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Paper-based cascade cationic isotachophoresis: Multiplex detection of cardiac markers

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Abstract

Paper-based analytical devices (PADs) are widely used in point-of-care testing (POCT) as they are cost-effective, simple and straightforward. However, poor sensitivity hinders their use in detecting diseases with low abundance biomarkers. The poor detection limit of PADs is mainly attributed to the low concentration of analytes, and the complexity of biological fluid, leading to insufficient interactions between analytes and capture antibodies. This study aims to overcome these difficulties by developing a paper-based cationic isotachophoresis (ITP) approach for simultaneously detecting pico-molar levels of two essential cardiac protein markers: acidic troponin T (cTnT) and basic troponin I (cTnI) spiked into human serum samples. The approach utilizes 3-aminopropyltrimethoxysilane (APTMS) treated glass fiber papers with decreasing cross-sectional area assembled on a 3D printed cartridge device. Our results showed that in the presence of cTnT monoclonal antibody (mAb), fluorescently labeled cTnI and cTnT could be effectively enriched in cationic ITP. Each individual target was captured subsequently by a test line in the detection zone where the capture mAb was immobilized. Detailed analysis suggests that the technology is capable of simultaneous on-board depletion of abundant plasma proteins and enrichment of cTnI/cTnT by ~1300-fold with a sensitivity of 0.6pmol/L for cTnT and a sensitivity of 1.5 pmol/L for cTnI in less than 6 min. The results demonstrate the potential of this technology for rapid, ultrasensitive and cost-effective analysis of multiplex protein markers in clinical serum samples at point of care.

Keywords

Isotachophoresis; Paper-based analytical devices; Point of care testing; Troponin

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Conflicts of interest

There are no conflicts to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2019.07.007>.

1. Introduction

Cardiovascular disease (CVD) is the most common cause of death globally. CVD is a group of different disorders that affect the functions of heart and blood vessels. The cause of CVD involves numerous complicated pathological conditions, including atherosclerosis, which can ultimately cause a heart attack or stroke and result in cardiac mortality and morbidity. Rapid clinical diagnosis of the cause of CVD, especially at the early stages, is a key step to prevent irreversible cardiac damage. However, conventional clinical assessment using electrocardiography alone may not provide enough information for an accurate and rapid diagnosis; delayed diagnosis often increases morbidity, cost, and mortality. Highly sensitive cardiac-specific biomarkers have been considered a good complement for clinical assessment and have emerged as strong and reliable risk predictors for CVD. According to American Heart Association Guideline [1], both cardiac troponin T (cTnT) and cardiac troponin I (cTnI) are regarded as the gold standard for diagnostic, prognostic, and risk stratification of CVD.

Cardiac troponin T and cardiac troponin I are regulatory proteins that are subunits of the cardiac troponin complex. Both cTnI and cTnT play key roles in regulating cardiac contraction and relaxation. During a cardiovascular event, the cell membranes of cardiac myocytes may rupture and release cTnI/cTnT into the bloodstream. Because both cTnI and cTnT are cardiac specific, the presence of cTnI/cTnT in the bloodstream is a strong indicator of the cardiovascular event, and the circulation levels of cTnI/cTnT are directly related to the degree of cardiovascular damage. However, at the early stages immediately following a cardiovascular event, the circulation concentration of cTnI/cTnT is extremely low (~ 0.4 pmol/L) [2,3]; detection of such low concentrations of circulating proteins require highly sensitive assay technologies. Current cTnI and cTnT based assays still rely on central laboratory platforms and resource intensive instruments, including mass spectrometry [4,5], and specialized biomarker analyzer [6–8], requiring skilled personnel to carry out the tests. Highly specialized assays significantly limit the accessibility for patients outside hospital laboratories and in underdeveloped regions [9]. Furthermore, there is a high demand for the development of accessible and cost-effective detection alternatives for early diagnosis, prognosis, and disease monitoring for general clinical settings and point of care testing (POCT).

To address these issues, paper-based analytical devices (PADs) have been a technology of choice, as they provide a cheap, easy to use, and environmentally-friendly [10–12] POCT platform. Several PADs for abundant analytes in biological fluids are commercially available, including 9-tetrahydrocannabinol (the psychoactive ingredient in marijuana), human chorionic gonadotropin (pregnancy hormone), cholesterol, glucose, and hepatitis B/C virus. However, the application of PADs in detecting disease markers with low abundance in biological fluids has been rare, due to their poor detection sensitivity. Many efforts have been made to improve the detection sensitivity of PADs. These include amplifying the detection signal [13–16], evaporative concentration [17], electrokinetic stacking [18,19], and isotachopheresis (ITP) [20,21]. Among these approaches, ITP is an ideal choice because of its ability to simultaneously separate, purify, and concentrate sample components [22,23]. ITP uses a discontinuous buffer system, comprised of a high-mobility leading electrolyte

(LE) buffer and a low-mobility trailing electrolyte (TE) buffer, to focus charged analytes with intermediate-mobility into a concentrated band between the LE and TE zones. Our previous study showed that integrating ITP into cascade microchip technology (cascade microfluidic ITP) provides a 10,000-fold increase in the concentration of the target protein [22]. Thus, we expected that the integration of ITP with PAD technology could greatly enhance the performance of PADs in POCT to detect low levels of disease biomarkers.

pH condition is critical to ITP focusing because it plays a determinant role in overall charges of target molecules. Depending on the pH of the LE/TE buffers ITP can operate under either an anionic or a cationic condition. Anionic ITP is known to preferentially focus negatively charged analytes, such as cardiac biomarker cTnT (with isoelectric point, $pI=5.0$), while cationic ITP can effectively concentrate positively charged analytes, such as cardiac biomarker cTnI ($pI=9.8$). When cationic ITP is used in protein analysis, positively charged proteins can be effectively separated from negatively charged components and stacked into a concentrated band as they move toward the anode [24]. Since most of the serum abundant proteins have isoelectric points less than 7, they are typically negatively charged and immobile under cationic ITP condition; thus, it would be advantageous to use cationic ITP for detecting positively charged biomarkers in serum samples. However, many disease protein biomarkers in serum samples are known to be negatively charged and immobile under the cationic ITP condition. It is a significant challenge to isotachophoretically separate the negatively charged protein biomarkers from the abundant serum albumins using ITP technology. To address this challenge and expand the use of cationic ITP in biological assays, we developed a technique to manipulate the electromobility of negatively charged target proteins through immunobinding under cationic ITP conditions [25]. Using this approach, we were able to achieve electrokinetic focusing of a heavily negatively charged protein under cationic ITP conditions. Here, we present a study using this novel approach to simultaneously detect two essential cardiac biomarkers, basic cTnI protein ($pI=9.8$) and acidic cTnT protein ($pI=5$) in serum sample on a paper-based cascade cationic ITP device. By combining a cost-effective and rapid paper-based assay with cascade cationic isotachophoretic focusing, and modifying electromobility of target protein via immunobinding, we were not only able to detect both positive and negative protein biomarkers simultaneously in a single sample analysis, with the more than 1000-fold enrichment factor, but also able to achieve limit of detection (LOD) of 1.5pmol/L for cTnI and 0.6pmol/L for cTnT with a total experiment time of less than 6 min. These results demonstrate a novel cost-effective approach for the rapid, sensitive and multiplex analysis of disease biomarkers in biological fluid samples.

2. Materials and methods

2.1. Materials

All chemicals, unless otherwise stated were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor 488 NHS Ester and Tetramethylrhodamine-5-Isothiocyanate (TAMRA) were purchased from Thermo Fisher Scientific, standard 14 glass fiber paper and Fusion 5 paper were purchased from GE Healthcare. To validate the concept of our design, rabbit polyclonal antibodies to cTnT and cTnI were used for target capture at test lines, and mouse

monoclonal antibody specific to cTnT was used for manipulating electromobility of cTnT. All antibodies used in this study were purchased from Abcam.

2.2. Protein purification and labeling

Human cTnI and cTnT were overexpressed in *E. coli* strain BL21 (DE3) cells, and protein purification and quantification were performed according to our previous studies [22,26]. The purified cTnI was labeled with TAMRA, and purified cTnT was labeled with Alexa Fluor 488, respectively. To achieve 1:1 label ratio of both proteins, an optimized protocol was developed based on manufacture suggestion (Thermo Fisher Scientific). Two-fold fluorescent dye was mixed with a known amount of protein sample at pH 8.6 for 30 min at room temperature. Then, the mixture was passed a DEAE Sepharose Fast Flow anion exchange column (GE Healthcare) to fractionate unlabeled, single-labeled, and multiple-labeled proteins [48]. Under our optimized labeling condition, only the single-labeled fraction was observed and eluted out. After extensive dialysis (4 changes of dialysis buffer) against PBS to remove free fluorescent probes, Absorption measurements were performed to determine the labeling ratio. The molar extinction coefficients of $65,000 \text{ cm}^{-1}\text{M}^{-1}$ at 555 nm and $73,000 \text{ cm}^{-1}\text{M}^{-1}$ at 495 nm were used to calculate the ratio of TAMRA and Alex Fluor 488 to the known amount cTnI and cTnT, respectively. The overall label ratio is 1:1 for both proteins.

2.3. LE and TE buffer preparation

The LE solution was prepared by adjusting 20 mmol/L KOH to pH 6.6 with the counterion MES. The LE solution also contained, 0.3 mol/L urea, 0.1% v/v Triton X-100, and 2% PVP. The TE solution consisted of 60 mmol/L glycine, 0.3 mol/L urea, 0.1% v/v Triton X-100, 2% PVP, and was titrated to pH 5.9 with MES. Both the LE and TE solutions contain PVP to suppress the electroosmosis. Triton X-100 was added to the electrolyte to facilitate sample movement and reduce nonspecific attachment. All solutions were prepared using nanopure water from Millipore.

2.4. Apparatus

The paper strip was wetted by adding a few drops of LE from the LE side to make four-fifths of the paper is wet. Then the strip was placed on the holder and both ends were dipped in reservoirs, which were filled with 300 μL TE (on the left) and LE (on the right), respectively (Fig. 1). The paper strip holder 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 $10\times$ objective lens for quantification and a $4\times$ objective lens for capturing representative images; the exposure time was 1 s. The reservoirs and platinum wires were rinsed with DI water three times before starting the experiments to reduce contamination. Protein labeled with fluorophore was excited by a Leica Microsystems EL 6000 light source using a Red/Green dual filter cube. Platinum electrodes were dipped in the anode and cathode reservoirs, respectively. The anode reservoir voltage was first set to 200 V, and the cathode reservoir was set to ground with an XHR 600–1 power supply (Xantrex Technology, Vancouver, Canada). A slightly higher voltage could result in a higher concentration effect and shorter assay time, however, high electromobility of targets within the test region leads to insufficient interactions between antigen and capture

antibody. Hence, we lowered the voltage to 70 V when focused target bands moved into the test region, allowing more time for antibody-antigen interaction. After the ITP band passed the test line, the voltage was increased back to the optimized condition. Images were taken at different positions along the paper strip and fluorescence intensities were quantified using ImageJ (NIH) as previously described [22].

3. Results

3.1. Design of paper-based cascade ITP for maximizing sample stacking

The design of paper-based ITP is shown in Fig. 1. A 3D printed cartridge, made from high-density acrylonitrile butadiene styrene (ABS), was used to produce buffer reservoirs and electrode seats. The device was also equipped with a hydrophobic treated 25×25 mm glass slide as the support for the paper strip. A 35 mm APTMS treated paper strip (see supporting information) was cut to form a structure with a decreasing cross-sectional area in the middle region of the paper strip, beginning a width of 8 mm at both ends, and tapering to a width of 2 mm in the detection region in the middle of the paper strip (Fig. 1). The rationale of this design is to maximize the electrokinetic concentration effect on the paper strip while providing enough capacity for sample loading and effective solution wicking to maintain effective material and heat transfer during the ITP process. Our previous microfluidic study demonstrated that reducing the cross-sectional area by factors of 50 resulted in an increase in the concentration of the analyte by 10000-fold [22]. We expected that this similar cascade design would significantly improve concentrating efficiency and performance of our paper-based device. To increase the analyte concentrating effect of the decreasing cross-sectional area of the paper strip and accommodate the high volume of sample loading, an $8 \text{ mm} \times 4 \text{ mm} \times 0.36 \text{ mm}$ ($L \times W \times D$) APTMS treated sample pad (Fusion 5, GE Healthcare) was overlaid on the paper strip at the anode end. By adjusting the size of the sample pad, up to 100 μL of the sample can be loaded. In this experiment, 50 μL of sample loading was used and the factor of the decreasing cross-sectional area of the paper strip is ten (that is, the cross-sectional area of the paper strip decreases tenfold between the widest and narrowest regions). The detection region was made hollow to minimize the light scattering and fluorescence background noise for quantification experiments.

3.2. Optimization of paper length and applied voltage

The length of the paper strip is an important parameter to consider in the design of a PAD. Paper strip length determines the available space for complicated designs such as multiple immobilizations of capture antibodies for multiplex assays, and more sample loading area which may improve the limit of detection. To optimize the length of the paper strip, we performed ITP measurements with samples containing cTnI-TAMRA on the paper strips with total lengths of 35 mm, 40 mm, and 45 mm while maintaining the length of the middle narrow detection region constant to 6 mm. After isotachopheric focusing under each experimental condition, the fluorescence intensity of the focused cTnI-TAMRA band was determined at the middle of the narrow detection region. As shown in Fig. 2, compared to the other lengths, the 45 mm paper strips showed the lowest fluorescence intensity independent of the applied voltage, suggesting that a long length of the paper strip does not benefit the ITP concentration effect. In contrast, the 35 mm paper length yielded the highest

fluorescence intensity, especially when a high voltage was applied. Regarding voltage effects, our results (Fig. 2) showed that the intensity of cTnI increased with increasing voltage because high voltage favors electrokinetic focusing of the target molecules into the sharp band between LE and TE. This electrokinetic focusing effect reached a plateau at 200 V, and further elevation of voltage to 225V did not improve the intensity. Fig. 2 also shows that as the total length of the paper strip was increased, the fluorescence intensity of the band decreased, meaning the concentrating effect decreased with increasing paper length, particularly for the ITP performed with 45 mm paper strips using 200V and 225V. These observations are most likely associated with limited heat and material transfer within the porous medium under high voltages. For electrophoresis on a porous paper strip, it is expected that higher voltage will elevate local electric fields in each zone of the strip, leading to accelerated heat production and evaporation, and resulting in paper drying. This high-voltage induced detrimental effect is prominent in the TE zone, because of its lower conductivity. With our design, the buffers wicking from both wide ends of the paper strip could mitigate the drying issue in the narrow detection region; however, for longer strips, this migrating effect becomes weaker. Based on these observations, we chose 200V with 35 mm long paper strips for the other experiments of this study.

3.3. Simultaneous detection of cTnT and cTnI

An optimal POCT platform should meet the requirements of miniaturization, cost-effectiveness, and the ability for simultaneous detection of multiple biomarkers. Compared to single analyte methods, multiplex assays reduce assay time, decrease sample consumption, and increase assay reliability. Ideally, multiplex assays could perform in parallel on a single device, thereby reducing test time. Moreover, a sensitive multiplex immunoassay requires optimized antigen-antibody interaction, and generally the optimum pH to for antigen-antibody interaction is around physiological pH. Antibody-antigen interaction is weaker at unfavorable pH conditions as studies showed the equilibrium constant at pH 9.5 or 5.0 was 100-fold lower than at 6.5–7.0 [27]. Unfavorable pH conditions may cause significant conformational changes in antibody and antigen, altering their complementarity [28], therefore, a nearly physiological pH condition is desirable for antibody-antigen interaction and best sensitivity results. Under physiological pH, cTnT is negatively charged with an isoelectric point of 5.0, while cTnI is positively charged with an isoelectric point of 9.8. It is a significant challenge for ITP technology to analyze cationic and anionic biomarkers simultaneously. Although it is possible to detect both positively and negatively charged proteins by running ITP at either extremely basic or acidic pH or running bidirectional ITP at neutral pH [29], such pH condition in the former is far from the preferred pH for immunoassay, while the latter requires a complex buffer system and experimental setup. As mentioned in the introduction, we address this challenge by enhancing electromobility of the negatively charged cTnT under cationic ITP through immunobinding [25]. Specifically, the electromobility of cTnT under cationic ITP conditions was modified in this study by incubating cTnT with the specific primary monoclonal antibody. Theoretically, when an antibody binds with antigen forming an antibody-antigen complex, the isoelectric point of the complex will be closer to the larger molecule. It is known that the isoelectric point of immunoglobulin G (IgG) type antibody is around 9.0 [30], with ~150kDa molecular weight, which is more than four times the size of cTnT (35

kDa). It is expected that the overall mobility of the immunocomplex of cTnT-antibody under cationic ITP conditions will primarily be dominated by the electromobility of IgG.

To verify this, we first performed cationic ITP experiments with individual cTnI protein labeled with red TAMRA and cTnT protein labeled with green Alexa Fluor 488, respectively, on the paper device. As expected, when the cationic ITP was performed with basic cTnI alone, the red labeled protein in a diluted sample (Fig. 3A) was effectively focused by ITP (Fig. 3B and C) before being captured at the test line by the immobilized anti-cTnI capture antibody (Fig. 3D), suggesting effectiveness of cationic ITP in electrodynamic focusing of basic protein. By calculating the ratio of the concentrations of the target with ITP and without ITP at the same fluorescence intensity, we can quantitatively assess the effectiveness of ITP on pre-concentrating the target before interacting with the capture antibody. In this study, we achieved 1300-fold enrichment for cTnI-TAMRA (data not shown). This result represents the maximum enrichment factor for cTnI analysis, i.e. the diluted cTnI can be concentrated by 1300-fold under our cationic ITP experimental conditions. When the cationic ITP was performed with acidic cTnT protein alone, as expected that no green focusing was observed, i.e. green target was immobile on the paper matrix under ITP conditions (data not shown). However, as shown in Fig. 3E–H, green labeled cTnT in a diluted sample (Fig. 3E) was effectively concentrated into bright fluorescent bands in the presence of the monoclonal antibody (Fig. 3F and G). The focused green target was then captured on the test line by the immobilized capture antibody (Fig. 3H). The results demonstrate the effectiveness of immunobinding to alter the isotachophoretic mobility of acidic protein under cationic ITP conditions. Similar to the cTnI experiment, the pre-concentration factor was calculated by the concentration ratio of the cTnT-Alexa Fluor 488 with ITP and without ITP at the same fluorescence intensity. In this study, a maximum enrichment factor of 1100 was achieved in the presence of immunobinding.

To demonstrate the multiplex potential of our paper-based device, we further performed the paper-based cascade cationic ITP with a cTnI/cTnT mixture. Red labeled cTnI (1 nmol/L) and green labeled cTnT (1 nmol/L) were first incubated with mouse monoclonal antibody specific against cTnT (0.4 µg/ml) for 15 min at room temperature. The ITP was performed after loading 50 µL of mixed sample on the sample pad of the device. The fluorescence signal of the sample was too low to be observed when ITP started (Fig. 3I), and the labeled proteins were rapidly focused to a band (Fig. 3J) after 1 min. Note that the color of the focusing band containing the mixture of red cTnI and green cTnT was yellowish green, due to the higher quantum yield of the green Alexa flour 488 than the red TAMRA dye under our microscopy conditions. Later, the focusing band became more concentrated (Fig. 3K and L), followed by capturing green cTnT-immunocomplex at the test line at 3.5 min (Fig. 3M) by the immobilized anti-cTnT antibody. The remaining band in yellow continued migrating toward the cathode. At the end of the ITP (5 min), red cTnI was captured by anti-cTnI antibody immobilized at the test line (Fig. 3N) with a small amount of uncaptured analytes in a faint band at the right side of the test line. Capture antibodies on the test lines were optimized to 0.5mg/ml for cTnT and 0.6mg/ml for cTnI to achieve the best compromise between high intensity and cost-effective use of the antibodies (see supplementary information). When analytes migrated close to the antibody capture region, the ITP voltage

was reduced to 70 V to decrease the velocity of band migration, thus increasing the interaction time between analytes and capture antibodies.

3.4. Quantitative evaluation of device performance with serum sample

To investigate the quantitative potential of the paper-based cascade ITP device, we performed ITP experiments to quantitatively detect cTnI and cTnT in a mixture spiked with human serum. Briefly, the stock samples were prepared by doping various amounts (0.3–150 pmol/L) of red cTnI-TAMRA and green cTnT-Alexa Fluor 488 into 10% human serum, which contains various physiological electrolytes, antibodies, antigens, and hormones. The doped mixture was then incubated with 0.4 µg/ml of monoclonal antibody specific to cTnT for 15 min before loading onto the sample pad of the device. To perform the quantification experiments, 50 µL of sample with different concentrations of labeled cTnT and cTnI mixtures were loaded directly onto the sample pad, and the voltage was applied immediately after to start ITP. The fluorescence signal for each target band was imaged while performing ITP. The fluorescence intensities of the images were then quantified using ImageJ (NIH). Linear relationships between the fluorescence intensity of each target measured at the end of ITP and its initial concentration were then plotted (red lines) in Fig. 4. The concentration of cTnT and cTnI was linearly correlated with the intensity within the concentration range of 0.6–50 pmol/L and 1.5–150 pmol/L, respectively. In this study, visual evaluation was used to determine the limit of detections (LOD) [49–51], which was equivalent to the minimal level of the target proteins that could be reliably detected in establishing the linear relationship between the known concentrations of targets and the fluorescence intensity of the images as shown Fig. 4. LOD was found to be 0.6 pmol/L for cTnT, and 1.5 pmol/L for cTnI by visual evaluation of the results shown in Fig. 4. These observed sensitivities were better than ELISA assays performed under similar assay condition with our cTnI and cTnT sample (data not shown), which gave LOD of 2.1 pmol/L for cTnI and 2.6 pmol/L for cTnT, respectively.

To assess the enrichment of paper-based cascade ITP for each target captured at the test line in presence of serum, we also plotted the fluorescence intensity of each target against its initial concentration without ITP (blue lines) in Fig. 4. The mean fluorescence intensity without ITP was determined by imaging paper membranes fully wetted by a known high concentration (0.2–100 nmol/L) of the cTnI-TAMRA and cTnT-Alexa Fluor 488, respectively. The enrichment factor for each target was then calculated by the ratio of the concentrations of the target with ITP (red line) and without ITP (blue line) at the same fluorescence intensity. For example, as shown in Fig. 4A, the fluorescence intensity of 0.4 intercepts with the blue line at 44.71 nmol/L (point a) and with the red line at 0.0615 nmol/L (point b), from which an enrichment factor of 727 can be calculated. The results suggest that diluted cTnI-TAMRA spiked in serum was enriched by 727-fold. Similar calculations from the ratio of the concentrations indicated by points c (10.81 nmol/L) and d (0.0187 nmol/L) shown in Fig. 4B gave an enrichment factor of 578 for cTnT-Alexa Fluor 488 capture and detection, suggesting that our device can enrich acidic cTnT bound to its antibody by 578-fold upon immunocapture at the test line. Note that the observed enrichment factors of 727 for cTnI and 578 for cTnT are lower than the maximum enrichment factors of 1100 for cTnT and 1300 for cTnI, respectively, since the maximum enrichment factor represents the highest

concentrating power of paper-based cascade ITP to enrich each analyte, which is calculated using the fluorescence intensity of the focused target band before immunocapture. In contrast, the enrichment factor at the test line is applicable for analyzing each analyte isolated from complex biological fluid, since not all focused target is captured at the test line, the fluorescence intensity at the test line is always lower than the intensity before immunocapture.

4. Discussion

Typical PAD based lateral flow immunoassays suffer from low sensitivity with the LOD of $\sim 10^{-11}$ mol/L at best [31], while the circulating concentration of biomarkers for many malicious diseases, especially in their early stages, being much lower than 10^{-11} mol/L; for instance, the concentration of released cTnI immediately after myocardial infarction can be below 0.01 ng/ml ($\sim 10^{-13}$ mol/L) [2,3]. At such low concentrations, conventional immunobinding alone will not be able to effectively capture target proteins in serum. The sensitivity of immunoassays is known to be determined by the binding affinity between antibody and antigen [32]. Antibody binding to its antigen is a reversible process, and the rate of the binding reaction is proportional to the concentrations of the reactants. The dissociation constants (K_D) of antibodies are in the range of 10^{-6} to 10^{-11} mol/L [33–35], which are well above the concentrations of many disease-associated biomarkers in serum; thus, the sensitivity of PADs based immunoassays of these targets is ultimately influenced by the binding affinity of antibody to its target [36]. Furthermore, when an assay is performed with serum samples, the LOD of PADs may be further decreased by non-specific binding of the various highly abundant proteins in serum. It is known that plasma contains up to 80 mg/mL protein and more than 10,000 different proteins in concentrations stretching over 12 orders of magnitude [37,38]. Approximately 99% of the protein weight in serum consists of only 22 different protein species and their isoforms, such as albumin and globulins [38,39], which can significantly interfere with the immunobinding of target proteins, which may only be present at ultralow concentrations.

Generally, the sensitivity of PAD based immunoassays can be improved by enhancing immuno-interactions between the target and the capture antibody and by signal amplification. Currently, most research efforts are focused on signal amplification, which has been quite successful at improving PAD sensitivity. For example, nanoparticle-based approaches, including use of gold nanoparticles [40], carbon nanoparticles [41], and quantum dots [42], as well as chemiluminescence [43] have all been used to improve PAD based immunoassay sensitivity to levels comparable with a large-scale instrument like ELISA and mass spectrometry. In terms of enhancing effective immunobinding at ultralow analyte concentration, an ideal technique should be capable of on-board depletion of abundant proteins to reduce their interference on immunobinding of the desired target, and should further be able to elevate concentration of the desired target before immunocapture. However, developing such ideal techniques is challenging because of the nature of lateral flow. The movement of sample molecules in traditional lateral flow immunoassays is known to solely depend on the capillary effect of the membrane matrix, hence, analyte molecules are evenly and extensively dispersed within the matrix. With such lateral flow attributes, it is difficult to perform on-board manipulation of sample, or separate abundant contaminant

protein from target protein, and further raise target protein concentration to enhance immunocapture at test lines. Generally, pre-treatment of samples before lateral flow assay is performed to deplete abundant proteins and elevate the concentration of target protein, which is typically time-consuming, laborious and not effective.

In contrast, analyte molecules in the ITP-based lateral flow assay, as reported in this study, can be controlled by isotachophoretic force. Through fine tuning LE and TE buffer conditions, it is expected that the technology can simultaneously achieve on-board depletion of abundant serum proteins and enrichment of target concentration for immunocapture. A fundamental advantage of cationic ITP implemented in our device is that all acidic abundant serum proteins can be depleted, as they do not migrate toward the test line under our cationic conditions, while basic target proteins can be isotachophoretically concentrated. As demonstrated by the results shown Fig. 3, by manipulating electromobility of acidic cTnT protein through immunobinding we were able to pull the target cTnT from serum sample and concentrate it together with cTnI to form a train of sharp bands under our favorable buffer conditions. In addition to being isotachophoretically focused, the levels of target proteins are further elevated by cross-section reduction of our paper strip design (Fig. 1). The combined effects from these unique functional features of our paper-based cascade ITP fluidic device dramatically increased levels of target cTnI and cTnT by 1100- and 1300-fold, respectively. Ultimately, our device enabled us to achieve LOD of 0.6 pmol/L for cTnT and 1.5 pmol/L for cTnI detection in serum spiked samples within 6 min. These observed sensitivities for detection of cTnI and cTnT make our technology comparable to, but more cost-effective than, currently available ultra-sensitive cardiac marker detection. Currently, two commercial ultra-sensitive assays are available for cTnI/cTnT detections, one is the hs-cTnI assay from Singulex [44], and the other is from Abbott Diagnostic [2]. The former employs a sophisticated single-molecule flow cytometry technique, while the latter utilizes large sample volumes and high concentrations of antibody, along with extra blocking steps to suppress background signals. Because of their complexity and labor-intensive protocol, it is very difficult to adopt these assays for a cost-effective and rapid detection of low concentration proteins in resource-limited settings. Finally, compared to other microfluidic detection platforms, our paper-based open format technology has more advantages. For example, microfluidic chips offer acceptable portability and sometimes good sensitivity, and have drawn great research efforts and plenty of applications have been reported [45]. Most microfluidic chips are fabricated from glass, silicon [46], poly(di-methylsiloxane) (PDMS), or poly(methyl methacrylate) (PMMA) [24,47], which is very hard to fabricate and recycle. Assay flow in the closed microfluidic channel requires directing different assay buffers accurately to the detection sites, through valves and inlets, which makes the construction of the assay complicated. Furthermore, it is a huge challenge to immobilize multiple capture antibodies within the detection channel for multiplex assays. These disadvantages in fabrication, complex flow controls, and lack of flexibility in target capture limit their broad applications. In contrast, the major component of our device is paper, which is accessible, disposable, easy to customize and improve, and which has great potential to be tailored into simple, sensitive yet cost-effective POCTs in the future.

5. Conclusions

In summary, we have successfully fabricated a cost-effective paper-based cascade ITP device, based on low-cost materials: 3D printed cartridge and lateral flow paper, for the simultaneous detection of human cardiac troponin T and cardiac troponin I. The device could detect and quantify cTnT in the concentration range of 0.6–50 pmol/L, with LOD of 0.6 pmol/L, cTnI in the concentration range of 1.5–150 pmol/L, with LOD of 1.5 pmol/L, with less than 6 min assay time. This device is simple, easy to fabricate, amenable to mass production, and provides proof of principle for detection and quantification of two essential cardiac biomarkers, which has potential utility in early detection at POCT of acute myocardial infarction and other cardiac events. To the best of our knowledge, no research has been reported on paper-based cascade ITP for multiplex analyte detection. This study also paves a new avenue to develop an ultra-sensitive and cost-effective technology for detecting other malicious diseases at their early stages via POCT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

ITP	isotachophoresis
POCT	point of care testing
PADs	paper-based analytical devices
pI	isoelectric point
LE	leading electrolytes
TE	trailing electrolytes
cTnI	cardiac troponin
cTnT	cardiac troponin T
CVD	cardiovascular disease

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Fig. 1. Schematic diagram illustrates the paper-based cascade ITP device.

A 3D printed cartridge was made from high-density acrylonitrile butadiene styrene and sealed by acetone vapor. The detection region was made hollow to minimize light scattering and background noise. A hydrophobic treated 25 * 25 mm glass slide was used as paper support.

Fig. 2. Optimization of paper length and applied voltage.

Paper-based ITP experiments were performed using cTnI-TAMRA on three lengths of paper strip (35 mm, 40 mm, and 45 mm) and four voltages (150V, 175V, 200V, and 225V) were compared, length of the narrow detection region was 6 mm for all experiments, and the intensity of cTnI-TAMRA was determined at the middle of the narrow detection region. Each intensity result was normalized as a percentage of the highest intensity band, and the error bar represents the standard deviation of three replicates (n=3).

Fig. 3. Representative images of analytes concentrating, detection, and capture on the paper-based cascade ITP.

A-D, cTnI-TAMRA only. A: sample dispersed after loading, B and C: analyte was focusing to a sharp band after voltage applied, D: analyte captured at the test line. E-H: cTnT-Alexa Fluor 488 only. E: sample dispersed after loading, F and G: cTnT-Alexa Fluor 488 was focusing to a sharp band after voltage applied, the band was further enhanced by cross-sectional reduction effect, H: analyte captured at the test line. I-O: a mixture of cTnT-Alexa Fluor 488 and cTnI-TAMRA, I, dim and dispersive sample after loading, J-L: analytes concentrating under applied voltage, M: cTnT-Alexa Fluor 488 was first captured, N: cTnI-TAMRA was later captured.

Fig. 4. (A) Calibration curves of cTnI detection. Comparison between direct imaging of cTnI on the paper (blue line) and paper-based cascade ITP (red line). Each intensity result was normalized as a percentage of the highest intensity band. (B) Calibration curves of cTnT detection. Comparison between direct imaging of cTnT on the paper (blue line) and paper-based cascade ITP (red line). Linear relationships of the fluorescence intensity of each target measured at the end of ITP varying with its concentration were plotted. Each intensity result was normalized as a percentage of the band with the highest intensity. Error bar represents

the standard deviation of three replicates ($n=3$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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