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Characterization of discontinuous buffer junctions using pH indicators in capillary electrophoresis for protein preconcentration

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Abstract

An effective sample preconcentration technique for proteins and peptides was recently developed using capillary electrophoresis (CE) with discontinuous buffers [C.A. Nesbitt, J.T.-M. Lo, K.K.-C. Yeung, J. Chromatogr. A 1073 (2005) 175]. Two buffers of different pH created a junction to trap the sample molecules at their isoelectric points and resulted in over 1000-fold preconcentration for myoglobin within 30 min. To study the formation of pH junctions in CE, a pH indicator, bromothymol blue, is used in this work to reveal the pH changes at the discontinuous buffer boundary. Bromothymol blue (BTB) exhibits a drastic change in its visible absorption spectrum (300–600 nm) going from the acidic to basic pH conditions, and is therefore ideal for visualizing the changes in pH at the junctions created by various buffer combinations. Preconcentration of myoglobin was performed in discontinuous buffers containing BTB. Major differences in the BTB absorption profiles were identified from buffer systems that differ significantly in preconcentration performance, which in turn, allowed for the identification of ideal buffers for sample preconcentration. Up to 2000-fold preconcentrations of myoglobin were achieved in the buffer systems studied in this work. In addition, the role of the electroosmotic flow (EOF) on the preconcentration performance was investigated. A low EOF was found to be desirable, as the pH junction could stay longer in the capillary for accumulation of proteins. The pH junction also displayed characteristics to resist bandbroadening. Potential laminar flow resulted from the mismatched residual EOFs under the two pH conditions within the discontinuous buffers appeared to have minimal effect on the preconcentration. In fact, external applied pressure can be used to control the migration of the pH junction without compromising the protein preconcentration.
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1. Introduction

Capillary-format separations, such as capillary liquid chromatography (LC) and capillary electrophoresis (CE) are becoming increasingly popular over the conventional techniques in protein analysis [2–4]. They offer superior efficiency and resolution, and interface seamlessly with the ultra sensitive nanoelectrospray [5] or nanoliter-microspot matrix-assisted laser desorption/ionization [6] mass spectrometry. Nevertheless, a major drawback with these capillary techniques is the small volume of sample handling or loading. Sample preconcentration, therefore, is a vital part of capillary-format separations. In capillary LC, sample preconcentration is most commonly performed by trapping a large volume of sample at the head of the column with a weak-eluting solvent. Then, by stepping up the elution strength, the sample is eluted in a small volume and thus is concentrated. While this approach is suitable for peptides, it is less effective for proteins. This is because the use of organic solvents to control the elution strength may not be compatible with proteins, as they are prone to precipitation or denaturation in non-aqueous environments. CE, on the other hand, generally does not require the use of organic solvents, and thus represents an attractive alternative for handling proteins.

Numerous reports on sample preconcentration for proteins and peptides using CE are available in the literature. A common technique is sample stacking, which results from a sudden change in the sample's electrophoretic mobility as the sample molecules cross the boundary between the injected sample plug and the running buffer [7,8]. Such a mobility change can

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be induced by a conductivity difference between the sample plug and the running buffer [9], e.g., by preparing the sample in a medium with lower conductivity than the running buffer. This procedure is referred to as field-amplified sample stacking [10,11] or large volume sample stacking [12,13]. Preconcentration factors of over 3000 were reported for peptides [14]. Transient isotachophoresis (t-ITP) has also been demonstrated as a sample preconcentration method [15,16]. A leading and a terminating electrolyte focus analytes that possess intermediate mobilities. Ionizable analytes, including proteins and peptides, were also concentrated using a transient moving chemical reaction boundary method [17].

Alternatively, sample preconcentration based on sudden mobility changes can be induced by a change in pH between the sample and buffer zones, which can effectively alter the net charges or mobilities of peptides and proteins [18,19]. For example, samples prepared in high pH solution concentrate at the boundary of an acidic running buffer [20]. In most cases, a dynamic pH junction is formed when the pH at the sample zone is titrated with the running buffer and the analytes are focused due to the electrophoretic mobility change in the two different pH environments [21]. After the sample pH was completely titrated by the running buffer, the pH junction would disappear (hence referred as "dynamic") and then regular electrophoresis would continue to resolve the concentrated components. The use of a dynamic or transient pH junction has been applied for the preconcentration of small organic and biological molecules [22–24]. Given that the sample enrichment and the separation need to be optimized simultaneously, moderately high preconcentration factors, 130 for peptides [25] and under 100 for proteins [26], were generally reported.

Recently a modified setup was reported by our research group to decouple the enrichment and separation steps, thereby sustaining the pH junction and prolonging the sample preconcentration process for higher degrees of enrichment [1]. In this method, an acidic buffer is placed at the anode and a basic buffer is placed at the cathode. Upon voltage application, the protons from the anode and hydroxide ions from the cathode continuously entered the capillary and maintained a pH junction at the boundary of the two buffers. The inner wall of the capillary was pre-modified with a zwitterionic phospholipid for EOF suppression, since the EOF in an unmodified fused silica capillary would have swept the pH junction out of the capillary in a few minutes. This setup is effective in concentrating proteins or peptides with p*I* values that fall within the pH range defined by the acidic and basic buffers. It has been tested in the preconcentration of proteins including myoglobin (p*I* 7.2) and carbonic anhydrase I (p*I* 6.6) [1]. A preconcentration factor of up to 1700 was reported in less than 30 min for myoglobin, when a pH 4.75 acetate (ammonium as counterion) was used as the acid and a pH 9.25 ammonium (acetate as counterion) was used as the base. Alternatively, the technique can be used to selectively exclude highly acidic or basic proteins, such as amyloglucosidase (p*I* 3.6) and cytochrome *c* (p*I* 10.6) in the preconcentration, by carefully selecting the pHs of the acidic and basic buffers [1]. Such on-capillary protein preconcentration using a discontinuous buffer operates in a very similar principle as the isoelectric sample trapping reported by Vigh and co-workers, which also isolates the protein of interest based on p*I*, but into separation compartments using isoelectric membranes [27–30]. The main difference between these techniques is the volume of sample handling; as the former focuses on microliter-volume samples, where as the latter is more suitable for preparative-scale operation (milliliter or more).

Based on the resulting sample signals and the preconcentration factors obtained with different buffer combinations in our previous work [1], the choice of buffers and pH was determined to play a critical role in the formation of the pH junction. Namely, the two buffers (acid and base) should have very good buffering capacity at the selected pH, i.e., very close to the pK_a, and at the same time, there should be very little buffering capacity at the gap between the two chosen pH conditions. In the above acetate–ammonium combination, the selected pH coincided with the pK_a of both buffers and there was essentially no buffering capacity at the gap (pH 6.5–7.5). Nevertheless, such buffer selection criteria, and even the formation of pH junction, have not been supported by any direct experimental evidence, other than monitoring the development of the enriched sample signals.

Numerous software and internet resources are available to model the electromigration of ions in capillary electrophoresis, as reviewed in ref. [31]. Specific modeling on dynamic pH junctions in CE has also been reported. Kim et al. conducted computer simulations on the focusing of phenol derivatives prepared in an acidic sample zone while using a basic background electrolyte [9]. Breadmore et al. stimulated the stacking of weak bases in a basic sample matrix and an acidic separation electrolyte [32]. Nevertheless, modeling results are typically specific to the experimental conditions such as analyte, buffer choices, buffer and sample pH, rather than universally applicable to all systems. In addition, both studies focused on transient pH boundaries that constantly transformed during the run. The pH junction created in this work, in contrast, was sustained throughout the entire run. It is therefore proposed herein to use a pH indicator as a probe to reveal the pH profile of the buffer junction. The use of pH indicators to monitor the pH changes in CE has been reported and shown to be very effective [9,33–35]. It is expected to generate visual representations of the pH junctions formed by various buffer combinations and hopefully provide insights on the protein preconcentration mechanism in discontinuous buffers.

2. Experimental

2.1. Apparatus

A Varian Cary 50 UV–vis absorption spectrophotometer (Palo Alto, CA, USA), with 1-cm pathlength cuvettes, was used to generate the absorption spectra for the pH indicators. All protein preconcentration and the online direct UV–vis absorption detection were performed with an Agilent 3D-CE capillary electrophoresis instrument (Palo Alto, CA, USA). Data acquisition was obtained through the ChemStation software by Agilent. Uncoated fused silica capillaries were purchased

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