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Removal of sample background buffering ions and myoglobin enrichment via a pH junction created by discontinuous buffers in capillary electrophoresis

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ABSTRACT

Traditional CE sample stacking is ineffective for samples containing a high concentration of salt and/or buffer. We recently reported the use of a discontinuous buffer system for protein enrichment that was applicable to samples containing millimolar concentrations of salt. In this paper, the technique was investigated for samples containing unwanted buffering ions, including TRIS, MES, and phosphate, which are commonly used in biological sample preparation. Using myoglobin as a model protein, the results demonstrated that background buffering ions can be effectively removed or separated from the enriched protein. The key is to use either the acid or the base of the discontinuous buffers to adjust the pH of the sample, such that the net charge of the unwanted buffering ions is near-zero. The successful isolation and enrichment of myoglobin from up to 100 mM TRIS and 50 mM MES was demonstrated. The enrichment factors remained at approximately 200. Removal of phosphate was more challenging because its net charge was anionic in both the acid and the base of the discontinuous buffers. The enrichment was only achievable up to 30 mM of sodium phosphate, the enrichment factors observed were significantly lower, below 50, and the process was delayed due to the higher ionic strength resulted from phosphate. The migration of phosphate during enrichment was studied using a UV-absorbing analogue, phenyl phosphate. In addition, Simul 5.0 was used to simulate the discontinuous buffers in the absence and presence of TRIS and phosphate. The stimulated TRIS and phosphate concentration profiles were generally in agreement with the experimental results. The simulation also provided a better understanding on the effect of phosphate on the formation of the pH junction.

1. Introduction

Capillary electrophoresis (CE) is best known for its superior separation efficiency and ability to handle miniscule, nanoliter sample volumes. While separation is the primary purpose for CE, additional sample preparation steps, such as sample clean-up (removal of unwanted background) and enrichment can also be performed using CE at these small sample volumes [1–4]. Enrichment and sample clean-up are vital for the success of CE separations and subsequent mass spectral analysis, especially for protein samples [5–7]. The short path length in the capillary for absorbance detection requires that the analyte is present at enriched concentrations. Background ions in the sample matrix can cause increased conductivity in the capillary leading to Joule heating [8]. These unwanted ions can also cause analyte ionization suppression in mass spectral (MS) analysis and reduce sensitivity [9].

Electrophoretic sample stacking is the most commonly used enrichment method in CE and recent developments are thoroughly categorized and explained within these reviews [2–4,6,7,10–12]. Stacking generally refers to a compression of the sample zone due to a reduction of analyte mobility as the molecules exit the sample zone into the electrolyte filling the remainder of the capillary. In isotachophoretic (ITP) sample stacking, the sample molecules are compressed between a leading electrolyte containing ions of highest mobility and a terminating electrolyte containing ions of lowest mobility [13–15]. Samples containing salt can be successfully enriched, but judicious attention must be paid to the selection of electrolytes for each specific sample [6,16,17]. Alternatively, stacking can be induced by a difference in conductivity between two zones, such as between a sample of low conductivity (yielding high field strength) and a buffer of high conductivity (low field strength). This form of stacking is very widely used in CE and can be referred to as field amplified

Abbreviations: PP, phenyl phosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane.

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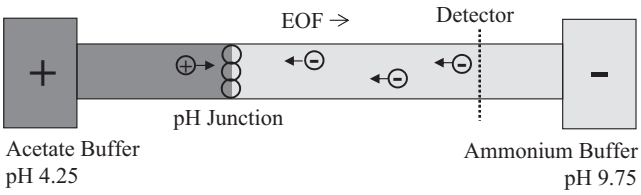

Fig. 1. Schematic of experimental setup. A step-pH junction is created by acetate and ammonium under voltage application. The myoglobin molecules (circles) inside the capillary electromigrate either as cations (in acetate) or anions (in ammonium) towards the junction and are trapped at the junction.

sample stacking (FASS) or large-volume sample stacking (LVSS) [18], depending upon the size of the sample plug [10,12]. Electrokinetic sample injection combined with the principles of FASS (field enhanced/amplified sample injection, FESI/FASI) has been successfully applied on proteins and peptides for enrichment prior to separation and MS detection [19,20]. However, these stacking techniques require the sample to be prepared in low conductivity, thus limiting their direct application on real samples of biological origin.

Rather than relying on conductivity differences in zones, one can achieve stacking of ionizable analytes, such as proteins and peptides, by manipulating their mobilities with a pH change between the sample zone and the electrolyte [11]. This pH manipulation approach is generally referred to as pH-mediated sample stacking [21]. In the literature, it has been described as stacking by dynamic pH junction [22–24], and transient pH boundary or transient moving chemical reaction boundary [25,26]. Because the analyte mobilities are primarily controlled by pH and not field strength, stacking has been successfully demonstrated and modeled with high salt concentrations in the sample zone [25,27,28]. However, a limitation of pH-mediated stacking is the need to customize the electrolyte pH to the pK_a of the analytes, making it difficult to simultaneously enrich analytes with a wide range of pK_a values. To address this issue, Pospíchal and co-workers reported the stacking of proteins at the junction of an acid and a base, created by the controlled electrolysis of non-buffering electrolytes. The technique was referred to as carrier ampholyte free isoelectric focusing (CAF-IEF) [29–31]. Alternatively, our group reported the use of discontinuous buffers to create a similar prolonged pH junction for protein enrichment [32]. Despite the differences in configuration, both of these approaches were described and generalized as examples of sample stacking by moving neutralization reaction boundary (NRB) in a review by Cao et al. [26].

The experimental setup of our discontinuous buffer system is illustrated in Fig. 1. The capillary was filled with two buffers, pH 9.75 ammonium and pH 4.25 acetate, which also served as the catholyte and anolyte respectively. The buffers were chosen to provide sufficiently constant concentrations of OH^- and H^+, and little buffer capacity at neutral pH. Upon voltage application, a sharp pH junction was formed and sustained by the OH^- and H^+ fluxes respectively from the catholyte and anolyte. When amphoteric molecules such as proteins, with isoelectric points between the two buffer pH (i.e., $4.75 > pI > 9.25$) were introduced, they possessed opposite net charges on either side of the NRB, and as a result were trapped and enriched at the pH junction. The rationale of the prolonged pH junction was to facilitate enrichment of large sample volumes. For example, an injection exceeding one full-capillary can be achieved by preparing the sample in the anolyte and/or catholyte [33]. Enrichment factors of up to 2000 were reported. Following the enrichment, the stacked protein or protein mixture molecules were subsequently separated using capillary zone electrophoresis (CZE) [34], spotted onto a MALDI target for MS analysis [35], or digested into peptides with trypsin prior to MS analysis [35,36].

To assess the ability of our discontinuous buffers in concentrating proteins from biological samples, the effect of salt on the pH junction was investigated [34]. Similar to pH-mediated stacking, the ionization of analytes, and in turn the mobilities, were mainly controlled by the pH. The salt appeared to have little effect on the pH junction, and successful enrichment was demonstrated in the presence of up to 50 millimolar ionic salts in the protein sample. However, the high ionic strength due to higher salt concentrations significantly impeded the migration of the analytes and hindered their enrichment. In addition to salt, extraction and/or solubilization protocols of proteins from biological samples typically require the use of buffers, such as Phosphate Buffered Saline (PBS), TRIS Buffered Saline (TBS), or the morpholine ring-containing buffers such as MES, at high millimolar concentrations. Unlike the non-buffering salt, the presence of background buffering ions in the sample could potentially react with the discontinuous buffers or alter the buffering capacity near the pH junction. Buffer ions present in the samples can significantly alter the local pH of the discontinuous buffers and thus potentially disrupt protein enrichment. In addition, the buffering ions can experience a change in their degree of ionization when crossing the pH junction. This could potentially lead to a mobility reduction and in turn, their stacking or accumulation near the pH junction. In this paper, the effects of TRIS, MES, and phosphate buffers on the enrichment of myoglobin are investigated. The migration behaviour of TRIS and phosphate ions (with the UV absorbing phenyl phosphate) at the pH junction is monitored by UV–visible absorption. The results presented herein demonstrate the capability of the pH junction in tolerating and removing the background ions during myoglobin enrichment.

2. Materials and methods

2.1. Apparatus

All myoglobin enrichment experiments were performed on an Agilent ^{3D}Capillary Electrophoresis instrument (Palo Alto, CA, USA) with a direct UV–visible absorbance detector. The Agilent ^{3D}CE ChemStation software was used for data collection. Unmodified fused silica capillaries of 50 μm i.d. and 364 μm o.d., were purchased from Polymicro Technologies (Phoenix, AZ, USA) and cut to a total length of 48.5 cm with an effective length of 40 cm. The capillary was thermostated to 25 °C during experiments. In order to suppress the electroosmotic flow (EOF) and prevent protein adsorption onto the capillary wall, a semi-permanent coating (1,2-dilauroyl-*sn*-glycero-3-phosphocholine, DLPC) was applied to the inner capillary wall [37].

2.2. Reagents

All solutions were prepared with deionized water (18.2 MΩ) from a Millipore water purification system (Bedford, MA, USA). Glacial acetic acid and ammonium hydroxide were purchased from EM Science (Gibbstown, NJ, USA) and used to make the buffer solutions. Sodium hydroxide (EM Science) was used to wash the new capillaries. Buffering ions examined included sodium phenyl phosphate dibasic dihydrate (Sigma, St. Louis, MO, USA), sodium phosphate dibasic, anhydrous (EM Science), sodium phosphate monobasic, monohydrate (Caledon), 2-(*N*-morpholino)ethanesulfonic acid (MES, Sigma), and tris(hydroxymethyl)aminomethane (TRIS, Sigma). Mesityl oxide (Aldrich) solutions were prepared in water at 20 mM and used to mark the EOF. Myoglobin from horse heart (Sigma) was used as a

model protein. The semi-permanent coating of phospholipid DLPC (Avanti Polar Lipids, Alabaster, AL, USA) was prepared as reported previously [37]. Briefly, DLPC (0.1 mM) was prepared in an aqueous solution of 20 mM TRIS buffer and 20 mM calcium chloride (Caledon Laboratories, Georgetown, ON, Canada) and adjusted to pH 7.2 by hydrochloric acid (EM Science). Solubilization of the DLPC required several 10-min, alternating cycles of sonication and magnetic bar stirring. Coating formation was performed by rinsing the capillary with this solution.

2.3. Enrichment of proteins by pH junction

Washing and coating of new silica capillaries was performed by pressure application (1 bar) at the capillary inlet with sodium hydroxide (0.1 M) for 10 min, followed by water for 10 min and DLPC solution for 20 min. To prepare the two discontinuous buffers, 10 mM ammonium hydroxide was adjusted to pH 9.75 with acetic acid, and 10 mM acetic acid was adjusted to pH 4.25 with ammonium hydroxide. The actual amount of acetic acid and ammonium hydroxide required to reach the desired pH was not measured. The enrichment experiments were performed as previously shown in Fig. 5C of Ref. [32] unless otherwise stated. Briefly, the capillary was filled with the sample solution of myoglobin (10 ng μL^{-1}) prepared in the 10 mM pH 9.75 ammonium buffer. The pH 4.25 acetate buffer was placed at the inlet (anode) and the pH 9.75 ammonium buffer was placed at outlet (cathode, near the detector). Voltage application was programmed to a constant voltage of 30 kV; however a maximum current limit of 100 μA was in place to prevent excessive Joule heating when salt-containing samples were used. With the DLPC capillary coating, the suppressed, forward EOF and the moving NRB slowly carried the enriched proteins towards the detector. Absorption detection was performed at both 200 nm (absorption by peptide bond) and 408 nm (absorption by heme). The capillary coating was regenerated in between runs by rinsing (1 bar) with the DLPC solution for 5–10 min.

Sample solutions of myoglobin were prepared at the specified concentration in either the acetate or ammonium buffer. To prepare samples containing background buffer ions (TRIS, MES or phosphate), monobasic phosphate and MES were added to the pH 4.25 acetate buffer, whereas dibasic phosphate and TRIS were added to the pH 9.75 ammonium buffer. The final pH was allowed to change without further adjustment. Nevertheless, the pH change was within 0.05 pH unit in all cases, except when 100 mM TRIS was added, the pH of ammonium buffer increased by 0.1 unit. The presence of TRIS was detected by UV-absorption at 200 nm. To study the migration of phosphate near the pH junction, a UV-absorbing analogue (also at 200 nm), phenyl phosphate, was used.

2.4. Computer simulation

Simul 5.0 developed by Bohuslav Gaš and co-workers was obtained online [38], and was used to computer simulate the formation of a pH junction and the movement of various ions during the enrichment of protein using discontinuous buffers. In the example provided with the software for isoelectric focusing (IEF_1.sna) [38], the anolyte and catholyte were entered as the terminating electrolyte (TE) and leading electrolyte (LE). Hence, for the simulation of our discontinuous buffer system, the anolyte, pH 4.25, 10 mM acetate buffer (pH adjusted with ammonium hydroxide) was input as 10 mM acetate with 2.33 mM ammonium, while the pH 9.75, 10 mM ammonium buffer (pH adjusted by acetic acid) catholyte was input as 10 mM ammonium with 2.33 mM acetate. For simulations in the presence of TRIS, MES and phosphate, these ions were entered as analytes present in either the LE or TE. Other simulation conditions are described in detail in the Supplementary Data.

3. Results and discussion

3.1. Protein enrichment and removal of TRIS

TRIS is one of the most commonly used buffers for protein extraction and/or solubilization. It is considered a MS-compatible buffer, since the degree of MS ionization suppression from TRIS is much less than that from ionic salt such as sodium. Nevertheless, significant TRIS-protein adduct formation was reported with conventional ESI from as low as 0.5 mM TRIS [39]. Herein, isolation of proteins from TRIS at sub-microliter sample volumes by discontinuous buffers is performed. Myoglobin is selected as the model protein in this work due to its unique UV absorption at 408 nm for identification. The enrichment of other proteins (carbonic anhydrase I, bovine serum albumin, lentil lectin, and β-casein) and peptides (tryptic digests of myoglobin, lentil lectin and β-casein, and endoproteinase Asp-N digest of myoglobin) with the same discontinuous buffer system has been demonstrated in previous reports [32,35,36].

The acid dissociation constant of TRIS (pK_a) is 8.1. It therefore exists as a cation in pH 4.25, or predominately as a neutral molecule in pH 9.75 (only 2% in the protonated form). To facilitate the removal of TRIS from the protein, the sample was prepared in the ammonium buffer (pH 9.75), and was injected to fill the entire capillary as illustrated in Fig. 2A. This setup allowed the protein (myoglobin, p*I* 7.2) and TRIS to be differentiated by net charges, as anionic and near-neutral molecules respectively. The experiment was performed with two TRIS concentrations, 10 and 100 mM, along with a blank (0 mM TRIS), and the results were shown in Fig. 2B. In the absence of TRIS (lower trace of Fig. 2B), myoglobin was enriched by the discontinuous buffers as expected and was carried by a slow EOF generated in the DLPC-modified capillary. This EOF, however, was not constant throughout the run. A cathodic EOF of roughly 1×10^{-4} cm^2 V^{-1} s^{-1} was measured in the pH 9.75 ammonium buffer and a slower anodic EOF of $<1 \times 10^{-5}$ cm^2 V^{-1} s^{-1} was observed in pH 4.25 acetate. As a result, the EOF varied between these two values during the experiment depending on the ratio of ammonium to acetate buffers inside the capillary. Most importantly, it mobilized the capillary content, including the pH junction and the enriched proteins, towards the detector near the cathode in approximately 22 min. In the presence of TRIS, similarly sharp peaks at approximately the same time as in the blank were observed. The identity of these peaks was confirmed by the heme absorption at 408 nm (data not shown). The TRIS molecules remained relatively stationary, and were detected as plateaus. A gap between the TRIS plateau and the myoglobin peak was also observed in Fig. 2B. However, this separation was only found in the about half of the 4–6 replicates on each of 0, 10 and 100 mM TRIS. When observed, the gap between the myoglobin peak and the TRIS plug ranged from 1 to 2 min. Computer simulation of the discontinuous buffers was performed in the presence and absence of TRIS (Supplementary Data, Fig. S1). The two pH junctions obtained were similar in both cases, supporting the successful myoglobin enrichment observed. The simulated TRIS concentration profile (Fig. S1B) confirmed that TRIS was immobile and stayed on the cathodic side of the pH junction. A gap between the myoglobin peak and the end of the TRIS plug observed in Fig. 2B (10 and 100 mM TRIS) was not evident in the simulation. The edge of the simulated TRIS profile only appeared to skew away from the pH junction. The replicate experiments of Fig. 2B (4–6 runs on each of 0, 10 and 100 mM TRIS) also revealed that the time of the myoglobin peak and the enrichment factor

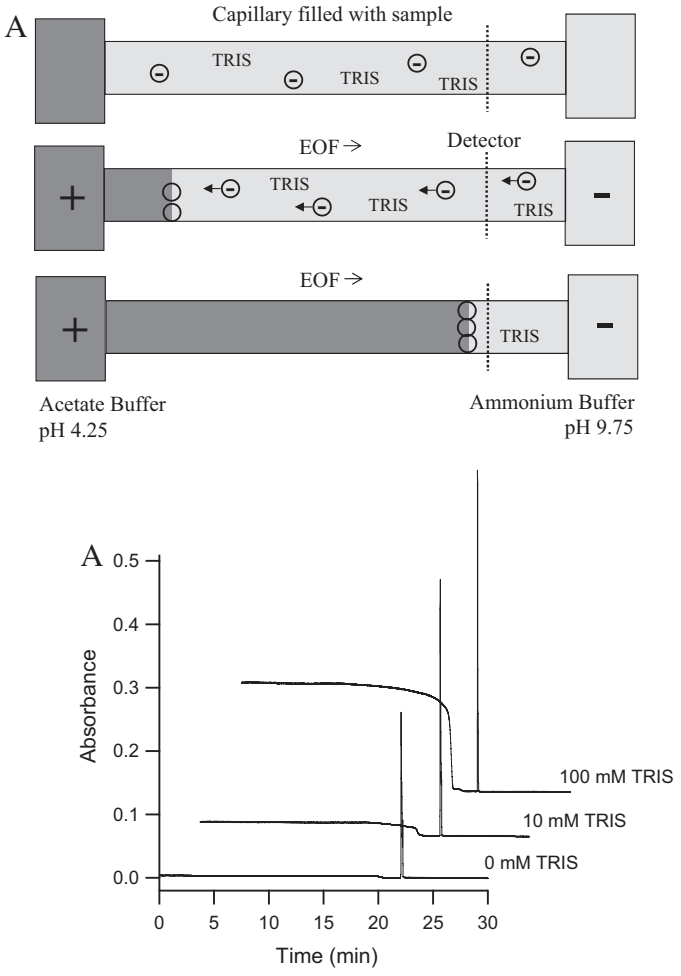

Fig. 2. Schematics representing the injection of 10 ng μL^{-1} myoglobin and TRIS prepared in pH 9.75 ammonium buffer to fill the capillary and the subsequent ion migration upon voltage application (A). UV-absorption signals recorded as the residual cathodic EOF mobilized the capillary content past the detector (30 kV) for 0, 10 and 100 mM TRIS prepared according to the above description (B).

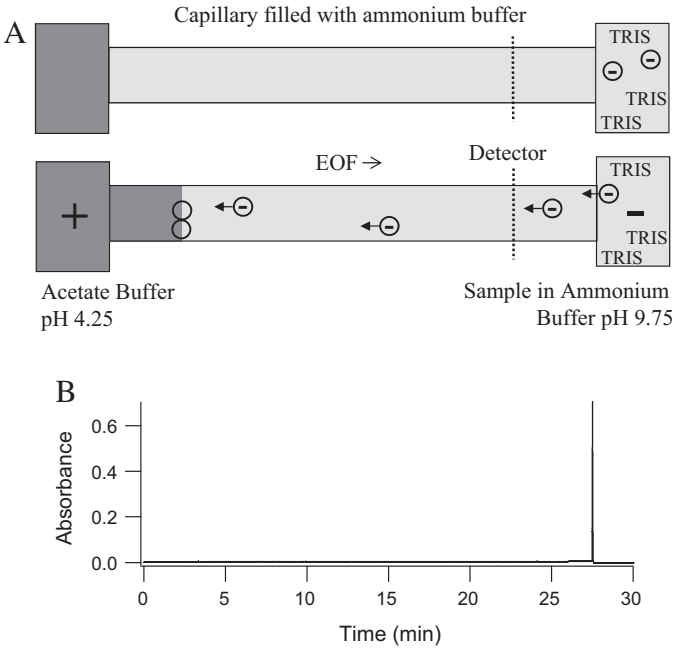

Fig. 3. Schematics representing the selective injection of 10 ng μL^{-1} myoglobin from 100 mM TRIS prepared in pH 9.75 ammonium buffer at the cathode and the sequential enrichment upon voltage application (A). The UV-signal recorded during voltage application (B).

were variable from run-to-run, likely due to fluctuations in the EOF. The myoglobin peak was observed between 13 and 30 min in replicates. The means, and standard deviations in parentheses, were 18 (3), 16 (4), and 19 (7) min respectively for 0, 10 and 100 mM TRIS. The enrichment factors from replicates ranged from 90 to 340. The means, and standard deviations in parentheses, were 230 (70), 210 (100) and 180 (100) respectively for 0, 10, and 100 mM TRIS. Based on these results, the enrichment factor appeared to be unaffected by the presence of TRIS up to 100 mM.

The setup in Fig. 2A corresponded to an injection of one capillary volume (0.95 μL). Injection of smaller sample volumes could easily be accommodated by partially filling the capillary with sample. Likewise the injection of sample volumes over 1 μL could also be achieved, but required an alteration of the experimental setup in which the sample was placed in a vial at the cathode (Fig. 3A). Under this condition, the anionic myoglobin molecules were introduced into the capillary during voltage application. They continued to migrate inside the capillary towards the anode until reaching the pH junction where they became trapped. The residual EOF eventually carried the pH junction with the enriched myoglobin to the detector, which was detected as a sharp peak (Fig. 3B). TRIS, present mainly as neutral molecules at pH 9.75 in the catholyte, was not drawn into the capillary by the voltage application. This selective injection experiment was performed in triplicates for each of 0, 10 and 100 mM TRIS. Based on the peak heights, the protein enrichment factors ranged from 130 to 470. The means, and standard deviations in parentheses, were 210 (70), 240 (130), and 260 (190) respectively for the three TRIS concentrations. The time of myoglobin peak was also found to vary in the replicates, ranging between 19 and 31 min. This time value was indeed the injection time of myoglobin. Hence, one should be able to increase myoglobin loading by slowing down the NRB movement and/or by using a longer capillary. Likewise, greater sample loading could be achieved by introducing a counter balancing flow to keep the protein band from exiting the capillary, as previously demonstrated [33]. In practice, this is limited by electrolysis, which can significantly alter the pH of the discontinuous buffers. The volume of the buffered sample and the concentration of the buffer will determine the maximum time of protein enrichment with selective injection. In the experiments performed in Fig. 3, 50–100 μL of sample solutions were placed at the cathode, and significant pH changes due to electrolysis were not evident in the presented experiments.

3.2. Protein enrichment and removal of MES

Similar to TRIS, MES is a monoprotic weak base (pK_a around 6), but MES also has a strongly acidic sulfonate group. As a result, MES is net anionic when fully deprotonated (at pH 9.75), and zwitterionic when fully protonated (at pH 4.25). In a similar fashion performed for TRIS, we propose the removal of MES as neutral, or zwitterionic in this case, molecules from myoglobin. The capillary was filled with the protein-MES sample prepared in pH 4.25 acetate (Fig. 4A). Upon voltage application, a pH junction was formed at the cathodic end of the capillary, the end closer to the detection point. The myoglobin molecules migrated as cations towards pH junction, while the zwitterionic MES molecules remained relatively stationary. At the beginning of the run, the capillary was filled with pH 4.25 acetate, resulting in a very small anodic (reversed) EOF. However, the anodic EOF was soon balanced by the cathodic EOF as the pH 9.75 ammonium buffer was drawn in from the cathode. A combination of voltage and pressure was therefore applied during the entire

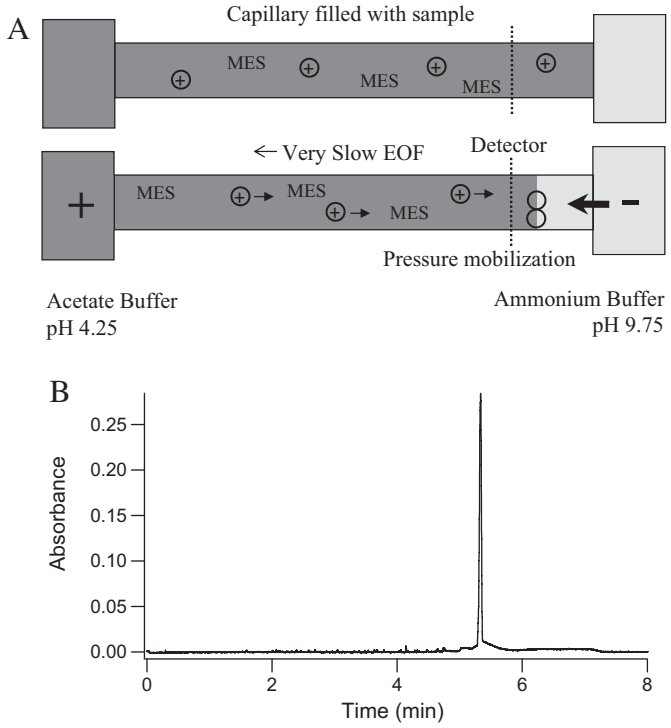

Fig. 4. Schematics representing the injection of 10 ng μL^{-1} myoglobin with 50 mM MES (prepared in pH 4.25 acetate) to fill the capillary and the subsequent ion migration (A). UV-absorption signal recorded when 30 kV and −5 mbar pressure at the anode was applied simultaneously during the entire run (B).

run to carry the myoglobin peak past the detection window. To compensate for EOF variation in replicate runs, the pressure at the anode was adjusted between −1 and −10 mbar, in order to bring the enriched proteins past the detector in approximately 5 min. Fig. 4B was produced with a pressure of −5 mbar. The enrichment factors from 3 replicates ranged from 150 to 220. A control experiment was performed under the same conditions as in Fig. 4B with MES absent from the sample. We observed a nearly identical peak with an absorbance of 0.24 (160-fold enrichment), which allowed us to conclude that the protein enrichment was not affected by MES.

While the peak heights observed in this experiment were lower than those in Figs. 2 and 3, the differences can be attributed to the different enrichment time elapsed at the detection point. In theory, this could be improved by reducing the applied pressure to below −1 mbar. In practice, our instrument was not capable of delivering reproducible pressure at such a low level.

3.3. Migration of phosphate at the pH junction

The removal of phosphate in the discontinuous buffers is less straight forward compared to TRIS and MES. Phosphate's approximate pK_a values are 2, 7, and 12 (the actual values depend on the ionic strength). Unlike TRIS and MES, the neutral form of phosphate (H_3PO_4) exists outside the operating pH of our discontinuous buffers, and thus it cannot be removed from the proteins as neutral molecules. Phosphate exists as the singly charged $H_2PO_4^-$ in pH 4.25, and as the doubly charged HPO_4^{2-} in pH 9.75. In other words, it remains as net anionic ions on either side of the pH junction, and does not experience charge reversal like proteins at the pH junction. We therefore hypothesized the removal of phosphate by its continuous, anodic electromigration across the pH junction, similar to the electromigration of non-buffering anions such as chloride observed previously in Ref. [34].

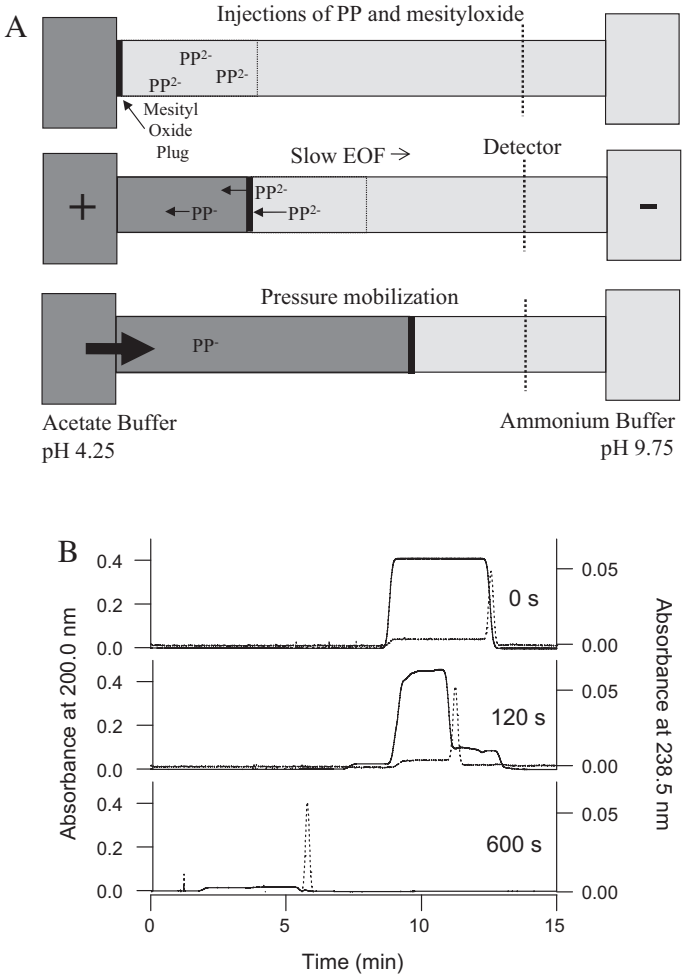

Fig. 5. Schematics of experimental setup and proposed migration behaviour of phenyl phosphate (PP) (A). Absorbance signals recorded during pressure mobilization of the capillary content after voltage application for the indicated time (B). Experimental conditions: sodium phenyl phosphate concentration, 10 mM prepared in 10 mM ammonium buffer; mesityl oxide concentration, 20 mM; voltage, 30 kV; mobilization pressure, 30 mbar; and detection, 200 nm for PP (solid line) and 238.5 nm for mesityl oxide (dashed line).

To experimentally confirm the migration behaviour of phosphate crossing the pH junction, the UV-absorbing analogue of phosphate, sodium phenyl phosphate (PP), was selected. PP has pK_a values (2.3 and 5.9) similar to those of phosphate [40], and thus also exists as 1- and 2-ions in the discontinuous buffers used in this work. The experiment began with placing a plug of phenyl phosphate solution prepared in the ammonium buffer next to the discontinuous buffer junction, marked by a mesityl oxide plug (Fig. 5A, top). The UV absorbance signals confirmed that the PP was on the cathodic side of the pH junction (pH 9.75), with the mesityl oxide peak marking the end of 1-capillary volume (0 s, Fig. 5B). Variable periods of voltage were applied to induce electromigration to various extents, followed by pressure mobilization of the capillary content past the detector. After 120 s of voltage application, the signal revealed that PP ions indeed crossed the pH junction. The absorbance observed was lower on the anodic side of the pH junction. The absorptivities of phenyl phosphate in pH 9.75 and 4.25 buffers were independently measured and verified to be essentially equal. In other words, the lower absorbance indicated a decrease in PP concentration after crossing the pH boundary.

The concentration decrease was hypothesized to be a result of electrodispersion due to the difference in conductivity between the two sides of the pH junction. The ionic strength at the cathodic side

of the pH boundary was higher due to the 10 mM PP. When the PP ions crossed the junction, they experienced a reduction in ionic strength (an increase in electrical field), and thus acquired a higher mobility in spite of the charge reduction. The higher mobility of the PP due to the ionic strength reduction was independently confirmed by mobility measurement in conventional capillary zone electrophoresis (data not shown). In addition, computer simulation by Simul 5.0 was used to predict the migration behaviour of phosphate when crossing the pH junction. The simulated phosphate signal (presented in Supplementary Data, Fig. S2) closely resembled the experimental phenyl phosphate signal observed in Fig. 5B. In addition, the simulated phosphate concentration drop coincided with the simulated conductivity drop, supporting our hypothesis on electrodispersion. Finally after 600 s of voltage application, the PP completely migrated out of the capillary and was not detected. The results importantly demonstrated that the phenyl phosphate ions crossed the pH junction and continued to electromigrate towards the anode, away from the enriched proteins at the pH junction. The experiment was repeated in triplicates with 10 mM PP for 0, 60, 120, and 600 s. In all cases, the same migration pattern was observed, with the phenyl phosphate crossing the pH junction by 600 s.

3.4. Phosphate removal during protein enrichment

In this section, the enrichment of myoglobin in the presence of sodium phosphate was performed. To monitor the migration of myoglobin near the pH junction, a plug of myoglobin was positioned next to the pH junction, and an increasing duration of voltage was applied followed by pressure mobilization for detection. We had a choice of preparing the myoglobin-phosphate sample in pH 9.75 with ammonium and placing the plug at the cathodic side of the junction, or by bringing the pH to 4.25 with acetate and placing it at the anodic side of the junction. Having phosphate on the anodic side, as illustrated in Fig. 6A, allows its migration out of the capillary without crossing the junction. This should minimize the effect of phosphate on the pH junction, and therefore was the chosen setup for this experiment. Voltage application was allowed to proceed for 0, 1, 5, or 10 min, followed by pressure mobilization of the capillary content past the detector. The results are shown in Fig. 6B. A control experiment, using a sample without sodium phosphate, was also performed under the same conditions (figure inset).

In the absence of phosphate, the enrichment was completed in 5 min of voltage application, in agreement with what was reported previously [32]. The accumulation of myoglobin occurred as expected at the left side of the plug (inset of Fig. 6B); i.e., at the junction of the sample plug (prepared in acetate) and the ammonium buffer. The residual cathodic EOF slowly moved the capillary content towards the detector and resulted in the peak shift towards the *y*-axis. When sodium phosphate (30 mM) was added to myoglobin, the enrichment process also proceeded with accumulation of myoglobin at the left side of the sample plug (Fig. 6B); however a second peak also appeared after 5 min of protein enrichment. The entire set of experiments was also repeated with 10 mM of sodium phosphate. Nearly identical protein enrichment behaviour was observed, although it occurred at a faster rate. For example, the 300 s trace from 10 mM phosphate resembled that of the 600 s trace from 30 mM phosphate (data not shown).

To address the double peak observed during enrichment, Simul 5.0 computer simulation was applied to model the system (presented in Supplementary Data, Fig. S3). The presence of phosphate ions and sodium ions contributed to the significantly higher conductivity in the sample zone. In the initial phase, the phosphate and sodium zones electromigrated in opposite directions away from the sample zone. Upon their exit, they were replaced with stacked acetate and ammonium ions. The conductivity around the

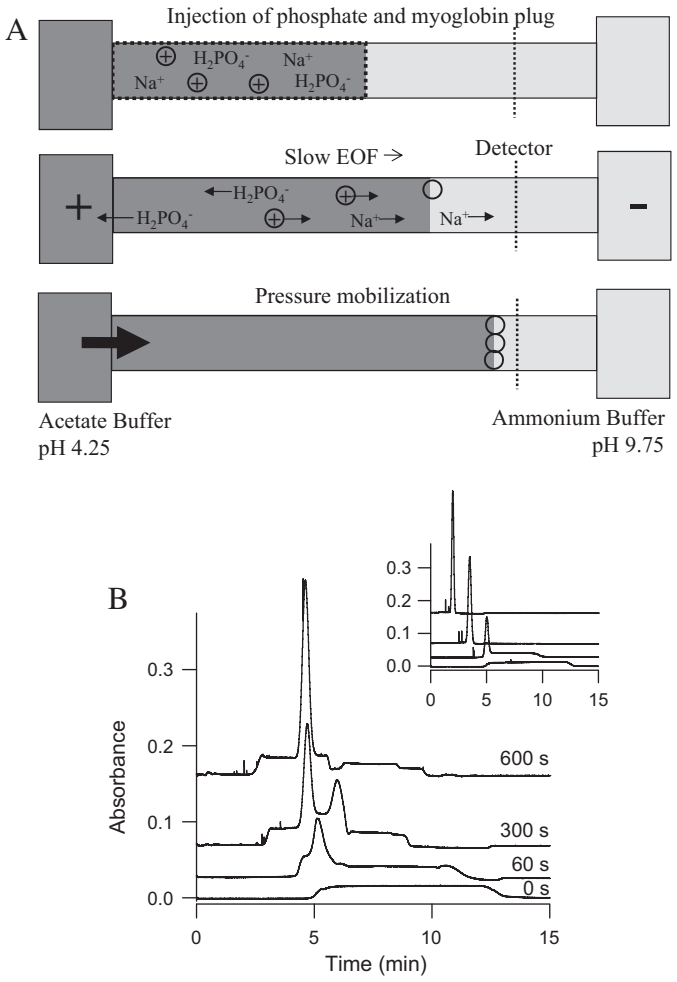

Fig. 6. Schematic showing the electrofocussing of a plug of myoglobin (100 ng μL^{-1}) and sodium phosphate (30 mM) prepared in 10 mM pH 4.25 acetate buffer (A). The UV-absorbance signals recorded during pressure mobilization (30 mbar) of capillary content containing the myoglobin-phosphate sample after various periods of voltage application: 0, 1, 5 and 10 min (B). (Inset showing the results of control experiments obtaining from myoglobin without phosphate. Voltage application time was 0, 1, 5 and 10 min from bottom to top.)

buffer junction remained very high, and thus hindered the formation of a sharp pH junction. Upon further voltage application, the zone passing between acetate and ammonium resulted in changes in conductivity profile, and as a result, a 2-step pH gradient was observed. This pH gradient profile was most likely responsible for the double-peak myoglobin signal observed in Fig. 6B (at 300 s). Finally after a longer period, nearly all sodium and phosphate ions exited the capillary. The acetate and ammonium ion distribution also evolved to the point where a single-step pH transition was observed. This likely is responsible for the single myoglobin peak observed in Fig. 6B (at 600 s). The absorbance of this peak was 0.23 (15-fold enrichment) which was lower than the absorbance of 0.25 and 0.38 (17- and 25-fold) from the control experiments respectively at 5 and 10 min. Nevertheless, the absorbance was recorded during pressure mobilization, and therefore the peak height was dependent on the position of the peak in reference to the detection point because of laminar flow bandbroadening. Taking this into account, we can conclude that comparable peak height values were observed in the enrichment of myoglobin with and without sodium phosphate.

To better compare the enrichment factors of myoglobin in the presence and absence of phosphate, experiments were repeated with the entire capillary filled with 100 ng μL^{-1} myoglobin pre-

pared in pH 4.25 acetate. Voltage was applied during enrichment (30 min) followed by pressure mobilization for detection (conditions otherwise same as Fig. 6). When 30 mM of phosphate was present in the sample, the enriched myoglobin band was found located at the anodic side of the detection window after 30 min of voltage application. It was detected as a sharp peak with an absorbance of 0.77 (51-fold enrichment) after 5 min of pressure mobilization at 30 mbar in the cathodic direction (data not shown). In the control experiment performed without phosphate, the myoglobin band stopped at the cathodic side of the detection point after voltage application, due to a different residual EOF obtained in the absence of phosphate. Pressure mobilization in the anodic direction at 30 mbar was used, and a peak with an absorbance of 0.63 was observed in approximately 3 min (42-fold enrichment, data not shown). Due to the use of pressure, the time and absorbance of the peaks were variable from run-to-run, and thus the enrichment factors should not be taken as precise values. Nevertheless, it is suffice to conclude that equally successful enrichment of myoglobin was obtained despite of the presence of phosphate.

4. Conclusions

Based on the results obtained with the model protein myoglobin, the pH junction created by our discontinuous buffer system is not only effective in enriching extremely small volumes of proteins, but is also effective in isolating the proteins from buffering ions as well as non-buffering salts. The flexibility of the experimental setup accommodates the removal of different ions. For TRIS, sample preparation in ammonium buffer allowed the negatively charged myoglobin to be removed from the predominately neutral TRIS. The removal of MES as zwitterions, on the other hand, required sample preparation in the acetate buffer. Phosphate does not exist as neutral molecules within the pH range of the discontinuous buffers, and therefore required a different removal mechanism. In this case, sample containing sodium phosphate was prepared in the acetate buffer. During voltage application, the phosphate ions migrated as anions away from the pH junction, while myoglobin migrated as cations towards the junction.

Despite of the successes reported in the paper, the developed method may not be readily applicable to any real samples, as compatibility with other commonly used additives or solubilization reagents in sample preparation such as urea and surfactants remains to be studied. The enrichment of proteins is also, in theory, limited to those with p*I* values between the two pH values of the discontinuous buffers. Nevertheless, the compatibility of our discontinuous buffers with buffering and non-buffering ions obviously represents an important step towards the handling of any samples from biological origins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.054.

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