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Temperature Difference between the Cooled and the Noncooled Parts of an Electrolyte in Capillary Electrophoresis

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Joule heating always accompanies electrophoresis and unavoidably leads to a temperature increase of the electrolyte. The elevated temperatures are known to adversely affect the quality of separation and detection. To minimize the temperature increase in capillary electrophoresis (CE), Joule heat is removed by actively cooling the capillary. However, there are always small parts of the capillary, such as its inlet, outlet, and detection window, which are not actively cooled. The noncooled capillary inlet has been recently proven to have an elevated temperature which is high enough to significantly affect CE-based quantitative affinity analyses. The temperature difference between the cooled and noncooled regions has never been determined due to the lack of a suitable method. Here, we report on the first experimental determination of temperature in the cooled part of the capillary and the noncooled inlet region of the capillary. We found that, under typical CE conditions, with a low-conductivity run buffer, the temperature in the noncooled inlet exceeded the temperature in the cooled region by more than 15 °C. High-conductivity buffers are anticipated to have even greater temperature differences between the noncooled and cooled capillary parts. Our results strongly suggest the potential effect of the noncooled capillary regions on the quality of CE-based analyses, which cannot be ignored. The simplest way to avoid potential errors is to move the sample to the cooled region by pressure or by applying a low electric field.

Joule heating in capillary electrophoresis (CE) unavoidably increases the electrolyte temperature, which can compromise the quality of CE-based analyses in a number of ways. For example, higher temperatures have been proven to cause (i) peak broadening,[1−6] (ii) drift in the analyte migration time,[7] (iii) baseline instability (when using indirect detection),[8] and (iv) analyte decomposition.[9−11] Affinity analyses are also sensitive to temperature changes of only a few degrees, which can lead to significant changes in the kinetic and thermodynamic parameters of affinity interactions.[12,13] To remove the Joule heat and control the electrolyte temperature, CE instruments are typically equipped with capillary thermostabilization systems. A capillary, for most of its length, is placed in contact with air, liquid, or a solid heat exchanger (Figure 1). However, the inlet and the outlet of the capillary, as well as the detection window, are not in contact with the heat exchanger. The temperature in these parts is not well controlled and is usually different from the temperature of the thermostabilized parts. In particular, the difference depends on the room temperature and the temperature of the heat exchanger. In most CE-based analyses, the heat exchanger temperature is within a few degrees of the room temperature and the temperature of the nonthermostabilized parts is obviously higher than the temperature of the thermostabilized parts (for simplicity we will refer to the thermostabilized and nonthermostabilized parts of the capillary as the cooled and noncooled regions, respectively).

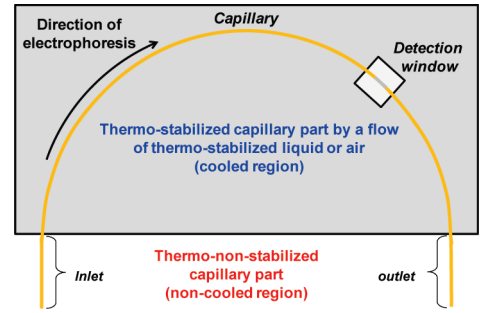

Figure 1. Schematic representation of a capillary cartridge assembly of a standard commercial CE instrument. The major portion of a capillary is actively cooled by a thermostated heat exchanger, which can be a flow of air, liquid, or capillary, or may be in contact with a metal surface. The inlet and outlet regions of the capillary, as well as the detection window, either are exposed to the ambient air or are in contact with the rubber or plastic instrument interface and are not subjected to any form of temperature control.

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The temperature in CE was previously studied by different methods, which was recently reviewed by Rathore.[14] Despite the appreciation of temperature importance in CE, the temperature difference between the cooled and noncooled regions has never been determined and its effect on the quality of CE-based analyses has largely been ignored. The temperature difference has not been determined due to the lack of a suitable method. The effects of this difference on CE-based analyses were ignored due to the general acceptance that such effects are negligible, owing to the short cumulative length of the noncooled regions with respect to that of the cooled ones. We have recently demonstrated that sample electromigration through the noncooled capillary inlet can lead to large systematic errors in CE-based quantitative affinity analyses.[15] Our results indirectly suggested a significant temperature difference between the cooled and noncooled capillary parts. The goal of the current work was to finally determine the temperature difference between the noncooled capillary inlet and the cooled part of the capillary.

To determine the temperatures in the cooled region and noncooled inlet region, we used two recently developed methods for temperature measurements inside the capillary: a diffusion-based method[16] and a reaction-rate-based method.[12] Our results showed that the temperature difference was more than 15 °C under typical CE conditions with a low-conductivity buffer (50 mM Tris−acetate). The difference will likely be greater for high-conductivity buffers.

EXPERIMENTAL SECTION

Materials. Recombinant MutS protein from *Escherichia coli* was purchased from Sigma-Aldrich (Oakville, ON, Canada). The HPLC-purified, fluorescently labeled DNA aptamer for MutS (5′-fluorescein-CTT CTG CCC GCC TCC TTC CTC GGG GTT AGA ACG TCG TGT AGG ACT CCT ATC GGT TTA TGG AGA CGA GAT AGG CGG ACA CT) was purchased from IDT DNA Technology Inc. (Coralville, IA). The DNA was dissolved in a TE buffer (10 mM Tris−HCl, 0.1 mM EDTA, pH 7.5) to obtain a 100 μM stock solution. The DNA stock solution was stored at −20 °C. All other chemicals were purchased from Sigma-Aldrich. Uncoated fused-silica capillaries with a 75 μm inner diameter (375 μm outer diameter) were purchased from Polymicro (Phoenix, AZ). The total length and the length from the injection end to the detection window were 50 and 40 cm, respectively. The capillary was mounted on a CE instrument (P/ACE MDQ, Beckman Coulter, Fullerton, CA) which was equipped with a temperature control unit for the capillary. The temperature of the coolant was controlled by the instrument with a precision of ±0.1 °C (as per the manufacturer's specifications). All solutions were made using deionized water and filtered through a 0.22 μm filter (Millipore, Nepean, ON, Canada).

Methods. The MutS aptamer was first prepared at a 1 μM concentration in a MutS sample buffer (50 mM Tris−acetate, pH 8.3, supplemented with 2.5 mM $MgCl_2$) and "annealed" by being heated to 80 °C for 3 min and cooled to 20 °C at a rate of 7.5 °C/min. The annealed aptamer and MutS were mixed in the MutS sample buffer to their final concentration of 50 and 100 nM, respectively. The mixture was incubated at 4 °C for 5 h to allow for equilibrium to be established. The equilibrium mixture was divided into two parts, and each part was placed in a separate Beckman CE-MDQ instrument. One instrument was used to perform nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) experiments required for temperature determination in the noncooled capillary regions. The second instrument was used to perform identical NECEEM experiments under the same set of electrophoretic conditions. NECEEM electropherograms from the second instrument were compared with each other to confirm that they were almost identical. This ensured that the "equilibrium mixture" was at equilibrium and all its components retained their concentrations and activity constants over the duration of all experiments.

Approximately 30 nL of the equilibrium mixture was injected into a capillary by a pressure pulse of 0.5 psi for 7 s. Then in experiments which included the noncooled region for separation, the electrophoresis was initiated by applying an electric field immediately after the injection. In experiments which excluded the noncooled region from separation, a second pressure pulse of 0.3 psi for 1 min was applied after the injection to move the sample along the first 5 cm of capillary length to reach a thermostabilized region of the capillary, and only then an electric field was applied to facilitate electrophoretic separation. In all MutS experiments the electrophoresis buffer was 50 mM Tris−acetate, pH 8.3, supplemented with 2.5 mM $MgCl_2$. Every experimental point in a graph of k_{off} versus the electric field strength was an average of 3−5 experiments. The order of experiments with different electric field strengths was shuffled to avoid any potential reagent-stability-related artifacts.

Electrophoresis Conditions. The CE running and sample buffer in all diffusion experiments was 50 mM Tris−acetate at pH 8.3. The CE running and sample buffer for MutS binding to its aptamer was 50 mM Tris−acetate at pH 8.3 supplemented with 2.5 mM $MgCl_2$. In the diffusion measurement experiments, the sample was introduced into the capillary by electroosmotic flow at 30 V/cm. The time of sample injection was 15 s for runs with an electric field strength of 300 V/s and below and 25 s for runs with an electric field strength above 300 V/s. In all other experiments, the sample was introduced by a pressure pulse of 0.5 psi (3.5 kPa) for 7 s. Prior to each run, the capillary was rinsed with 100 mM NaOH, 100 mM HCl, and deionized water at 20 psi (138 kPa) for 2 min each, followed by a 20 psi, 2 min rinse with the running buffer.

RESULTS AND DISCUSSION

Temperature Measurements in the Cooled Region. To measure the electrolyte temperature in the cooled region of the capillary, we employed a previously described diffusion-based method.[16] In this method, a short plug of a fluorophore is moved through the detector to record the initial concentration profile, then left for a prolonged period of time to diffuse, and finally moved back through the detector to record the concentration profile after diffusion. From the resultant electropherogram, the diffusion coefficient (D) is calculated using a numerical algorithm. Since the diffusion coefficient is a function of the molecular probe and its environment and the temperature, the temperature can be obtained from measurements of D. The method is ideal for

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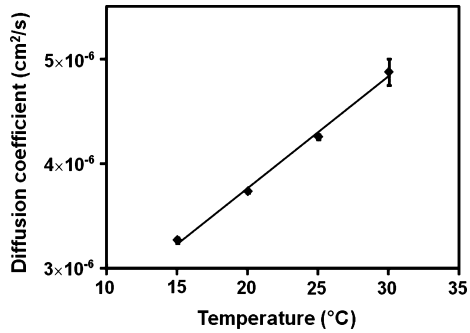

Figure 2. Calibration curve of the diffusion coefficient versus temperature. The probe was 100 nM fluorescein in 50 mM Tris−acetate buffer at pH 8.3. An electric field of 400 V/cm was used to move the probe through the detection window.

measuring the temperature in the cooled region since we can precisely control the migration distance of the probe along the capillary and ensure that it migrates only within the cooled region.

First, we built a calibration curve of D versus temperature using fluorescein as a diffusion probe. The following experiment for determination of D was conducted at different temperatures of the cooled region. A plug of fluorescein was moved by the electroosmotic flow (EOF) past the noncooled region and past the detector; the prediffusion concentration profile of fluorescein was recorded while it passed the detector. The EOF was then stopped to allow for lateral dispersion due to passive diffusion not affected by any electromigration artifacts. Then the plug was moved back through the detector by the reversed electroosmotic flow, and the postdiffusion concentration profile of fluorescein was recorded. It is important to note that, to eliminate the possible effect of dispersion due to electrophoresis-related artifacts such as Joule heating, the ratio between the time when the field was zero (no flow), $t_{\text{no flow}}$, and the total time of diffusion (time between the two passages through the detector), t_{dif}, was kept at about 0.95. This ratio is high enough for accurate measurement of the diffusion coefficient at the temperature set by the instrument, as demonstrated previously.[16] The resultant calibration curve is shown in Figure 2.

Second, to measure the temperature in the cooled region of the capillary in the presence of an electric field, the zero electric field step was eliminated. The plug was carried by EOF past the detector in one direction and further through the actively cooled region of the capillary. Then the EOF was reversed without any delay, and the plug was carried back past the detector. To eliminate possible influence of the noncooled region, the EOF was reversed while the plug was well away from the boundary between the two regions. Thus, the dispersion of the concentration profile occurred only within the cooled region and under the influence of a constant electric field. The resultant temperature increase due to Joule heating in the cooled region is shown in Figure 3. At the highest applied electric field of 600 V/cm, the temperature inside the cooled region of the capillary increased by 3.4 ± 0.8 °C with respect to that at the zero field. This relatively small temperature increase in the cooled region supports the previously reported observations.[16,17]

Temperature Measurements in the Noncooled Region. To measure the electrolyte temperature in the noncooled inlet region

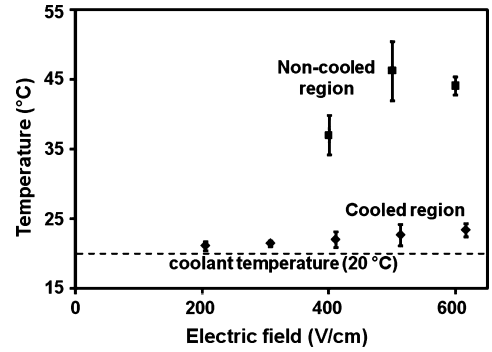

Figure 3. Temperature increase due to Joule heating inside the cooled and noncooled regions of the capillary. The temperature measurements for the cooled capillary region were done using diffusion of fluorescein in 50 mM Tris−acetate buffer at pH 8.3. The measurements of temperature in the noncooled region were done using k_{off} values of MutS/aptamer complexes measured by NECEEM. The equilibrium mixture contained 100 nM MutS and 50 nM fluorescein-labeled aptamer. The incubation and CE run buffer was 50 mM Tris−acetate. The cooled capillary region was washed with a liquid heat exchanger thermostabilized at 20 °C.

of the capillary, we used the dependence of the dissociation rate constant (k_{off}) on the temperature, which was previously used for temperature determination inside the capillary.[12] NECEEM was used to measure k_{off}. In NECEEM, a plug of equilibrium mixture containing unbound reactants and their noncovalent complex is injected into the capillary and an electric field is applied to start the separation. During the separation of free components from the complex, equilibrium is no longer maintained. Under nonequilibrium electrophoretic conditions the complex dissociates with the production of unbound reactants. The dissociation kinetics is "recorded" in the resultant electropherogram from which k_{off} values can be calculated. During NECEEM separation, the complex travels through both noncooled inlet and cooled regions, which have different temperatures. The kinetics of complex dissociation can be described by the following equation:

$$C = C_0 e^{-k_{\text{off}_c} t_c} e^{-k_{\text{off}_n} t_n} \quad (1)$$

where C_0 is the amount of complex in the equilibrium mixture, C is the amount of intact complex which reached the detector, k_{off_c} and k_{off_n} are the dissociation rate constants at the temperatures of the cooled and noncooled regions, respectively, and t_c and t_n are the times that the complex spends dissociating in the cooled and noncooled regions, respectively. Rearranging eq 1 gives the following expression for k_{off_n}:

$$k_{\text{off}_n} = \frac{\ln(C_0/C) - k_{\text{off}_c} t_c}{t_n} \quad (2)$$

The k_{off_c} value can be measured from the NECEEM experiment in which electromigration through the noncooled inlet region is eliminated by propagating the equilibrium mixture through this region by pressure and applying the electric field only when the equilibrium mixture reaches the cooled region. Assuming that the velocities of the complex are equal in the cooled and noncooled regions, we can write

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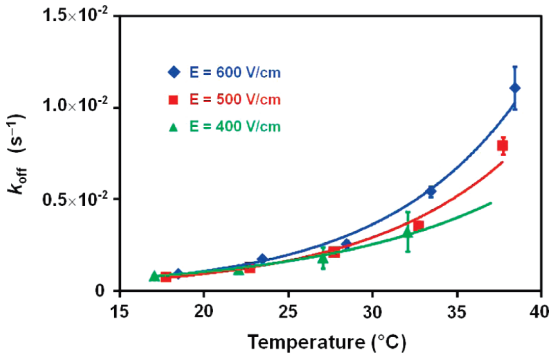

Figure 4. Calibration curves of k_{off} versus temperature at different electric fields for a complex of MutS protein with its DNA aptamer. Electrophoretic separation was performed in the cooled region alone. The temperature scale was adjusted to account for the electric field associated temperature increase. The values of the corresponding temperature increase due to the electric field were added to the values of the coolant temperature. The electric filed associated temperature increase at different electric fields was measured in a separate set of experiments using diffusion coefficients (see Figure 3). The equilibrium mixture contained 100 nM MutS and 50 nM fluoresceintly labeled aptamer. The incubation and CE run buffer was 50 mM Tris−acetate, 2.5 mM $MgCl_2$ at pH 8.3. The cooled capillary region was washed with a thermostabilized liquid heat exchanger.

$$t_c/t_n = L_c/L_n \tag{3}$$

where L_c and L_n are the lengths of the cooled and noncooled regions, respectively. On the other hand, the sum of the two times is equal to the experimentally determined migration time of the complex, $t_{complex}$:

$$t_c + t_n = t_{complex} \tag{4}$$

By combining eqs 3 and 4, we obtain the following expressions for the two times:

$$t_c = \frac{L_c}{L_n + L_c} t_{complex} \quad \text{and} \quad t_n = \frac{L_n}{L_n + L_c} t_{complex} \tag{5}$$

Finally, by combining eqs 2 and 5, we can write the following equation for finding k_{off_n}:

$$k_{off_n} = \frac{(L_c + L_n)\ln(C/C_0) - L_c k_{off_c} t_{complex}}{L_n t_{complex}} \tag{6}$$

It is important to note that, to compensate for the effect of the electric field itself on the rate of complex dissociation,[18] the individual calibration curves for the k_{off} dependence on the temperature were constructed for different strengths of the electric field (Figure 4).

Using the above procedure, we found that the temperature increase in the noncooled capillary region was significant even at low field strengths with a maximum of 24 ± 1.3 °C at 600 V/cm (Figure 3). The temperature measurements in the noncooled inlet region show a relatively large experimental error because the time that the complex migrates through the noncooled region is significantly shorter than the time it migrates in the cooled region. Therefore, small variations in the injected plug lengths as well as calculation errors of peak integration can introduce significant errors in the calculated temperature.

The temperature values for the noncooled region were experimentally measured for the inlet region of the capillary only. We also expect that the noncooled detection window and capillary outlet will experience similar temperature increases. In the majority of CE applications, however, these regions will contribute much less to the results than the capillary inlet. The detection window is typically shorter than the noncooled inlet and outlet, and the time it takes the analyte to go from the entrance of the detection window to the detection point is short. Moreover, the separation of every analyte is complete by the time it reaches the detection window. If any analyte degrades in the detection window, the product of degradation will have no time for separation from the analyte. The outlet region is beyond the detection window, and therefore, in the majority of CE applications, the elevated temperature in this region will not affect the results. Thus, the inlet region is expected to be the primary source of the temperature-related artifacts.

Practically, the detrimental effect of the noncooled regions may be easily eliminated by introducing an additional step before the start of CE separation. During this additional step, the injected sample is moved through the noncooled region by pressure, and the electric field is applied only when the sample reaches the cooled region. We used this approach for an NECEEM-based measurement of k_{off} in the cooled section of the capillary (k_{off_c}) described above. The propagation of the sample by pressure produces a parabolic flow profile inside the capillary, which leads to sample dispersion and decreasing resolution of the analytes. To avoid the decreased resolution, the sample can be moved through the noncooled region using a low-strength electric field, which does not produce a significant temperature increase in the noncooled capillary region. The temperature increase depends on many parameters such as the composition of the run buffer, capillary diameter, and geometry of the instrument. Therefore, the temperature increase must be determined experimentally for every new set of experimental conditions.

To conclude, our results suggest that Joule heating can significantly increase the electrolyte temperature in the short noncooled capillary inlet. The heated electrolyte, in turn, can cause sample degradation for temperature-sensitive samples and lead to systematic errors in CE-based quantitative analyses. Our results emphasize the need for a simpler and more generic experimental method capable of measuring the temperature in the noncooled capillary inlet. When such a method is available, the temperatures could be measured for different buffers, capillaries, and CE instruments and tabulated for simple use by the large community of CE users.

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