Controlling the Separation of Native Proteins With Temperature in Thermal Gel Transient Isotachophoresis

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ABSTRACT

Polyacrylamide gel electrophoresis (PAGE) is a ubiquitous technique used in biochemical research laboratories to characterize protein samples. Despite its popularity, PAGE is relatively slow and provides limited separation resolution, especially for native proteins. This report describes the development of a microfluidic thermal gel transient isotachophoresis (TG-tITP) method to rapidly separate native proteins with high resolution. Thermal gels were employed as a separations matrix because of their unique ability to change viscosity in response to temperature. Proteins were added into thermal gel and loaded into a microfluidic device. Electrolyte optimization was conducted to achieve robust tITP to isotachophoretically preconcentrate proteins and then electrophoretically separate them. Electropherograms were collected through both time and distance to enable both small and large proteins to be measured within a single analysis. The effects of temperature were evaluated and found to exhibit a pronounced effect on the separation. Temperature gradients were then employed to alter thermal gel viscosity over time to maximize separation resolution between proteins. The results herein demonstrate how gradient TG-tITP achieves rapid, high-performance separations of native proteins. This analysis provided a wide mass range (6–464 kDa) with two-fold higher resolution

than native PAGE while requiring 15,000-fold less protein loading and providing five-fold faster analysis times.

INTRODUCTION

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revolutionized biological research by providing a rapid and inexpensive means of characterizing protein samples (1, 2). Consequently, SDS-PAGE has become the standard quality control technique for routine screenings of sample purity in research laboratories. Despite the ubiquity of SDS-PAGE, the mechanism by which it operates is not well known. Modern SDS-PAGE analyses are not technically gel electrophoresis but rather transient isotachophoresis (tITP) (3, 4). SDS-PAGE gels are composed of a stacking gel and a resolving gel that are in physical contact (5, 6). Isotachophoretic preconcentration is first performed in the stacking gel to enrich all proteins into a narrow zone between a trailing electrolyte (TE) and a leading electrolyte (LE) (7, 8). This analyte zone then enters the resolving gel where the narrow gel pores slow protein migration, thus disrupting ITP and initiating the electrophoretic separation of proteins into distinct bands. Voltage is stopped after a fixed time, and the migration distances of analytes are measured to estimate their molecular weights (9). The percentage of polyacrylamide in the gel dictates the gel pore size and, consequently, the mass range and resolution of the separation. Multiple electrolytes can be employed in the gel depending on the masses of the proteins of interest. The traditional Laemmli tris-glycine buffer system is most common, as it is suitable for proteins over a large mass range (6-400 kDa). Alternatively, tris-tricine and tris-acetate buffer systems are optimal for low (1-40 kDa) and high (40-500 kDa) molecular weight proteins, respectively (10).

Most researchers operate PAGE under denaturing conditions where proteins are first heated with SDS. This sample preparation converts proteins into their linear forms which subsequently electrophorese as narrow, well-defined bands (1, 11). However, all information is lost on multimeric proteins, biological complexes, and three-dimensional structure because denaturation disrupts the association of protein subunits (2). Alternatively, native PAGE can be employed to preserve the tertiary and quaternary structures of proteins for analysis (12). Despite the advantages of assessing native structure, native gels often suffer from smeared bands, which diminish separation resolution and afford poor detection limits (13-15). Consequently, researchers prefer denaturing gels because of their superior performance. However, given that proteins must possess the correct higher order structure to exhibit their intended biological activities, accurate validations of protein integrity must evaluate native structure to ensure protein subunits remain

assembled. Better gel electrophoresis analyses of native proteins are needed for robust sample validation.

Thermal gels provide a potentially beneficial separations matrix for electrophoretic analyses of native proteins (16-20). Thermal gels are aqueous solutions of temperature-responsive polymers whose viscosity can be controlled with temperature (21, 22). For example, Pluronic F-127 (PF-127) is a thermal gel that exists as a low viscosity liquid at low temperature (e.g. 10 °C) and a high viscosity solid at warm temperature (e.g. 25 °C) (23-25). The ability to vary gel viscosity by >1000-fold and reversibly convert between liquid and solid phases affords a unique opportunity to tune analytical performance using temperature as an adjustable parameter (16, 25-28). Temperature in both SDS and native PAGE is typically held below room temperature (e.g. 4 °C) to minimize the detrimental effects of Joule heating (13, 29). Thermal gels, however, can be operated at or above room temperature because of their ability to attenuate separation current (30). Our group has previously employed PF-127 thermal gel to separate protein variants, miRNAs, DNAs, and small molecules in microfluidic channels (16, 31, 32), but we have not evaluated whether protein tITP can be achieved in this gel matrix. Furthermore, systematic evaluations of the effect of temperature on protein tITP have not been conducted. We assert that temperature can be optimized to enhance protein separations, and that monitoring proteins in both time (as in microchip electrophoresis) and distance (as in both SDS and native PAGE) will enable the detection of analytes across a wide mass range.

This report describes a microfluidic thermal gel tITP (TG-tITP) method to analyze native proteins and determine the mass range and separation resolution of the analysis. Experimental conditions were first optimized to achieve robust ITP preconcentration of proteins across a broad mass range (6–464 kDa). The enriched protein band was then introduced into a separation channel to electrophoretically separate the individual proteins. Proteins were detected in both time and distance domains to increase the accessible mass range. Significant peak dispersion was observed in the analysis using standard tITP electrolytes. To overcome this problem, a pseudo-ITP scheme was employed in the separation channel where ITP electrolyte zones were established between which proteins separated into narrower bands. Temperature was then evaluated to vary gel viscosity, which exhibited a pronounced effect on protein migration. A temperature gradient was incorporated into the analysis to maximize resolution across the mass range. These experiments demonstrate that TG-tITP is a fast and inexpensive technique for native protein analysis that enables temperature to be used as an adjustable parameter to tune analytical performance, unlike in standard gel electrophoresis. The optimized method possessed

sufficient separation resolution to measure both intact native proteins and their dissociated subunits and has great potential for subsequent bioanalytical applications.

METHODS AND MATERIALS

Reagents

PF-127, trizma, glycine, tricine, ammonium acetate, and β-galactosidase were purchased from Millipore Sigma (Burlington, MA). Tris-HCl, phosphate buffered saline (PBS), AlexaFluor 594-labeled ovalbumin, and R-phycoerythrin were obtained from ThermoFisher (Waltham, MA). Pluronic F-68 (PF-68) was purchased from MP Biomedicals. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was procured from Gold Biotechnology (St. Louis, MO). Recombinant murine epidermal growth factor (EGF) was purchased from BioVision (Milpitas, CA). AZDye 594 NHS ester was obtained from Fluoroprobes (Scottsdale, AZ). SU-8 photoresist and SU-8 developer were purchased from Kayaku Advanced Materials (Westborough, MA). Polydimethylsiloxane (PDMS) was obtained from Ellsworth Adhesives (Germantown, WI).

Protein Labeling

Proteins were covalently labeled with dye to ensure they could be readily detected during separations method development studies. β-galactosidase and EGF were fluorescently labeled by incubating 400 μg of each protein with AZDye 594 1:10 in 100 mM HEPES, pH 8.2 for 1.5 h at room temperature. Excess dye was removed using Amicon ultra centrifugal filters (Millipore Sigma) by washing with 10 mM Tris-HCl and PBS. Protein stock solutions were aliquoted in PBS and stored at −20 °C. All aqueous solutions were prepared using 18.2 MΩ·cm resistance water (Purelab Classic, ELGA LabWater, High Wycombe, UK).

Microchip Fabrication and Loading

PDMS devices were fabricated in-house using standard soft lithography (24). Briefly, SU-8 was spin-coated onto a 4-inch silicon wafer (University Wafer, Boston, MA). A photomask with channel features (Great Lakes Engineering, North Maple Grove, MN) was aligned over the wafer and exposed to UV light. The wafer was developed using SU-8 developer and then baked for 30 min at 100 °C. The final features on this master wafer were measured with a profilometer (Mitutoyo, Aurora, IL) to be 100 μm in width and 20 μm in height. The three sidearms of the device were 1 cm in length while the separation channel was 3 cm (Figure 1). A 10:1 degassed PDMS

mixture was poured onto the master wafer and cured for 2 h at 70 °C. The PDMS layer was removed from the wafer and cut into individual devices. A biopsy punch (Ted Pella, Redding, CA) was used to create reservoirs. Finally, the PDMS devices were placed on glass microscope slides (AmScope, Irvine, CA) to form enclosed channels.

Thermal gels with distinct compositions were loaded in each channel of a microfluidic device. A protein sample mixture containing 500 nM EGF (0.06 ng), 20 nM ovalbumin (0.018 ng), 100 nM β-galactosidase (0.93 ng), and 0.8 nM phycoerythrin (0.004 ng) was prepared in a stacking gel containing 15% (w/v) PF-127 in 5 mM tris-HCl. A resolving gel was prepared to contain 30% (w/v) 9:1 PF-127:PF-68 in 5 mM tris-HCl and 10 mM ammonium acetate. An analysis gel was also made that contained 30% (w/v) PF-127 in 25 mM tris-HCl and 25 mM tricine. The sample-containing stacking gel, resolving gel, and analysis gel were loaded into the West, East, and South reservoirs of a microfluidic device, respectively (Figure 1). Vacuum was applied to the North reservoir to load each gel solution into its respective channel. Devices were filled at 5 °C to maintain the thermal gels in their liquid states. Excess gel was then removed from each reservoir and replaced with 10 μL of electrolyte solution. The North and East reservoirs contained 30% (w/v) PF-127 in 25 mM tris-HCl while the South reservoir contained 25 mM tris-HCl. The West reservoir contained 100 mM glycine and 50 mM tris-HCl.
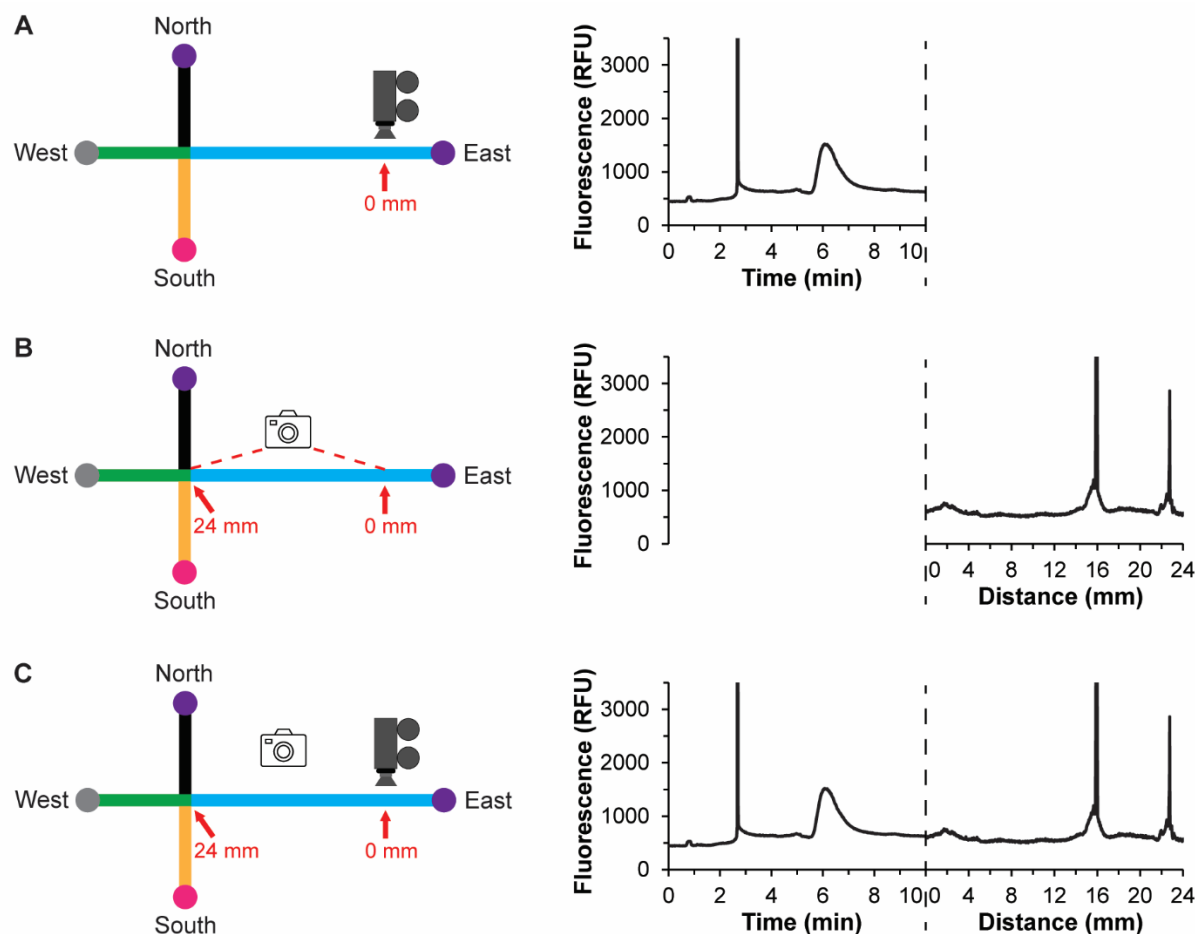
Figure 1. Schematic of the microfluidic device. Sample-containing stacking gel is loaded in the West channel (green), resolving gel in the East channel (blue), and analysis gel in the South channel (yellow). The reservoirs are then filled with electrolyte solutions for the analysis. **(A)** Fluorescence is measured at the detection point for 10 min to monitor the "time domain". **(B)** Voltage is stopped, and the channel is imaged upstream to detect proteins remaining in the "distance domain". **(C)** Integrated measurements of the time and distance domains expands the accessible mass range of the analysis.

Analysis and Data Processing

Loaded devices were placed on an AZ100 epifluorescent microscope (Nikon Instruments Inc., Melville, NY) with a motorized stage (Prior Scientific, Rockland, MA). Temperature of the stage was controlled with a Peltier (TEC1-12730, Amazon.com) and a thermoelectric controller (Wavelength Electronics, Bozeman, MT). A custom LabView program (National Instruments, Austin, TX) was used to control stage temperature and tITP voltages. Devices were equilibrated at desired temperature for 2 min before applying voltage with a high voltage power supply

(Advanced Energy, Ronkonkoma, NY). Analytes were detected with an ORCA Fusion sCMOS camera (Hamamatsu Corp., Bridgewater, NJ). A SOLA Light Engine (Lumencore, Beaverton, OR) with a Texas Red filter cube (560/630 nm) was used for illumination. Image acquisition was controlled by μManager (33).

Sample stacking was performed by applying 750 V cm^{-1} between the West and East reservoirs for 1.2 min. Analytes were then injected into the East separation channel, and the voltage changed to initiate the separation by applying 750 V cm^{-1} between the South and East reservoirs for 8.8 min. Images were collected 24 mm from the channel intersection for 10 min at 3.6x magnification with 150 ms exposure times at 1 s intervals (Figure 1A). These images were used to monitor analyte migration through the "time domain". After voltage application ceased, the East channel was imaged upstream from the detection point at 2.4x magnification to detect proteins that did not reach 24 mm within the 10-min runtime (Figure 1B). These images monitored analyte migration in the "distance domain". Combining time and distance domains enabled the entire analysis to be conducted in a relatively short (10 min) time. All images were processed using FIJI to measure fluorescence intensity versus either time or distance (34). The resulting electropherograms shown in the figures herein plot both time and distance domains together on an arbitrary x-axis with time and distance domains weighted 1:1 (Figure 1C). Depicting data in this manner shows all proteins across the mass range in one trace regardless of whether they reached the detection point. Electropherograms were input into Chromophoreasy to calculate separation resolutions between proteins based on width at base (35). Separation resolutions were only calculated for analytes within a single domain (i.e. time or distance).

Native PAGE Controls

Protein samples were analyzed with polyacrylamide gels to benchmark performance of the microfluidic thermal gel separations. Samples were electrophoresed through Novex Wedge Well Tris-Glycine gels (6%, 10%, 16%, and 4-20%) (ThermoFisher) using a Mini Gel Tank (ThermoFisher) and a PowerPac Basic voltage supply (Bio-Rad, Hercules, CA). Proteins were prepared in PAGE native sample buffer and analyzed in PAGE running buffer (ThermoFisher). The protein sample mixture contained 500 ng of each EGF (4.2 μM), ovalbumin (550 nM), and β-galactosidase (54 nM) and 100 ng of phycoerythrin (20 nM) to provide readily detectable signal for each protein band. A protein ladder (ThermoFisher) was also analyzed on each gel. Gels were run for ~45 min at 4 °C to minimize Joule heating-induced band-broadening. Protein bands were

imaged using a Typhoon FLA 9500 biomolecular imager (GE Healthcare Life Sciences, Piscataway, NJ).

RESULTS AND DISCUSSION

Optimizing Protein Preconcentration and Separation

Four model proteins were selected to assess method performance over a wide mass range: EGF (6 kDa), ovalbumin (45 kDa), R-phycoerythrin (250 kDa), and β-galactosidase (464 kDa). Initial characterizations were performed using native PAGE (Figure S1) to determine the expected numbers of bands from each protein and provide a benchmark for microfluidic TG-tITP. Three comigrating bands were observed for EGF, which were attributed to dye-labeling variants of the protein. Ovalbumin produced one primary band and a small secondary band at higher mass, which was attributed to dimer formation. Phycoerythrin exhibited one prominent band at the expected molecular weight, but also produced multiple minor low intensity bands that correspond to its subunits. β-galactosidase is a homotetramer and was found to produce four bands. Comparisons with the protein ladder showed that bands matched with the expected molecular weights of the monomer, dimer, trimer subunits as well as the intact tetramer. Migration order of the bands in the protein mixture (Table 1) was used to tentatively identify peaks in subsequent microfluidic analyses. Although thermal gel and polyacrylamide polymers are different, the electrophoretic mobilities of the proteins were expected to be comparable between analyses so that the relative migration order would be maintained.

Table 1. The migration order of each species in the protein mixture is shown. Protein bands were identified based on their molecular weights from single-component native PAGE analyses.

Migration Order	Protein	Molecular Weight (kDa)
1	epidermal growth factor (EGF)	6
2	ovalbumin	45
3	R-phycoerythrin subunit	78
4	ovalbumin dimer	88
5	β-galactosidase monomer subunit	116
6	β-galactosidase dimer subunit	232
7	R-phycoerythrin (intact heptamer)	250
8	β-galactosidase trimer subunit	348
9	β-galactosidase (intact tetramer)	464

The protein mixture was then translated on-chip to assess the performance of thermal gel. First, however, the functionalities of native PAGE gels needed to be adapted into a microfluidic format. Native PAGE employs tITP as the analysis mechanism, so we first sought to optimize isotachophoretic preconcentration to enrich dilute proteins into a high-concentration band (32). A tris-glycine buffer system was employed similar to those used in PAGE (3, 36). Initial results using a 30% (w/v) PF-127 stacking gel exhibited a premature separation between proteins, and high-mass proteins did not have sufficient mobility to reach the separation channel. To obtain all proteins into a single enriched band, a 15% (w/v) PF-127 stacking gel was used instead. This low-viscosity gel provided robust enrichment for all proteins across the mass range (6–464 kDa) using a single set of electrolytes (Figure S2).

ITP enrichment was achieved by applying voltage between reservoirs containing TE and LE (i.e. West and East). Once analytes were injected into the separation channel, the voltage scheme was switched to stop ITP and initiate electrophoresis by now applying voltage between reservoirs containing only LE (i.e. South and East). However, this homogenous electrolyte composition resulted in dispersed, closely migrating bands for the lower mass proteins (Figure 2A, 0 mM). Our group has encountered this problem before when conducting microfluidic tITP to analyze DNA (32). We found that an unconventional pseudo-ITP electrolyte scheme was required to minimize band dispersion during the electrophoretic separation by adding TE into the analysis gel (South channel). A similar approach was evaluated here for protein analyses. Tris-tricine gels are conventionally used in native PAGE for analyzing small proteins (36, 37). Therefore, tricine (TE) was added to the South channel – but not the South reservoir – to attempt to minimize broadening of EGF (Peak 1). Inclusion of this TE in the analysis gel resulted in a more intense band for EGF with less dispersion (Figure 2A, Time). The addition of tricine also helped better resolve EGF from ovalbumin (Peaks 1 and 2). Despite the improved performance with this unique electrolyte scheme, the higher mass proteins (i.e. phycoerythrin and the β-galactosidase monomer, dimer, trimer, and tetramer) still did not reach the detection point before electrolytes were depleted and the separation current dropped to a low level where analytes could no longer migrate (e.g. 20 min).
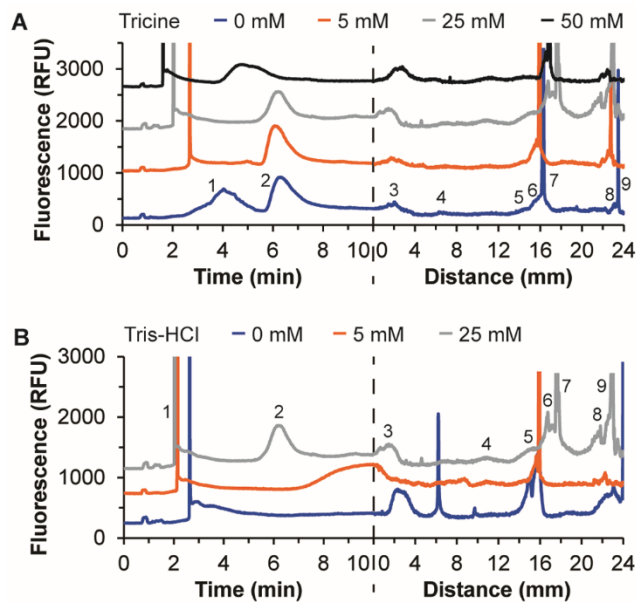
Figure 2. Analysis gel optimization in the South channel. **(A)** Tricine concentrations were evaluated in gels also containing 25 mM tris-HCl. **(B)** Tris-HCl was evaluated in gels also containing 25 mM tricine. Peak numbers correspond to the proteins listed in Table 1.

To overcome the low mobilities of large proteins, an alternative approach was employed for the analysis. Rather than exclusively monitor proteins through time – as is standard for microchip electrophoresis – proteins were also monitored through distance, like in native PAGE. Voltage was applied for 10 min to collect time-domain electropherograms (Figure 2A, Time). After voltage cessation, images were acquired along the separation channel back to the t-intersection to obtain distance-domain electropherograms (Figure 2A, Distance), akin to native PAGE analyses. Integrated electropherograms were made by plotting both time and distance domains on one continuous axis. This approach enabled proteins across the entire mass range to all be detected in a 10-min analysis, over which time the separation current was stable.

Having established detection of the full mass range, electrolyte optimization of the analysis gel then continued. Inclusion of a TE (i.e. tricine) provided good peak shapes for proteins migrating in the time domain, but late-migrating proteins still experienced comigration. Increasing TE concentrations improved the separation of later bands (25 mM tricine) up to a point where dispersion increased again (50 mM) (Figure 2A). TE was also evaluated in the South reservoir, but ITP reestablished with this electrolyte arrangement and precluded analyte separation. Thus, TE was only included in the South channel. The tris-HCl concentration in the analysis gel was also found to exhibit a significant effect on separation performance (Figure 2B). Excluding tris-HCl resulted in very late migration times where only EGF (Peak 1) was observed in the time

domain and β-galactosidase (Peak 9) had not left the channel intersection. Increasing the tris-HCl concentration increased protein migration and improved resolution between the protein bands. An optimal tris-HCl concentration of 25 mM was selected, as no further improvement was observed with 50 mM tris-HCl (data not shown).

Next, the electrolyte composition of the resolving gel (i.e. East channel) was evaluated and found to exhibit an effect on resolution between the protein bands. Exclusive use of tris-HCl caused peak intensities to be relatively low for Peaks 2–5 (Figure 3A, 0 mM). To attempt to increase peak intensities, ammonium acetate was added to the gel because of its utility as a LE in native PAGE to analyze larger proteins. Low concentrations of ammonium acetate (e.g. 10 mM) improved the analysis; however, higher concentrations (e.g. 25 mM), compressed the analyte zone, causing comigration. The tris-HCl concentration in the resolving gel was also found to impact the analysis (Figure 3B). A low concentration of tris-HCl (e.g. 5 mM) was needed to sharpen the bands, although too much (25 mM) caused recollection of the protein bands into a single comigrated peak.
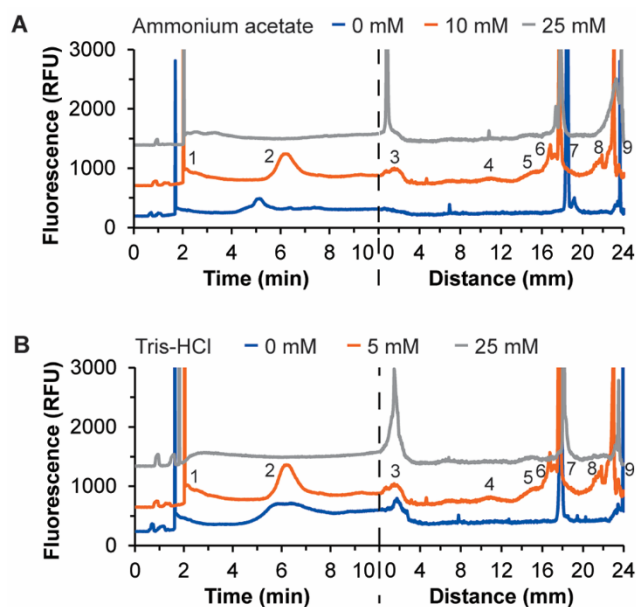

Figure 3. Resolving gel optimization in the East channel. **(A)** Ammonium acetate was evaluated in gels also containing 5 mM tris-HCl. **(B)** Tris-HCl was evaluated in gels also containing 10 mM ammonium acetate.

Collectively, the results from these electrolyte optimization studies indicated that 5 mM tris-HCl and 10 mM ammonium acetate were required in the East channel with 25 mM tris-HCl and 25 mM tricine in the South channel. This scheme established a finite zone of TE behind the

injected protein plug which provided a separation while maintaining good peak shapes during electrophoresis. Additionally, a finite zone of ammonium acetate in front of the injected proteins was required to sharpen the bands, although too much caused some analytes to refocus and comigrate. We speculate that this unconventional electrolyte scheme enables analytes to electrophorese between distinct electrolyte zones, which improves separation resolution while minimizing dispersion. This is consistent with observations from our previous analyses of nucleic acids in thermal gel (31, 32). The integration of time and distance domains was found to benefit the analysis, as the entire protein mass range could now be detected in a single analysis. Furthermore, the ability to analyze proteins in their native states revealed intrinsic dissociation of phycoerythrin and β-galactosidase subunits. Denaturing gels are blind to this dissociation, but monitoring for this is critical to properly validating sample integrity. Although dissociation was not quantified in this study, determining the fraction of protein in its biologically active structure is crucial to ensure accuracy in subsequent biological research studies.

Effects of Temperature on TG-tITP Resolution

The mass range and separation resolution in native PAGE is controlled by altering the percentage of acrylamide in the gel. More acrylamide decreases the pore sizes in the gel (3, 38). Narrower pores better resolve low-mass proteins but restrict migration of higher mass proteins, causing comigration. Conversely, less acrylamide creates gels with large pores. Wide pores afford higher resolution between higher mass proteins, but smaller proteins have limited interaction with the gel and thus do not adequately resolve. We hypothesized that similar effects could be attained in TG-tITP using a single thermal gel composition, but by controlling gel viscosity through a thermal dimension to adjust mass range and separation resolution.

The four-protein mixture was analyzed over a range of temperatures using the optimized TG-tITP method. Temperature was kept below physiological temperature to ensure proteins were not inadvertently denatured by excessive heat. Electropherograms were collected in both time and distance domains to monitor migration of proteins across the mass range. Results showed that peaks were sharper at higher temperatures (e.g. 35 and 30 °C) than at cooler temperatures (Figure 4). The high resolution between EGF and ovalbumin (Peaks 1 and 2) at warmer temperatures was expected because smaller proteins were expected to resolve better at higher gel viscosity. Larger proteins were expected to exhibit comigration at high viscosity, but interestingly, these species were generally well resolved, except for the β-galactosidase dimer subunit and R-phycoerythrin (Peaks 6 and 7). At cooler temperatures, lower resolution between lower mass proteins was expected because of the reduced gel viscosity; however, this was

generally not observed. Rather, significant peak dispersion of lower mass proteins occurred at 20 and 25 °C, which was attributed to higher rates of diffusion through lower viscosity thermal gel, and consequently, more band broadening (24). Higher mass proteins remained well resolved at 25 °C. Interestingly, the β-galactosidase dimer subunit exhibited lower mobility at this temperature causing the migration order to reverse for Peaks 6 and 7. This result can be observed as the low-intensity band of β-galactosidase dimer began to migrate after the high-intensity band of phycoerythrin at lower temperature. At 20 °C, all proteins across the mass range experienced significant dispersion through the liquid-phase gel. These results demonstrate that even though thermal gel viscosity is reduced at lower temperatures (e.g. 20 °C) – which in principle would facilitate protein migration due to lower fluid resistance – the lower viscosity could not overcome the decreased electrophoretic mobilities of proteins at low temperatures (20).
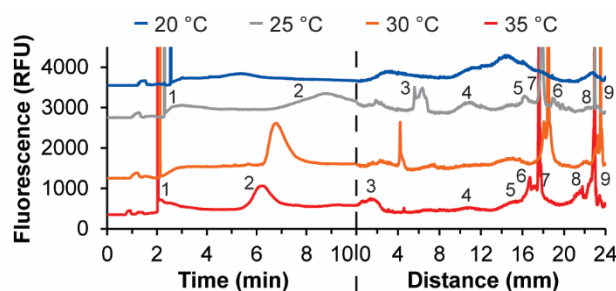

Figure 4. Effect of temperature on protein migration through thermal gel. The East channel contained 5 mM tris-HCl and 10 mM ammonium acetate and the South channel contained 25 mM tris-HCl and 25 mM tricine.

The temperature-dependent results in thermal gels demonstrated that analytical performance could be adjusted by varying gel viscosity through a thermal dimension. As native PAGE obtains similar effects by varying gel pore sizes (39, 40), we sought to establish equivalencies between thermal gel temperature and PAGE acrylamide percentages. The four-protein mixture was analyzed using a series of native PAGE gels with 6%, 10%, and 16% acrylamide to provide points of comparison. Separation resolution was calculated between EGF–ovalbumin (Peaks 1 and 2), β-galactosidase dimer subunit–phycoerythrin (Peaks 6 and 7), and phycoerythrin–tetrameric β-galactosidase (Peaks 7 and 9). Resolution values were compared between the native PAGE gels and the TG-tITP temperature series (Table 2). Resolution between EGF and ovalbumin increased on-chip as temperature increased from 25 to 35 °C, with values similar to the 10% acrylamide gels. No resolution was obtained in 6% polyacrylamide because gel pores were too wide to interact with the proteins and provide resolution, as expected.

Separation resolution between β-galactosidase dimer subunit and intact phycoerythrin were similar at 25 and 35 °C. Resolution was reduced at 30 °C, as the peaks began to invert their migration order. However, resolution even under these nonoptimal conditions was still superior to the best resolution achieved by native PAGE. Interestingly, these peaks could only be resolved in PAGE using the 10% acrylamide gel. This suggests that pore sizes in the 6% gel were too wide to provide resolution, whereas proteins could not migrate sufficiently through the 16% gel to resolve.

Table 2. Separation resolution values were calculated between pairs of proteins. Resolution is compared between different temperatures in TG-tITP and different acrylamide percentages in native PAGE. Values reflect the average and standard deviation of three replicate analyses.

Proteins	TG-tITP – Temperature			Native PAGE – Acrylamide		
	25 °C	30 °C	35 °C	6%	10%	16%
EGF - ovalbumin	1.9 ± 0.4	2.4 ± 0.2	2.7 ± 0.4	0	2.3 ± 0.1	1.88 ± 0.05
β-gal dimer - phycoerythrin	1.7 ± 0.3	0.93 ± 0.03	2.0 ± 0.4	0	0.58 ± 0.07	0
phycoerythrin - β-gal	9 ± 3	15 ± 2	17 ± 2	8.8 ± 0.1	7.5 ± 0.5	6.7 ± 0.2

Separation resolution between intact phycoerythrin and β-galactosidase was lowest in TG-tITP at 25 °C, matching the highest value obtained in native PAGE using the 6% acrylamide gel. Interestingly, increasing temperature on-chip improved resolution, whereas poorer resolution was achieved with more acrylamide. It was expected that both higher temperature in TG-tITP and higher acrylamide percentages in native PAGE would reduce resolution because larger proteins would experience more resistance and not migrate as far through the gel. However, this was only observed in native PAGE. We attribute the high resolution in TG-tITP to higher protein mobilities at warmer temperatures that imparted sufficient mobility to transit through the high-viscosity gel. Similar results could not be achieved in native PAGE because proteins became trapped in the narrow gel pores. These findings illustrate an interesting difference between analyte migration through the rigid crosslinked pores of polyacrylamide and the dynamic micellar structure of PF-127 thermal gel. Collectively, these results demonstrate that, although there was no direct correlation between TG temperature and acrylamide percentage, TG-tITP provided superior resolution than native PAGE for all measured proteins.

Temperature Gradient Analysis

A limitation of PAGE gels is that once the gel is cast, its pore size – and consequently the accessible protein mass range and resolution – is fixed. With thermal gels, the separation performance can be tuned on-demand by employing a temperature gradient to dynamically adjust the gel viscosity over time (16, 41). Isothermal analyses at 35 °C provided the best resolution between Peaks 1 and 2. However, better resolution and peak shapes were observed for Peaks 3–7 at 25 °C. Therefore, a temperature gradient was evaluated to analyze all proteins at their optimal temperature.

ITP focusing was carried out at 35 °C, and then the temperature of the microscope stage was ramped from 35 to 25 °C during the separation. This analysis strategy resulted in high resolution between both high- and low-mass proteins (Figure 5, solid). Performing the focusing step at 35 °C helped maintain good peak shapes for the lower mass proteins, which did not occur in 25 °C isothermal analysis. The same inversion of Peaks 6 and 7 was also seen at 25 °C, which indicated that analysis in distance domain proceeded similarly as isothermal runs. Temperature ramps of 2 and 4 min produced similar results (Figure S3). However, an 8-min ramp caused more pronounced dispersion of the lower mass proteins and reduced resolution between the β-galactosidase trimer and tetramer (Peaks 8 and 9). This was attributed to proteins migrating through lower viscosity gel for longer and undergoing additional band-broadening. Thus, the 2-min gradient was selected as optimal. Our previous studies demonstrated that thermal gels undergo a phase change in response to decreasing temperature in <5 s within microfluidic devices of similar dimensions (30). This rapid response time is significantly shorter than the duration of the temperature gradient, so we assert that the in-channel gel temperature closely mirrored the setpoint stage temperature. Although temperature gradients are common in gas chromatography, they also provide a promising means of tailoring electrophoretic separations within thermal gels to accommodate the analytes of interest.
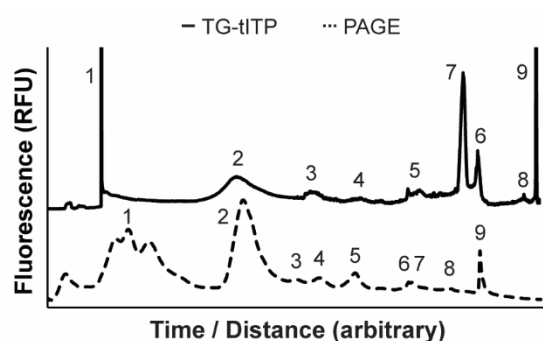

Figure 5. Proteins were analyzed using either **(top)** TG-tITP using a temperature gradient from 35 to 25 °C or **(bottom)** native PAGE using a 4-20% gradient gel.

To benchmark the performance of gradient TG-tITP, analysis of the protein mixture was compared against gradient native PAGE. Figure 5 depicts the 2-min temperature gradient from TG-tITP (solid line) and the line profile from the PAGE gel from Figure S1, Mixture (dashed line). The thermal gel method was found to exhibit superior resolution between the lower mass proteins (e.g. EGF-ovalbumin) as well as some of the higher mass proteins (e.g. phycoerythrin-β-galactosidase dimer). This is consistent with the resolution values discussed above. Additionally, the enhanced performance of TG-tITP is compounded by the low amount of protein required (~15,000-fold less protein mass than native PAGE) and rapid analysis times (>4-fold faster than native PAGE). Operating the analysis under native conditions also enabled determinations of protein dissociation. The intensities of the subunit bands from phycoerythrin and β-galactosidase indicate the extent of dissociation of the protein multimers. Although evaluating dissociation was not the goal of this work, TG-tITP could be used in future studies to determine the fraction of protein in its active complex to validate protein integrity.

CONCLUSIONS

This report successfully demonstrates the translation of tITP into a microfluidic device for the analysis of native proteins using a thermal gel separations matrix. Proteins ranging in molecular weight from 6–464 kDa were separated by integrating both time and distance domains into a single analysis. Optimal separations were obtained by including tricine and ammonium acetate in distinct locations in the microfluidic device to produce narrower peaks during electrophoresis. Separation resolutions in TG-tITP were two-fold higher than control analyses using native PAGE while requiring significantly less protein mass loading (15,000-fold less) and shorter analysis times (5-fold less). Temperature was found to have a significant impact on the analysis, as gel viscosity played a pivotal role in the protein separation. The introduction of a temperature gradient (35 to 25 °C over 2 min) showed the ability to adjust gel viscosity through a thermal dimension, which affords additional flexibility that cannot be attained in conventional static gels. This dynamic nature of thermal gels to tune separation performance exhibit their utility for bioanalytical measurements.

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CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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