Tm value of each sample was calculated from a differentiated plot with an RT-PCR program as noted in the Supplementary Information (Table S2). All samples were triplicated.

3. Results and discussion

X-DNA is simply formed by a combination of four different oligonucleotides designed to anneal with one other (Fig. 1A, Table S1). The formation and sizes of the X-DNA were confirmed using a gel electrophoretic mobility shift assay (Fig. 1B). The formation of X-DNA was confirmed by observing the stepwise band increase after sequential addition of each oligonucleotide. To provide further insight, self-assembly of the oligonucleotides was evaluated using RT-PCR. The component oligonucleotides of the X-DNA in solution were stained with SYBR® Green I. Next, the solution was exposed to certain temperature variations to induce hybridization between the oligonucleotides. The change in fluorescence intensity and its differential values were recorded (solid and dotted lines, respectively, in Fig. 1C). The peaks observed among the differential value curves, presented as annealing curves, indicate the melting temperatures (Tm) of the synthesized DNA nanostructures.

3.1. Effects of temperature decrease rate

To determine the effect of temperature decrease rate on the formation of junctional DNA nanostructures, the DNA solutions were initially heated to 95 °C and subsequently cooled to 20 °C over different periods of time. Three different cases were investigated, wherein the temperature was decreased over periods of 5, 10, or 30 min (Fig. 2A). In the cases where the temperature was decreased over a 10 or 30 min period, approximately the same peak value was observed in the annealing curve (Fig. 2B). However, when the temperature was decreased over 5 min, the peak value was lower than the other two cases. As peak values in the annealing curve indicate the degree of hybridization, there was a significant decrease in hybridization for the 5 min case. Thus, for the effective formation of X-DNA, the rate of temperature decrease should be slower than 7.5 °C per minute.

3.2. Effects of salt concentration

Once the rate of temperature decrease was fixed, salt concentration was varied in order to determine its effect on DNA nanostructure formation. Conventionally, during the DNA annealing processes, sodium chloride (NaCl) was added to the solution. This compound is generally known to balance the ionic strength among

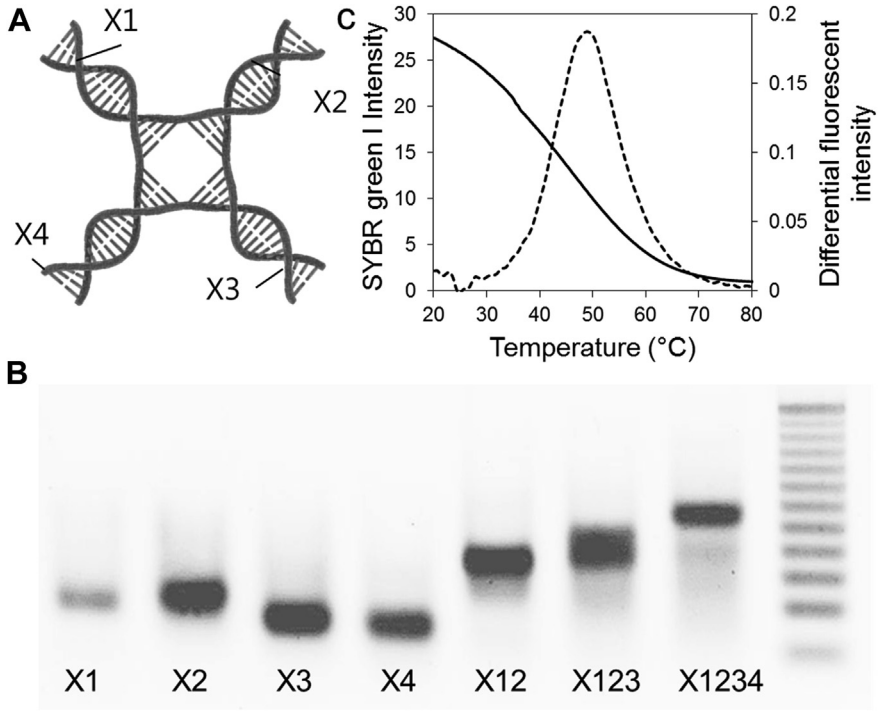

Fig. 1. Schematic drawing and characterization of X-DNA. (A) Four oligonucleotides (X1, X2, X3, and X4) hybridize with each other to form an X-shaped DNA nanostructure. (B) Evaluation of X-DNA formation and its component oligonucleotides through gel electrophoretic mobility shift assay. Stepwise increases in the bands upon sequential addition of component oligonucleotides indicate the formation of the X-DNA. (C) Annealing curve (dotted line) derived by differentiating fluorescence intensity variation (solid line) measured using RT-PCR. The peak in the annealing curve indicates the melting temperature.

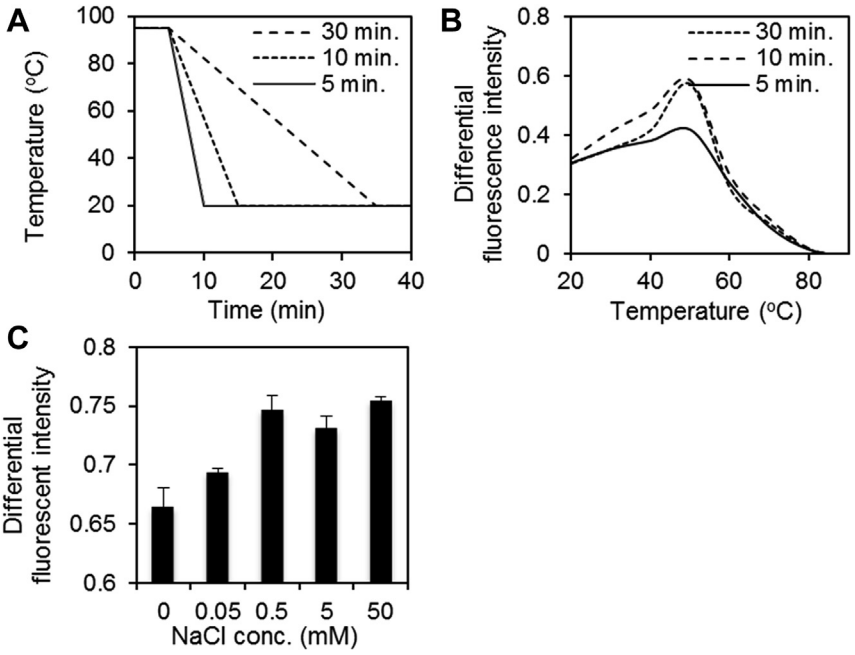

Fig. 2. Investigation of X-DNA formation for various temperature and salt conditions. (A) Design of temperature variation experiment for X-DNA formation. The solution containing the component oligonucleotides was first heated to 95 °C and then decreased to 20 °C over three different time periods: 5, 10, or 30 min. (B) Differential annealing curve of the X-DNA formed in three different conditions. The peak values upon integration represent the amount of X-DNA created. (C) Peak values of annealing curves at different NaCl concentration conditions.

the oligonucleotide strands used [18]. Therefore, NaCl was also added to test solutions at different molar concentrations, and relevant peak values of the differential annealing curve were measured (Fig. 2C). According to the results, gradual escalation of NaCl concentration leads to an increased amount of X-DNA formation, reaching a plateau when more than 0.5 mM of NaCl is added. The data obtained suggest that both temperature variation rate and salt concentration have a profound influence on DNA nanostructure formation.

3.3. Formation mechanism of junctional DNA nanostructures

The mechanism of oligonucleotide hybridization in X-DNA was further investigated by comparing the annealing curves of single strands or their complexes (Table S2). In principle, it is known that Tm value is determined according to base species and sequence order within the strand [18]. It is thus expected that each pair of oligonucleotides used in hybridization will have a characteristic Tm value. In the case of X2 and X3 strands, each single strand has an intrinsic Tm value corresponding to its distinctive secondary structure (approximately 37 °C and 40 °C, respectively). Upon mixing the two single strands, which contain a sequence that is partially complementary to that of the other, (Fig. 3A), a new single Tm (Tm′) emerged, as indicated by a higher differential peak value at 47 °C. Here, the Tm value produced by the hybridized structure is referred to as Tm′ to distinguish it from the Tm value of the single strand itself. Interestingly, it was observed that the shape of the annealing curve is determined by the relative positions of Tm and Tm′. For instance, in the case of hybridization between X3 and X4, in which Tm′ is higher than Tm for each strand, the overall shape of the annealing curve has only one peak corresponding to the Tm′ (Fig. 3B). The shape of the graph indicates that the secondary structure of oligonucleotides degraded early in hybridization, whereas if Tm′ is placed between the two Tm values of the oligonucleotide precursors, two peaks corresponding to Tm of one oligonucleotide and Tm′ are observed. It is therefore assumed that inter-strand hybridization takes place before intra-strand folding during the temperature decrease owing to its higher melting temperature. Therefore, once the single strands are incorporated into a hybridized structure, their respective peaks in the annealing curve decrease significantly to the point that they are almost unrecognizable. However, if Tm′ is between the Tm values of the two component oligonucleotides, two peaks in the annealing curve, one of which corresponds to the oligonucleotides with a higher Tm and the other to Tm′, are observed. Such a case can be observed when X1 and X2 hybridize (Fig. 3C). In the initial stage of cooling (approximately in the range of 50 °C–95 °C), the annealing curve of X1 and X2 hybridization overlaps with that of X1 alone. As the temperature further decreases, the annealing curve of X1 and X2 hybridization shows a peak at a temperature point (42 °C) higher than that exhibited by the X2 oligonucleotide (39 °C). This phenomenon indicates that, until hybridization occurs between complementary oligonucleotides, each oligonucleotide retains its unique properties. This independence is observed in cases where non-hybridizing oligonucleotides are mixed (note the complexes of X1–X3 or X2–X4 and see Fig. 3D–E). For example, the annealing curve of a X1 and X3 mixture (solid line) was almost identical to the outer lines of individual X1 and X3. Therefore, it is assumed that, when two oligonucleotides hybridize, a new melting temperature is produced along with a higher annealing peak value.

According to the previous results, junctional DNA structures show similar trends to conventional DNA hybridization under various environmental conditions. Proper annealing time and salt concentration should be maintained to achieve efficient formation of junctional DNA nanostructures. Further, during hybridization, there were significant differences between conventional DNA hybridization and junctional DNA nanostructure formation.

In the case of X-DNA, it was expected that there would be multiple Tm values observed when the annealing curve was measured, as the X-DNA consists of four different arms of double-stranded DNA (Fig. 4A). However, instead of multiple peaks

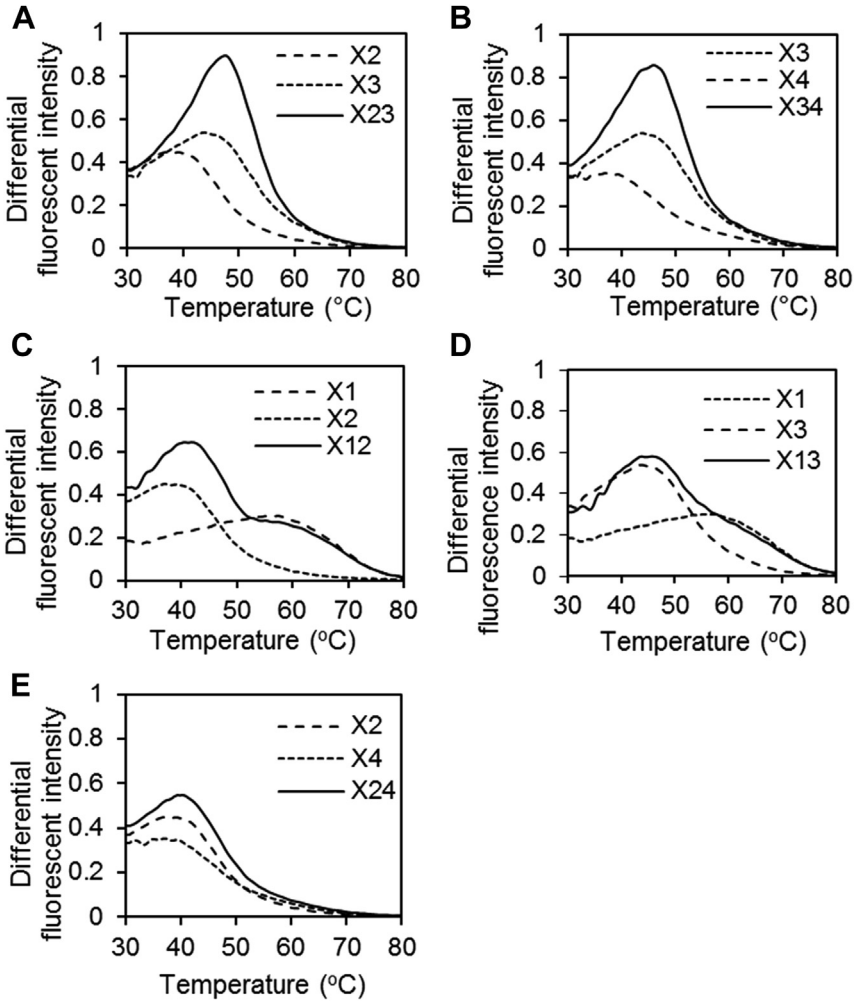

Fig. 3. Annealing curves of single and combinations of oligonucleotides. (A) A T_m' is observed upon mixing two oligonucleotides with complementary sequences. X2 and X3 oligonucleotides, having T_m values of 37 °C and 40 °C, respectively, hybridize into a double-stranded structure (X23) for which a T_m' is observed at 47 °C. (B) T_m may appear at a higher temperature or (C) in between the T_m' values of the component oligonucleotides. If the oligonucleotides are not designed to hybridize with each other, the annealing curve exhibits forms that (D) merely follow or (E) integrate the outlines of those of the component oligonucleotides.

appearing within the temperature range of 44 °C–47 °C, where the Tm′ values of the four double helixes were measured, only one peak was observed at 49 °C (Fig. 4B). This indicates that the formation of the X-DNA occurs in a concerted manner, rather than independently with regard to the individual portions of a double strand. This phenomenon may be due to the interactions among all oligonucleotides comprising the X-DNA. We presume that, when the junctional DNA nanostructure is formed, pre-interacted strands cause the collection of other leftover strands. That is, once an initial hybridization between any two oligonucleotides takes place, there is a higher probability of inducing residual hybridization across the entire structure. Therefore, a cascade of in situ hybridization occurs

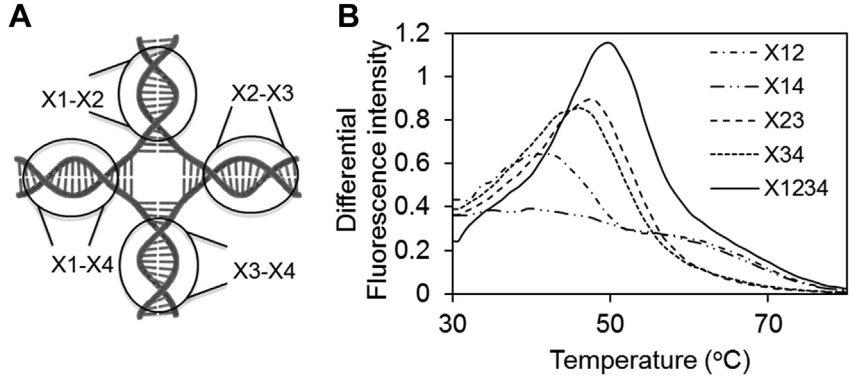

Fig. 4. Characterization of complete X-DNA hybridization. (A) Expected regions in the X-DNA structure where four different T_m' values were measured. (B) Actual form of the annealing curve when all four component oligonucleotides were mixed to create a complete X-DNA structure. Only one peak was observed (solid line) at 49 °C.

for all oligonucleotides, which increases the rate of reaction, leading to the rapid formation of a complete X-DNA structure. When X3 and X4 strands interact with each other, their combination has the highest Tm value, and the complex structure of X3 and X4 enhances the hybridization of other single strands. Therefore, it is likely that X-DNA has a differential annealing curve with a single peak. It may be assumed that, if a number of oligonucleotides that are designed to be complementary to one another form a complex junctional DNA structure, the hybridization of the oligonucleotides occurs simultaneously, generating a new Tm′ value that corresponds to the whole junctional structure. Such a phenomenon may be observed during the formation of more complex DNA nanostructures where hundreds of oligonucleotides hybridize during cooling [14].

We used X-DNA as a model junctional DNA nanostructure and investigated two factors that influence X-DNA formation: rate of temperature variation and salt concentration in solution. We found that decreasing the temperature at a rate slower than 7.5 °C per minute while adding more than 0.5 mM NaCl is essential for complete annealing of the compositional oligonucleotides.

Also, a new Tm was observed after several oligonucleotides hybridized to form a complex double-stranded structure. As such, a higher yield of final DNA nanostructures may be achieved by considering the Tm values of all the compositional oligonucleotides and intermediate double-stranded structures when designing the annealing conditions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.020.

Transparency document

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