1000-fold sample focusing on paper-based microfluidic devices†

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We present an experimental and analytical study of a novel paper-based analytical device (µPAD) for isotachophoretic sample focusing. Guided by a simple heat transfer model, we further developed wax printing fabrication to enable the creation of shallow channels, which are critical in providing sufficient dissipation of Joule heat, and thus enable the use of high electric fields and short analysis time. This results in a device that is self-contained on a simple piece of filter paper and does not require any specialized enclosures or cooling devices to combat evaporation at high temperatures. Furthermore, we provide an analytical model for isotachophoretic sample accumulation in porous media, introduce a simple figure of merit for evaluating and comparing the efficiency of such devices, and present experimental validation in both paper and glass channels. Using this device we demonstrate the processing of 30 µL of sample achieving 1000-fold increase in peak concentration in 6 min. We believe that this method and device can serve as a guide to the design of low-cost, rapid and highly sensitive paper-based diagnostic platforms.

1. Introduction

Microfluidic paper-based analytical devices (µPADs) have recently gained significant attention due to their potential as low-cost, durable, multiplexed, and easy-to-use diagnostic platforms. µPADs, first introduced by Martinez et al.,1 are formed by patterning paper into hydrophilic regions, bounded by regions of a hydrophobic material. A variety of methods, including wax printing, CO2 laser cutting, and photolithography, now exist for fabrication of such devices,2–3 and they have found use in a variety of biochemical applications including glucose monitoring, detection of heavy metals, nanoparticle-based detection, total protein measurements, and ELISA.2,4–8 However, despite the use of well-identified biomarkers, many diagnostic needs cannot be met by the current sensitivity of such assays.8,9 The application of low-cost and rapid assays capable of accurately and sensitively detecting disease at the point-of-care could have a significant impact on global health, enabling access to advanced molecular diagnostics even in under-resourced and rural areas. We herein present a method for significantly increasing the sensitivity of such tests by coupling them with isotachophoresis (ITP).

ITP is an electrophoresis technique which allows simultaneous separation and preconcentration of analytes based on their effective electrophoretic mobility. ITP has its roots in the fundamentals developed by Kohlrausch10 over a century ago and in moving-boundary electrophoresis (MBE) developed in the 1920’s and 30’s.11–13 Notably, filter paper was used as a substrate for electrophoresis as early as 1951,14 before the introduction of gel electrophoresis15 or capillary-based electrophoresis.16 In the 70’s and 80’s, renewed interest in ITP led to its first implementation on paper substrates including cellulose acetate membrane (CAM) and filter paper.17–19 ITP has been used to directly focus or separate proteins of interest from urine,20 as well as to establish electroosmotic flow (EOF) patterns for delivery of target proteins to immnosensing sites. To mitigate excessive Joule heating and evaporation, experimental setups either housed the membrane in a closed chamber,21,22 used external cooling components as part of their apparatus,17,21 or used cellogel film which holds high water content in its gel matrix.23 In addition, experiments were run at relatively low electric fields, resulting in several hours of analysis time.20

Other electrokinetic techniques have been demonstrated on paper substrates. Mandal et al.23 demonstrated electrokinetic control of liquid transport in a paper-based device, and Ge et al.24 were the first to demonstrate electrophoretic separation on µPAD. Recently (in work published during preparation of this manuscript), Moghadam et al.25 have demonstrated ITP focusing on a nitrocellulose membrane housed in an acrylic device containing reservoirs. The authors have addressed the challenge of evaporation by augmenting their main channel with a cross channel which is dipped in solution and provides additional hydration to

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the membrane. We herein present a different approach and introduce a novel \( \mu \)PAD for isotachophoresis focusing, which is self-contained on a simple piece of paper, does not require any specialized enclosures or cooling devices and enables over 1000-fold enhancement by focusing a 30 \( \mu \)L sample for 10 min. More importantly, we overcome Joule heating by designing our fabrication process such that a thick layer of wax deposited at the bottom of the channel enables the creation of shallow channels, resulting in sufficiently rapid heat dissipation.

In section 2.1, we present a simple model for the rate of sample accumulation under ITP in porous media and define convenient figures of merit which allow direct comparison of extraction efficiency between standard microchannels and paper-based devices. We further provide scaling arguments for the generation and dissipation of heat on such devices, which directly guide the fabrication process. In section 3.1, we provide detailed information on the fabrication of shallow channels, and finally in section 4 we characterize the performance of our devices, demonstrating lossless focusing of finite sample injections and over 1000-fold enhancement by sample focusing in less than 6 min for infinite sample injections.

2. Theory

2.1. Sample accumulation under ITP in porous media

ITP is an electrophoresis technique that uses a discontinuous buffer system to focus sample ions of interest at a sharp electric field gradient formed between high electrophoretic mobility leading ions (LE) and low electrophoretic mobility trailing ions (TE). We herein focus our discussion on peak-mode ITP, which is characterized by sample concentrations that are significantly lower than those of the background buffers and thus do not affect the conductivity or the electric field in the channel.

A useful model for porous media used in several applications is based on reducing the complex geometry to a network of independent tortuous capillaries (or ‘pores’) embedded in a solid. Analysis is simplified to consider a single pore and is then extended to the entire area by summation over all pores.

Assuming an array of \( n \) cylindrical pores, the porosity of the porous media, \( \varepsilon \), is expressed as the ratio of the void volume of all the pores to the total volume of the substrate,

\[
\varepsilon = \frac{V_{\text{void}}}{V_{\text{total}}} = \frac{nA_l}{AL} \tag{1}
\]

where \( A_p \) is the average pore cross-section area, \( l \) is the average pore length, and \( A \) and \( L \) are the physical cross-section area and length of the porous media, respectively (see Fig. 1). Tortuosity is defined as

\[
\tau = \left( \frac{l}{L} \right)^2 \tag{2}
\]

which can then be used to express the effective area of the porous media as a function of measurable parameters

\[
A_{\varepsilon} = nA_p = \frac{\varepsilon}{\sqrt{\tau}} A \tag{3}
\]

Taking a moving control volume around the ITP interface within a single pore, the rate of analyte ions entering the LE–TE interface is governed by the difference between the analyte ions’ velocity in the adjusted TE zone and the velocity of the ITP interface. The sample accumulation rate in peak-mode ITP within one pore is given by

\[
\frac{dN_a}{dt}_{\text{pore}} = (V_{a,\text{ITP}} - V_a) c_a A_p \tag{4}
\]

where \( c_a \) is the concentration of the analyte in the adjusted TE zone. We note that eqn (4) holds irrespective of the electrolysmotic flow (EOF) in the system, which equally affects all velocity components. Defining the electrophoretic mobility of species X in zone Y as \( \mu_X^Y \) and the electric field in zone Y as \( E^Y \), substituting the ITP condition \( V_{\text{ITP}} = \mu_{\text{LE}} E^\text{LE} = \mu_{\text{TE}} E^\text{TE} \), and using the relation \( V_a = \mu_a E \), eqn (4) can be expressed as

\[
\frac{dN_a}{dt}_{\text{pore}} = \left( \frac{\mu_{\text{TE}}}{\mu_a} - 1 \right) \mu_{\text{LE}} E^{\text{LE}} c_a A_p \tag{5}
\]

Summing for all \( n \) pores to express the sample accumulation rate in the entire channel, and relating the concentration of the analyte in the TE to that in the reservoir, \( c_a = \frac{\sigma_T^{\text{TE}}}{\sigma_T^{\text{well}}} c_{a,\text{well}} \), we obtain,

\[
\frac{dN_a}{dt} = \sum_t \frac{dN_a}{dt}_{\text{pore}} = \left( \frac{\mu_{\text{TE}}}{\mu_a} - 1 \right) \frac{\sigma_T^{\text{TE}}}{\sigma_T^{\text{well}}} \mu_{\text{LE}} E^{\text{LE}} c_{a,\text{well}} \varepsilon \sqrt{\tau} A \tag{6}
\]
$V_i$, applied across the channel, can be expressed as a function of the electric field in the TE and the LE zones via

$$V = E^{TE} I^{TE} + E^{LE} I^{LE}$$  \hspace{1cm} (7)$$

where $I^{TE}$ and $I^{LE}$ are the (time varying) lengths of the pore segments filled with TE and LE, respectively.

Substituting the TE electric field from the current conservation condition, $E^{TE} = \frac{\sigma_{LE}}{\sigma_{TE}} E^{LE}$, the electric field in the LE zone can be expressed as

$$E^{LE} = \frac{\sqrt{V}}{\sqrt{\sigma_{LE}^{LE} L^{TE} / \sigma_{TE}^{TE} L^{LE} + 1}}$$  \hspace{1cm} (8)$$

Substituting the electric field into eqn (6), we obtain

$$\frac{dN}{dt} = \left( \frac{\mu^{TE}}{\mu^{TE} - 1} \right) \frac{\sigma_{TE}^{TE}}{\sigma_{TE}^{LE}} \frac{I^{LE}}{L^{LE}} \int \frac{\varepsilon AV}{\sigma_{LE}^{LE} L^{TE} / \sigma_{TE}^{TE} L^{LE} + 1} \, c_{well}^{LE} \, dt$$  \hspace{1cm} (9)$$

Eqn (9) represents the focusing rate at a given voltage and decreases in time as the ITP interface propagates and $L_{TE}$ increases. As expected, it shows direct dependence on the porosity and tortuosity of the media. However, noting that the expression in brackets is precisely the electric current across the entire channel, $I_t$,

$$I(t) = \sum_{i} I_i(t) = \sum_{i} \left( \frac{\mu^{TE}}{\mu^{TE} - 1} \right) \frac{\sigma_{TE}^{TE}}{\sigma_{TE}^{LE}} \frac{I^{LE}}{L^{LE}} \int \frac{\varepsilon AV}{\sigma_{LE}^{LE} L^{TE} / \sigma_{TE}^{TE} L^{LE} + 1} \, c_{well}^{LE} \, dt$$  \hspace{1cm} (10)$$

allows further simplification of the expression to the form

$$N_s(t) = \left( \frac{\mu^{TE}}{\mu^{TE} - 1} \right) \frac{\mu^{LE}}{\mu^{LE} - 1} \frac{\sigma_{TE}^{TE}}{\sigma_{TE}^{LE}} \frac{I^{LE}}{L^{LE}} \int \frac{\varepsilon AV}{\sigma_{LE}^{LE} L^{TE} / \sigma_{TE}^{TE} L^{LE} + 1} \, c_{well}^{LE} \, dt$$  \hspace{1cm} (11)$$

More importantly, when expressed as a function of electric current, $I$, sample accumulation shows no direct dependence on the cross-section area, or on porous media parameters. Hence, this expression can be used to describe sample accumulation in both porous and non-porous substrates (e.g. typical glass microchannels). For ideal conditions, where the parameters in the reservoir remain constant over time, and the analyte concentration in the reservoir equals its initial concentration, $c_0$, we may write

$$N_s(t) = \left( \frac{\mu^{TE}}{\mu^{TE} - 1} \right) \frac{\mu^{LE}}{\mu^{LE} - 1} \frac{\sigma_{TE}^{TE}}{\sigma_{TE}^{LE}} \int c_0^{well} \, I(t) \, dt$$  \hspace{1cm} (12)$$

We thus choose to define

$$\eta = \frac{N_s(t)}{c_0^{well} \int I(t) \, dt}$$  \hspace{1cm} (13)$$

as an indicator for ITP focusing efficiency. $\eta$ has units of $\text{L A}^{-1} \text{s}^{-1}$ and reflects the ability of the assay to process a certain sample volume per unit current and time. For constant reservoir conditions, as indicated by eqn (12), the value of $\eta$ is constant and can be estimated numerically using simulation software such as PeakMaster, Simul or Spresso. Such estimations must account though for dependencies on effects such as pH, ionic strength, or temperature. Alternatively, as we have shown in section 4.2, it can be measured experimentally by monitoring both the amount of the accumulated sample and the integral of the current. We emphasize that while we expect variations in EOF, channel geometry and topology to be reflected in electric current measurements for an ideal system with no changes in reservoir conditions, the value of $\eta$ should, in theory, remain constant for a given system of species, regardless of geometry or substrate material.

In section 4.2, we refer again to this ratio for evaluation of the efficiency of our paper-based devices compared to that of conventional glass microchannels.

**Deviation from the ideal model.** When implemented in a standard microchannel or microcapillary, the volume of the channel is typically on the order of ~10 nL, significantly lower than the ~10 µL volume of the reservoir, and thus conditions in the reservoir may be assumed constant. However, our paper-based devices are intended for processing a large volume and may hold several µL. As a result, both depletion of sample ions from the TE reservoir and dilution of the TE reservoir by incoming electroosmotic flow (for anionic ITP) may result in a decrease in anolyte concentration,

$$c_{well}^{LE}(t) = \frac{c_0 V_o - \int \frac{dN}{dt} \, dr'}{V_o + \int u_{EOF} A dr'}$$  \hspace{1cm} (14)$$

where $V_o$ is the initial volume of the TE reservoir, and $u_{EOF}$ is the area-averaged velocity due to EOF. In addition to these effects, in the absence of convection, the concentration of the sample at the entrance to the channel may be diffusion limited, i.e. the diffusion rate of new sample ions to the channel entrance may not be sufficient to compensate for the high flux into the channel. Since we apply relatively high electric currents, the conductivity in the well may also change in time as a result of electrolysis. Finally, mobilities and conductivities are a strong function of temperature and their values may be affected by Joule heating.

**2.2. Joule heating in µPAD**

In our initial attempts to perform ITP on paper, we created channels which were based on the entire thickness of the filter paper (approximately 150 µm deep). Such designs exhibited high temperatures, leading to rapid evaporation of
the liquid and occasionally even autoignition of the paper. Thus, better thermal management of paper-based devices was required for electrokinetic applications. Aiming to maintain a simple and low-cost device, we did not wish to add any external heat-removing devices and instead studied the potential for a geometrical design that would prevent excessive heating. We herein present our model for intra-paper temperature under an applied electric field, whose results guided the fabrication process described in section 3.1.

As illustrated in Fig. 2, we consider the cross section of a paper channel sealed from bottom and top with wax and masking tape, respectively. We divide the model into two different regions; the paper channel is denoted as A, and the sealing material (e.g. masking tape) is marked as B. Moreover, we assume that the width of the channel, $W$ (typically on the order of several mm to several cm), is much larger than its depth, $H_A$ (typically in the order of 10–100 μm), and therefore the model can be treated as a 1D model in the y (depth) direction. The governing equations for the model can thus be written as

$$\frac{d^2T_A}{dy^2} = -\frac{E^2\sigma}{k_A} 0 \leq y \leq H_A,$$

$$\frac{d^2T_B}{dy^2} = 0  \quad H_A \leq y \leq H_A + H_B,$$

where $T_X$ is the temperature within region X, $k_A$ is the thermal conductivity of the liquid in the channel, $Q_A = E^2\sigma$ is the heat per unit volume generated during electrophoresis in the channel (region A), $E$ is the electric field, and $\sigma$ is the conductivity of the buffer. No heat is generated in region B. $H_A$ is the depth of the paper channel containing the liquid, and $H_B$ is the thickness of the masking tape.

The Biot number, $Bi = \frac{hH}{k}$, relates the timescales of heat conduction inside the body and heat convection from its surface, with $h$ denoting the heat transfer coefficient (typically in the range of 2–25 W m$^{-2}$ K$^{-1}$ (ref. 32) for the free convection of gases) and $H$ representing the characteristic length (here the depth of the channel is on the order of 10–100 μm). The thermal conductivity $k$ of water, waxes and polymers used in this work are all on the order of ~0.1–1 W m$^{-1}$ K$^{-1}$.\textsuperscript{32} resulting in a very small Biot number, $Bi \sim 10^{-2}$–$10^{-1}$, which is $\ll 1$. This indicates that convection from the surface is significantly slower than conduction within the body, and thus the temperatures within each of the regions, $T_A$ and $T_B$, can be assumed to be uniform.

Equating the heat fluxes at the interface between region A and B and at the interface between region B and air and using the assumption $Bi \ll 1$ yield the simple solution

$$\Delta T = T_A - T_e = \frac{\sigma E^2 H_A}{h} \quad (16)$$

where $T_e$ is the temperature far from the device (room temperature). The solution shows that the increase in intra-paper temperature, $T_A$, is proportional to the square electric field, the conductivity of the electrolyte, and the depth of the channel and is inversely proportional to the heat transfer coefficient, $h$. The product $\sigma E^2$ is highest in the TE region. The conductivity $\sigma$ of our buffer is of the order ~0.1 S m$^{-1}$, and the electric field $E$ is on the order of $\sim 10^4$ V m$^{-1}$. Assuming a heat transfer coefficient of 10 W m$^{-2}$ K$^{-1}$, the temperature difference $\Delta T$ is then given roughly by $\sim 10^6 H_A$ (where $H_A$ is in units of meters), i.e. 150 μm thick paper would result in a temperature increase of roughly 150 K.

Clearly, the temperature can be most efficiently reduced by lowering the electric field (by decreasing the voltage used). However, this will also result in a diffused ITP interface and an increase in total analysis time. Lower LE concentration (resulting in lower conductivities) could also be used. However, this would also eliminate TE concentration adaptation which is a source of significant increase in the focusing rate. Thus, in order to maintain high electric fields in the system without excessive Joule heating, one should reduce the paper channel depth, $H_A$, as much as possible. As described in detail in the Experimental section, reducing the thickness of the paper from 150 μm to the range of 10–50 μm is sufficient to reduce the temperature to an operational range. Our fabrication process, as described in section 3.1, was specifically designed to achieve this goal.

3. Experimental

3.1. Fabrication of shallow-channel μPADs

The method of fabricating μPADs by wax printing\textsuperscript{33,34} has now been widely used owing to its low cost and simplicity. The technique is based on patterning a hydrophilic paper (or other porous membranes)\textsuperscript{35} with hydrophobic wax barriers.
Upon heating, the wax melts and penetrates by capillary action through the entire thickness of the paper and serves as side walls for the paper channel. We further developed this technique to be compatible with electrokinetic assays. Instead of printing only one layer of wax that wicks through the entire thickness of the paper, we printed wax on both sides of the paper. Upon heating, both layers wick into the paper until they meet, resulting in channels that are significantly shallower (~50 μm) than the original thickness of the paper. Such shallow channels are critical in providing sufficient dissipation of Joule heat, as detailed in section 2.2, and thus enable the use of high electric fields and short analysis time.

We used cellulose filter paper (125 mm diameter, grade 595, Whatman, GE Healthcare) as our substrate, as it is relatively thin (150 μm thickness) and provides medium-fast flow rate compared to other filter papers. We chose cellulose as our substrate as it does not contain active functional groups and thus is expected to have only weak interaction with biomolecules.36 We cut the paper to match with the width of an A6 paper size using a guillotine (3020, KW-trio, Changua, Taiwan). We then designed the device’s geometry using Autodesk AutoCAD 2013 (Autodesk Inc, San Rafael, CA) and fabricated the microfluidic paper-chip by printing (ColorQube 8570DN, Xerox Corporation, Norwalk, CT) the channel side wall template on one side of the paper, followed by a layer of wax on the opposite side, forming the bottom of the channel. After printing, the two layers are not yet in contact. In order to create a closed channel, we inserted the paper into a laminating pouch, and then heated the paper using a temperature-controlled lamination machine (335 R6, SKYDBS Co., Seoul, Korea), which provides uniform heating and can be controlled to provide penetration of the wax to the desired depth.

Different temperatures provide different channel depths. At low temperatures, less than 75 °C, the wax penetration level is insufficient, and the side walls created do not reach the bottom layer of wax. This results in leakage from the channel upon filling. At high temperatures, over 105 °C, the bottom layer of wax may penetrate the paper entirely and block the channel. We found the optimal lamination temperature, providing shallow yet unblocked channels, to be 95 °C, the wax penetration 50 μm depth. The channel is connected on either end to 6.5 mm radius round reservoirs. We then passed the paper through a hydrophobic wax barrier, which stops the flow of the LE and serves as a repeatable starting point for ITP. (c) Raw fluorescence images of the paper cross section showing the effect of lamination temperature on the penetration of wax into the paper, resulting in control of the channel depth.

3.2. Experimental setup

We obtained images using an upright epifluorescence microscope (Eclipse Ci-L, Nikon, Tokyo, Japan) equipped with a 660 nm LED light source (M660L3-C3, Thorlabs Inc., Newton, NJ) and a filter cube (Cy5-4040C-000, 628/40 nm excitation, 692/40 nm emission and 660 nm dichroic mirror, Semrock Inc., Rochester, NY). We used a 1X objective (NA = 0.04, WD = 3.2 mm, Plan UW, Nikon, Tokyo, Japan) for the experiments in paper devices and a 10X objective (NA = 0.3, WD = 16 mm, Plan Fluor, Nikon, Tokyo, Japan) for the experiments in glass channels. Images were captured using a 14 bit, 1392 × 1040 pixel array CCD camera (Clara DR-2584, Andor, Belfast, Ireland) cooled to ~19.5 °C. Images of the ITP focusing were taken using an exposure time of 100 ms. When not imaging, the light source was shuttered to prevent photobleaching of the dye. We controlled the camera using NIS Elements software (Version 4.11, Nikon, Tokyo, Japan) and processed the images with MATLAB (R2011b, MathWorks, Natick, MA). All ITP experiments were performed at constant voltage using a high-voltage sourcemeter (model 2410, Keithley Instruments, Cleveland, OH).

3.3. Isotachophoresis assay and choice of experimental conditions

In all experiments, we used 100 mM HCl, 200 mM BisTris, and 1% 1.3 MDa polyvinylpyrrolidone (PVP) as the LE solution. Our analyte was DyLight 650 (NHS Ester, Thermo Fisher Scientific, Waltham, MA) which has its peak fluorescence at an excitation wavelength of 652 nm. We mixed the analyte
with the TE solution to get an initial concentration of 10 nM DyLight 650 in the reservoir. We used two sets of TE solutions; the first TE set was composed of 100 mM Heps, 200 mM BisTris, and 1% PVP; the second TE set was composed of 10 mM Tricine, 20 mM BisTris, and 1% PVP. The former set was used for the experiments comparing the focusing efficiency of paper devices to that of glass channels. The latter set was used for demonstration of maximum focusing in paper. PVP was added to the LE and TE solutions for suppression of electroosmotic flow (EOF). We used high ionic strength LE to maximize the focusing rate of species and to ensure a thin double layer for further reduction in EOF. The TE buffer consisting of 10 mM Tricine provides higher accumulation rates at the expense of lower buffering capacity. Lower concentration could not be used as the assay's repeatability and robustness is compromised. Heps, Tricine, BisTris, and PVP were obtained from Sigma-Aldrich (St. Louis, MO). HCl was obtained from Merck (Darmstadt, Germany). All buffer solutions were made using deionized water (DI) from a Millipore Milli-Q system (Billerica, MA). We measured buffer conductivities using a conductivity meter (PC700, Eutech Instruments, The Netherlands).

The experiments in a glass channel were performed on a commercially available isotropically etched microchip (NS12A, PerkinElmer, Waltham, MA) having channel dimensions of 90 μm × 20 μm (width × depth). Our chip consisted of four reservoirs connected by channels; the West reservoir was connected to the longest channel (45.59 mm long) which intersected three shorter channels, termed North (15.1 mm long), South (3.92 mm long) and East (7.38 mm long). We first cleaned the channel by flowing 200 mM NaOH for 1 min, followed by 1 M HCl for another 1 min, and then rinsed the channel with DI for 1 min. In each experiment, we applied vacuum to the West reservoir, and filled the North, East and South reservoirs with 20 μL of LE. Once the channel was filled, we rinsed the West reservoir with DI water and filled it with 20 μL of the TE-analyte mixture. We then placed the positive electrode to the East reservoir and grounded the West reservoir. We applied a voltage of 400 V across the channel and simultaneously recorded the resulting electric current. The focused sample was imaged at eight stations, located 4.7, 9.7, 14.7, 25.7, 30.7, 35.7, 40.7 and 44.7 mm from the TE reservoir. At each station, the images were background corrected (background was taken in the LE solution before the ITP plug arrives).

The process of running ITP on our fabricated µPAD is presented in Fig. 4. For convenience, we separated the process into two steps to allow filling of multiple channels simultaneously. We began by adding 150 μL of LE to the right reservoir and relied on capillary action for filling the channel with LE solution (Fig. 4b, c). We allowed sufficient time (~10 min) for the liquid to reach a designed wax barrier, where it stopped (Fig. 4d). We then located the chip on the microscope, placed the electrodes in each of the reservoirs, and added another 150 μL of LE to the right reservoir. We then filled the left reservoir with 300 μL of a TE-analyte mixture (Fig. 4e, f). Importantly, the left-most part of the channel is initially exposed to air (i.e. is not covered with tape), and after adding the buffers it serves as the initial contact point of the LE and TE. We applied 200 V across the channel to initiate ITP. Fig. 4g and h present the resulting focused ITP plug, as imaged by a consumer-grade camera (SX510 HS, Canon, Tokyo, Japan) and under the microscope, respectively. The focused sample was imaged at eight stations, located at 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25 and 2.5 cm from the TE reservoir (stations are printed as 1–8 on the paper-chip in Fig. 4). At each station, the images were background corrected (background was taken in the LE solution before the ITP plug arrives).

To enable processing of large sample volumes, we designed our paper channel to be 2.5 mm wide. Under an applied voltage of 200 V, the electric currents established are on the order of 100 to 1000 μA. Large reservoir volumes are thus required for three primary reasons: (i) to provide sufficient hydration for the paper, compensating for any residual evaporation; (ii) to provide sufficient buffering from electrolysis;37 and (iii) to provide a sufficient sample volume to be processed by the ITP channel. Our design supports a volume of 300 μL in each of the reservoirs, which, as further demonstrated in the Results and discussion section, provides a sufficient amount of sample to maintain a repeatable and stable process for over 10 min.
4. Results and discussion

4.1. ITP focusing on μPAD

As illustrated qualitatively in Fig. 4g, at high initial concentrations (>1 μM) the ITP plug can be seen by naked eye or imaged by a consumer-grade camera. However, the quantitative analysis presented in this and the subsequent sections utilizes significantly lower concentrations and has been performed based on microscopy imaging.

Fig. 5 presents quantitative experimental results of ITP focusing using our μPADs in a continuous injection scheme (i.e. sample mixed in the TE reservoir). Fig. 5c presents raw fluorescence images of the ITP front at different stations along the paper channel. While the sample zone appears significantly more dispersed compared to sample focusing observed in standard glass microchannels, focusing is nevertheless clearly evident and the ITP plug is well contained and steadily electromigrates along the paper channel. Fig. 5a and b present the width averaged concentration along the channel during ITP and the total sample accumulation, respectively. We converted each image from intensity values to concentrations through the calibration curve of each substrate (see the ESI† for complete details) and averaged those concentrations across the width of the channel. We then calculated the total accumulated sample at each station by integrating all concentrations greater than 10% of the maximum and multiplying by the width, depth, and porosity of the channel (for details regarding porosity measurements see the ESI†). Fig. 5b presents the total accumulated sample as a function of time with the solid line representing a fit to \( N_a(t) \) expressed by eqn (13), with \( \eta \) serving as the free fitting parameter.

4.2. Characterization of ITP extraction efficiency on the μPAD

The value of \( \eta \), defined in eqn (13), describes the ratio between the number of sample molecules accumulated and the total amount of current passed in the system during ITP and the initial concentration of the analyte in the well. Since \( \eta \) depends only on the properties of the participating species (i.e. buffers and analyte), for a given system of chemistries, the value of \( \eta \) should be identical for glass and paper. However, a discrepancy in measured values of \( \eta \) may occur as a result of the substrate itself (e.g. due to adsorption) or of the physical setup (e.g. pH changes in the reservoirs, or depletion of the sample from the reservoir). Thus, \( \eta \) (units of \( \text{L A}^{-1} \text{s}^{-1} \)) can serve as an excellent indicator for the relative efficiency, or processing ability, of a specific substrate or setup.

Assuming a fully ionized analyte mobility of \( 36 \times 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1} \), as estimated by Milanova et al.,28 we used Spresso31 to compute the value of \( \eta \) for the TE chemistry consisting of 100 mM Hepes and 200 mM BisTris and obtained a value of \( 1.3 \times 10^{-4} \text{ L A}^{-1} \text{s}^{-1} \). Fig. 6a presents experimental results for this chemistry, showing the value of \( \eta \) as a function of time for both the glass channel and the paper channels. More importantly, both setups yield a near constant value at steady state, consistent with the analytical model. The mean value obtained in the glass channel is \( 1.3 \times 10^{-4} \text{ L A}^{-1} \text{s}^{-1} \), which is identical to the numerical prediction. We thus consider the experiments in glass to be the accurate reference value. For the paper, however, while theory predicts the same \( \eta \) value, in practice we obtained a measured value of \( 0.88 \times 10^{-5} \text{ L A}^{-1} \text{s}^{-1} \), which is an order of magnitude lower than that obtained in glass.

Three main reasons could cause this behavior and reduce paper efficiency compared to that of glass: (i) adsorption of the analyte on the paper and/or wax, (ii) decrease of analyte concentration in the TE reservoir, and (iii) decrease in the influx of sample ions into the channel due to diffusion rate limitations. We will herein address each of these possibilities: (i) we have ruled out adsorption by performing several ITP experiments in which we have injected a finite amount of the sample to the paper channel and monitored the total amount of the focused sample in time (see the ESI† for complete details). Results clearly showed no decrease in the focused sample, indicating no adsorption of the dye on the μPAD. (ii) Since in our design, the volume of the channels (in the order of several μL) is not negligible compared to the reservoir volume (in the order of hundreds of μL), dilution of the sample in the reservoir could arise both from EOF delivering more liquid to the TE reservoir and from rapid depletion of sample ions as they enter the channel. This change in
concentration is expressed by eqn (14). From observing the electromigration velocity, we estimated the upper bound for EOF to be \( \eta_{\text{EOF}} \approx -5 \times 10^{-4} \text{ m s}^{-1} \). Assuming a cross-section area of \( 8 \times 10^{-8} \text{ m}^2 \) and a total run time of \( \approx 700 \text{ s} \), the total volume that enters the TE reservoir as a result of EOF during our typical experiment is \( \approx 30 \mu\text{L} \). While this is a significant volume, it is still small compared to the 300 \( \mu\text{L} \) volume of the reservoir. Thus we obtain \( V_0 > \int_0^t \eta_{\text{EOF}} A \text{d}t' \). Furthermore, our experimental results show the accumulated sample, \( N_a \), to be on the order of \( 3 \times 10^{-13} \text{ mol} \), while the amount of the sample in the reservoir, \( N_o \), is \( 3 \times 10^{-12} \text{ mol} \), and we may therefore assume \( N_o > \int_0^1 \frac{\text{d}N}{\text{d}t'} \). The average concentration in the reservoir can thus be approximated to be constant. (iii) The large size (width) of the channel may result in a situation where the diffusion rate of new sample ions to the channel entrance is not sufficient to compensate for the high flux into the channel. Taking a characteristic length scale of \( h = 1 \text{ mm} \) (corresponding to both the characteristic diameter and the height of the reservoir) into consideration, one can define the characteristic electromigration time as \( \tau_E = h/\mu_{\text{TE}}E \) and the characteristic diffusion time as \( \tau_D = h^2/D \). Estimating the diffusion coefficient from the Einstein relation as \( D = 8.5 \times 10^{-10} \text{ m}^2 \text{ s}^{-1} \) and assuming an electric field \( E = 10^3 \text{ V m}^{-1} \), we obtain \( \tau_D \approx 1 \text{s} \) vs. \( \tau_E \approx 10^2 \text{s} \). This analysis neglects free convection which likely exists in the reservoir to some extent, but nevertheless shows a discrepancy in the timescales which may result in depletion of the sample from the entrance to the channel. As we have ruled out other factors, we hypothesize that diffusion limits indeed play a dominant role in the efficiency of the assay. It is however important to note that while efficiency is lower, the absolute volume being processed by the paper channel is significantly larger than that of the glass microchannel due to its larger dimensions.

4.3. Demonstration of 1000-fold sample focusing

For a given analyte mobility, in order to maximize the amount of the sample focused at the ITP interface, eqn (12) suggests minimizing the TE mobility as well as reducing the TE concentration to allow a larger increase in analyte concentration across the TE adaptation region. We thus replaced Heps (\( \text{pK}_a \approx 7.5 \)) by Tricine (\( \text{pK}_a \approx 8.15 \)), resulting in an expected reduction in effective TE mobility from \( 7.4 \times 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1} \) to \( 5.6 \times 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1} \) and in the TE concentration from 100 mM to 10 mM. Fig. 6b presents the experimentally measured \( \eta \) for the two TE chemistries, showing a 34-fold improvement (3 \( \times \) \( 10^{-4} \text{ mol A}^{-1} \text{ s}^{-1} \)) vs. 8.8 \( \times \) \( 10^{-6} \text{ mol A}^{-1} \text{ s}^{-1} \) in sample accumulation with the 10 mM Tricine chemistry.

Fig. 6c presents the total accumulated sample (in moles) as registered at each station using 10 mM Tricine and 20 mM BisTris TE chemistry. The total sample accumulated is approximately 3 \( \times \) \( 10^{-13} \text{ mol} \), which was achieved after 700 s. Since the initial concentration of the analyte in the TE reservoir is 10 nM, the total sample volume which was processed by ITP, evaluated using \( \frac{N(t)}{c_o} \), is 30 \( \mu\text{L} \).

We have thus far addressed the total amount of the sample accumulated at the ITP interface. Fig. 7 provides a quantitative evaluation of the peak (maximum) and the average concentration of the focused sample. The peak value is important for detection and imaging applications where focusing is used to directly increase the signal to be detected (e.g. of a fluorescent molecule). However, in applications where ITP is used to accelerate the reaction between co-focusing species, it is the average concentration which, to first order approximation, determines the rate of reaction.39

Fig. 7a presents a typical raw fluorescence image of the focused sample. At each station, to reduce the noise associated with this measurement, we applied 5 \( \times \) 5 binning to the ITP plug image and converted it to concentrations via the
The analyte in paper ITP. (a) Raw fluorescence image of the focusing zone. The analyte’s maximum concentration is denoted by $C_{\text{peak}}$. (b) We calculated the width averaged concentration (blue line) of the analyte and denoted $\delta(t)$ as the full width of the profile at 10% of the maximum value. We denoted the average concentration in that region as $C_{\text{average}}$. (c) Measurements of $C_{\text{peak}}$ and $C_{\text{average}}$ based on four repeats. Each marker shape represents an individual experiment, and 4th order polynomial fits based on the average of all experiments are presented solely to guide the eye. Results show that paper-based ITP provides a 200-fold average concentration enhancement, while the peak concentration is increased by 1000-fold in less than 6 minutes.

Fig. 7 Experimental measurements of the focusing ratio achieved in paper ITP. (a) Raw fluorescence image of the focusing zone. The analyte’s maximum concentration is denoted by $C_{\text{peak}}$. (b) We calculated the width averaged concentration (blue line) of the analyte and denoted $\delta(t)$ as the full width of the profile at 10% of the maximum value. We denoted the average concentration in that region as $C_{\text{average}}$. (c) Measurements of $C_{\text{peak}}$ and $C_{\text{average}}$ based on four repeats. Each marker shape represents an individual experiment, and 4th order polynomial fits based on the average of all experiments are presented solely to guide the eye. Results show that paper-based ITP provides a 200-fold average concentration enhancement, while the peak concentration is increased by 1000-fold in less than 6 minutes.

5. Conclusions

We presented an experimental and analytical study of a novel paper-based analytical device for sample focusing using isotachophoresis. We showed that although dispersion is much more significant in paper than in glass, peak enhancement by substantial sample focusing (on the order of 1000-fold) can be achieved in several minutes. Choosing or modifying the parameters of the porous media in order to minimize this dispersion remains an interesting and potentially important direction for research. Obtaining high sample concentrations in paper has direct implications in accelerating reaction kinetics and creating low-cost devices with much enhanced sensitivity.

Another benefit of paper-based ITP is the ability to process large sample volumes. While microchannels are excellent platforms for ITP, their small dimensions typically limit their application to the analysis or processing of small sample volumes. Implementation of ITP in larger channels or larger diameter capillaries is challenging due to hydrodynamic instabilities and excessive Joule heating. Paper (and porous media in general) offers the ability to process large sample volumes while maintaining high hydrodynamic resistance in a planar format. In our work, we used 2.5 mm wide channels and demonstrated processing of a 30 μL sample in several minutes. However, we see no fundamental reason why the width of the channel could not be substantially increased to enable processing of hundreds of μL and even mL of sample volume. This would open the door to the use of ITP for detection of extremely dilute samples (e.g. detection of bacteria at 10–100 copies per mL).

Managing Joule heat and limiting evaporation are key challenges in integrating electrophoretic techniques with paper-based devices. While many other techniques for fabrication of paper-based devices exist, we find that wax printing is particularly suitable for obtaining shallow channels which result in a higher surface to volume ratio of the channels and thus faster dissipation of heat. Nevertheless, one could envision other methods for achieving this goal, the most simple of which is the use of paper sheets with smaller initial thickness. However, to the best of our knowledge, such substrates are currently not commercially available.

Our analytical model provides a convenient figure of merit for evaluating the efficiency of ITP focusing. Using this figure we showed that, despite the demonstrated gains, the efficiency of our device is roughly 10%, i.e. the amount of sample accumulated is only a fraction of the theoretical limit. By eliminating other potential reasons, we showed that this reduced efficiency is likely due to diffusion limits within the sample reservoir. Further investigation of these limitations is warranted, but it suggests that an order of magnitude improvement on all parameters is possible with further optimization.

We believe that the device and the analysis we have presented can serve as a guide to the design of low-cost, rapid and highly sensitive paper-based diagnostic platforms.

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