Published in final edited form as:

Electrophoresis. 2014 July ; 35(14): 2029–2038. doi:10.1002/elps.201400009.

Cationic isotachopheresis separation of the biomarker cardiac troponin I from a high-abundance contaminant, serum albumin

Thomas Jacroux¹, Danny Bottenus¹, Bennett Rieck¹, Cornelius F. Ivory¹, and Wen-ji Dong^{1,2}

¹Gene and Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, Pullman, WA, USA

²The Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman, WA, USA

Abstract

Cationic ITP was used to separate and concentrate fluorescently tagged cardiac troponin I (cTnI) from two proteins with similar isoelectric properties in a PMMA straight-channel microfluidic chip. In an initial set of experiments, cTnI was effectively separated from R-Phycoerythrin using cationic ITP in a pH 8 buffer system. Then, a second set of experiments was conducted in which cTnI was separated from a serum contaminant, albumin. Each experiment took ~ 10 min or less at low electric field strengths (34 V/cm) and demonstrated that cationic ITP could be used as an on-chip removal technique to isolate cTnI from albumin. In addition to the experimental work, a 1D numerical simulation of our cationic ITP experiments has been included to qualitatively validate experimental observations.

Keywords

Cardiac troponin I; Cationic isotachopheresis; Microfluidics; Preconcentration; Proteins

1 Introduction

Recent advances in microfluidic electrophoresis have matured to a point where detecting the low-abundant cardiac protein biomarker cardiac troponin I (cTnI) [1–10] using ITP as a pre-separation, preconcentration method has shown considerable promise for clinical application [11,12]. ITP-on-chip applications offer several advantages such as low reagent consumption, high throughput, and rapid analysis. ITP requires a discontinuous electrolyte system comprised of a leading electrolyte (LE) and a terminating electrolyte (TE) [13]. ITP can concentrate sample components of interest by several orders of magnitude while separating them into stacked, pure zones in order of their electrophoretic mobilities between

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Correspondence: Dr. Wen-ji Dong, Gene and Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, P.O. Box 642710, Pullman, WA 99164-2710, USA wdong@vetmed.wsu.edu.

Additional supporting information may be found in the online version of this article at the publisher's web-site

The authors have declared no conflict of interest.

a slow terminating ion and a fast leading ion within minutes [14–16]. Furthermore, ITP can be easily coupled to several different analytical techniques such as CZE [17], IEF [18], gel electrophoresis [19], MS [20], and an on-chip sandwich immunoassay [21].

One major challenge in targeting cTnI with ITP-on-chip for clinical applications is isolating cTnI from highly abundant serum proteins such as albumin. Albumin makes up 60–70% of the human serum profile [22] and the concentration is orders of magnitude higher than most serum proteins. Therefore, it is essential to remove albumin from serum in order to detect lower abundant proteins such as cTnI. In our previous study, we limited albumin's effect in serum samples by preprocessing off-chip because of the lack of resolving power by the chip in the chosen electrolyte system [12]. This was attributed to geometrical limitations, as the projected width of the albumin zone was greater than the entire length of the separation channel in the microchip [12]. A more attractive solution to off-chip removal would be to do on-chip processing to isolate cTnI from albumin and potentially apply it to clinical samples containing whole serum. One mode of ITP that could be used to isolate cTnI from albumin is cationic ITP at high pH. Since cTnI has a pI , or point of zero net charge at a $pH > 10$ and most serum proteins including albumin have pI 's < 7 , an electrolyte ITP system with an intermediate pH could isolate cTnI from serum-contaminating proteins.

Over the last three decades, cationic ITP has been used in the preconcentration, separation, detection, and identification of different kinds of cationic proteins and their building blocks such as metal-binding proteins [23], model peptides (i.e., human angiotensins) [24], and basic amino acids (arginine, histidine, and lysine) in pharmaceutical preparations [25]. In the seminal work by Janssen et al. [26], application of both cationic ITP and HPLC was performed to control the peptide purity of insulin, adrenocorticotrophic hormone, and β -endorphin. In more recent work involving cationic ITP separations of proteins, the chosen electrolyte systems were comprised primarily of acetate-based chemistry for both the LE and TE in the pH range of ~4.4–5 [24, 25, 27]. These ITP electrolyte systems were able to successfully isotachophoretically separate basic amino acids (arginine, histidine, and lysine) [25] and hormones [27] (Lecirelin) in complex buffer solutions, and could be also be coupled with other detection schemes such as MS to lower detection limits [24]. However, even with all the advances and applications involving cationic ITP separations of proteins, to the best of our knowledge, cationic ITP has never been run at pH 8 to demonstrate the effective separation of a low-abundant serum protein (cTnI) from a high-abundant serum protein (albumin).

In this proof-of-concept study, we perform cationic ITP experiments to demonstrate the effective separation of fluorescently tagged total cTnI from two different proteins; first R-Phycoerythrin (PE) and then FITC-labeled albumin in a 2.2 cm straight-channel PMMA microchip. In the first set of experiments, we performed proof of principle cationic ITP experiments showing the isolation of cTnI from PE using a pH 8 buffer system. PE was used as a model protein because it has similar chemical properties, such as pI (4.4) [28], and charge (–14) [29] to albumin (pI ~4.4 and charge –18.3) [29, 30] at the pH of our working electrolyte system. Next, PE was replaced by FITC-albumin to provide an example of a clinically relevant contaminant. These experiments took ~10 min or less and demonstrate that cationic ITP shows promise as an on-chip pre-separation technique to isolate cTnI from

albumin. In addition to the experimental work, we have included a 1D numerical simulation modeling our cationic ITP experiments to qualitatively validate experimental observations.

2 Materials and methods

2.1 Materials

All chemicals, unless otherwise stated, including FITC-albumin, were purchased from Sigma-Aldrich (St. Louis, MO, USA). PE and Pacific Blue™ C5-maleimide were purchased from Molecular Probes (Eugene, OR, USA). A single stock of human cardiac troponin I was purified and then labeled with Pacific Blue™ C-5 maleimide-thiol chemistry as outlined in our previous report [11].

2.2 Fabrication of microfluidic chips

The PMMA straight-channel microchips used in these experiments were manufactured as reported previously with slight modifications [31] and the manufacturing process is given in more detail in the accompanying Supporting Information. Briefly, the fabrication process consisted of (i) photolithography to produce an SU-8 image on a polyetherimide substrate, (ii) hot embossing the SU-8 image into a UV transparent PMMA substrate to form the channel, (iii) finalizing the microchip by drilling holes for the anode and cathode reservoirs in the embossed PMMA, and (iv) bonding a PMMA piece with microchip features to a blank PMMA substrate using solvent or surface modification-assisted bonding techniques. The microchip channel dimensions are 2.2 cm in length, 150 μm wide, and 20 μm deep. The cathode and anode reservoirs are both 3 mm in diameter.

2.3 LE and TE solutions

The LE solution was prepared by adjusting 20 mM KOH to pH 8.0 with the counterion HEPES. The LE solution also contained 0.5 M urea, 1% v/v triton X-100, and 2% PVP. The TE solution consisted of 60 mM histidine, 0.5 M urea, 1% v/v triton X-100, 2% PVP, and was titrated to pH 7.2 with HEPES. 2% PVP was added to both the LE and TE solutions in order to suppress the EOF [32]. Urea and triton X-100 were added to the electrolyte solutions to prevent cTnI from precipitating out of solution. All electrolyte solutions were prepared using nanopure water from a Barnstead Ther-molyne Nanopure Infinity UV/system (Dubuque, IA, USA) and degassed with a CPS-8B vacuum pump (US Vacuum Pumps LLC, Canton, TX, USA).

2.4 Coating and loading the microchip

Prior to testing of any samples, the microchannel walls that are initially negatively charged at the electrolyte pH were coated with a surface modifier to prevent ionic adsorption of the positively charged cTnI. cTnI is positively charged because its pI is ~ 10 , which is above the pH of the LE. The choice of an appropriate surface modifier was thus limited to those that would have a positive charge at the pH of the LE; this would provide a repulsive electrostatic force to discourage cTnI adsorption onto the microchannel walls. In addition to the charge, another important factor was the presence of amino acid groups which could react with the available methyl esters of the PMMA under basic conditions, thus forming strong covalent bonds on the surface of the PMMA. Based on these criterion and the prior

work of Kitagawa et al. [33] high-molecular mass branched polyethylenimine (PEIn) was chosen as the surface modifier since it has a large number of amino groups. It also has a positive net charge over a wide pH range that includes that of the LE in this current work, thus providing a good basis for cationic repulsion between PEIn and cTnI. It was also shown in the Kitagawa study that coating PMMA microchannel walls with PEIn created an anodic EOF which was stable for up to 32 days and over 50 runs [33]. This anodic EOF was shown to be very low ($-1.25 \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$) [34] and the EOF was about four times lower and opposite in direction to that of untreated PMMA ($4.93 \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$) [34].

A modification of the PEIn-coating procedure used by Kitagawa et al. [33] was used here. Following immobilization protocols by Kitagawa et al. [33], we anticipated that the one-step immobilization reaction between the acyl carbon of PMMA and secondary amino groups in PEIn dissolved in basic aqueous solution proceeded as follows. First, the PMMA microchip was flushed with a 0.1 M NaOH solution for 15 min at a flow rate of 0.4 mL/h using a syringe pump (Stoelting, Wood Dale, IL, USA) to pretreat the microchannel surfaces. Next, the chip was flushed with ddH₂O for 15 min at the same flow rate to remove residual NaOH solution. Then, a solution of 10% w/w PEIn in 100 mM borate buffer (pH 11.5) was pumped through the microchannel for 1 h and flushed again with ddH₂O for 15 min at the flow rate used previously. Finally, the PMMA microchip was baked at 30°C for 2 h to complete the PEIn-coating process.

After successful coating of the microchannel walls, the microchips were ready to load samples and perform cationic ITP. Prior to loading, two different sample stock solutions were made. In the preliminary experiments, labeled cTnI and PE were diluted in LE solution to concentrations of 8 and 40 µg/mL, respectively. The sample solution also contained 150 mM NaCl which was added to the sample to simulate the physiological salt concentration found in serum. The rationale for choosing the electrolyte system used in the experiments will be discussed in more detail in the theory section of this manuscript. For the cTnI/FITC-albumin cationic ITP experiments, labeled cTnI and FITC-albumin were also diluted with LE solution containing 150 mM NaCl to concentrations of 8 and 10 µg/mL, respectively. After the samples were made, the microchip was immersed in LE solution for 1 h prior to running any experiments to allow the microchip to equilibrate in LE. Since the LE solution contained 2% PVP, it provided a dynamic wall coating on top of the PEIn coating to further reduce the EOF of the system. Next, sample solution was loaded into the cathode reservoir and pushed into the microchannel using a 3-mL syringe. Then, excess sample was removed from the anode and cathode reservoirs, rinsed, and filled with TE and LE, respectively. After each ITP experiment, the microchip was cleaned by flushing once with 1% triton X-100 and twice with filtered ddH₂O (MILLEX-HV₁₃, EMD Millipore, Billerica, MA, USA). In between each flush, house air was used to blow out residual chemicals and dry the microchip. At the end of the flushing stage, the next trial was reloaded as previously described.

The loaded microchip was secured onto the stage of a Leica DM 2000 fluorescence microscope equipped with a DFC310 digital color camera (Leica Microsystems, Bannockburn, IL, USA) underneath a 4× objective lens. All proteins were excited by UV light with a Leica Microsystems EL 6000 light source using an A-type filter cube. Platinum electrodes

were then immersed in the anode and cathode reservoirs, respectively. The anode reservoir voltage was set to 75 V and the cathode reservoir was set to ground with an XHR 600-1 power supply (Xantrex Technology, Vancouver, Canada). The camera was configured and controlled with the provided Leica Application Suite (LAS) V3.6 software to collect images of the labeled cTnI and PE/FITC-albumin as they migrated through the microfluidic chip by cationic ITP to their respective reservoirs. Images were taken at different positions along the length of the microchannel and at the end of the channel just before either cTnI or PE/FITC-albumin reached the cathode or anode reservoirs, respectively. Over a 100 images were acquired for each successive trial. These images were compiled using Windows Movie Maker 2.6. The images close to the end of each experiment were imported into ImageJ (<http://rsb.info.nih.gov/ij>) software to produce electropherograms to be used for further analysis.

2.5 Theory and COMSOL simulation

AID model of cationic ITP in the presence of high salt (150 mM) was generated in COMSOL Multiphysics v.4.3a (COMSOL, Palo Alto, CA, USA) to qualitatively compare with experimental observations. The simulations were run on a Windows Server 2008 R2 Enterprise (64-bit) operating system that included two Intel Xeon X5690 @ 3.47 GHz processors and 144 GB of installed memory (RAM). The 1D numerical model was described using the following modified Nernst–Planck-based transport equation, excluding the convection term, available in COMSOL:

$$\frac{\partial c_i}{\partial t} + \nabla \cdot (-D_i \nabla c_i - \mu_i c_i \nabla \psi) = R_i, \quad (1)$$

where c_i is the concentration, D_i is the diffusion coefficient, μ_i is the electrophoretic mobility, ψ is the electric potential, and R_i is the rate of production of component i . The electrical potential is calculated in COMSOL using the conservation of electric charge equation shown below in Eq. (2) as:

$$\nabla \cdot i = F \sum_{i=1}^n z_i R_i, \quad (2)$$

where i is the current density, F is the Faraday constant, and z_i is the charge of component i . The parameters necessary to solve Eq. (1) are given in Table 1. In addition, all of the buffering reactions and corresponding pK values that were included in the simulations are shown in Table 1. Protein titration curves were not included in the 1D numerical simulations. Instead each protein was assigned a single charge and mobility based on values obtained experimentally or from the literature as close as possible to the LE pH [13, 29, 35–38].

Two simulations were performed; one that included the proteins cTnI (protein of interest) and PE (model protein—contaminant), and the other that included cTnI and serum albumin (high-abundant protein in blood—contaminant). The idea of performing ITP at pH 8 was to isolate and concentrate cTnI from the contaminating proteins, PE and/or serum albumin. This could be accomplished because under the experimental conditions, cTnI is positively

charged ($pI > pH$) and the contaminating proteins are negatively charged ($pI < pH$). The cationic ITP system which formed in the presence or absence of NaCl included potassium ion as the leading ion, histidine ion as the terminating ion, and cTnI as the sample zone. In cationic ITP, at high concentrations of sodium, the sodium actually displaced the leading ion, potassium. Both sample proteins in both ITP systems ran in peak mode ITP [39].

The simulated geometry was a 2.2 cm line. On the line itself, the initial conditions were set-up so that 0.05 cm of the line was occupied by LE (from right to left), 2.1 cm of the line was occupied by sample, and 0.05 cm of the line was occupied by TE. The LE region was closest to the cathode which was set to ground; and the TE region was closest to the anode which was set to 75 V. In between the LE and TE was sample which initially occupied over 95% of the channel in the simulation. The specific concentrations for each component in each region are shown in Table 1.

3 Results and discussion

3.1 Cationic ITP electrolyte system

Potassium was chosen as the leading ion for the cationic ITP system since it has a higher effective electrophoretic mobility than cTnI. Histidine was selected as the terminating ion because histidine ($pK = 6.04$) has a slight positive charge at the pH of the TE (pH 7.2), and it has a lower effective electrophoretic mobility than cTnI. 150 mM NaCl was added to the sample to represent the physiological contribution of sodium chloride found in human serum [40]. HEPES ($pK_a = 7.55$) served as the counterion for cationic ITP since it has an overall negative charge at the pH of the LE (pH 8). In order to prevent cTnI from precipitating out of solution, triton X-100 (1%) and urea (0.5 M) were present in both electrolyte solutions. A concentration of 0.5 M for urea was selected due to studies done by Morjana and Tal [41] in which the partial unfolding of human cTnI from the native to the denatured state begins at concentrations above 0.5 M urea. This selection of urea molar concentration will be important in later studies that use capture antibodies to detect cTnI after ITP pretreatment. Urea concentrations in excess of 0.5 M can significantly disrupt the antigen-antibody interaction [41], thus severely limiting the sensitivity of the detection scheme used.

3.2 Cationic ITP experiments

In our first set of experiments, cationic ITP was performed to show the isotachophoretic separation of cTnI from PE. Visualization of the two fluorescent proteins was not observed until the approximate midpoint (1.1 cm) of the microchannel, ~4 min after the start of the experiment. The initial separation distance between cTnI (blue) and PE (yellow) was ~1.6 mm. An image of this is shown in Fig. 1 from one of the three trials performed. The proteins continued to migrate toward their respective reservoirs with cTnI migrating toward the cathode and PE migrating toward the anode and beginning to accumulate mass resulting in an increase in their fluorescence intensities. Additional representative images were captured at different time points showing the proteins just prior to exiting the microchannel into their respective reservoirs (Fig. 1). These images were collected during different trials to provide the best illustration of the complete electrophoretic separation between the two proteins. cTnI on average reached the cathode at $\sim 10 \pm 0.5$ min and PE entered the anode reservoir on

average at $\sim 16 \pm 1$ min. Therefore, each experiment showing the separation of cTnI from PE could be completed in ~ 10 min or less.

Following a successful demonstration of the electrophoretic removal of PE from cTnI using cationic ITP, we applied the same experimental technique to concentrate and isolate cTnI from the major clinical serum contaminant, albumin. The albumin used was labeled with FITC for fluorescent visualization. Again, protein migration was not initially visualized until the midpoint of the microchannel. An initial image of the proteins at the point of first visualization is illustrated in the middle of the image in Fig. 2. The initial separation distance between FITC-albumin and cTnI is ~ 2.1 mm after ~ 4 min of applied voltage. The FITC-albumin band (green) showed up very faint in comparison to the cTnI band (blue). Thus, the image was slightly modified in Adobe Photoshop CS 5.1 to enhance the signal of albumin by modifying the brightness/contrast features to more clearly visualize albumin.

Representative images captured at different time frames showing the proteins just before they migrate into their respective reservoirs are also shown in Fig. 2. These images were collected during different trials to provide the best quality representative images of the electrophoretic separation between the two proteins. cTnI again averaged a migration time of $\sim 10 \pm 0.5$ min to reach the cathode while FITC-albumin averaged a migration time of $\sim 18 \pm 3$ min to reach the anode. Again, each trial showing the separation of cTnI from FITC-albumin could be completed in ~ 10 min or less at an electric field strength of only 34 V/cm.

A mass balance was performed on each sample to determine cTnI's final concentration following cationic ITP while in peak mode. This mass balance is shown in Eq. (3) below:

$$c_i = \frac{M_i}{w_i A}, \quad (3)$$

where c_i is the concentration of the sample component, M_i is the total mass of species i , w_i is the peak width of species i , and A is the cross-sectional area ($3 \times 10^{-5} \text{ cm}^2$) of the separation channel. Calculation of the peak width and total mass will be discussed in subsequent sections and in the accompanying Supporting Information.

Moment analysis was performed from electropherograms for each trial to determine the peak width, w_i , of each sample component as previously described [11, 12].

Electropherograms for the analysis of peak widths were obtained from cTnI images located near the cathode. These locations in the microchannel generated the maximum fluorescence intensities and subsequent maximum protein concentrations for cTnI achieved by cationic ITP during each trial. Each image acquired was processed in Adobe Photoshop to remove background noise by modifying the brightness and contrast settings and saving it as a TIF file with 16 bits/channel. Electropherograms were produced from these processed images using ImageJ software by plotting the average intensity across the channel width as a function of distance (μm) along the length of the channel relative to the camera's field of view. Electropherograms of three successive trials for the cTnI/PE experiments are shown in Fig. 3. Figure 3 shows the electropherograms of three trials for fluorescent cTnI. Likewise, Fig. 4 illustrates the cTnI electropherograms for three successive trials for the cTnI/albumin experiments.

For each set of trials, the electropherograms were plotted in Origin Pro 9.0 and translated along the x-axis (distance (μm) relative to the field of view of camera) so that the maximum peak intensity for each trial within the trial set occurred at the same x -coordinate position. All the electropherograms indicate a near-Gaussian peak shape, signifying that ITP ran in “peak mode,” and that none of the proteins reached their maximum concentration, that is, the plateau concentration [42].

Next, moment analysis was applied to the electropherogram data to determine band attributes (e.g., peak width) obtained by ITP in the straight-channel microchip. The method of analysis is described in more detail by Bottenus et al. [11, 12]. Briefly, the n th moment for discrete positions using the trapezoidal rule is given by Eq. (4):

$$m_n = \sum_i \frac{1}{2} (I(x_{i+1}) \cdot x_{i+1}^n + I(x_i) \cdot x_i^n) (x_{i+1} - x_i), \quad (4)$$

where $I(x)$ is the intensity signal, assumed to be proportional to protein concentration as discussed in our previous reports [11,12], and x is the spatial position (relative to the field of view of the camera). The variance σ^2 and resulting peak width, w_i , were calculated using n^{th} moments according to Eqs. (5) and (6) given below:

$$\sigma^2 = \frac{m_2}{m_0} - \left(\frac{m_1}{m_0} \right)^2, \quad (5)$$

$$w_i = 4\sigma, \quad (6)$$

The peak widths calculated for three cTnI/PE trials are given in Table 2. The average cTnI peak width calculated from moment analysis was $98.34 \pm 19.06 \mu\text{m}$. Likewise, shown in Table 2, for the cTnI/FITC-albumin experiments, the calculated peak width for cTnI was $207.29 \pm 50.09 \mu\text{m}$. Reasons for the higher variance in the peak widths from the cTnI/FITC-albumin trials in comparison to the cTnI/PE trials are related to a greater potential of nonspecific binding occurring between albumin/cTnI than PE/cTnI. Another reason could be from possible deformation in the protein peaks associated with residual bubbles left over from sample loading. These reasons are discussed in more detail later in the manuscript. The calculated peak widths will be used later to determine the final concentrations for each protein from Eq. (3). First, however, the total mass of each protein that makes up each peak is needed to determine the final concentrations and will be discussed below.

In order to accurately determine M_i , the mass of protein that makes up each peak, it was necessary to calculate the mass lost during ITP. The total mass can then be calculated from the initial mass loaded onto the microchip minus the mass lost. In our earlier studies, we used a tee channel [11,12] to control the mass of protein loaded onto the microchip and assumed that the total mass loaded onto the microchip was equivalent to the total mass that made up each peak. The total mass loaded on the microchip could be calculated using Eq. (7) given below:

$$M_i = c_i^0 LA, \quad (7)$$

where c_i^0 is the initial concentration of protein (8 µg/mL for cTnI, 40 µg/mL for PE, and 8 µg/mL for FITC-albumin), L is the length of the separation channel (22 mm), and A is the cross-sectional area of the channel ($3 \times 10^{-5} \text{ cm}^2$). However, since this study was a proof-of-concept application to show the electrophoretic separation of cTnI from a highly abundant clinical contaminant, we used a simple straight-channel design. The inherent design flaw in a straight-channel chip as opposed to a microchip with a tee junction is that not all of the initial mass loaded onto the microchip is equivalent to the mass of protein that makes up each protein peak via ITP. Instead mass losses will occur due to zonal electrophoresis occurring ahead of the stack as ITP progresses down the channel. For instance, initially while the LE, sample, and TE are forming their respective zones, residual sample in the LE zone is not stacking in the sample zone, but migrating by zonal electrophoresis out of the channel into its respective reservoir. This leads to a certain amount of sample loss. An in-depth analysis of how the mass loss was calculated for each protein following ITP is given in the accompanying Supporting Information.

After calculating the mass loss for each protein, the final mass associated with cTnI in the cTnI/PE system was 0.16 ng. For the cTnI/FITC-albumin system, the final mass was 0.19 ng for cTnI.

Finally, Eq. (3) could be used to calculate the final protein concentrations now that the final mass and peak widths were determined. The final protein concentration of the cTnI/PE system was $0.45 \pm 0.08 \text{ mg/mL}$ for cTnI (shown in Table 2). The final protein concentration of the cTnI/FITC-albumin system was $0.28 \pm 0.07 \text{ mg/mL}$ for cTnI (shown in Table 2). The concentration factors were calculated by dividing the final concentration by the initial concentration. For cTnI/PE, the concentration factor for cTnI was 56 ± 10 (shown in Table 2). For cTnI/FITC-albumin, the concentration factor for cTnI was 35 ± 8 (shown in Table 2).

Based on our experimental results, one can observe that cTnI did not concentrate as well in the presence of FITC-albumin as it did in comparison to PE following cationic ITP. One major reason for this may be related to how “sticky” albumin is due its large number of hydrophobic patches [43]. Potential nonspecific binding between cTnI and FITC-albumin could have prevented some of the cTnI from clearing out completely from the anodic components and forming its own ITP cathodic stack. Another contributing factor that could explain the differences in final concentrations between the two systems during different trials is residual bubbles left over from sample loading. Although each solution used in the experiments was properly degassed and carefully loaded into the microchip, small bubbles would remain in the channel and be present when performing some of the trials. Residual bubbles in the channel did cause some distortion of the bands as they passed through each bubble. However, these distortions were quickly eliminated due to ITP’s self-sharpening effect. It was expected though that some minute percentage of mass was probably lost due to bubble deformation but this was a small contributing factor to the overall mass loss.

3.3 Cationic ITP COMSOL simulation

The simulation served to qualitatively confirm the observation of cationic ITP in the experiments. The model results for the cTnI/PE and cTnI/albumin systems are shown in Fig. 5 at a time of 5 min. The concentration of the leading ion was divided by a factor of 5000 while the concentration of the positive terminating ion was divided by a factor of 2000 to help visualize all of the cationic ITP components on the same scale. Figure 5 illustrates the cationic ITP of cTnI between sodium which displaces the leading ion, potassium, during the experiment and the terminating ion, histidine. The contaminant proteins, albumin, and/or PE migrated in the opposite direction toward the anode and did not interfere with the cTnI ITP cathodic stack. The only difference between the two simulations is that one includes PE and the other includes albumin. As such, the cTnI migration and final concentration were identical in both cases which is different than the observations seen experimentally. Reasons for the differences in the cTnI concentrations determined by the simulation and experimental components were outlined in the previous section.

The initial concentrations of the proteins in the simulation were equivalent to the initial concentrations used in the experiment. In addition, the final concentrations and subsequent concentration factors for cTnI, the molecule of interest, from the simulation were close to those obtained in the cationic ITP experiments. For example, the final cTnI concentration determined from the simulation was ~0.55 mg/mL (0.023 mM) which is close to within the range of the final concentration determined experimentally 0.45 ± 0.08 mg/mL in the cTnI/PE system (shown in Table 2). The simulation also matched the experimental results in that all of the protein peaks were Gaussian-like, implying that ITP was being performed in peak mode.

We are aware that the initial concentration of cTnI and conditions used in our experiments and simulations is significantly higher (10 000 $\times$) and different than what is found in serum/clinical levels. However, this paper describes a potential method that could be applied to isolate and concentrate a low abundance protein (cTnI) from a highly abundant serum protein such as albumin under serum conditions. Removal of contaminants such as albumin from serum can help to lower the limit of detection and raise the sensitivity of current detection units used to quantify cTnI isoforms in clinical settings.

4 Concluding remarks

This paper demonstrates the possibility of using cationic ITP to purify and preconcentrate a cardiac biomarker, cTnI, from both a model protein, PE, and from a highly abundant serum protein, albumin. We were able to concentrate cTnI in the presence of PE or from FITC-albumin in a 2.2 cm PMMA straight-channel microfluidic chip using a pH 8 buffer system under LE buffer conditions. These experiments took ~10 min or less and illustrate that cationic ITP could be used as an on-chip depletion technique to isolate cTnI from PE and/or albumin. In addition to the experimental work, we have included a 1D COMSOL numerical simulation modeling our cationic ITP experiments to qualitatively validate experimental observations.

Our future research activities include performing cationic ITP at pH 8 of cTnI isoforms such as phosphorylated cTnI in human serum samples using a cascade PMMA microchip with decreased cross-sectional area reductions (1000-10 000×). Furthermore, we are looking to incorporate an on-chip ITP immunoassay that includes immobilized captured antibodies that will be specific to a particular cTnI isoform and then using secondary detection antibodies to quantify these isoforms and increase limits of detection. We believe that by incorporating a preconcentration/purification step such as cationic ITP with an immunoassay, we can use this integrated technology in the detection of low-abundant biomarkers found in other complex matrices such as blood, saliva, urine, or cerebrospinal fluid.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported, in whole or in part, by National Institutes of Health Grants HL80186 and HL80186-5S1 (to W.-J. D.). The research was also supported by Life Science Discovery Fund and the Washington State University National Institutes of Health Protein Biotechnology Training Program Award Number T32GM008336 from the NIGMS.

Abbreviations

LE	leading electrolyte
PEIn	polyethylenimine
TE	terminating electrolyte

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Magid D, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, Moy CS, Mussolino ME, Nichol G, Paynter NR, Schreiner RJ, Sorlie RD, Stein J, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. *Circulation*. 2013; 727:e6–e245. [PubMed: 23239837]
2. Dunlay SM, Shah ND, Shi Q, Morlan B, Van-Houten H, Long KH, Roger VL. *Circ. Cardiovasc. Qual. Outcomes*. 2011; 4:68–75. [PubMed: 21139091]
3. Mohammed MI, Desmulliez MP. *Lab. Chip*. 2011; 11:569–595. [PubMed: 21180774]
4. La Vecchia L, Mezzena G, Zanolla L, Paccanaro M, Varotto L, Bonanno C, Ometto R. *J. Heart Lung Transplant*. 2000; 79:644–652. [PubMed: 10930813]
5. Logeart D, Beyne P, Cusson C, Tokmakova M, Leban M, Guiti C, Bourgoin R, Solal AC. *Am Heart J*. 2001; 747:247–253. [PubMed: 11174339]
6. Peacock, WFT; De Marco, T.; Fonarow, GC.; Diercks, D.; Wynne, J.; Apple, FS.; Wu, AH. *N. Engl. J. Med*. 2008; 358:2117–2126. [PubMed: 18480204]
7. Latini R, Masson S, Anand IS, Missov E, Carlson M, Vago T, Angelici L, Barlera S, Parrinello G, Maggioni AP, Tognoni G, Cohn JN. *Circulation*. 2007; 776:1242–1249. [PubMed: 17698733]
8. Miller WL, Hartman KA, Burritt MF, Grill DE, Rodeheffer RJ, Burnett JC Jr, Jaffe AS. *Circulation*. 2001; 776:249–257.
9. Perna ER, Macin SM, Canella JR, Augier N, Stival JL, Cialzeta JP, Pitzus AE, Garcia EH, Obregon P, Brizuela M, Barbagelata A. *Circulation*. 2004; 770:2376–2382. [PubMed: 15477403]

10. Perna ER, Macin SM, Cimbaro Canella JP, Al-varenga RM, Rios NG, Pantich R, Augier N, Farias EF, Jantus E, Brizuela M, Medina F. *Int. J. Cardiol.* 2005; 99:253–261. [PubMed: 15749184]
11. Bottenus D, Jubery TZ, Ouyang Y, Dong WJ, Dutta R, Ivory CF. *Lab. Chip.* 2011; 77:890–898. [PubMed: 21416810]
12. Bottenus D, Hossan MR, Ouyang Y, Dong WJ, Dutta P, Ivory CF. *Lab. Chip.* 2011; 77:3793–3801. [PubMed: 21935555]
13. Mosher, RA.; Saville, DA.; Thormann, W. *The Dynamics of Electrophoresis.* New York: VCH, Weinheim; 1992.
14. Wainright A, Williams SJ, Ciambone G, Xue Q, Wei J, Harris D. *J. Chromatogr. A.* 2002; 979:69–80. [PubMed: 12498234]
15. Jung B, Bharadwaj R, Santiago JG. *Anal. Chem.* 2006; 78:2319–2327. [PubMed: 16579615]
16. Gebauer P, Mala Z, Bocek P. *Electrophoresis.* 2009; 30:29–35. [PubMed: 19101930]
17. Jung B, Zhu Y, Santiago JG. *Anal. Chem.* 2007; 79:345–349. [PubMed: 17194159]
18. Mohan D, Lee CS. *Electrophoresis.* 2002; 23:3160–3167. [PubMed: 12298088]
19. Mohamadi MR, Kaji N, Tokeshi M, Baba Y. *Anal. Chem.* 2007; 79:3667–3672. [PubMed: 17437335]
20. Tomas R, Koval M, Foret F. *J. Chromatogr. A.* 2010; 7277:4144–4149. [PubMed: 20219201]
21. Kawabata T, Wada HG, Watanabe M, Satomura S. *Electrophoresis.* 2008; 29:1399–1406. [PubMed: 18384019]
22. Rengarajan K, de Smet MD, Wiggert B. *Biotechniques.* 1996; 20:30–32. [PubMed: 8770400]
23. Stutz H, Bordin G, Rodriguez AR. *Electrophoresis.* 2004; 25:1071–1089. [PubMed: 15095450]
24. Kler PA, Posch IN, Pattky M, Tiggelaar RM, Huhn C. *J. Chromatogr. A.* 2013; 7297:204–212. [PubMed: 23706548]
25. Kubacak P, Mikus P, Valaskova I, Havranek E. *Arch. Pharm.* 2006; 339:96–99.
26. Janssen PS, van Nispen JW, van Zeeland MJ, Melgers PA. *J. Chromatogr.* 1989; 470:171–183. [PubMed: 2544620]
27. Sazelova P, Kasicka V, Solinova V, Koval D. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2006; 847:145–151.
28. Cui H, Horiuchi K, Dutta P, Ivory CF. *Anal. Chem.* 2005; 77:1303–1309. [PubMed: 15732911]
29. Inglis DW, Goldys EM, Calander NP. *Angew. Chem. Int. Ed. Engl.* 2011; 50:7546–7550. [PubMed: 21710667]
30. Liu R, Pidikiti R, Ha CE, Petersen CE, Bhaga-van NV, Eckenhoff RG. *J. Biol. Chem.* 2002; 277:36373–36379. [PubMed: 12118010]
31. Jubery TZ, Hossan MR, Bottenus DR, Ivory CE, Dong W, Dutta P. *Biomicrofluidics.* 2012; 6:16503–1650313. [PubMed: 22396722]
32. Kaneta T, Ueda T, Hata K, Imasaka T. *J. Chromatogr. A.* 2006; 7706:52–55. [PubMed: 16443452]
33. Kitagawa F, Kubota K, Sueyoshi K, Otsuka K. *Sci. Technol. Adv. Mat.* 2006; 7:558–565.
34. Currie CA, Shim JS, Lee SH, Ahn C, Limbach PA, Halsall HB, Heineman WR. *Electrophoresis.* 2009; 30:4245–4250. [PubMed: 20013912]
35. Bahga SS, Bercovici M, Santiago JG. *Electrophoresis.* 2010; 31:910–919. [PubMed: 20191554]
36. Bocek P.; Demi, M.; Gebauer, P.; Dolnik, V. *Analytical Isotachophoresis.* Germany: VCH, Weinheim; 1988.
37. Douglas NG, Humffray AA, Pratt HRC, Stevens GW. *Chem. Eng. Sci.* 1995; 50:743–754.
38. Viskari PJ, Colyer CL. *J. Chromatogr. A.* 2002; 972:269–276. [PubMed: 12416885]
39. Khurana TK, Santiago JG. *Anal. Chem.* 2008; 80:6300–6307. [PubMed: 18642874]
40. Standley, L. [accessed April 17,2013] *Common. Lab. Values In Electrolytes.*
www.drstandley.com/labvalues_electrolytes.shtml
41. Morjana N, Tal R. *Biotechnol. Appl. Biochem.* 1998; 28(1):7–17. [PubMed: 9693083]
42. Everaerts, FM.; Mikkers, FEP.; Verheggen, TP. *Analytical Isotachophoresis: Proceedings of the 2nd International Symposium on Isotachophoresis.* New York: Eindhoven, Elsevier; 1981.

43. Livesey JH, Nicholls MG. J. Immunoassay Immunochem. 2012; 33:302–313. [PubMed: 22738653]

Figure 1.

Cationic ITP separation of cTnI from PE. Protein migration was not visualized until approximately halfway down the microchip, 1.1 cm, after 4 min of applied voltage. At (1) this point, both cTnI (blue) and PE (yellow) were first observed at an initial separation distance of ~1.6 mm. Following the initial separation, cTnI began migrating toward the cathode while PE migrated toward the anode. Representative images captured at different time points show the proteins just reaching their respective reservoirs as shown above. cTnI on average reached the cathode at around ~10 min (2) while PE finished at the anode on average at around ~16 min (3). Thus, each experiment showing the complete separation of cTnI from PE could be completed in ~10 min or less.

Figure 2.

Cationic ITP separation of cTnI from FITC-albumin. Following the same procedure as done in the previous experiments, protein migration was initially visualized at about the midpoint of the microchannel. This initial image as illustrated above (1) shows an ~2.1 mm separation distance between cTnI and FITC-albumin. The white arrows on either side of the channel represent the initial formation of the FITC-albumin band (green) which is very faint in comparison to the cTnI (blue) band. This image was taken approximately 4 min after starting the experiment. To enhance the exposure of the albumin band and image, the image was modified in Adobe Photoshop CS 5.1 using the brightness/contrast features to brighten the image. Representative images captured at different time frames show the proteins just prior to reaching their respective reservoirs as shown (2 and 3) above. cTnI again averaged a migration time of ~10 min (2) to reach the cathode while FITC-albumin averaged a migration time of ~18 min (3) to reach the anode. Again, each trial showing the complete separation of cTnI from FITC-albumin could be completed in ~10 min or less.

Figure 3.

Electropherograms of cTnI during cationic ITP experiments. Electropherograms for the analysis of peak widths for cTnI were obtained from images taken of cTnI located close to the cathode. Each image acquired was processed in Adobe Photoshop to remove background noise by modifying the brightness and contrast settings and saving as a TIF file with 16 bits/channel. Electropherograms were then produced from processed images using Image J software by plotting the average intensity across the channel width as a function of distance (μm) along the length of the channel relative to the camera's field of view.

Figure 4.

Electropherograms of cTnI during cationic ITP experiments. For each set of trials, the electropherograms were plotted in Origin Pro 9.0 and translated along the x -axis (distance (μm) relative to the field of view of camera) so that the maximum peak intensity for each trial within the set occurred at the same x -coordinate position.

Figure 5.

Cationic ITP simulation at leading electrolyte pH 8.0 of cTnI after 5 min of applied voltage. The simulation shows the cationic ITP stacking of cTnI between sodium which displaces the leading ion, potassium, and the terminating ion histidine. The sodium represents the physiological amount of salt present in human serum. The concentration of the leading ion and the terminating ion have been divided by a factor of 5000 and 2000, respectively, to easily visualize the ITP stacking on the same concentration scale. The contaminant proteins, albumin, and/or PE migrate in the opposite direction toward the anode and do not interfere with the cTnI ITP stack. The cTnI peak is Gaussian-like indicating the cationic ITP is being performed in peak mode ITP which is consistent with experimental observations.

Table 1

List of species, charge (*z*), diffusion coefficient (*D*), electrophoretic mobility (*μ*), reactions, *pK*s, and initial concentrations in the leading electrolyte, sample zone, and trailing electrolyte using the cationic ITP simulation

Species	<i>z</i>	<i>D</i> (10 ⁻⁹ m ² /s) ^a	<i>μ</i> (10 ⁻⁹ m ² /V·s)	LE concentration (mM)	Sample concentration (mM)	TE concentration (mM)	Reaction	<i>pK</i>
HEPES ⁻	-1	0.20	-7.80 ²⁷	20	20	56.12	HEPES ⁰ ⇌ HEPES ⁻	7.5 ^g
HEPES ⁰	0	0.20	0	6.321	6.32	111.97	+H ₃ O ⁺	
Histidine ⁺	+1	0.76	29.6 ³⁶	0	0	56.12	His ⁺ ⇌ His ⁰ +H ₃ O ⁺	6.04 ^g
Histidine ⁰	0	0.76	0	0	0	3.88		
Potassium ⁺	+1	1.96	76.2 ³⁶	20	20	0		
Sodium ⁺	+1	1.33	51.9 ³⁶	0	150	0		
Chloride ⁻	-1	2.03	-79.1 ³⁶	0	150	0		
Hydronium ⁺	+1	9.31	362.5 ³⁶	1 × 10 ⁻⁵	1 × 10 ⁻⁵	6.31 × 10 ⁻⁵	2H ₂ O ⇌ H ₃ O ⁺ +OH ⁻	
Hydroxide ⁻	-1	5.28	-205.5 ³⁶	1 × 10 ⁻³	1 × 10 ⁻³	1.58 × 10 ⁻⁴		
Albumin ^{-b}	-18.3 ³⁰	0.03	-20.5 ³⁷	0	1.49 × 10 ⁻⁴	0		
cTnI ⁺	8.9 ^d	0.10	35.4 ^e	0	3.32 × 10 ⁻⁴	0		
PE ^{-c}	-14 ²⁹	0.05	-28.2 ^{38f}	0	1.67 × 10 ⁻⁴	0		

^aThe diffusion coefficients were calculated by the equation, $D_i = \mu_i \cdot T \cdot (Z_i \cdot F)^{-1}$, where *R* is the universal gas constant, *T* is the temperature, and *F* is Faraday's constant.

^bOnly used in albumin/cTnI simulation.

^cOnly used in PE/cTnI simulation.

^dDetermined from Protein Calculator (<http://www.scripps.edu/~cdputnam/protcalc.html>) using sequence information.

^eCalculated in independent experiments.

^fCalculated from data in Viskari and Colyer [38].

^gValue obtained from Sigma-Aldrich product information sheets.

Table 2

Summary of peak widths determined from moment analysis, experimental (calculated from Eq. (6)) and simulation (calculated from COMSOL) final concentrations, and concentration factors (experimental and simulation) for cTnl in the cTnl/PE and cTnl/albumin systems, respectively

Trial no.	cTnl experimental-cTnl/PE			cTnl-simulation-cTnl/PE			cTnl experimental-cTnl/Alb			cTnl simulation-cTnl/Alb		
	Peak width (μm)	Concentration (mg/mL)	Concentration factor	Concentration (mg/mL)	Concentration factor	Concentration factor	Peak width (μm)	Concentration (mg/mL)	Concentration factor	Concentration (mg/mL)	Concentration factor	Concentration factor
1	82.34	0.53	65.79	0.55	68.75		161.30	0.35	43.16	0.55	68.75	
2	93.27	0.46	58.07				199.91	0.28	34.83			
3	119.42	0.36	45.36				260.67	0.21	26.71			
Average	98.34	0.45	56.41				207.29	0.28	34.90			
Standard deviation	19.06	0.08	10.33				50.09	0.07	8.23			