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Determination of DNA Melting Temperatures in Diffusion-Generated Chemical Gradients

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For fast and reliable determination of DNA melting temperatures with single-nucleotide resolution in a microfluidic setup, stable gradients of the denaturing agent formamide were generated by means of diffusion. Formamide lowers the melting temperature of DNA, and a given formamide concentration can be mapped to a corresponding virtual temperature along the formamide gradient. We applied this concept to determine the melting temperatures of five sets of dye- and quencher-labeled oligonucleotides of different lengths. Differences in the length of complementary sequences of only one nucleotide as well as a single nucleotide mismatch can be detected with this method. Comparison with conventional melting temperature measurements based on temperature scans yields very good agreement.

Due to their central role in biochemistry and molecular biology, the interactions between single strands of DNA with complementary sequences are extremely well studied. At low temperatures, and under favorable buffer conditions, two complementary DNA strands will usually form a double-helical duplex structure, which is stabilized by the hydrogen bonds between these bases and by stacking interactions between neighboring base pairs. A variety of factors such as temperature or denaturing agents, however, can destabilize the DNA double helix. The separation of the duplex into single strands can be regarded as a phase transition and is referred to as "melting" of the double helix. The so-called "melting temperature" T_M of a DNA duplex is defined as the temperature at which half of the double strands are separated into single strands. T_M is a convenient measure for the stability of a DNA duplex, which is important in a variety of contexts. The stability of a DNA duplex will also be reduced when the two single strands composing the duplex are not fully sequence-complementary. A single base mismatch may already lead to a significant reduction in melting temperature. Another source of destabilization is the presence of denaturing chemicals such as formamide or urea, which may also form hydrogen bonds with the bases and therefore compete with Watson−Crick pairing.

Knowledge of the melting temperature T_M of a given DNA or RNA sequence is important for many biotechnological applications. For example, it is crucial for polymerase chain reaction (PCR) experiments, as the PCR amplification principle is based upon DNA denaturation and renaturation cycles. As the melting temperature of a duplex is very sensitive to base mismatches, determination of T_M is also of great interest for the detection of sequence mutations or variations such as single-nucleotide polymorphisms (SNPs). Knowledge of the stability of DNA structures is also of great importance in the emerging area of DNA nanotechnology, in which DNA is used as a building material for artificial supramolecular structures and devices.[1]

In many cases, the T_M of a DNA or RNA duplex can be accurately determined within a theoretical model. While for sequences shorter than 15 bases the simple Wallace rule[2] often yields satisfactory results, for longer sequences more complex models have to be used. One of the most accurate models presented by SantaLucia and co-workers is based on the nearest neighbor thermodynamic parameters for Watson−Crick pairs.[3,4] In many cases, however, a computational approach is not feasible or accurate, e.g., when nonstandard buffer conditions are used, or when two DNA strands are mismatched or contain extensive secondary structure. In these cases, experimental determination of T_M is necessary.

The common experimental method to determine T_M is to slowly heat a sample of buffer solution containing the duplex of interest, while recording its absorbance at a wavelength of 260 nm. Absorption at this wavelength is due to in-plane transitions of the π electrons of the bases. Since the absorbance of two single strands is higher than the absorbance of the same strands forming a double helix, the cooperative melting of a DNA duplex can be monitored as an increase of absorption with temperature. For accurate T_M measurements a slow temperature sweep—typically 0.1−0.5 °C/min—is important to allow the system to equilibrate. Measuring the melting temperature of a given sequence between room temperature and 90 °C thus can easily take 12 h. A further disadvantage of the absorbance method is its relatively high sample consumption. Thermal cycler platforms containing an integrated microvolume fluorometer work with sample volumes of only 10 μL and allow for the fast analysis of PCR products during their amplification.[5−7] An essential disadvantage of such devices is their high acquisition cost. Dodge et al. used a microfluidic

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setup with an integrated heating unit to perform dehybridization experiments with surface-bound DNA.[8]

Here we present a method to determine the T_M of DNA duplexes in solution in an inexpensive, reusable, and easy-to-build microfluidic setup. The concept is based on the fact that formamide lowers the melting temperature of DNA double strands 0.6 °C per 1% of formamide in the buffer.[9,10] By building up a stable, linear gradient of formamide in a solution containing the DNA strands of interest, it is possible to read out T_M by analyzing the ratio of double-stranded and single-stranded DNA along the formamide gradient. For practical reasons, we did not monitor the melting transition of DNA by measuring the absorbance at 260 nm. Instead, the dissociation of fluorescently labeled duplex DNA into single strands was monitored with Förster resonance energy transfer (FRET)[11] (cf. the Results and Discussion), both in the microfluidic setup on an epifluorescence microscope and using a fluorescence spectrometer. The obvious disadvantage of this method is the expensive labeling of the corresponding DNA strands with proper FRET donors and acceptors. However, the setup used here could in principle be adapted to allow also for absorbance measurements.

EXPERIMENTAL SECTION

Preparation of Poly(dimethylsiloxane) (PDMS) Chambers. We fabricated multilayer microfluidic chambers containing on−off valves as described in ref 12.

Two silicon wafers—one for the control channels and one for the flow channels—were prepared by exposing a 20 μm thick layer (spin coating for 45 s at 900 rpm, soft bake for 2 min at 60 °C, 5 min at 115 °C, and 2 min at 60 °C) of SPR 220-7.0 (Microresist, Germany) for 25 s to light from a mercury lamp projected through a photoplotted transparency (Zitzmann GmbH, Germany) using a standard mask aligner (Carl Suss, Germany). Exposed wafers were developed for 3 min in SD 334 (Microresist, Germany), washed in H_2O, and dried with N_2. Prior to PDMS contact, the processed wafers were exposed to the vapor of trichloro(perfluorooctyl)silane (Sigma-Aldrich, Germany) for 2 min to facilitate the later removal of the cured PDMS.

A 2 cm thick 5:1 (A:B) mixture of PDMS (Sylgard 184, Dow Corning, Germany) was poured onto the wafer bearing the structure for the control channels. After a 60 min curing step at 80 °C this control layer was peeled off from the wafer. The second wafer supporting the flow channels was covered by a layer of PDMS (20:1), which was spin-coated (60 s, 2100 rpm) and cured (30 min, 80 °C). After punching the holes for the air-control tubing into the control layer, the control layer was aligned manually onto the flow-channel wafer. During additional curing for 30 min, the flow layer conjoins with the control layer and the joint layers were peeled off the wafer. After punching the holes for the flow tubing, the joint layers were placed on a glass object carrier (50 mm × 75 mm, Marienfeld, Germany), which was covered by a layer of spin-coated (60 s, 2400 rpm) and cured (30 min, 80 °C) PDMS (20:1). The whole microfluidic chamber was cured another 2 h at 80 °C, cooled down, and left overnight.

Chemical Gradient Measurements. A home-built valve controller with electrically actuated microvalves (LHDA1211111H, Lee Valves, Germany) as core elements was used to address the microfluidic chamber. The control channels and the tubes connected to the common ports of the valves were filled with water. A few microliters of PBS buffer (150 mM NaCl, 10 mM KCl) containing 2.5 μM of strand M with the sequence 5′ RG-CCC TAA CCC TAA CCC TAA CCC 3′, 2.5 μM and one of the five quencher-labeled strands (M'_{11}, 5′ GTT AGG GTT AG-BHQ-1 3′; M'_{12}, 5′ G GTT AGG GTT AG-BHQ-1 3′; M'_{14}, 5′ AGG GTT AGG GTT AG-BHQ-1 3′; M'_{14mm}, 5′ AGG GT**A** AGG GTT AG-BHQ-1 3′; M'_{17}, 5′ GTT AGG GTT AGG GTT AG-BHQ-1 3′) were filled into the reservoirs, separated by the pushed down valve in the middle (oligos purchased from biomers.net, Germany). The buffer in the right chamber additionally contained 75% formamide. After closing both inlet and both outlet valves the middle valve was opened, which led to a fast, diffusion-driven buildup of a stable formamide gradient in the connection channel between the two reservoirs. Fluorescence microscopy was performed with an Olympus IX 71 microscope using a 10× objective. Images were recorded with a CCD camera (Photometrics CoolSnap HQ). Below 250 nM concentration of labeled DNA the extracted line scans became noisy and hard to interpret. However, the sensitivity of our device could be enhanced by an improved optical setup.

Temperature Sweep Measurements. The temperature scans were performed with a fluorescence spectrometer (Fluorolog-3, Jobin Yvon, Germany). For each scan a solution of 300 μL of PBS buffer containing 2.5 μM of the RG-labeled oligo, 2.5 μM of one of the three quencher-labeled strands, 150 mM NaCl, and 10 mM KCl was filled into a glass cuvette, which was placed in the excitation beam (480 nm). While slowly heating the sample from 20 to 65 °C (1 °C every 3 min) the fluorescence was recorded at 525 nm.

T_M Calculations. Theoretical values were obtained using the melting temperature calculator provided by IDT (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx) using the following settings: target type, DNA; oligo concentration (2.5 μM + 2.5 μM)/4; NaCl, 150 mM. The model used for the calculations is described in ref 3.

RESULTS AND DISCUSSION

To create a stable concentration gradient, we prepared a multilayer microfluidic chip[12] (Figure 1) and mounted it on a fluorescence microscope. The flow layer consists of two inlets and two outlets accessing two reservoirs, which are separated by a connecting channel. If pressure is applied to the channels of the control layer, which cross the flow channels at designated points, the PDMS membrane separating the two layers is pushed down and acts as a closing valve at these points. If the valve between the two reservoirs is pushed down during the filling process, the liquids in the two reservoirs stay separated.[13] In a calibration experiment (Figure 2) we filled the left reservoir with PBS buffer alone and the right reservoir with PBS buffer containing 2.5 μM

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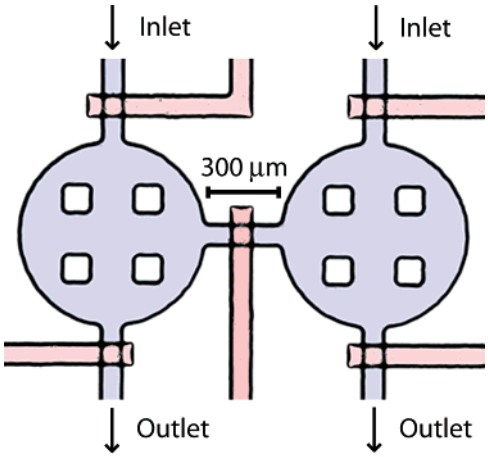

Figure 1. Schematic representation of the two-layered microfluidic setup. The blue flow channels and reservoirs are located beneath the red-colored control channels, separated at the intersections by a thin layer of PDMS. All channels are 100 μm wide and 20 μm high. The diameter of both reservoirs is 750 μm. After subtracting the volume occupied by the supporting columns of 110 μm edge length (white squares), the volume of each reservoir is 7.9 nL, whereas the volume of the 300 μm long connecting channel is only 0.6 nL.

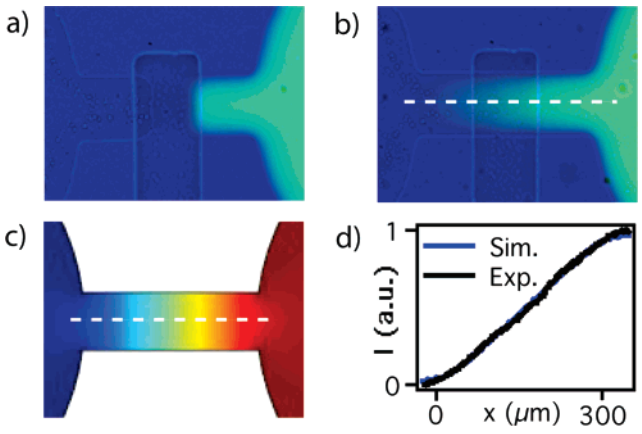

Figure 2. Calibration of the device. (a and b) Overlaid fluorescence (green) and bright-field (blue) images of the reservoirs and the connection channel. (a) The left reservoir was filled with buffer, and the right reservoir was filled with buffer containing a fluorescently labeled oligonucleotide while the valve in the middle was closed. (b) One minute after opening the valve a stable, linear gradient is established between the reservoirs. (c) FEMLAB simulation of the concentration gradient. (d) Intensity profiles along the dashed lines in (b) and (c).

of the 21-mer DNA strand M labeled at the 5′ end with the fluorescent dye rhodamine green (RG). Within 1 min after opening the intersecting valve, the two solutions mixed diffusively and a linear gradient of the labeled oligo along the connection channel was established, which could be monitored with the fluorescence microscope. Since the reservoirs are large in comparison to the connection channel, the concentrations of the analytes in the reservoirs did not change significantly during the first minutes while the gradient was already established. Due to the low Reynolds numbers in microfluidic systems, convection is negligible in our setup.

In a simplified model, the channel can be considered as a one-dimensional (1D) tube of length L, containing molecules or particles with a constant diffusion coefficient D and fixed concentrations $c(x = 0, t) = 0$ and $c(x = L, t) = c_0$ at its ends. With these boundary conditions, the solution to the 1D diffusion equation $\partial c(x, t)/\partial t = D\ \partial^2 c(x, t)/\partial x^2$ is time independent and reads $c(x) = c_0 x/L$ for $0 \le x \le L$. Hence, a linear gradient is expected in the channel. In order to make sure that this is also true for our experimental channel geometry, the two-dimensional diffusion equation was solved using the finite element simulation program package FEMLAB. The boundary condition along the margin of the left reservoir was set to a constant concentration of zero, while the concentration along the margin of the right reservoir was set to one. Indeed, the simulation revealed a linear concentration gradient along the connection channel, which was reproduced perfectly in the experiment (Figure 2d). The equilibration of the concentrations in the two reservoirs during experiments took 40 min for the small formamide molecule and 6.1 h for the dye-labeled 21-mer. This shows that, in principle, our microfluidic setup could also be used to determine the diffusion constant of a molecule of interest. Changing the dimensions of the connection channel and reservoirs can shorten or lengthen the equilibration times and can therefore be adjusted to the requirements of the application aimed at. The continuity of the gradient and the fact that sustained input of liquid is not needed to maintain the gradient distinguishes our approach from previously proposed methods, which mostly create stepwise gradients under continuous flow.[14,15]

To determine the melting temperatures of strand M paired with either of four complementary strands M'_{11}, M'_{12}, M'_{14}, M'_{17}, consisting of 11, 12, 14, and 17 nucleotides and labeled with the quencher BHQ-1 at their 3′ ends we prepared two buffer solutions for each pair of oligonucleotides: one contained 0%, the other 75% formamide, while both contained the same amount of DNA. At low formamide concentrations the DNA was present in the double-stranded conformation, and the dye at the 5′ end of strand M and the quencher at the 3′ ends of strands M'_{11}, M'_{12}, M'_{14}, M'_{17} were in close proximity, which led to strong fluorescence quenching due to FRET. At high formamide concentrations the duplexes dissociated into single strands, and the fluorescence was unquenched due to the spatial separation of dyes and quenchers (Figure 3a).

In contrast to conventional temperature scan experiments, where typically several hundred microliters of buffer are needed, the separated reservoirs of the microfluidic setup could be filled with only a few microliters of the two corresponding solutions. After the separating valve was opened a formamide gradient established, and meaningful fluorescence images could already be recorded after 1 min. The intensity profiles along the connecting channel were extracted, normalized, and plotted and the linear formamide gradient along the channel was mapped onto a temperature scale. From this plot the melting temperature of the DNA duplexes could be determined (Figure 3b).

We also determined conventional DNA melting curves with a fluorescence spectrometer, with which the fluorescence of the

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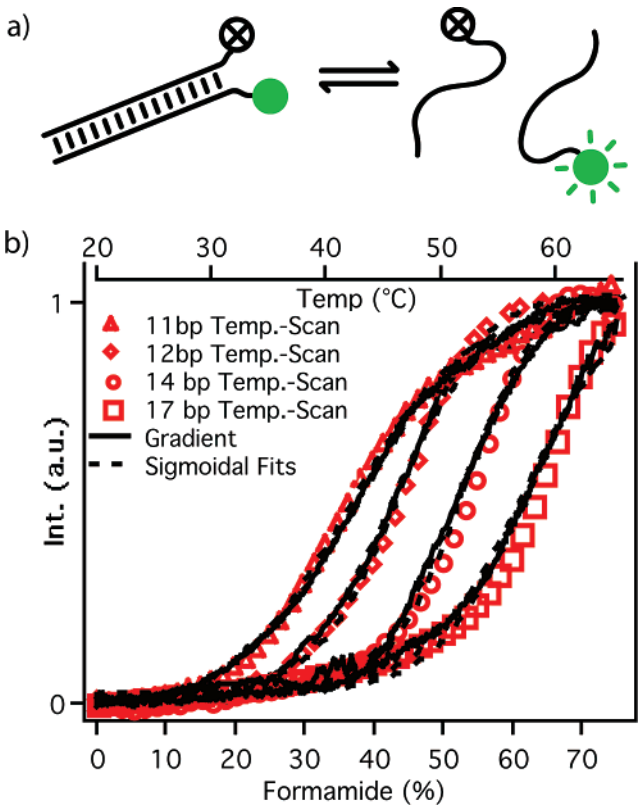

Figure 3. (a) Two complementary DNA strands—one labeled with a fluorophore, the other with a dark quencher—form a double-helical duplex structure at low temperatures and low formamide concentration (left). The quencher and the fluorophore are in close proximity, and the fluorescence is quenched. At high temperatures and high formamide concentrations, the DNA duplex dissociates into single strands and no quenching occurs. (b) Melting temperature measurements from conventional temperature scans in a fluorescence spectrometer compared to measurements performed in diffusion-generated gradients of formamide in the microfluidic setup.

Table 1. Melting Temperatures of Four Pairs of Oligonucleotides of Different Lengths Obtained from Formamide Gradient Measurements, Conventional Temperature Scans, and Theoretical Calculations

no. complementary oligonucleotides	temperature scan (°C)	formamide gradient (°C)	calculation (°C)
11	41.9 ± 0.2	42.5 ± 0.1	37.9 ± 1.4
12	46.4 ± 0.2	45.4 ± 0.1	43.9 ± 1.4
14	52.2 ± 0.2	51.5 ± 0.1	52.0 ± 1.4
17	58.5 ± 0.6	57.5 ± 0.3	55.5 ± 1.4

dye-labeled DNA strands was recorded while slowly sweeping the temperature. Plotting these melting curves in the same graph as the curves obtained from the gradient measurements revealed very good agreement of the two different techniques. Sigmoidal fits yielded the melting temperatures listed in Table 1. The deviations between the values obtained from the two techniques were always less than 2%. The values for T_M calculated with the nearest neighbor method[3] agreed well with the experimental data for all strands but for the shortest M'_{11} and M'_{12}. The deviation of 10% between the calculated value and the value measured with both methods—temperature sweep and gradient experiment—

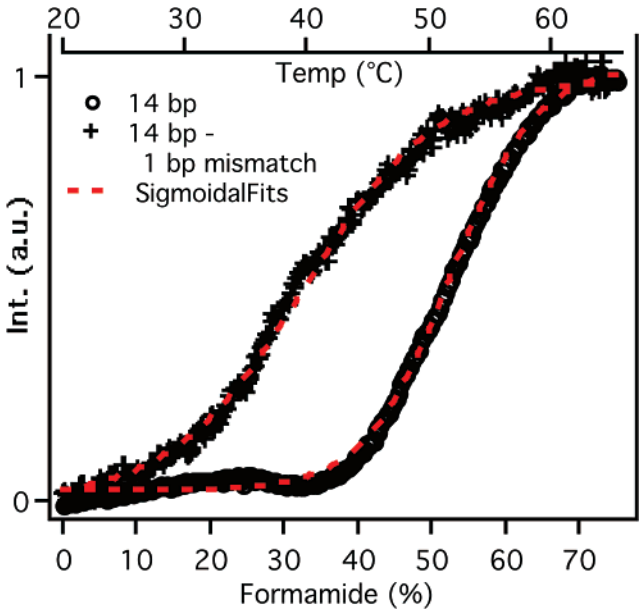

Figure 4. Detection of a single base mismatch. The 14 bp scan was performed with the DNA strand M'_{14}. Introduction of single nucleotide mismatch into the sequence of strand M'_{14mm} (5′ AGG GT**A** AGG GTT AG-BHQ-1 3′) can be easily detected in the diffusion-generated formamide gradient. T_M for the strand M'_{14mm} is 43.8 ± 0.1 °C in contrast to 51.5 ± 0.1 °C for the strand M'_{14}.

demonstrates the need for experimental determination of T_M under certain circumstances.

We also performed an experiment in which a single nucleotide mismatch was introduced into the sequence of one of two otherwise complementary strands. The resulting change in the melting temperature could be easily detected in the diffusion-generated formamide gradient (Figure 4). This demonstrates the potential of our method for the detection of point mutations or SNPs.

CONCLUSION

We have shown that the melting temperature of DNA duplexes can be determined with a linear formamide gradient created in a microfluidic chip. The low sample consumption of the method, the ability to detect single base mismatches and length differences, and the fact that all gradient measurements displayed in this work were done within less than 2 h inside the same chamber without any loss in performance clarifies the potential of the method presented here. The characterization of longer oligonucleotides sequences with higher melting temperatures could be accomplished by maintaining the microfluidic chip at a higher temperature, or increasing the maximum formamide concentration, or both. Further improvements of this technique are necessary to circumvent the time and cost-intensive labeling of the DNA strands and could include the use of the double-strand-specific dye SYBR green I,[6] the utilization of molecular beacons,[16,17] or the application of UV absorption measurements along the gradient. The generated gradients are stable, linear, and continuous and should be applicable to other solutes. Measurements on protein and RNA

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degradation[18] are conceivable as well as studies of biological morphogenesis, which relies on diffusive gradients of gene transcription factors.

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