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Immunobinding-induced alteration in the electrophoretic mobility of proteins: An approach to studying the preconcentration of an acidic protein under cationic isotachophoresis

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Abstract

The objective of this study is to explore an approach for analyzing negatively charged proteins using paper-based cationic ITP. The rationale of electrophoretic focusing the target protein with negative charges under unfavorable cationic ITP condition is to modify the electrophoretic mobility of the target protein through antigen-antibody immunobinding. Cationic ITP was performed on a paper-based analytical device that was fabricated using fiberglass paper. The paper matrix was modified with (3-aminopropyl)trimethoxysilane to minimize sample attraction to the surface for cationic ITP. Negatively charged BSA was used as the model target protein for the cationic ITP experiments. No electrophoretic mobility was observed for BSA-only samples during cationic ITP experimental condition. However, the presence of a primary antibody to BSA significantly improved the electrokinetic behavior of the target protein. Adding a secondary antibody conjugated with amine-rich quantum dots to the sample further facilitated the concentrating effect of ITP, reduced experiment time, and elevated the stacking ratio. Under our optimized experimental conditions, the cationic ITP-based paper device electrophoretically stacked 94% of loaded BSA in less than 7 min. Our results demonstrate that the technique has a broad potential for rapid and cost-effective isotachphoretic analysis of multiplex protein biomarkers in serum samples at the point of care.

Keywords

Cationic isotachophoresis; Paper-based analytical devices; Point of care; Proteins

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1 Introduction

Paper is affordable, abundant, portable, disposable, and compatible with large-scale manufacturing processes for microfluidic devices. Because of these advantages, paper-based analytical devices (PADs) are by far the most common diagnostic tools for point-of-care applications; in particular, they are widely used in pregnancy and influenza tests. It has been suggested that PADs not only have great potential as medical tools in remote and undeveloped areas, but can also be developed into an appropriate technology for a wide variety of diagnostic applications [1–3]. Because of their porous structure and easy fabrication, filter papers, including nitrocellulose paper and fiberglass paper, provide a unique matrix for accommodating multiplex assays, which is critical for the reliable early diagnosis of cancers and other malignant diseases. Developing portable, disposable PADs capable of multiplex detection for general clinical settings and point of care (POC) applications can significantly improve patient care management, thus leading to positive impacts on the quality of life of patients and reduced healthcare costs.

Despite their potential social impact and clinical significance, the application of PADs in POC diagnosis of malignant diseases at their early stages is hindered by the poor sensitivity and detection limit of the technology. It is known that typical PADs based on lateral flow immunoassay have relatively poor detection limits ($\sim 10^{-11}$ mol/L) [4], while the serum concentrations of many biomarkers of malignant diseases, such as cardiovascular diseases and cancers, are much lower (10^{-12} to 10^{-16} mol/L) at their early stages [5–8]. When PADs are used in serum sample tests, the sensitivity and detection limits of the technology are further hindered by the millimolar concentrations of abundant serum proteins, such as albumin and globulin, which can significantly interfere with immunobinding of the relevant biomarkers, which may be present in nano-/pico-molar concentrations. Lack of adequate sensitivity and an inadequate detection limit make general PADs unreliable for quantitative result on these disease biomarkers in serum samples.

To expand the POC applications of PADs in the early diagnosis of malignant diseases, significant efforts have been made to improve the sensitivity of PAD technology. These efforts include detection signal amplification [9–12], evaporative concentration [13], electrokinetic stacking [14,15], ion concentration polarization [16,17], and ITP [18–20]. Among these approaches, ITP has been one of the best choices because of its ability to rapidly concentrate sample components and the ease with which ITP can be coupled with other analytical techniques [21,22]. ITP is an electrophoretic technique that allows simultaneous separation, purification, concentration, and quantification of analytes based on their effective electrophoretic mobility. ITP uses a discontinuous buffer system to focus an analyte in a sharp electric field gradient formed between high electrophoretic mobility leading electrolytes (LE) in the leading buffer and low electrophoretic mobility trailing electrolytes (TE) in the trailing buffer. It has been demonstrated that ITP can increase the concentration of analyte by several orders of magnitude. 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 [21]. Thus, it is expected that integration of ITP into PAD technology will greatly expand the usability of PADs for the POC detection of disease biomarkers.

For general biological analysis, ITP can be operated under either anionic or cationic conditions, which are defined by the pH of the LE/TE buffers used for ITP. Cationic ITP can effectively concentrate positively charged analytes [23], while anionic ITP will preferentially concentrate negatively charged analytes. Recently, bidirectional ITP was also explored for electrophoretically concentrating both positively charged and negatively charged components under complicated buffer combination [24]. Since the majority of biological components, including DNA, RNA, and proteins, are negatively charged under normal physiological conditions, anionic ITP has been widely used for analyzing RNA/DNA and various low-abundance negatively charged proteins in biological samples, including serum samples [24]. However, it is known that the dynamic range of nucleotide and protein concentrations stretches over 12 orders of magnitude in biological samples [25, 26]. For example, plasma contains up to 80 mg of protein per mL and more than 10,000 different proteins. Approximately 99% of the protein weight consists of only 22 different protein species and their isoforms; these include albumin, globulins, transferrin, haptoglobin, and lipoproteins [26,27]. The remaining 1% of the proteins, including the protein biomarkers of interest, are present at very low concentrations. The presence of the abundant proteins can significantly compromise the effectiveness of anionic ITP analysis for the low-abundance proteins in the sample. Therefore, sample pretreatment is required to analyze the extra-low concentration of low-abundance proteins using anionic ITP [23]. In contrast, cationic ITP provides a favorable electrophoretic condition for analyzing low-abundance target proteins in serum samples without interference from the abundant negatively charged serum proteins. In our recent study, by performing cationic ITP on a cascade microfluidic chip having a 100-fold cross-sectional area reduction, the most abundant serum protein, albumin, was successfully depleted and simultaneously concentrated cardiac troponin I (a positively charged protein) by 8200-fold in less than 10 minutes [28]. This result demonstrates the power of cationic ITP in the on-board depletion of abundant serum proteins and simultaneous concentration of extra-low-abundance target proteins. However, the potential of the cationic ITP technique is limited by the fact that it can only be used to analyze target proteins with positive charges, which are much less common than the negatively charged protein biomarkers in the biological samples. The objective of this study is to address this issue and expand the applications of cationic ITP by developing a technology for the electrophoretic analysis of negatively charged protein biomarkers under cationic ITP conditions. The rationale of the current study is to modify the electrophoretic mobility of negatively charged proteins via immunobinding to favor cationic ITP conditions. The electrophoretic mobility of a protein under a certain ITP condition is dominated by the pI and molecular mass of the protein [28]. Under our previous cationic ITP conditions (pH of LE=8, pH of TE =7.2) [23], proteins with $pI > 7$ (such as troponin I) can be electrophoretically stacked, while acidic proteins, $pI < 7$ (such as albumin), will be immobile or migrate toward the opposite direction. To alter the electrophoretic mobility of acidic proteins, antibodies specific to these target proteins can be used. It is known that a typical antibody has a relatively large molecular mass (~ 150 kDa) and basic isoelectric point ($pI \sim 9.0$) [29]. It is expected that the immunobinding of a basic antibody and an acidic protein can significantly alter the electrophoretic mobility of the protein.

To test this hypothesis, the current study employs bovine serum albumin, which has a molecular mass of 66.5 kDa and a $pI \sim 5$, as an example of an acidic protein to demonstrate that paper-based cationic ITP combined with immunobinding can be an effective tool for preconcentrating negatively charged protein. Our results show that the electrophoretic mobility of BSA is negligible under cationic ITP conditions. However, in the presence of the BSA-specific monoclonal antibody, fluorescently labeled BSA starts migrating toward the anode on a fiberglass paper strip. Adding a secondary antibody conjugated with amine-rich quantum dots (QD) to the sample further increases the electrophoretic mobility of BSA and improves the stacking efficiency of the protein, suggesting the effectiveness of immunobinding at increasing the electrophoretic mobility of an acidic protein under cationic ITP conditions. The approach developed in this study paves a new avenue to the utilization of cationic ITP to preconcentrate a broad range of target proteins, while simultaneously depleting the abundant proteins in serum samples. Therefore, the technique is potentially useful for developing cationic ITP-based multiplex PADs for POC applications.

2 Materials and methods

2.1 Chemicals and samples

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488 NHS Ester, tetramethylrhodamine-5-isothiocyanate, rabbit anti-mouse IgG labeled with Alexa Fluor 488, and BSA polyclonal antibody were purchased from ThermoFisher. TriLite™ fluorescent nanocrystal 540-nm amine (QDs-540-Amine) was purchased from Cytodiagnostics, Inc. (Burlington, Ontario). Mouse monoclonal antibody to human cardiac troponin T (cTnT), rabbit polyclonal antibody to human cTnT, and mouse monoclonal antibody against BSA was purchased from Abcam (Cambridge, MA).

2.2 Protein labeling

BSA was labeled with tetramethylrhodamine-5-isothiocyanate at a ratio of 1:2 at pH 8.6. The labeled mixture was passed four times before measurements through a DEAE Sepharose (GE Healthcare) column to remove extra fluorescent probes and dialysis against PBS. Unlabeled goat anti-mouse IgG (Jackson ImmunoResearch) was conjugated to QDs-540-amine using the appropriate LYNX rapid conjugation kit (AbD Serotec). Human cTnT were over expressed in *E. coli* strain BL21(DE3) cells and purified according to our previous studies [30] and then labeled Alexa Fluor 488, with a ratio of 1:2 at pH 8.6, the labeled mixture was first passed a DEAE Sepharose (GE Healthcare) column to remove extra fluorescent probe and dialysis against PBS for four times before measurements.

2.3 LE and TE solutions

The LE solution was prepared by adjusting 20 mmol/L KOH to pH 6.4 with the counterion, MES. The LE solution also contained 0.1% v/v Triton X-100 and 2% PVP. The TE solution consisted of 60 mmol/L glycine, 0.1% v/v Triton X-100, and 2% PVP and was titrated to pH 5.8 with MES. Both the LE and TE solutions contained PVP to suppress the EOF [31]. Triton X-100 was added to the electrolyte to facilitate sample movement. All electrolyte solutions were prepared using Nanopure water from Millipore.

2.4 Device operation

The APTMS treated paper strip (see S1) was wetted by adding a few drops of LE from the LE side to make four-fifths of the paper wet. Then the strip was placed on the holder and both ends were dipped in reservoirs, which were filled with 300 μ L of TE (on the left) and LE (on the right). The paper strip holder was secured on the stage of a Leica DM 2000 fluorescence microscope equipped with a DFC310 digital color camera (Leica Microsystems, Bannockburn, IL) underneath a 4 $\times$ objective lens. The reservoirs and platinum wires were rinsed with DI water three times before the experiments started to reduce contamination. Protein labeled with a fluorophore was excited by UV light from a Leica Microsystems EL 6000 light source using an A-type filter cube. Platinum electrodes were dipped in the anode and cathode reservoirs, respectively. The anode reservoir voltage was set to 150 V, and the cathode reservoir was set to ground with an XHR 600–1 power supply (Xantrex Technology, Vancouver, Canada). Images were taken at various positions along the paper strip.

3 Results

3.1 Electrokinetics of BSA and antibody proteins in cationic ITP-based PAD

Our cationic ITP-based PAD is schematically shown in Fig. 1. The paper strip is cut from GE standard 14 glass fiber paper that was treated with 0.033% APTMS (see S2) and loaded onto a 3D printed ITP device. The objective of our device is to focus positively charged proteins electrophoretically using cationic ITP. The paper strip with the best performance should have the shortest ITP stacking time and the highest fluorescence intensity, optimization of paper property, and buffer conditions were shown in supporting information. Before testing our hypothesis that the electrophoretic mobility of BSA can be altered by immunobinding, we examined the electrokinetic behavior of individual BSA and antibody proteins in our cationic ITP-based PAD. With $pI \sim 5$ and a molecular mass of 66.5 kDa, BSA is expected to carry a net negative charge under our buffer conditions, and for that reason, it was expected that BSA would not electrophoretically stack under cationic ITP conditions. In contrast, antibody protein, with pI of 7.0–9.0 and molecular mass of ~ 150 kDa, is expected to carry a net positive charge and be stacked under cationic ITP-based PAD. To verify these expectations, we performed cationic ITP on our PAD device, as shown in Fig. 1, with a mixture of 20 ng/mL of the green fluorescent antibody protein (rabbit anti-mouse IgG labeled with Alexa Fluor 488) and 1 nmol/L red fluorescent BSA-TAMRA. The green fluorescent antibody protein (Fig. 2) started forming a green band as ITP proceeded (Fig. 2B), whereas the distribution of the red fluorescent BSA on the paper strip remained unchanged. As ITP continued, the green antibody band passed through the red region, migrating toward the anode (Fig. 2C), indicating the antibody protein has little interaction with either BSA-TAMRA or the paper matrix and can be focused by ITP. Figure 2D–F are inverted color images that corresponding to Fig. 2A–C.

3.2 Alteration of electrophoretic mobility of BSA by immunobinding

We then tested our hypothesis by investigating the electrokinetic behavior of BSA on our cationic ITP-based PAD in the presence of the antibody that specifically binds to BSA. Before the experiment, the sample was prepared by mixing mouse anti-BSA antibody (0.4

μg/mL) with 1 nmol/L fluorescent BSA-TAMRA for 15 min at room temperature. One microliter of the mixed sample was then added onto the anode side of the paper strip to perform the ITP experiment. Under the cationic ITP conditions used in the experiment described in Fig. 2A, at which BSA was immobile, a large portion of the red fluorescent BSA-TAMRA was stacked into an ITP plug in the presence of the specific antibody. However, a considerable amount of BSA still lagged behind the focused region and was resistant to migrating (Fig. 3B). At the end of the experiment, only 43% of the analytes were stacked into a band (Fig. 3C). Increasing the amount of the antibody in the sample mixture and the ITP voltage had a negligible effect on the result. The observed partial BSA stacking suggests that by binding to BSA the positively charged antibody can indeed neutralize negative charges of BSA but that the effect is not enough to mobilize all BSA under our ITP conditions.

To further improve the performance of BSA under cationic ITP conditions, we added a secondary antibody, commercial goat anti-mouse IgG (2 μg/mL), to the sample mixture, in which an immunocomplex of BSA/primary Ab/secondary Ab would form. The rationale was that, with a basic *pI*, and five times more mass than BSA, the two Ab proteins could effectively neutralize the negative charges of BSA and make increase the electrophoretic mobility of the complex under cationic ITP conditions. To perform the experiment, the BSA-TAMRA was mixed first with the primary antibody and then with the secondary antibody of goat anti-mouse IgG. One microliter of the sample mixture was then added to the anode end of the paper strip, followed by conduction of the cationic ITP experiment. Figure 3D–F shows the results after 10 minutes of ITP, nearly 80% of the red fluorescent BSA was stacked in the presence of the primary and secondary antibodies (Fig. 3F). The band intensity in Fig. 3F is 68% stronger in comparison with band intensity in Fig. 3C.

3.3 Further improving the electrophoretic mobility of the BSA-Ab complex using a quantum dot modified secondary Ab

The results shown in Fig. 3 pave a new avenue to altering the electrophoretic mobility of acidic proteins under cationic ITP conditions. Since the formation of an immunocomplex in the presence of a secondary Ab is the key to altering the electrophoretic mobility of the target protein effectively, it is expected that the secondary Ab can be an effective mobility modifier and general probe for cationic ITP assays. To test this hypothesis, we used the secondary antibody of goat anti-mouse IgG conjugated with amine-rich green QD replacing the unlabeled secondary Ab in the experiments of Fig. 3. It was expected that abundant amine groups on QD would ensure ample positive charges in the immunocomplex, which would facilitate an effective stacking of the complex in cationic ITP. In a separate cationic ITP experiment with the amine-rich QD alone, it took less than 5 minutes for the amine-rich QD to migrate the full length of our paper-based ITP device with little attachment (data not shown). To repeat the Fig. 3 experiments in the presence of the green QD-modified secondary Ab, stock sample was prepared by mixing BSA-TAMRA with the primary antibody, followed by incubating QD-modified goat anti-mouse IgG for 15 minutes. One microliter of the sample mixture was then loaded onto the anode end of the paper strip, followed by cationic ITP analysis. Because of the presence of an excess amount of QD-modified Ab proteins, the sample was dominated by green fluorescence (Fig. 4). As cationic

ITP proceeded, a green band with a yellowish center started to form (Fig. 4A). The yellowish color is the result of an overlap of red (BSA) and green (QD-modified secondary Ab), suggesting the target BSA proteins migrate toward the anode as immunocomplexes. As ITP continued, the plug was further concentrated with more yellowish components (Fig. 4B). After the fluorescent plug passed the test line, where BSA polyclonal antibody was immobilized, the yellowish component (BSA immunocomplex) was captured as the unbound green QD-Ab continued migrating toward the anode (Fig. 4C). The whole process took less than 7 minutes. The result demonstrated that the secondary Ab conjugated with amine-rich QD not only can be an effective modifier of the electrophoretic mobility of an acidic target protein under cationic ITP conditions, but also can be a general probe for target quantification, which can be a powerful approach to multiplex ITP assay design in the future.

3.4 Cationic paper-based ITP to detect cardiac troponin T, an acidic biomarker

To further test the performance of our device in the analysis of negatively charged protein in serum sample, we performed cationic paper-based ITP with cardiac troponin T (cTnT) labeled with Alexa Fluor 488 spiked into human serum sample. Under physiological condition, cTnT is a negatively charged protein, with a molecular weight of 37 kDa and $pI \sim 5$. To perform the experiment and test the effect of serum impurities on target analysis, 0.6 nmol/L of cTnT-Alexa Fluor 488 was spiked into 10% human serum, then incubated with mouse monoclonal antibody against cTnT (0.4 $\mu\text{g/mL}$) for 15 minutes at room temperature. One microliter of the mixture was loaded to the device, followed by ITP analysis as described in Fig. 5. Similar to BSA, cTnT alone was immobile on the paper strip under our cationic ITP conditions (data not shown). However, after incubation with the primary antibody against cTnT, green fluorescently labeled cTnT was effectively concentrated (Fig. 5B). At the end of the experiment, the majority of cTnT-Alexa Fluor 488 was captured on the test line where a capture antibody against cTnT was immobilized (Fig. 5C), and the remaining uncaptured proteins were continuously moving forward. Interestingly, in BSA analysis (Figs. 3 and 4) both primary and secondary antibodies are required to mobilize BSA on the paper strip. However, in cTnT analysis the presence of the primary antibody alone is sufficient to alter the mobility of cTnT; this may due to the molecular weight of cTnT is only 56% of BSA.

4 Discussion

This study reports our efforts to develop a paper-based cationic ITP technology for the analysis of acidic proteins. Three techniques were developed and integrated into our final device: surface modification of the selected filter paper as a solution-holding matrix, alteration of the electrophoretic mobility of target proteins via immunobinding, and use of an amine-rich QD-labeled secondary Ab as a general electrophoretic mobility modifier and fluorescent probe for cationic ITP assay.

The paper matrix provides an environment for solution holding, sample focusing, and material transfer, and plays a vital role in determining the stacking ratio for a given sample. Recently, Posner's group explored using ITP to improve the performance of a nitrocellulose

paper-based lateral flow assay, leading to 100-fold improvement in the detection limit [19]. However, the study encountered a challenging issue, specifically paper dryness during ITP, which leads to a significant decrease in the concentrating ability of ITP as the target component starts to reach its concentration peak. The reason is that the porous structure and thickness of commercial nitrocellulose paper are optimally designed for lateral flow assays, and under isotachophoretic conditions, the nitrocellulose paper exhibits relatively poor transfer of materials, heat, and charges, through the porous structure of the paper. The poor transfer properties of the nitrocellulose lead to Joule heat accumulation, which decreases the electrophoretic mobility of the components and eventually causes the analytes to stick on the porous surface. Therefore, choosing porous filter paper capable of effective transfer of heat and material under electrophoretic conditions is a viable option for addressing the dryness issue encountered in these studies [18–20]. To test this hypothesis, this study used fiberglass papers with a large porous structure to fabricate an ITP-based PAD. Diverse pore sizes (0.3–8 μm) of commercial fiberglass papers enable excellent water capacity and wicking speed, which leads to better performance in material transfer and heat dissipation under our cationic ITP conditions.

Another unique advantage of fiberglass paper is that the charge of the porous surface can be easily modified through salinization [31, 32], which makes the fiberglass paper a versatile matrix for electrophoretic analysis. Fine-tuning the surface charge of the pore structure of the papers (data provided in Supporting Information Fig. 1) using modifiers such as APTMS can accommodate material transfer of target proteins with different charges under electrophoretic conditions; therefore, it may find new uses in the future in electrophoretic assay designs. In contrast, cellulose membranes are impervious to surface modification via mild chemical reactions, limiting its broad use in charge-based electrophoretic analysis.

As an electrokinetic focusing technology, the ITP focusing effect is only applicable to singular charged analytes, i.e. it is difficult to concentrate negatively charged analytes in a cationic ITP or positively charged analytes in an anionic ITP. We overcame this limitation by modifying the electrophoretic mobility of target proteins through antibody-antigen interactions. By investigating electrokinetic focusing of negatively charged BSA in the presence of BSA-specific antibodies under cationic ITP conditions (Fig. 3). We showed that binding a large positively charged IgG to a small negatively charged protein target significantly improved cationic ITP-based electromobility and stack of the target. Our result demonstrates a novel electrophoretic technique for analyzing negatively charged proteins under a cationic ITP condition. This technique provides an effective approach to overcome the notorious interference from the majority of the negatively charged abundant serum proteins in isotachophoretic analysis of low-abundance acidic target proteins in serum samples [24]. This is further demonstrated by cationic ITP analysis of negatively charged cTnT spiked in serum sample as shown in Fig. 5. Collectively, our results suggest that cationic ITP combining with immune-binding can serve as an effective approach for isotachophoretic analysis of low-abundance target proteins regardless of their charges without worry about interference from the abundant negatively charged serum proteins. This expectation is demonstrated by one of our recent studies, in which a paper-based cascade cationic ITP was performed on a serum sample to sensitively detect ultra-low levels of

multi-cardiac markers carrying both positive and negative charges. Due to space limitation, the results of the study will be presented in a different report.

Another unique feature of this technique is using a secondary antibody conjugated with amine-rich QD as both a charge modifier to effectively alter the overall electrophoretic mobility of the antigen-antibody complex and a fluorescent probe to monitor the mobility and capture of the target (Fig. 4). Because of the abundant positive amine groups on the surface of QD, it is expected that tertiary immuno-complex consisting of a QD-conjugated secondary Ab can effectively facilitate the electrophoretic stacking of target proteins with broad sizes and positive charges under cationic ITP conditions. Furthermore, integrating a sandwich immunoassay scheme into the cationic ITP design, as illustrated in Fig. 4C, the QD-conjugated secondary Ab can be used as a universal fluorescent probe for a multiplex biomarker assay in a single test, which is essential for POC applications to accurately diagnose malignant diseases in their early stages. In a hypothetical scenario, proteins interleukin-6, interleukin-8, and interleukin-1- β are strongly recommended as multiplex biomarker panel for diagnosis of oral cancer, especially at an early stage [33]. These multiple biomarkers in a serum sample can be electrophoretically stacked individually under our cationic ITP condition in the presence of three primary Abs specific for each target protein and a universal QD-conjugated secondary Ab for these primary Abs. Once the stacked target immunocomplexes migrate into the detection zone of the PAD device, each target complex will be captured by an immobilized capture Ab specific to that target. The QD fluorescence intensity of each capture spot will provide concentration information on each target in the sample. It is very likely that the technique developed in this study will pave a new avenue to the design of a rapid, cost-effective ITP-based PAD technology for POC multiplex assays and general clinical applications.

Comparing to many other traditional analytic technologies for protein analysis, such as ELISA, Western blot, surface plasmon resonance, microcantilever, and other nanotechnology-based assays, our paper-based ITP has many advantages and great potentials for POC applications. ELISA and Western blot are best known for their sensitivity of protein detections, but are time consuming, require specialized laboratory equipment and significant expertise to carry out. Multiple-step sample treatments not only require extensive experimental time but also often introduce errors in protein analysis. The paper-based ITP enables online separation of analyte from other interferant molecules, thus eliminates the labor-intensive and repetitive incubation and washing procedures, achieving rapid analysis. More importantly, the concentrating power of ITP can significantly enhance antigen-antibody interaction during isotachophoretic analysis, leading to improved sensitivity and LoD of PAD. Surface plasmon resonance, electrochemical detection, or microcantilever are also preferred for protein analysis because they enable label-free analytes detection. However, studies showed their LoD usually worse 1–2 orders of magnitude than labeled assay settings [34]. This is mainly due to nonspecific binding caused by interfering molecules. With our cationic paper-based ITP device, however, most acidic interfering molecules are immobile and trapped in the sample loading region of the paper strip. Thus, with the the power of online preseparation and concentration, our cationic paper-based ITP provides more favorable conditions for target capture and detection. Also, some nano-technologies based assays, such as carbon nanotube and nanowire technologies [35], can

achieve high sensitivity and specificity, but complex fabrication and requirement of expensive equipment make them less suitable for POC application.

5 Concluding remarks

In summary, we have developed a novel approach to modify the overall electrophoretic mobility of target proteins on APTMS-modified fiberglass paper strips through antibody-antigen immunobinding. We demonstrated that with this technique negatively charged BSA, our model target protein could be effectively stacked on a fiberglass paper-based PAD under cationic conditions. Under optimized experimental conditions, 94% of the loaded target protein can be focused into a stacking band on the paper strip in less than 7 minutes. Our approach makes it possible to broaden the applications of cationic ITP in analyzing target proteins with different charge properties. In particular, including a universal secondary Ab conjugated with amine-rich QD in the assay significantly improves the electrophoretic focusing effect and opens the door to rapid multiplex biomarker detection in a single test. Therefore, the technology developed based on this approach may find broad application in POC diagnostics and general clinical care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ab	antibody
LE	leading electrolytes
PAD	paper-based analytical device
POC	point of care
QD	quantum dots
TE	trailing electrolytes

6 References

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Figure 1.

A 3D printed cartridge, made from high density acrylonitrile butadiene styrene (ABS), was used as buffer well and electrode seat. The device also equipped with a hydrophobic treated 25×25 mm glass slide as paper support. We made the detection region of the part hollow to minimize the light scattering and background.

Figure 2. Paper-based cationic ITP for proteins with different surface charges. (A) BSA-TAMRA only. (B) Mixture of BSA-TAMRA and rabbit anti-mouse IgG labeled with Alexa Fluor 488. (C) ITP plug formed from IgG labeled with green dye. (D–F) are inverted color images that corresponding to (A–C).

Figure 3. Paper-based cationic ITP for BSA incubated with antibodies. (A–C) BSA incubated with primary antibody alone. (D–F) BSA incubated with primary antibody and secondary antibody. The band intensity in Fig. 3F is 68% stronger than that in Fig. 3C.

Figure 4.

Snapshots taken at three different times showing that the BSA-TAMRA immunocomplex was captured at the test line: (A) as the mixture was effectively forming an ITP plug, (B) as the concentrated band formed, and (C) as BSA-TAMRA was captured at the test line in yellow, while the rest of the QDot and QDot-antibody was moving toward the cathode side.

Figure 5.

Snapshots taken at three different times showing that the acidic protein cTnT-Alexa Fluor 488 was effectively concentrated under cationic ITP: (A) cTnT-Alexa Fluor 488 incubated anti-cTnT antibody was loaded onto the paper, (B) under current, the immunocomplex was concentrating by cationic ITP, and (C) cTnT-Alexa Fluor 488 was later captured as shown in the first green line, with a small amount was moving toward cathode.