Fast Molecular Beacon Hybridization in Organic Solvents with Improved Target Specificity

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DNA hybridization is of tremendous importance in biology, bionanotechnology, and biophysics. Molecular beacons are engineered DNA hairpins with a fluorophore and a quencher labeled on each of the two ends. A target DNA can open the hairpin to give an increased fluorescence signal. To date, the majority of molecular beacon detections have been performed only in aqueous buffers. We describe herein DNA detection in nine different organic solvents, methanol, ethanol, isopropanol, acetonitrile, formamide, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), ethylene glycol, and glycerol, varying each up to 75% (v/v). In comparison with detection in water, the detection in organic solvents showed several important features. First, the molecular beacon hybridizes to its target DNA in the presence of all nine solvents up to a certain percentage. Second, the rate of this hybridization was significantly faster in most organic solvents compared with water. For example, in 56% ethanol, the beacon showed a 70-fold rate enhancement. Third, the ability of the molecular beacon to discriminate single-base mismatch is still maintained. Lastly, the DNA melting temperature in the organic solvents showed a solvent concentration-dependent decrease. This study suggests that molecular beacons can be used for applications where organic solvents must be involved or organic solvents can be intentionally added to improve the molecular beacon performance.

Introduction

DNA hybridization is of tremendous importance in biology, bionanotechnology, and biophysics.[1−7] The relative ease in DNA synthesis and modification has allowed a variety of DNA probes to be used in genomics, disease diagnosis, bioanalytical chemistry, and nanotechnology.[8−16] Analytical tools including gene chips,[17] polymerase chain reactions,[18] molecular beacons,[15,19] and functionalized nanoparticles[20] have all been developed using DNA hybridization properties. To date, such assays have been almost exclusively performed in aqueous buffers, which could be related to the perception that DNA has poor solubility and stability in organic solvents. As in the case of ethanol, it is routinely used for DNA precipitation, and it is well known that ethanol can decrease DNA melting temperature (T_m) and facilitate DNA denaturation.[21] Protein enzymes have been systematically studied in organic solvents, and their applications in making pharmaceutical compounds and fine chemicals have been well-documented.[22,23] Studying DNA hybridization in organic solvents may extend the application of DNA-based tools and assays to many other fields of research, such as the monitoring of organic wastes,[24] studying chemical reactions in organic solvents with DNA,[25] and understanding nanomaterials with DNA probes.[26] In addition, we may gain a better biophysical understanding of DNA hybridization. One important aspect related to practical applications is the kinetics of hybridization where a higher rate of reaction would allow for a quicker sensor response for homogeneous assays. For immobilized DNA sensors, however, other kinetic processes such as diffusion may be the rate-limiting step.

Using DNA-functionalized gold nanoparticles (AuNPs), we recently reported that small amounts of alcohol (e.g., <30%) in low ionic strength buffers can accelerate DNA-directed nanoparticle assembly.[27] However, the self-aggregation of AuNPs at high alcohol and salt concentrations did not allow a complete understanding of the role of organic solvents in the assembly process. The tendency of AuNP self-aggregation in solvents with a low dielectric constant likely occurs because of the high density of negatively charged DNA immobilized on the AuNP surface resulting in strong electrostatic interactions. We may observe a faster hybridization rate with nonimmobilized probes such as molecular beacons. Molecular beacons are engineered DNA hairpins with the two ends labeled with a fluorophore and a quencher, respectively.[15,19,28] The beacon in the closed hairpin form has low fluorescence. In the presence of target DNA, the hairpin is opened, resulting in an increase in the fluorescence. Molecular beacons have been widely used for DNA/RNA detection in medical diagnosis,[19,28] real-time PCR, and biosensor development.[29] Over the past 15 years, a number of improvements have been made on the beacon fluorophore,[30,31] quencher,[32−34] and the DNA backbone[35−37] to enhance signal, decrease background, and increase specificity and stability. Varying the solvent composition may provide another factor for molecular beacon engineering.

There are numerous studies regarding the hybridization of DNA in water.[1,38−42] Because DNA is a polyanion, increasing the salt concentration screens the negative charge. resulting in a faster hybridization rate, whereas increasing the temperature can sometimes decrease the rate.[43,44] Few reports exist regarding DNA hybridization in various organic solvents. In one study, formamide was shown to accelerate DNA hybridization.[45−47] In another, the formation of an emulsion with the addition of phenol to water also increased the hybridization rate, which was attributed to interfacial adsorption and diffusion.[48,49] Because of its complexity, however, the mechanism of DNA hybridization is still under debate.[50]

Herein we report molecular beacon hybridization in nine different organic solvents up to 75% (v/v). Hybridization of the target DNA with the beacon is achieved in all nine solvents. In

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TABLE 1: DNA Sequences and Modifications Used in This Work[a]

name	sequences and modifications (listed from 5′ to 3′)
beacon	FAM-GCGAGCCAGGTTCTCTTCACAGATGCGCTCGC-BHQ1
target	ACGCATCTGTGAAGAGAACCTGGG
Mis1	ACGCATCTTTGAAGAGAACCTGGG
Mis2	ACGCATCTCTGAAGAGAACCTGGG
Mis3	ACGCATCTATGAAGAGAACCTGGG
Mis4	CTTCTTTCTTCCCCTTGTTTGTTG
34-mer	ACACAACGCATCTGTGAAGAGAACCTGGGACACA
44-mer	ACACAACACAACGCATCTGTGAAGAGAACCTGGG ACACAACACA
60-mer	ACAACACAACACAACACA ACGCATCTGTGAAGAGAACCTGGG ACACAACACAACACAACA

[a] BHQ1 denotes Black Hole Quencher 1. The mutated nucleotides in Mis1-3 are highlighted by an underline. For the last three entries, the target DNA sequence is embedded in the middle highlighted by an underline.

particular, the rate of hybridization is significantly accelerated in most solvents (e.g., up to 70-fold in 56% ethanol). At the same time, DNA melting temperature (T_m) decreases in all nine solvents. The increased beacon response and decreased T_m allow better discrimination of mismatched DNA targets. The fast hybridization rates can be explained by the decreased activation energy in the presence of organic solvents.

Materials and Methods

DNA and Chemicals. The molecular beacon DNA was purchased from Gene Link (Hawthorne, NY). The other DNA samples were purchased from Integrated DNA Technologies (Coralville, IA). The DNA sequences and modifications used are shown in Table 1. NaCl and HEPES for making the buffers were purchased from Mandel Scientific (Guelph, Ontario, Canada). All of the buffers and solutions were made using Millipore water. All of the organic solvents were purchased from VWR (Mississauga, Ontario, Canada), and solvent percentages added are in v/v.

Kinetic Studies. Most of the kinetic studies were performed using a Molecular Device Spetramax M5 plate reader. The excitation wavelength was set at 485 nm, and emission at 520 nm was monitored. All operations were performed at room temperature (26 °C). For the majority of the experiments, 50 nM molecular beacon was dissolved in 300 mM NaCl, 10 mM HEPES, pH 7.6 with varying percentages of organic solvents. The fluorescence of this sample was monitored for 2 min. Immediately to this sample, 2 μL of target DNA was added (final target concentration 200 nM in a volume of 100 μL), and the fluorescence signal was monitored every 10 s for 5 min. Approximately 16 s elapsed from the time the target DNA was added to the first reading of the machine. This elapsed time was included for both the kinetic calculations and plotting purposes. All of the kinetic studies were performed in triplicate. The kinetic data shown in Figure 2B were obtained using a PTI fluorometer every 1 s after the addition of the target DNA. The beacon and target DNA concentrations were 1.25 and 12.5 nM, respectively.

DNA Melting Curves. The melting curves were collected with a sample volume of 11 μL containing 1 μM of the molecular beacon and 4 μM of the target DNA. The mixture also contained 300 mM NaCl and 10 mM HEPES, pH 7.6. The samples were loaded in a 96-well PCR plate and sealed with a heated (105 °C) plastic cover to avoid evaporation. The samples were analyzed using a Bio-Rad CFX-96 real-time PCR thermocycler. The LED excitation range was set to be 450−490 nm, and the emission at the 520 nm region was collected (the FAM channel). The instrument was programmed to first anneal the samples from 85 to 6 °C; then, the melting curves were measured from 6 to 95 °C with 1 °C increment and 10 s holding time.

Results and Discussion

System Design. For this study, we employed a molecular beacon with a six-base-pair stem and a 20-nucleotide loop. The presence of a target DNA is expected to open the hairpin, therefore producing a fluorescence signal increase. To test the effect of an organic solvent on molecular beacon hybridization, we chose to use ethanol for most of the studies because ethanol is a common laboratory solvent, is nontoxic, and is routinely used for nucleic acid extraction and precipitation. To understand the mechanism of hybridization rate change in organic solvents, DNA melting studies have also been performed, and there are two types of melting in the molecular beacon system: the beacon alone melts at a temperature lower than the duplex DNA formed by the beacon and the target DNA, which is the thermodynamic basis for molecular beacon detection. The T_m of the beacon DNA alone is independent of its concentration because this is an intramolecular transition. However, the T_m of the duplex DNA is a function of DNA concentration.[51] Therefore, the beacon and target DNA concentrations have been kept constant for most of the experiments.

Effect of Varying Ethanol Percentage. To study the molecular beacon hybridization kinetics, the ethanol concentration was first varied in 300 mM NaCl with 10 mM HEPES, pH 7.6. In a typical experiment, the fluorescence of 50 nM molecular beacon was monitored for 2 min prior to the addition of 200 nM target DNA. In the aqueous buffer (no ethanol), it takes >10 min for the fluorescence signal to plateau, as shown in Figure 1A (black curve) and Figure 2B (the 300 mM trace). This rate is typical for molecular beacon detection in aqueous buffers.[19] The hybridization kinetics become progressively faster as the ethanol content is increased to 56% (Figure 1A), where the signal saturates in less than 16 s (the fastest time that can be achieved by manual pipetting and the fluorescence plate reader). In the case of 75% ethanol (pink curve), no fluorescence increase was observed, and the background fluorescence was lower compared with that of the other samples. This suggests that most of the beacon DNA was in the hairpin conformation, and the addition of the target DNA failed to form the duplex structure. DNA is known to dehydrate and aggregate in the presence of high amounts of alcohol and salt rather than forming Watson−Crick base-pairing interactions.[52] As a result, we chose to perform most studies using 56% ethanol to achieve both high reaction rates as well as specificity.

When the ethanol percentage was varied from 0 to 56%, the final fluorescence intensities after addition of the target DNA saturated at similar intensities if enough time was allowed to elapse. In addition, the initial fluorescence intensities are also similar for these samples (Figure 1A and Figure 2B, the 300 mM trace). Because the observed fluorescence intensities are directly related to the equilibrium between the hairpin and the duplex state, ethanol did not significantly shift the equilibrium of the molecular beacon/target hybridization reaction. The rapid fluorescence increase suggests that the hybridization rate is increased. To maintain a similar equilibrium constant, the reverse

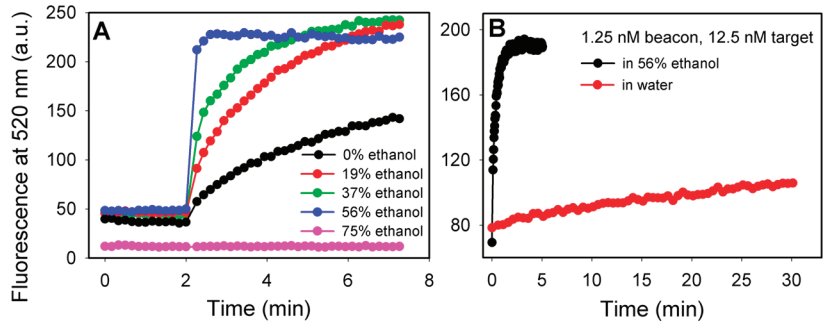

Figure 1. Kinetics of molecular beacon hybridization in 300 mM NaCl 10 mM HEPES, pH 7.6 with different ethanol percentages. (A) With 50 nM molecular beacon and 200 nM target DNA. DNA was added at time of 2 min. (B) With 1.25 nM molecular beacon and 12.5 nM target DNA.

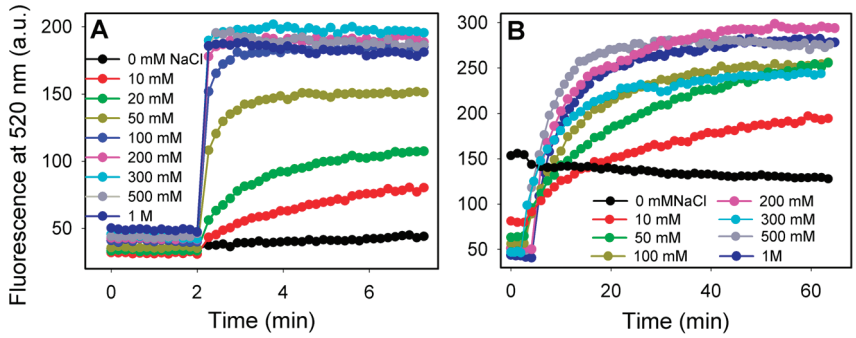

Figure 2. Effect of increasing NaCl concentration on DNA hybridization (A) in the aqueous buffer and (B) in 56% ethanol. Note that fluorescence was monitored after the addition of target DNA for (A) 5 min or (B) 1 h.

reaction rate must also increase to a similar extent. Therefore, ethanol functions as a catalyst for molecular beacon hybridization.

Because with a respective concentration of 50 and 200 nM for the molecular beacon and target DNA, the reaction was completed in the first 16 s, to understand quantitatively the rapid kinetics of hybridization, we decreased the concentration of the beacon and target DNA to obtain data points in the initial stage of the reaction. The concentration of the molecular beacon was decreased to 1.25 nM, and the target DNA was decreased to 12.5 nM in 56% ethanol. As shown in Figure 1B (black curve), the decreased DNA concentrations allowed us to observe the initial kinetic profile. In particular, this pseudo-first-order reaction condition (target DNA/molecular beacon 10:1) allowed us to fit the kinetics data with the equation $y = y_0 + a(1 - e^{-bt})$, where b is the rate of the reaction. A rate of 2.5 min^{-1} for DNA hybridization was determined for the sample in 56% ethanol, and a rate of 0.035 min^{-1} was determined with no ethanol ($R^2 = 0.98$ for both fittings), giving a rate enhancement of ~70-fold in 56% ethanol.

Effect of Salt Concentration. Because DNA is a polyanion, electrolytes such as NaCl play a crucial role in the hybridization kinetics. With the optimal ethanol concentration of 56%, we tested the effect of salt. As shown in Figure 2A, very little signal increase was observed if no NaCl was present, and this is consistent with the notion that salt is required for DNA hybridization. With increasing NaCl concentration, the final fluorescence signal increased, suggesting that NaCl can control the reaction equilibrium and a higher salt concentration favors duplex formation. The kinetics of hybridization were very fast with >100 mM NaCl. For comparison, Figure 2B shows the salt-dependent experiments in the aqueous buffer (no ethanol). The rates were much slower, requiring the hybridization reaction to be monitored for 1 h. The hybridization rate increased until NaCl was increased to 200 mM. Further increase in NaCl had very little effect on the rate or final fluorescence intensity. Therefore, increasing NaCl in water can accelerate molecular beacon hybridization, but there is a limit (~200 mM in this case). Importantly, ethanol-induced acceleration shown in Figure 1A (with 300 mM NaCl) is on top of the NaCl effect. We also noticed that the final fluorescence intensities in high salt buffers (e.g., >100 mM in Figure 2) were higher for water than for ethanol. This was attributed to the decreased T_m in ethanol and therefore less duplex DNA was formed (vide infra).

Mismatched DNA Targets. For sequence-selective DNA detection, an important aspect is to distinguish single-base mismatches. To test the target specificity of our molecular beacon in ethanol, four DNA strands containing mismatches were also studied. As shown in Figure 3A, fast hybridization rates were observed for the three target DNAs containing single-base mismatches (Mis1−3, see Table 1 for the location of mismatches). However, the final fluorescence intensities were lower compared with the perfectly matched target. This experiment further supported that the reaction equilibrium can be quickly established in ethanol. For a completely nontarget sequence (Mis4), no fluorescence enhancement was observed. Therefore, the molecular beacon in ethanol was capable of selectively binding to its target DNA, and the observed fluorescence enhancement was due to specific DNA hybridization instead of nonspecific interactions. Interestingly, the mismatched and matched DNA targets behaved quite similarly in the aqueous buffer. As shown in Figure 3B, two of the mismatched targets (Mis2 and Mis3) appear to hybridize slower compared with the perfectly matched one. However, Mis1 showed a similar hybridization rate to the matched target. It is also difficult to distinguish these DNAs containing single-base mismatches from the perfect target in water by monitoring the final fluorescence; they all showed a similar final fluorescence (Figure 3B). These results indicate that ethanol also allows better recognition of mismatched DNA strands.

To better understand the observed effects, DNA melting experiments have been performed. First, the beacon was allowed to form a duplex with the target DNA; then, the T_m of this duplex

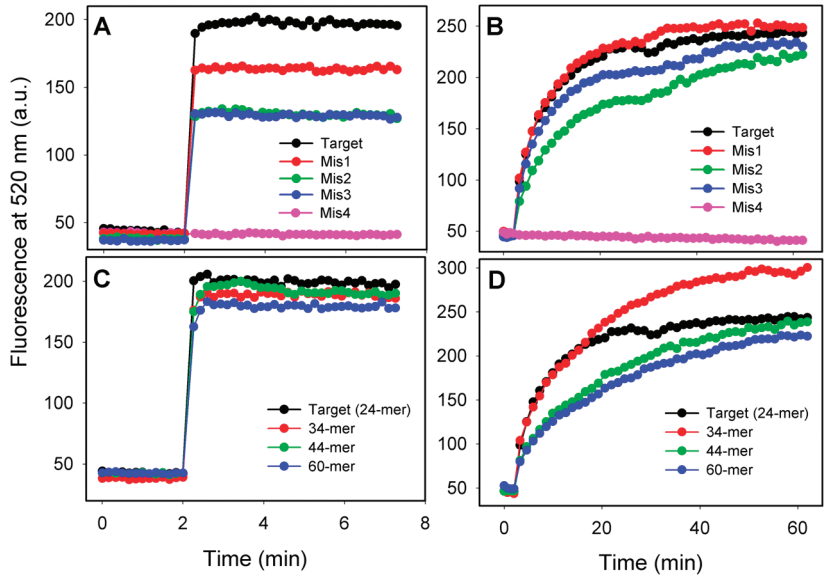

Figure 3. Detection of target DNA sequences containing mismatches in (A) 56% ethanol and (B) water. Detection of long target DNA sequences in (C) 56% ethanol and (D) water. All of the samples contained 300 mM NaCl, 10 mM HEPES, pH 7.6.

was measured. A T_m value of 71 °C in the 300 mM NaCl aqueous buffer was obtained for the target DNA. When DNA strands contained single-base mismatches, the T_m values dropped to ~60 °C. Because these T_m values were much higher than room temperature (26 °C), at which the experiment was conducted, the decreased stability brought by the mismatches was not obvious. In 56% ethanol, a T_m of 50 °C was observed with the perfectly matched target DNA, and for mismatched targets, T_m values close to 38 °C were obtained, which were close to the experimental temperature of 26 °C. Therefore, the fraction of the beacon in the duplex form was much more sensitive to the T_m in ethanol. Whereas it is possible to decrease the T_m of DNA by simply decreasing the salt concentration, the hybridization kinetics also decreases. In ethanol, however, we have achieved increased hybridization kinetics with high target specificity.

For detection of biological DNA samples, it is likely that the molecular beacon can probe only a small portion within a long DNA target. To test the effect of target DNA length, DNA strands made up of 60, 44, and 32-mer with the target sequence embedded within the strands were also tested. As shown in Figure 3C, all of the DNA showed a fast hybridization rate and similar final fluorescence intensity in 56% ethanol. For comparison purposes, the hybridization rates were also monitored in water (Figure 3D). The hybridization rates were slightly faster with two shorter targets compared with the longest one, but overall, the rates were significantly slower compared with that in ethanol. The fast hybridization could be important for very long DNA samples to allow a shorter incubation time.

Molecular Beacon Hybridization in Other Organic Solvents. Whereas ethanol is an important solvent for DNA manipulation, DNA can also be dissolved in many other organic solvents. To test the effect of organic solvents on hybridization kinetics systematically, we performed solvent concentration-dependent assays using eight other common laboratory solvents (Figure 4). Significant rate enhancements were observed for most solvents within a certain concentration range. Methanol was slightly less effective compared with ethanol (Figure 4A). Fast hybridization was observed only at 75%, and the final fluorescence intensity was also lower. Although isopropanol showed a rapid enhancement at 56%, the final fluorescence at that alcohol concentration was also quite low (Figure 4B). Acetonitrile showed rapid hybridization even at 37% (Figure 4C). Although acetonitrile is miscible with water alone, phase separation occurs at percentages >56% in the presence of 300 mM NaCl. Therefore, higher concentrations were not tested for this particular solvent. Formamide, dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) showed a quite similar behavior (Figure 4 D−F); all gave an optimal rate at 37%. Ethylene glycol showed only a moderate rate enhancement compared with other solvents, whereas glycerol did not show any obvious rate enhancement. These experiments indicate that the molecular beacon detection can occur in many organic solvents within a certain percentage range, and a significant rate increase can be observed. However, if the percentage of an organic solvent is too high, then hybridization is inhibited.

Melting Curves. To understand further the observed hybridization kinetics, the melting curves of the beacon-target duplex were measured in 300 mM NaCl, 10 mM HEPES, pH 7.6 with varying percentages of organic solvents. There is a decrease in T_m with increasing solvent percentages, and this trend appears to be linear for most solvents (Figure 5). This is in good agreement with the literature reports where T_m was measured by other methods.[53,54] Only four solvents have been measured up to 75% (methanol, glycerol, ethylene glycol, and formamide). The rest of the solvents failed to show a melting transition at high concentrations, and the plots were stopped at the highest solvent concentration, where a melting transition can still be observed.

In general, solvents that were effective in decreasing the T_m of DNA, such as the alcohols, formamide, ACN, and DMF, were also quite effective in accelerating the hybridization kinetics. Ethylene glycol and glycerol showed a smaller T_m slope, and the kinetics increase was also small. Viscosity may also play an important role for some of the solvents such as glycerol. Drastically increasing the viscosity should disfavor hybridization, which may explain why glycerol showed little increase in the hybridization rate even with decreased T_m. Some solvents such as those tested alcohols are known to precipitate DNA and cause DNA aggregation at high solvent concentrations.[21,52] For most tested solvents at 75%, for example, very little fluorescence change was observed in the presence of the

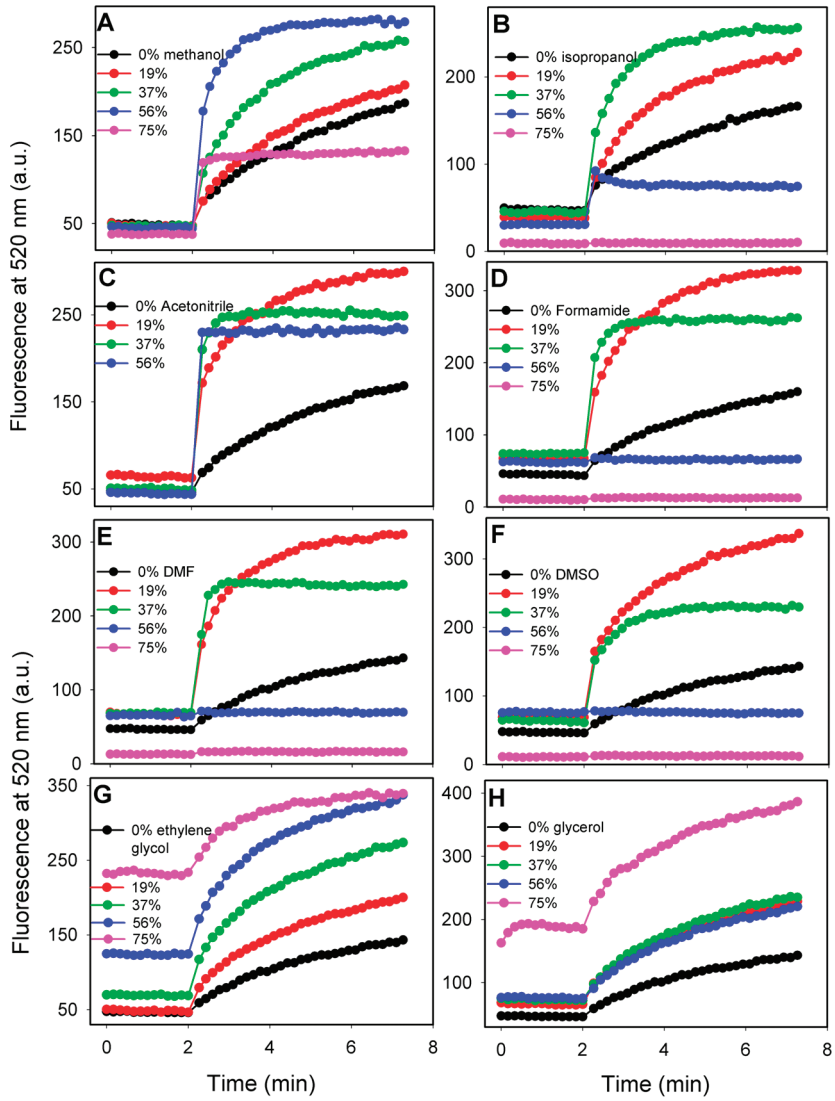

Figure 4. Kinetics of molecular beacon and target DNA hybridization in various percentages of organic solvents in 300 mM NaCl, 10 mM HEPES, pH 7.6. The target DNA was added after 2 min.

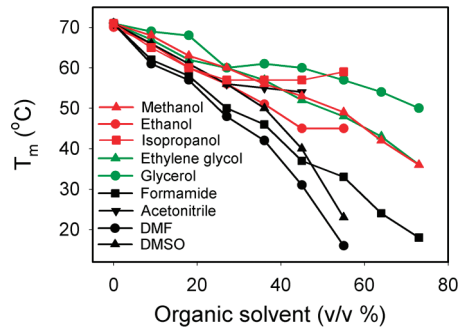

Figure 5. Melting temperatures of the molecular beacon hybridized to the target DNA in 300 mM NaCl, 10 mM HEPES, pH 7.6 with various percentages of organic solvents.

target DNA (Figure 4), possibly due to after DNA aggregation/precipitation, and the diffusion and binding become more difficult. For most tested solvents under relatively dilute conditions (e.g., <50−60% ethanol),[52] DNA is not known to aggregate; therefore, the observed kinetics change should be independent of such processes. On the basis of the T_m and kinetic data, the effect of organic solvents on the hybridization rate appears to stem from the decrease in activation energy for the hybridization and dehybridization (or melting) reactions to a similar extent, such that the rate is enhanced without significantly shifting the reaction equilibrium.

Conclusions

In summary, molecular beacon hybridization can be carried out in a wide range of organic solvents. A significant hybridization rate enhancement was observed for most solvents. These accelerated hybridization reactions are attributed to the reduced activation energy barrier for the hybridization reaction. At the same time, the reaction specificity is still maintained. This work suggests that DNA-based probes can be used for applications where organic solvents must be involved, and at the same time, organic solvents can be intentionally added to improve the performance of such assays.

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