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Capillary isoelectric focusing with pH 9.7 cathode for the analysis of gastric biopsies

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Abstract Capillary isoelectric focusing tends to suffer from poor reproducibility, particularly for the analysis of complex protein samples from cellular or tissue homogenates. This poor reproducibility appears to be associated with erratic variations in electroosmotic flow. One cause of electroosmotic flow variation is degradation of the capillary coating caused by the extremely basic solution commonly used during mobilization and focusing; this degradation of the capillary coating can be reduced by employing a CAPS mobilization buffer at pH 9. Another cause of variation is protein adsorption to the capillary wall, which causes an increase in electroosmotic flow. The effects of protein adsorption can be reduced by use of surfactants in the buffer and by employing an extremely low sample loading. We report the use of CAPS mobilization buffer in combination with an ultrasensitive laser-induced fluorescence detector for the reproducible analysis of ~2 ng of protein from a Barrett's esophagus biopsy.

Keywords Capillary isoelectric focusing · Laser-induced fluorescence · Capillary coatings · Electrophoretic reproducibility

Introduction

Among capillary electrophoresis separation methods, capillary isoelectric focusing (cIEF) provides the greatest resolving power but has typically been the most challenging technique to perform [1–5]. In cIEF, ampholytes establish a pH gradient in the capillary, and the analytes focus to their isoelectric points. Either of two detection methods are then employed: whole-column imaging, in which a camera is used to image the focused contents of the capillary, or end-column detection, where the focused analytes are mobilized past a fixed detector near one end of the capillary. End-column detection allows use of high sensitivity laser-induced fluorescence or information-rich mass spectrometry as detectors.

There are several approaches to mobilization. Chemical mobilization is arguably the most efficient [6–9] and is performed by adding additional salt ions to either the anode or cathode. The addition of salt ions at one end disrupts the pH gradient and causes the analytes to migrate past the detector.

cIEF has most commonly been used to analyze simple samples, usually containing a single protein or a handful of standard proteins. Hemoglobin and recombinant proteins appear to be particularly commonly analytes [10–13]. However, the analysis of complex samples is much less common. Several publications employed cIEF for separation of complex peptide mixtures generated from tryptic digest of yeast or *Escherichia coli* homogenates [14–18]. However, there are very few studies employing cIEF for

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analysis of complex protein samples prepared from a cellular or tissue homogenate. As one example, we have published an example of the analysis of a homogenate prepared from a Barrett's esophagus cell line, which resolved many dozen components [19]. Nevertheless, the small number of publications describing the application of cIEF to the analysis of complex protein samples is striking.

Run-to-run irreproducibility is a major issue plaguing cIEF for analysis of complex protein samples. In general, electroosmosis must be reduced to very low levels during focusing to achieve high efficiency separations. Covalent attachment of a polymer to the capillary wall is commonly employed to control electroosmosis, but over time, the level of electroosmosis may change due to either coating degradation or protein adsorption. There are two mechanisms that degrade the performance of the coating. First, the coating can be contaminated by residual proteins from earlier runs. If charged, these proteins will introduce electroosmotic flow during focusing, degrading the separation. These proteins also provide sites for interaction with other proteins in the sample. Second, the high pH used during mobilization can etch the capillary wall, removing the coating and exposing the silica capillary, again inducing electroosmotic flow and providing sites for proteins to adsorb to the surface.

Many protocols for capillary coating have been published [20–28]. Several different acrylamide-based polymers have been used to coat the capillary walls, of which a common choice is linear polyacrylamide (LPA), first used in cIEF by Hjérten [20]. However, the high pH at the cathode can hydrolyze polyacrylamide to polyacrylate [29], which itself can create electroosmotic flow, or if the basal layer connecting the LPA to the silica is susceptible to hydrolysis (as is the case for silane-based reagents such as γ-methacryloxypropyltrimethoxysilane), the LPA can be removed under basic conditions, exposing silanol groups and increasing electroosmotic flow. This problem can be remedied by attaching a vinyl group directly to the capillary wall via a Grignard reagent, and then anchoring the LPA to this vinyl group [26]. However, these coatings eventually degrade after exposure to high pH.

One way to eliminate coating failure is to use a catholyte with a pH less than 10. Rodriguez-Diaz and Wehr suggested replacement of NaOH as the cathode with a zwitterion that is electrically neutral in the proper solution conditions [9]. They performed cIEF on a hemoglobin sample and used taurine (500 mM, pH 8.8) as the catholyte. Righetti also investigated the use of zwitterions at the cathode and performed cIEF with lysine (pH 9.7, 50 mM) as the catholyte [21]. In both cases, the separation efficiency was not affected by switching the cathode from NaOH to a zwiterionic molecule, but the stability of the capillary coating improved.

Coating the capillary also helps to minimize protein adsorption, keeping electroosmotic flow to a minimum [30, 31]. Righetti's group has suggested coating the capillary with *N*-acryloylaminopropanol (AAP) to decrease the protein interaction to the capillary wall [21, 25]. Righetti and Vigh also recommended the use of additives such as neutral surfactants, short chain LPA, and urea to the sample to further decrease the protein adsorption [21, 31]. Righetti, Lui, and Watzig all suggest proper cleaning of the capillary between runs with HCl, urea, or a surfactant to remove proteins that have adsorbed to the walls [21, 24, 32, 33]. By following these steps, Lui et al. were able to perform cIEF on a set of standard proteins and achieve 80 cIEF runs on one capillary [24]. Watzig was able to achieve an impressive 86 runs with a relative standard deviation for migration time of less than 10% for the analysis of three standard proteins [32].

In this paper, we describe a cIEF-LIF procedure that is consistent with the constraints mentioned above and that provides a reproducible separation of a complex protein homogenate. To achieve this goal, we coated capillaries with a Grignard reagent basal layer [23, 25] followed by polymerizing *N*-acryoylaminopropanol. We eliminated the use of NaOH at the cathode and instead used CAPS (100 mM, pH 9.7); in addition, urea, Tween 20, and Triton-X 100 were used as buffer additives to help reduce protein precipitation and adsorption to the capillary wall. The use of an ultrasensitive laser-induced fluorescence detector allowed dramatically decreased sample loading compared to those employed with UV absorbance detection.

Experimental section

Reagents and materials Unless stated, all reagents were purchased from Sigma-Aldrich and used without further purification. Solutions were made with distilled deionized water and vacuum filtered through a 0.22-μm filter. Tween 20 was from Fisher Scientific. Pharmalytes were purchased from GE-health and were photobleached using a bank of high-power green light emitting diodes [19]. Chromeo P540 was purchased from Active Motif. Uncoated 50/140 μm ID/OD fused silica capillaries as well as LPA-coated 50/140 μm ID/OD fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). Acrylamide and ammonium persulfate were purchased from BioRad (Hercules, CA, USA).

Capillary coating Capillaries were coated in a manner similar to that reported by Cobb et al. and Gelfi et al. [25, 26]. Briefly, silanols on the capillary wall were chlorinated with thionyl chloride, and then a Grignard reagent (vinylmagnesium bromide) was added to replace each silanol

with an alkene. This treatment was followed by radical polymerization of 3-acryloylamino-1-propanol inside the capillary.

The procedure was as follows: several 4-m-long capillaries were washed for 2 h by flowing a 1-M NaOH solution at 30 psi, followed by H_2O for 1 h, and then methanol for 1 h. A device similar to that reported by Gao and Liu was used to push fluids through the capillaries with dry nitrogen [24]. The bulk of the capillaries (everything except for roughly 10 cm at the inlet end) was then threaded into an oven and dried overnight at 140–150 °C with 20 psi N_2. The oven temperature was then reduced to 60 °C, and the outlet of the capillary was threaded through the oven and into an Eppendorf tube with a small hole punched on the top, which served as a collection vessel. $SOCl_2$ was then pumped through the capillaries for 5 h at 30 psi. The capillaries were then purged with 30 psi N_2 until bubbles could be seen coming from the outflow end of the capillary. Vinylmagnesium bromide (1 M in tetrahydrofuran, Sigma-Aldrich) was then loaded into the reservoir at the inlet end and pumped through the capillary for 5 h. During this time, the outflow end was placed in a container of methanol, to avoid becoming clogged with the precipitate that forms when vinylmagnesium bromide reacts with air. Furthermore, the outflow ends were checked hourly to ensure that the capillaries had not clogged. If any capillary was not clearly flowing (THF has a different index of refraction than methanol), several centimeters were cut off the end to attempt to restore flow.

After 5 h, the oven was turned off, and dry tetrahydrofuran was then pumped through the capillaries for 2 h at 30 psi, followed by H_2O for 2 h. It was during these two steps that the capillaries would most frequently clog—if they did, several centimeters would be cut off the outlet end, then the inlet end, then the outlet end again, repeating until flow was restored. Solutions of 10% APS, 10% TEMED, and 1% 3-acryloylamino-1-propanol were degassed and mixed at a volumetric ratio of 10:10:980, and the mixture immediately started flowing through the capillaries at 15 psi. Higher concentrations of monomer (2–3%) were found to quickly clog the capillaries. Solution was left flowing through the capillaries overnight, and the following day, the capillaries were flushed with H_2O for 1 h, and then stored filled with H_2O at 4 °C.

Instrumentation Our laser-induced fluorescence instrument has been described in detail elsewhere [34–41]. Briefly, a multipurpose injection block was connected to a CZE1000R high-voltage power supply (Spellman). Analytes were detected using a post-column sheath-flow cuvette. Fluorescence was excited by an 8-mW, 532-nm diode-pumped solid-state laser beam (Crystalaser), collected with an M-PLAN 60×, 0.7 NA microscope objective, and filtered with a 550–600-nm bandpass filter. Light was detected by an avalanche photodiode single-photon counting module. Voltage programming and fluorescence measurement were controlled by home-built software written in Labview (National Instruments).

Sample Barrett's esophagus biopsy samples were generously donated by the Brian Reid lab at the Fred Hutchison Cancer Research Center. Endoscopies were performed on patients in the Seattle Barrett's Esophagus Study with a large-channel endoscope and "jumbo" biopsy forceps using a turn-and-suction technique. Tissues were fixed in 70% ethanol within 15 s of sampling. The biopsy was homogenized using a PowerGen 125 homogenizer (Fisher) in 500 μL of a 1% Triton-X solution. The homogenized samples were aliquoted and stored at −80 °C. The Seattle Barrett's Esophagus Study was approved by the Human Subjects Division of the University of Washington in 1983 and renewed annually thereafter with reciprocity from the IRB of the Fred Hutchinson Cancer Research Center (FHCRC) from 1993 to 2001. Since 2001, the study has been approved annually by the IRB of the FHCRC with reciprocity from the Human Subject Division of the University of Washington.

Sample and ampholyte preparation A fresh biopsy aliquot was thawed each day and labeled with Chromeo P540. Each week, a 100-nmol aliquot of P540 was dissolved in methanol (100 μL). A 20-μL labeling solution contained 5 μL P540, 5 μL of homogenate sample, and 10 μL CAPS (100 mM). The labeling solution was placed at 38 °C for 30 min and then diluted to a final volume of 100 μL with dd-H_2O. Unless stated, before each run, the sample was first diluted 10:1 in a solution that contains Triton-X 100 (1.0%), Tween 20 (0.1%), and urea (2.5 M). The sample was again diluted 20:1 in a solution that contains Triton-X 100 (1.0%), Tween 20 (0.1%), urea (2.5 M), pharmalytes pH 5–8 (4% *v*/*v*), and pharmalytes 3–10 (2% *v*/*v*).

Capillary isoelectric focusing An ampholyte/sample solution (described above) was injected for 2 min at 5 psi to fill the entire capillary. cIEF was performed in two steps: focusing and mobilization. During focusing, 10 mM phosphoric acid (anode) made up the sheath flow, and the injection end of the capillary was placed in either CAPS (100 mM, pH 9.8) or sodium hydroxide (40 mM, pH 12; cathode). A voltage of −20 kV is applied across the capillary for 12 min, during which time, the current decreased from 7.4 to 0.06 μA. After focusing, mobilization occurred by keeping the voltage at −20 kV and switching the sheath flow to ammonium hydroxide (40 mM, pH 9) for anodic detection. All solutions where filtered through a 0.1-μM Millex syringe driven filter unit

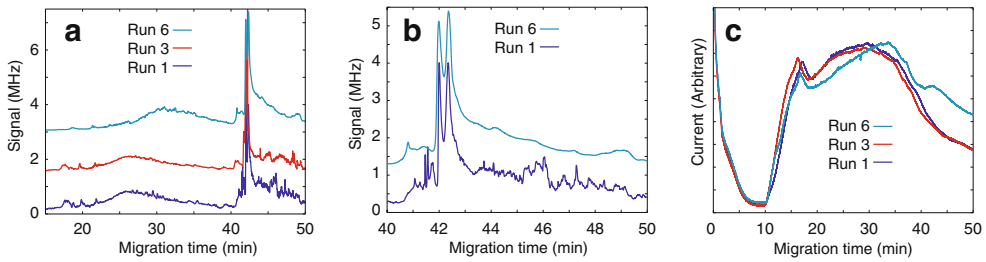

Fig. 1 Capillary isoelectric focusing separation of proteins from a biopsy sample, labeled with Chromeo P540. Sodium hydroxide was employed at the cathode end. The sheath flow consisted of phosphoric acid (2 mmM, pH 3). Proteins were mobilized with NH_4OH. All experiments were performed on an AAP coated capillary. **a** Runs 1, 3, and 6. **b** Close-up of runs 1 and 6. **c** Current profiles for the three runs

and centrifuged for 5 min. After each run, the capillary was flushed with 3 M HCl, followed by 4 M urea, and finally dd-H_2O all for 5 min at 5 psi.

Data processing Data were collected at 10 Hz. The data was processed using SigMan 1.2.6 (http://sourceforge.net/projects/sigman/) and plotted in Matlab (Mathworks). The data were corrected for photodetector nonlinearity before further processing [40, 41]. The corrected data were treated with a five-point median filter to remove spikes caused by the passage of particles through the laser beam and then smoothed by convolution with a Gaussian function that had a five-point (0.5 s) standard deviation. Electropherograms were shifted to bring the peak near 42 min into alignment.

Results and discussion

Capillary coating For best results in cIEF, electroosmotic mobility should be around 1×10^{-6} cm^2 $V^{-1}s^{-1}$ [21]. The electroosmotic mobility for the commercial LPA coated capillary was measured to be 2.7×10^{-5} cm^2 $V^{-1}s^{-1}$ using Zare's conductivity method [15]. cIEF separations of the homogenized sample performed on LPA coated capillary are shown in the Electronic Supplementary Material.

Several in-house capillary coating methods were investigated. The most stable coating was polymeric AAP attached to the silica via a vinyl group deposited by a Grignard reaction. The electroosmotic mobility for the AAP was measured to be 1.1×10^{-6} cm^2 $V^{-1}s^{-1}$, which is significantly lower than the commercially coated capillaries. Results from this capillary are shown below.

cIEF cocktail Unlike other CE separation modes where a small plug is injected, in cIEF, the entire capillary is filled with a sample and ampholyte mixture. The cIEF mixture should contain additives such as neutral surfactants to improve protein solubility and minimize adsorption to the capillary wall. The mixture cannot contain any charged ions that disrupt the pH gradient [38]. Unfortunately, some common additives lead to an increase in background and loss in sensitivity in our laser-induced fluorescence detector. For example, ethylene glycol creates a change in index of refraction in the detector, which leads to an increase in background signal. Glycine is another common additive. Unfortunately, it contains a primary amine and would be labeled with unreacted dye in the sample. We observed that the best combination of additives was Tween 20, Triton-X, and urea in the sample/ampholyte mixture.

Laser-induced fluorescent detection Laser-induced fluorescents can provide exquisite detection limits, which allows reduced sample loadings compared with UV absorbance detection. Reducing sample loadings both minimizes protein precipitation and protein adsorption to the capillary walls.

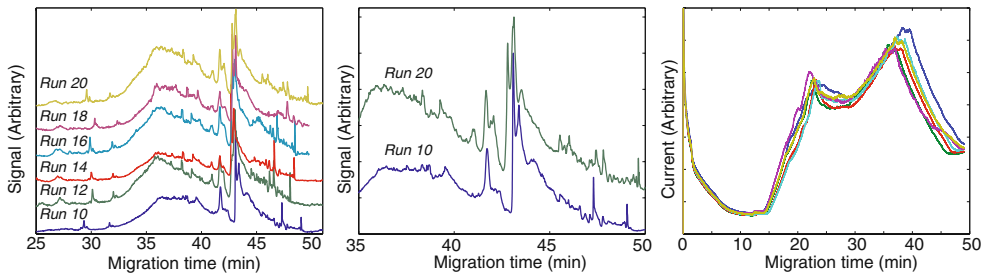

Fig. 2 Capillary isoelectric focusing separation of proteins from a biopsy sample, labeled with Chromeo P540. Sodium hydroxide was replaced with CAPS at the cathode end. The sheath flow consisted of phosphoric acid (2 mmM, pH 3). Proteins were mobilized with NH_4OH. All experiments were performed on an AAP coated capillary. **a** Five runs taken over a 3-day period. **b** Close-up of runs 10 and 20. **c** Current profiles for the runs

We employ an ultrasensitive laser-induced fluorescence detector that provides zeptomole detection limits in cIEF. Several innovations are required to achieve this sensitivity, including the use of a sheath-flow cuvette to minimize the contribution from scattered light to the background, the use of photobleached ampholytes to minimize the contribution of fluorescent impurities to the background, the use of cationic fluorogenic reagents to minimize perturbation of labeling reagent on the isoelectric point of the protein, and the use of a reagent that is excited at relatively long wavelengths to further decrease the background signal.

Biopsy analysis Improved cIEF reproducibility and capillary longevity was seen when NaOH was eliminated at the cathode and replaced with CAPs buffer (Figs. 1 and 2). Fluorescent labeling using neutral or anionic reagents disrupts both the zone electrophoresis and the isoelectric focusing profile of standards [42–44]. We employed the cationic fluorogenic reagent Chromeo P540 in this experiment; this reagent converts cationic lysine residues to cationic products, generating clean electrophoretic profiles [5, 17].

Labeled biopsies were analyzed with cIEF first with the NaOH and then with CAPs at the cathode. All experimental details were the same except for the cathode solution. In both experimental protocols, a fresh AAP-coated capillary was used.

Figure 1A shows three electropherograms generated from cIEF of a homogenized biopsy with the use of NaOH at the cathode. The top trace is the first run followed by the third and fourth runs, and the bottom trace is the sixth run on the capillary. After only six runs, the sharp peaks seen in the first run are gone, and only broad features can be seen (Fig. 1B).

Figure 2A shows six electropherograms generated from cIEF of a homogenized biopsy sample with the use of CAPS at the cathode. The bottom trace is from day 1, the middle four traces are from day 2, and the top trace is from day 3. There is very little change in the protein profile across these runs.

Figures 1C and 2C show the current profile from the different runs. If all the solutions and the capillary coating remain constant from run to run, the current profile should not change. The initial current value, the time it takes to focus, and the current value before mobilization should be the same for each runs, indicating the ampholyte mixture is consistent, and the anode/cathode solutions have not lost their buffer capacity. During mobilization, the current increases and then levels out, which should occur at the same rate for each run, indicating that the proteins are moving off the capillary at the same rate. A more consistent current profile is seen when CAPS is used at the cathode. With NaOH at the cathode, focusing took 8 min and 30 s, but focusing took 4 min longer with CAPS at the cathode.

Rodriguez-Diaz and Wehr demonstrated the use of taurine at the cathode but suggested several other neutral buffering solutions including CAPS [27]. Several zwitterion buffers were considered. However, CAPS was the only solution that generated isoelectric focusing separations comparable to those generated by NaOH. We found a 100-mM concentration of CAPS worked best. Lower concentrations resulted in incomplete focusing where the current does not drop to 10% of its initial value, which is typically the case when ampholytes reach a neutral state.

Switching to anodic detection was the final cIEF procedural change that made reproducible detection of biopsies possible. Anodic detection was better suited for this experimental set-up particularly because of our sheath flow off column detection. For cathodic detection, CAPS is in the sheath flow, and phosphoric acid is at the injection end. During mobilization, CAPS is switched to an acidic zwitterion. CAPS in the sheath flow often results in the formation of crystals on the tip of the capillary. CAPS buffering capacity caused longer and inconsistent transition times from focusing to mobilization. With phosphoric acid in the sheath flow, the mobilization transition is smoother. The set-up also kept the capillary from being exposed to harsh acidic conditions for the entire experiment; instead, the capillary is mainly exposed to a solution with a pH around 9.5.

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