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Notes & Tips

A real-time assay for DNA sticky-end pairing using molecular beacons

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DNA sticky-end pairing (SEP)¹ plays an important role in cellular processes and various biotechnologies [1–4] and has been studied with different methods including ultracentrifuge, gel electrophoresis, electron microscopy, and atomic force microscopy [5–8]. However, these methods need product separation, DNA ligation, and surface deposition, all of which tend to shift the reaction equilibrium of SEP. Moreover, these methods are discontinuous, time consuming, and laborious. Therefore, there has been a clear need for in situ, continuous and convenient, real-time SEP assays. Here we introduce an effective real-time assay, based on the molecular beacon (MB), for SEP observation. The MB is a newly developed ssDNA probe and has found wide applications from gene detection to protein quantitation [9–13]. Among the many advantages of MBs are “detection without separation” and ultrasensitive analysis in homogeneous solution [11], which fits well for real-time SEP study.

Fig. 1 shows the mechanism of applying MB to SEP observation. A MB molecule and its three cDNAs (complementary DNA) have been synthesized. The MB contains a 16-base loop and a 6-bp stem, with a fluorophore and a quencher linked to its 5' and 3' end, respectively. Without target molecules, the MB's stem keeps the fluorophore and the quencher close to each other, causing fluorescence quenching. In the presence of a complementary DNA, the MB opens and forms a double-stranded hybrid, leading to the restoration of fluorescence. With its three cDNAs, the MB can form three different types of hybrids, each with a unique sticky-end structure.

Hybrid MB–18-mer(a) possesses two complementary 5-base-long sticky ends, while the other two have only one sticky end each. Consequently, SEP can occur only with MB–18-mer(a), while SEP will not occur with MB–18-mer(b) nor with MB–23-mer. SEP between two MB–18-mer(a) hybrid molecules results in the formation of a 5-bp double helix, which draws together a fluorophore–quencher pair, leading to a fluorescence quenching again. By recording the fluorescence intensity (FI) of fluorophores in the hybridization solution, we will be able to monitor SEP process in real time.

An abnormal hybridization behavior was observed when MB was hybridized to 18-mer(a) in the presence of high ion concentration. The 18-mer(a) was added to the MB solution, which contained different $[Mg^{2+}]$, and a spontaneous fluorogenic response was recorded (Fig. 2a). With 1 mM Mg^{2+} , the addition of 18-mer(a) gave rise to an increase in FI in the initial stage, indicating the progress of MB–18mer hybridization. As time increased, the FI gradually reached a plateau, indicating the completion of the hybridization (Fig. 2a, curve 1). This was a normal hybridization curve, similar to those observed by others [9,10]. When Mg^{2+} concentration was increased to 10 mM, however, an unusual hybridization curve was observed (Fig. 2a, curve 2): the FI decreased abruptly after an initial increase, leaving a peak in the curve. This drop in FI suggested that SEP occurred. At initial stage, hybridization was quick, and SEP was relatively slow due to low concentration of hybrids. As time passed, the concentration of free MB was decreased, resulting in a slower hybridization rate, while the concentration of MB hybrid was increased, accelerating SEP rate. At a certain time point, the SEP rate surpassed the MB hybridization rate, leading to the drop in FI. The stability of the short double helix forming through SEP was lowered with the decrease in

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¹ Abbreviations used: SEP, sticky-end pairing; MB, molecular beacon; FI, fluorescence intensity.

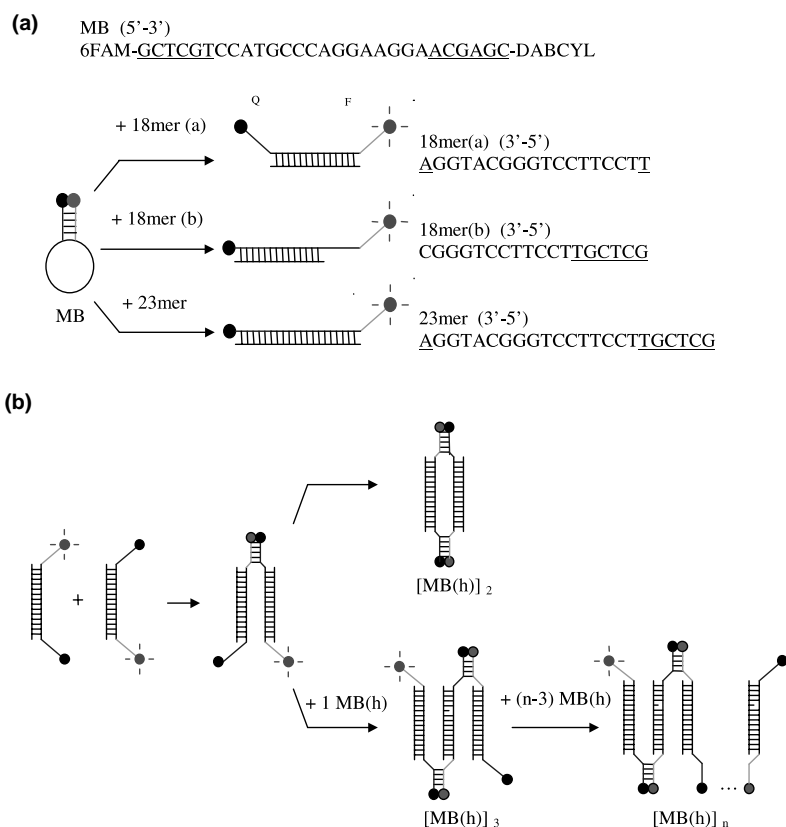


Fig. 1. (a) Structures of MB and its hybrids with three complementary DNAs. Upon hybridization, the MB lights up, and forms hybrids with different sticky-end structures. Underlined are sequences at stem or complementary to stem. FAM and DABCYL are used as fluorophore (F) and quencher (Q), respectively. (b) SEP mechanism. Two complementary sticky ends from two MB-18-mer(a) hybrids (MB(h)) can pair to form a short double helix, leading to association of the two hybrids at one end. These two MB(h) can form a closed structure, [MB(h)]₂, by pairing the other two sticky ends or polymerize into a multimolecular structure, [MB(h)]_n ($n \geq 3$), by pairing with more hybrids. With sticky-end pairing, F and Q are drawn together again, causing fluorescence quenching.

Mg^{2+} concentration, and thus SEP did not occur significantly at low $[Mg^{2+}]$. That was why no apparent FI decrease was observed with 1 mM Mg^{2+} . To confirm that the abnormal hybridization curve was indeed caused by SEP, control experiments with the 23-mer were conducted. The MB-23-mer hybridization gave a normal hybridization curve in both 1 and 10 mM Mg^{2+} solutions (Fig. 2a, curves 3 and 4). Moreover, the final FI, instead of going down, went up slightly when $[Mg^{2+}]$ was increased from 1 to 10 mM, which might be due to a higher stability of MB-23-mer hybrid in the presence of higher ion concentrations. Each MB-23-mer hybrid had only one sticky end, making SEP impossible (Fig. 1a). The sharp contrast between hybridization behaviors of the two types of hybrids confirmed that SEP caused the abnormal hybridization observed in Fig. 2a. It is noteworthy that, even with 1 mM Mg^{2+} , the final FI of MB-18-mer(a) solution is still lower than that of the MB-23-mer solution. Three factors contributed to this difference. First, even at low $[Mg^{2+}]$, there was still a small amount of SEP in MB-18-mer(a) solution (see below). Second, the double helix of MB-18-mer(a) was shorter and thus less stable than that of MB-23-mer.

Third, the fluorophore–quencher separation in MB-18-mer(a) was smaller than that in MB-23-mer; therefore, the fluorescence of the fluorophores in the MB-18-mer(a) hybrids was weaker than that of the MB-23-mer hybrids.

To observe SEP process without interference by the initial hybridization, we prepared MB-18-mer(a) hybrid in solution with low ion concentration and then initiated SEP by raising the ion concentration (Fig. 2b, curve 1). FI was decreased significantly after $[Mg^{2+}]$ was increased. The FI drop was smaller than that when 10 mM Mg^{2+} was present (Fig. 2a). The higher the ion concentration was, the stronger the SEP effect would be. As expected, no FI drop was observed when MB-18-mer(b) was examined (Fig. 2b, curve 1). Instead, a slight FI increase was observed due to a higher stability of MB-18-mer(b) hybrid with higher ion concentration. Here the 18-mer(b), instead of the 23-mer, was chosen since MB-18-mer(b) was a better control because of its same double-helix length and stability similar to that of MB-18-mer(a). The FI of MB-18-mer(a) hybrid was lower than that of MB-18-mer(b) hybrid even before $[Mg^{2+}]$ was raised, indicating that SEP also occurred at low ion concentration, confirming the explanation for the results in Fig. 2a.

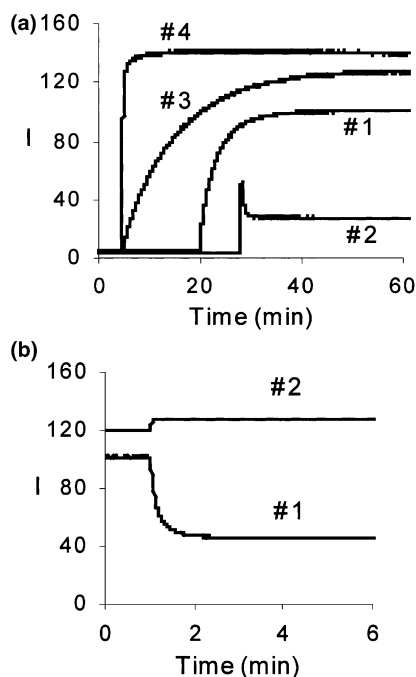


Fig. 2. (a) Time courses of hybridization between MB and cDNAs. For each curve, excess cDNA was added into 1 μ M MB solution, and the fluorescence intensity was recorded before and after the addition of the cDNA: curve 1, 18-mer(a), 1 mM Mg^{2+} ; 2, 18-mer(a), 10 mM Mg^{2+} ; 3, 23-mer, 1 mM Mg^{2+} ; 4, 23-mer, 10 mM Mg^{2+} . Note that, in the four curves, time lengths for the background are intentionally set differently for the convenience of displaying the difference in the curves' shapes. (b) Time courses of SEP. 1 μ M MB–18-mer(a) hybrid was prepared in 1 mM $MgCl_2$ buffer, and SEP was triggered by increasing Mg^{2+} concentration to 5 mM (curve 1). MB–18-mer(b) hybrid was run as a control (curve 2).

SEP efficiency can be accurately quantified. Fig. 3 shows one example of how to calculate the pairing percentage of MB–18-mer(a) as a function of $[Mg^{2+}]$. To quantify, two reference states, the totally paired state and totally unpaired state, need to be considered. Since the 5-bp helix structure remained the same in both MB and the paired sticky ends (Fig. 1a), we used MB as a totally paired reference and MB–18-mer(b) as the totally unpaired one. FIs for MB, MB–18-mer(a), and MB–18-mer(b) were measured under different $[MgCl_2]$ (Fig. 3a). As expected, FI of MB–18-mer(a) was dropped sharply with the rise of Mg^{2+} concentration, while that of MB–18-mer(b) was increased slightly. FI of the MB was decreased slightly with the increase in Mg^{2+} concentration, due to a higher stability of the stem of the MB with higher ion concentration. With the FIs of the above three samples available, pairing percentage was calculated according to the equation shown in the legend to Fig. 3. The result is presented in Fig. 3b. Even 1 mM Mg^{2+} caused 16% SEP, which was consistent with the result in Fig. 2b. SEP was enhanced with the increase of ion concentration. When $[Mg^{2+}]$ exceeded 100 mM, over 93% of sticky ends were paired. Many other factors could influence SEP efficiency, including ion type, pH, temperature, MB concentration, sticky-end length, and sequence.

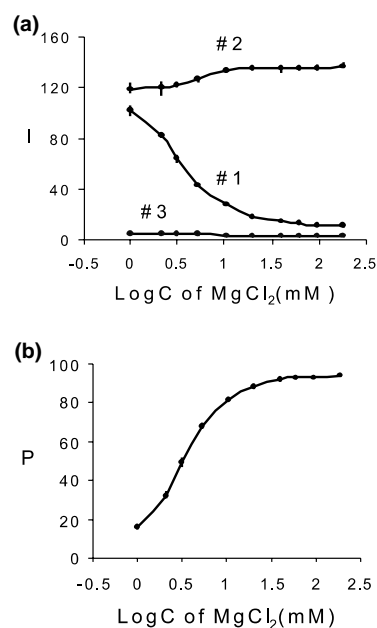


Fig. 3. (a) Effect of $MgCl_2$ concentration on SEP. FI of MB–18-mer(a) solution (curve 1) was measured at different $MgCl_2$ concentrations. As references, FIs of MB–18-mer(b) (curve 2) and MB (curve 3) solutions were also recorded. (b) Percentage SEP at different $MgCl_2$ concentrations. The percentage was calculated using the following equation: $p = (I_0 - I_s)/(I_0 - I_c)$, where p is percentage of SEP and I_0 , I_s , and I_c represent FI of solutions of MB–18-mer(b), MB–18-mer(a), and MB, respectively. The error bars represent standard deviations of the measurements.

This is the first time that DNA sticky-end pairing has been observed in real time in a homogeneous solution. In addition to the real-time measurements and high sensitivity, there are other advantages in using MB for SEP studies. First, it provides great convenience for SEP studies. With only one labeled DNA probe synthesized, a group of SEP data can be obtained by designing different cDNA probes. Moreover, sticky-end length can be easily adjusted by changing the length or position of the cDNA, permitting study of the sticky-end length effect using only one MB. Second, compared with conventional methods, thermodynamic and kinetic information on SEP can be obtained accurately, easily, and simultaneously by recording SEP time courses.

In addition to providing a general method for SEP observation, this report is useful in several other areas. First, it provides an approach for developing more sensitive MB. Our observation indicates that MB hybridization is accompanied by SEP, which leads to severe loss in fluorescent signal. Thus, SEP needs to be eliminated in order to achieve high sensitivities in gene analysis. One efficient way to accomplish this is to cover one sticky end, as shown in Fig. 1a. This strategy has actually been applied to an initial trial in MB design for ultrasensitive DNA analysis in our lab. Second, SEP observation can provide guidance for SEP-based DNA self-assembly by facilitating optimization of experimental parameters [4].

Third, the SEP assay will be useful for mechanism studies of nonhomologous end-joining. As a major pathway for the repair of DNA double-stranded breaks, nonhomologous end-joining has been studied intensely; yet limited information is known about its mechanism at this time. SEP was suggested to be an important step in most nonhomologous end-joining reactions [14]. Our result here is the first direct solution-phase evidence for the strong tendency of intermolecular pairing of short sticky ends without help by enzymes or proteins. Further study is necessary to understand the roles of SEP in nonhomologous end-joining, in which sticky-end length and sequences, physiological factors, and pairing mode (side by side or tandem) all need to be considered.

In summary, a novel real-time assay, for the first time, was developed for the observation and quantitation of DNA sticky-end pairing. The simplicity, convenience, and high sensitivity of this assay will make it widely useful for the characterization of DNA sticky-end pairing and for the study and understanding of various biophysical processes that involve DNA sticky-end pairing, from nonhomologous end-joining to DNA self-assembly. Our observation clearly indicates that DNA sticky-end pairing may cause severe loss in the fluorescent signal in molecular beacon DNA probes during hybridization. Sticky-end pairing needs to be avoided in order to achieve high sensitivity. Using one of the stems of the molecular beacon for sequence specificity will result in a higher analytical sensitivity in bioanalysis and biotechnological studies.

Acknowledgments

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