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Microchip Electrophoresis at Elevated Temperatures and High Separation Field Strengths

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Abstract

We report free-solution microchip electrophoresis performed at elevated temperatures and high separation field strengths. We used microfluidic devices with 11-cm long separation channels to conduct separations at temperatures between 22 (ambient) and 45 °C and field strengths from 100 to 1000 V/cm. To evaluate separation performance, N-glycans were used as a model system and labeled with 8-aminopyrene-1,3,6-trisulfonic acid to impart charge for electrophoresis and render them fluorescent. Typically, increased diffusivity at higher temperatures leads to increased axial dispersion and poor separation performance; however, we demonstrate that sufficiently high separation field strengths can be used to offset the impact of increased diffusivity in order to maintain separation efficiency. Efficiencies for these free-solution separations are the same at temperatures of 25, 35, and 45 °C with separation field strengths 500 V/cm.

Keywords

carbohydrate; glycan; microchip electrophoresis; microfluidics; temperature

1 Introduction

Separations at elevated temperatures see improved efficiencies and speed for liquid chromatography (LC) [1] and capillary electrochromatography (CEC) [2] due to faster mass transfer and reduced buffer viscosity. Separation efficiencies for capillary gel electrophoresis (CGE) of DNA can also be enhanced at elevated temperatures. Higher temperatures help to keep DNA denatured and decrease the viscosity of the sieving gel, which leads to a significant reduction in analysis times compared to operation at ambient temperatures [3]. Higher temperatures also increase the read length of DNA fragments separated by CGE [4]. Interestingly, improved efficiencies for peptide separations by capillary electrophoresis (CE) are observed at subambient temperatures; however, the improvement in resolution was greater than what was expected from the enhancement in separation efficiency alone and, thus, attributed to the enhanced selectivity [5]. For CE, use of buffers with some organic content lowers viscosity and, consequently, increases electrophoretic mobility, but the contribution of axial diffusion to band broadening is typically higher than with aqueous buffers [6]. Moreover, due to the lower thermal

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conductivity of organic buffers, the effects of Joule heating can be more pronounced [7]. We are interested in high efficiency separations of carbohydrates, which are important in protein stabilization and folding, cell differentiation and development, cellular communication, and disease [8]. Due to the complexity of carbohydrate structures, i.e., chemical composition and structural isomers, separation techniques such as CE [9–11], CGE [12–13], CEC [14–15], and LC [16–17] are needed for highly efficient, quantitative measurements. Our group and others have analyzed carbohydrate mixtures by microchip electrophoresis [18–21], microchip gel electrophoresis [22], and microchip LC [23] and have observed faster analysis times and, in some cases, higher separation efficiencies compared to conventional CE.

In this study, we report the effects of elevated temperatures and separation field strengths on the efficiency of microchip electrophoresis of N-glycans derived from ribonuclease B. N-Glycan samples are labeled with 8-aminopyrene-1,3,6-trisulfonic acid and analyzed on microfluidic devices with 11-cm long separation channels with asymmetrically tapered, 180° turns. Electrophoretic mobilities and, therefore, migration velocities increase by ~35%, and separation efficiencies are maintained at elevated temperatures when sufficiently high separation field strengths are used.

2 Methods and Materials

Materials

We purchased acetic acid, acetone, ammonium persulfate, citric acid, dimethylsulfoxide (DMSO), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), β -mercaptoethanol, γ -methacryloxypropyltrimethoxysilane (MAPTOS), methanol, potassium phosphate monobasic, ribonuclease B (RNase B), sodium cyanoborohydride, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) from Sigma-Aldrich Co.; acrylamide from Bio-Rad Laboratories, Inc.; ammonium hydroxide from J.T. Baker; high-purity 8-aminopyrene-1,3,6-trisulfonic acid (APTS) from Beckman Coulter, Inc.; peptide-N-glycosidase F (PNGase F) of *Chryseobacterium menigosepticum* (EC 3.2.2.18) from Northstar BioProducts; sodium hydroxide from Fisher Scientific; hydrogen peroxide from Macron Fine Chemicals; Microposit MF-319 developer from MicroChem Corp.; 353NDT epoxy from Epoxy Technology; chromium etchants 8002-A and 1020 and buffered oxide etchant (BOE) from Transene Co., Inc.; B270 mask blanks and cover plates from Telic Co.; and 1000-Da cut-off cellulose dialysis tubes from Harvard Apparatus.

Sample Preparation

N-glycans were enzymatically cleaved from RNaseB with PNGase F [24]. Extracted N-glycans were labeled with APTS [25] to impart charge for electrophoresis and render the carbohydrates fluorescent. Briefly, 200- μ g amounts of RNaseB were suspended in 2.5 μ L phosphate buffer (pH 7) with 1% β -mercaptoethanol and thermally denatured at 95 °C for 10 min. After the solution reached ambient temperature, a 0.5- μ L aliquot of PNGase F in phosphate buffer was added, and the reaction mixture was incubated at 37 °C overnight. The released N-glycans were dried with a vacuum CentriVap Concentrator (Labconco Corp.) and labeled with APTS by addition of a 2- μ L aliquot of 100 mM APTS solution prepared in 0.9 M citric acid and a 1- μ L aliquot of 1 M sodium cyanoborohydride prepared in DMSO.

The reaction proceeded for 2 h at 55 °C. Derivatized samples were dialyzed overnight at room temperature with cellulose membranes with a 1000-Da cut-off to reduce the amount of unreacted APTS in the samples. The dialyzed mixtures were dried and resuspended in 100 μ L of 1 mM phosphate and 20 mM HEPES buffer (pH 6.8) prior to electrophoretic analysis.

Microfluidic Device Fabrication

Microfluidic devices were fabricated as previously reported [19]. B270 glass substrates coated with 120 nm of Cr and 530 nm of AZ1518 photoresist were exposed to UV radiation (200 mJ/cm²) through a photomask (HTA Photomask) on a mask aligner (205S, Optical Associates, Inc.), developed for 2 min in MF-319 developer, and rinsed with water. Cr etchant 8002-A transferred the pattern into the chromium layer, and BOE etched the channels into the substrate to a depth of 14 μ m. Channel dimensions were measured with a stylus-based profiler (Dektak 6M, Veeco Instruments, Inc.). The straight sections of the channel were 90- μ m wide, and the asymmetrically tapered, 180° turns were 30- μ m wide. The taper from the straight channel section into the turn had a taper ratio of 3 [26]. Access holes were drilled at the ends of the channels with a sandblaster (AEC Air Eraser, Paasche Airbrush Co.). The remaining photoresist and Cr layers were removed sequentially by rinsing in acetone and etching in Cr etchant 1020. Etched substrates and cover plates were hydrolyzed in a solution of NH₄OH, H₂O₂, and H₂O (2:1:2), sonicated in H₂O, brought into contact with each other, dried at 90 °C for 2 h, and annealed at 550 °C for 10 h in a furnace. Short segments of glass tubing (6-mm o.d., 4-mm i.d., and 6-mm tall) were affixed over the sandblasted holes with 353NDT epoxy.

Microchannel Coating

The microchannels were coated with linear poly(acrylamide) to minimize electroosmotic flow and prevent analyte adsorption. The microchannels were cleaned with 1.0 M sodium hydroxide, water, and methanol for 20 min each, filled with 45 μ L MAPTOS dissolved in 1.5 mL methanol with 0.02 M acetic acid, and allowed to react for 45 min. The microchannels were rinsed with methanol and water for 15 min each to remove residual silane solution, filled with an aqueous solution containing 2.4% (w/w) acrylamide, 1.0 μ L/mL TEMED, and 1.0 mg/mL ammonium persulfate, and allowed to react for 2 h. The microchannels were rinsed with water and filled with a 1 mM phosphate and 20 mM HEPES buffer (pH 6.8) prior to electrophoresis experiments.

Microchip Electrophoresis

A schematic of the microfluidic device with a serpentine separation channel that was 11-cm long and had asymmetrically tapered, 180° turns is shown in Figure 1. For electrophoretic separations, a 1 mM phosphate and 20 mM HEPES buffer (pH 6.8) was used, and sample was introduced into the separation region by a standard pinched injection [27]. A fast-slewing high voltage power supply (0–10 kV), developed in-house, was used to apply potentials to the sample, buffer, and waste reservoirs. A commercial high voltage power supply (0–30 kV; CZE 1000R, Spellman High Voltage Electronics Corp.) was used to apply a potential at the analysis reservoir. The high voltage outputs were controlled through an analog output board (PCI-6713, National Instruments Corp.) by a program written in LabVIEW 8.0 (National Instruments Corp.).

An inverted optical microscope (TE-2000U, Nikon, Inc.) was used to monitor the electrophoretic separations and was equipped with a 20x objective and HQ FITC filter cube (excitation bandpass filter: 480 ± 20 nm; dichroic filter: 505 nm; emission bandpass filter: 535 ± 25 nm; Chroma Technology Corp.). The 488-nm line of an argon ion laser (Melles Griot, Inc.) was attenuated to 1.0 mW with neutral density filters and focused to a point in the analysis channel 11 cm downstream from the cross intersection. The fluorescence signal was spectrally filtered by the emission bandpass filter, spatially filtered by a 600- μ m pinhole, detected with a photomultiplier tube (H5783-01, Hamamatsu Corp.), amplified by a low-noise current preamplifier (SR570, Stanford Research Systems, Inc.), and recorded with a multifunction data acquisition board (PCI-6032E, National Instruments Corp.) and the software written with LabVIEW at a sampling frequency of 100 Hz. A microscope-stage thermoplate heater (MATS-U505R30, Tokai Hit Co. Ltd.) was used to control temperature for electrophoretic separations.

Data Analysis

OriginPro 8.5 software (OriginLab Corp.) was used to fit peaks from electropherograms with a Gaussian function. Migration times and peak variance obtained from three replicate electropherograms were used to calculate electrophoretic mobilities, diffusion coefficients, and plate heights.

3 Results and Discussion

Electrophoretic Separations of N-Glycans

Microfluidic devices with 11-cm long separation channels and asymmetrically tapered, 180° turns with taper ratio 3, as depicted in Figure 1, were used for electrophoretic analysis of the high-mannose N-glycans derived from ribonuclease B (RNase B). Separation performance was evaluated at 22 (ambient), 25, 35, and 45 °C and electric field strengths of 100, 250, 500, 750, and 1000 V/cm. Electropherograms of the N-glycans are shown in Figure 2, where the electric field strength was 1000 V/cm and the temperature was varied from 22 to 45 °C. Mannose 5 (M5) derived from RNase B was used to evaluate separation performance. Below, we report mobilities and diffusion coefficients measured for temperatures from 25 to 45 °C because temperature control was most stable in this range. The microscope stage heater is passively cooled, which limits the lower end to above room temperature (22 °C), and has a maximum temperature of 50 °C.

Separation Efficiency

Plate height is plotted as a function of linear velocity for mannose 5 at 25, 35, and 45 °C in Figure 3a. For these separations, the two primary contributions to the plate height (H) are axial dispersion and the length of the injection. The asymmetric turns with a taper ratio of 3 do not contribute significantly to the plate height [26]. For the data in Figure 3a, the plate heights for each temperature were fitted with Equation 1.

$$H = \frac{\sigma_{diff}^2}{L} + \frac{\sigma_{inj}^2}{L} = \frac{2D}{\mu_{ep}E_{sep}} + \frac{l_{inj}^2}{12L} \quad (1)$$

where σ_{diff}^2 and σ_{inj}^2 are the variance for diffusion and injection, respectively, L is the separation length, D is the diffusion coefficient, μ_{ep} is the electrophoretic mobility, E_{sep} is the separation field strength, and l_{inj} is the injection length. As seen in the figure, the separation efficiencies for all three temperatures asymptotically approach a minimum at velocities > 6 cm/min, which corresponds to $E_{sep} = 500$ V/cm.

Because the primary contribution to the plate height is axial dispersion, Equation 1 can be expressed as:

$$H = \frac{2D}{\mu_{ep} E_{sep}} = \frac{2RT}{Nq E_{sep}} \quad (2)$$

where

$$\mu_{ep} = \frac{q}{6\pi\eta r} \quad (3)$$

$$D = \frac{RT}{6\pi N\eta r} \quad (4)$$

and q is the charge on the molecule, η is the viscosity, r is the radius of the molecule, N is Avogadro's number, R is the gas constant, and T is the temperature. Equation 2 shows that H is proportional to T and inversely proportional to E_{sep} . Consequently, the negative impact of diffusion at higher temperatures is offset by a concurrent increase in the separation field strength. Figure 3b clearly shows that at sufficiently high separation field strengths ($E_{sep} = 500$ V/cm), which corresponds to migration velocities > 6 cm/min, the separation efficiency was maintained at elevated temperatures.

The diffusion coefficients D were obtained by fitting Equation 1 to the data in Figure 3a. Both D and μ_{ep} are inversely proportional to the viscosity of the separation medium. Combining Equations 3 and 4 yields

$$D = \frac{RT}{Nq} \mu_{ep} \quad (5)$$

As shown in Figure 4, the diffusion coefficients derived from the data are proportional to the electrophoretic mobilities, and electrophoretic mobilities increased with temperature due to decreased buffer viscosities. At 45 °C, the electrophoretic mobility and, therefore, migration velocity for mannose 5 increased by ~35% when compared to electrophoretic mobility and migration velocity at 25 °C.

4 Conclusion

In summary, this study demonstrated that if elevated temperatures are used for capillary and microchip electrophoresis, separation field strengths of sufficient magnitude are needed to offset the increased diffusivity of the analyte and to maintain separation efficiency. More

generally, because the diffusion coefficient and electrophoretic mobility are proportional, a lower mobility ion requires a higher separation field strength than a higher mobility ion of the same charge to have a comparable separation efficiency.

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Figure 1.

Schematic of a microfluidic device with an 11-cm serpentine separation channel used for electrophoretic analysis of N-glycans derived from RNase B. The inset is a bright-field image of an asymmetrically tapered, 180° turn with taper ratio 3. The straight channel width and turn width are 90 and 30 μm, respectively.

Figure 2. Electropherograms of N-glycans derived from RNaseB and analyzed at 22, 25, 35, and 45 °C. The labeled peaks are the high-mannose N-glycans, mannose 5 (M5), mannose 6 (M6), mannose 7 (M7), mannose 8 (M8), and mannose 9 (M9). The electric field strength was 1000 V/cm, separation length was 11 cm, and separation medium was 1 mM phosphate and 20 mM HEPES buffer at pH 6.8. For laser-induced fluorescence, the APTS label was excited at 488 nm, and emission was collected through a bandpass filter (535 ± 25 nm).

Figure 3.

(a) Variation of plate height with velocity for mannose 5 at 25, 35, and 45 $^{\circ}\text{C}$. (b) Variation of plate height with temperature for mannose 5 at electric field strengths of 100, 250, 500, 750, and 1000 V/cm . The error bars are $\pm\sigma$ for $n = 3$ for both plate height and velocity.

Figure 4.

Variation of the diffusion coefficient (D) with electrophoretic mobility (μ_{ep}) for mannose 5 at 25, 35, and 45 °C. D is determined from a fit of Equation 1 to the data in Figure 3a, and μ_{ep} is derived from electropherograms similar to Figure 2. The solid line is a linear fit with a correlation coefficient >0.99 .